PRiMER

A high PRotein Mediterranean-style diet and resistance Exercise for cardiac Rehabilitation

Richard Kirwan

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Contents

Declaration

I declare that the work contained within this thesis is my own. No portion of the work referred to in the thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.

Dedication

To my parents, Jim and Anne, for supporting me no matter what notions I take in life, and to Georgia, for her love and for getting me through everything. I can never thank you enough.

Ní bhíonn an rath ach mar a mbionn an smacht

継続は力なり

From discipline, success

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List of publications

Publications or manuscripts resulting from this programme of research

- **Kirwan R**, McCullough D, Butler T, Perez de Heredia F, Davies IG, Stewart C. Sarcopenia during COVID-19 lockdown restrictions: long-term health effects of short-term muscle loss. *Geroscience*. 2020;42(6):1547-1578. https://doi.org/10.1007/s11357-020-00272-3
- **Kirwan RP**, Mazidi M, García CR, Lane KE, Jafari A, Butler T, Perez de Heredia F, Davies IG. Protein interventions augment the effect of resistance exercise on appendicular lean mass and handgrip strength in older adults: a systematic review and meta-analysis of randomized controlled trials. *American Journal of Clinical Nutrition*. 2022;115(3):897-913. https://doi.org/10.1093/ajcn/nqab355
- **Kirwan R**, Isanejad M, Davies IG, Mazidi M. Genetically Determined Serum 25-Hydroxyvitamin D Is Associated with Total, Trunk, and Arm Fat-Free Mass: A Mendelian Randomization Study. *The Journal of Nutrition, Health & Aging*. 2022;26(1):46-51. https://doi.org/10.1007/s12603-021-1696-1
- **Kirwan RP**, Mazidi M, Butler T, Perez de Heredia F, Davies IG. The association of appendicular lean mass and grip strength with LDL, VLDL and HDL particle diameter: a Mendelian randomization study of the UK Biobank cohort. *Awaiting submission*.
- **Kirwan R**, Perez de Heredia F, McCullough D, Butler T, Davies IG. Impact of COVID-19 lockdown restrictions on cardiac rehabilitation participation and behaviours in the United Kingdom. *BMC Sports Science, Medicine and Rehabilitation*. 2022;14(1):67. https://doi.org/10.1186/s13102-022-00459-5
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- **Kirwan R**, Newson L, McCullough D, Butler T, Davies IG, Perez de Heredia F. Developing a high-protein Mediterranean-style diet and resistance exercise protocol for cardiac rehabilitation patients: a mixed-methods study with patient and public involvement. *Awaiting submission*

Other publications completed by the candidate during the PhD tenure

- Ma L, Mazidi M, Li K, Li Y, Chen S, **Kirwan R**, Zhou H, Yan N, Rahman A, Wang W, Wang Y. Prevalence of mental health problems among children and adolescents during the COVID-19 pandemic: A systematic review and meta-analysis. *Journal of Affective Disorders*. 2021;293:78-89. https://doi.org/10.1016/j.jad.2021.06.021
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- Sun X, Mazidi M, **Kirwan R**, Li Y, Zhao B, Kandiah J, Wang Y. Increased COVID-19 Infection Susceptibility and Adverse Outcomes Due to Obesity: A Systematic Review and Meta-analysis. *Preprint only.* 2021. https://doi.org/10.21203/rs.3.rs-244649/v1
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- Mazidi M, Kengne AP, Siervo M and **Kirwan R**. Association of dietary intakes and genetically determined serum concentrations of mono and poly unsaturated fatty acids on chronic kidney disease: insights from dietary analysis and Mendelian randomization. *Nutrients*. 2022;14(6):1231. https://doi.org/10.3390/nu14061231

- **Kirwan R**, Mallett GS, Ellis L, Flanagan A. Limitations of self-reported health status and metabolic markers among adults consuming a “Carnivore diet”. *Current Developments in Nutrition*. 2022;6(5):nzac037. https://doi.org/10.1093/cdn/nzac037
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Conference communications

- **Kirwan R**, Perez De Heredia F, Davies I, Butler T. A high-protein Mediterranean diet and resistance training on the amelioration of cardiometabolic risk markers in cardiac rehabilitation patients with sarcopenic obesity: study protocol for a randomized control trial. *European Congress on Obesity*. April 2019.Glasgow, UK. [Poster]
- **Kirwan R**, Perez De Heredia F, Davies I, Butler T. A high-protein Mediterranean diet and resistance training on the amelioration of cardiometabolic risk markers in cardiac rehabilitation patients with sarcopenic obesity: study protocol for a randomized control trial. *Obesity facts, the European Journal of Obesity*. 2019;12:134. [Abstract]
- **Kirwan R**, Perez De Heredia F, Davies I, Butler T. A high-protein Mediterranean diet and resistance exercise for cardiac rehabilitation: a pilot randomised controlled trial. *FENS European Nutrition Conference*. October 2019. Dublin, Ireland. [Poster with oral presentation]
- **Kirwan R**, McCullough D, Butler T, Perez de Heredia F, Davies IG, Stewart C. Sarcopenia during COVID lockdown restrictions. Health Service Executive Ireland ‘*Older Persons Impacted by COVID - Time to Get Moving Again’ Webinar*. May 2021. Virtual [Oral presentation]

- **Kirwan R**, García CR, Mazidi M, Lane KE, Butler T, Perez de Heredia F, Davies IG. Protein interventions augment the effect of resistance exercise on lean mass and strength in older adults: a systematic review and meta-analysis of randomized controlled trials. *Communications to the International Sarcopenia Translational Research Conference*. September 2021. Virtual [Poster]
- **Kirwan R**, Isanejad M, Davies IG, Mazidi M. Genetically Determined Serum 25-Hydroxyvitamin D Is Associated with Total, Trunk, and Arm Fat-Free Mass: A Mendelian Randomization Study. *LJMU Postgraduate Research Festival*. May 2022. Liverpool, UK. [Poster]

Abbreviations

25(OH)D	25-hydroxy vitamin D
AE	Aerobic exercise
ALM	Appendicular lean mass
AR	Anabolic resistance
BMI	Body mass index
CAD	Coronary artery disease
CHD	Coronary heart disease
CM	Cardiometabolic
CR	Cardiac rehabilitation
CRP	C-reactive protein
CVD	Cardiovascular disease
DXA	Dual-energy X-ray absorptiometry
EAA	Essential amino acid
FFM	Fat-free mass
FM	Fat mass
GWAS	Genome wide association study
HDL	High-density lipoprotein
HGS	Hand grip strength
HPMD	High-protein Mediterranean Diet
IDL	Intermediate density lipoprotein
ICU	Intensive care unit
IGF-I	Insulin-like growth factor I
IL	Interleukin
IMAT	Intramuscular adipose tissue
IMV	Invasive mechanical ventilation
IQR	Interquartile range
IR	Insulin resistance
IVW	Inverse variance weighted
KCCS	Knowsley Community Cardiovascular Services
KE	Knee extension
LBM	Lean body mass
LDL	Low-density lipoprotein

LHCH	Liverpool Heart & Chest Hospital
MedDiet	Mediterranean diet
MetS	Metabolic syndrome
MR	Mendelian randomization
MPB	Muscle protein breakdown
MPS	Muscle protein synthesis
mTORc1	Mammalian target of rapamycin complex 1
NAFLD	Non-alcoholic fatty liver disease
NHS	National Health Service
PA	Physical activity
PPI	Patient participant involvement
Q	Cochrane Q statistic
RAPS	Robust adjusted profile score
RCT	Randomised controlled trial
RE	Resistance exercise
RM	Repetition maximum
SD	Standard deviation
sdLDL	Small dense low-density lipoprotein
SE	Standard error
SNP	Single nucleotide polymorphism
SO	Sarcopenic obesity
T2DM	Type-2 diabetes mellitus
TNF-α	Tumor necrosis factor alpha
UPF	Ultra-processed foods
VAT	Visceral adipose tissue
VDR	Vitamin D receptor
VLDL	Very low-density lipoprotein
WM	Weighted median
WMD	Weighted mean difference
WHR	Waist-to-hip ratio
WC	Waist circumference

1 Chapter 1: Introduction

1.1 Background

1.1.1 The Burden of Cardiovascular Disease

Cardiovascular disease (CVD) is responsible for more than 1 in 4 deaths in the UK amounting to over 170,000 deaths/year (British Heart Foundation, 2019). CVD is an umbrella term for all diseases of the heart and circulation, both congenital and those that develop in later life, including coronary heart disease (CHD) (formally known as ischaemic heart disease), atrial fibrillation, heart failure, stroke and vascular dementia (British Heart Foundation, 2019). CHD, resulting from the build-up of atherosclerotic plaque in the coronary arteries, which leads to impairments in blood flow, is the greatest contributor to years of life lost in the UK (Steel et al., 2018) and the leading cause of death worldwide (World Health Organization, 2018). In addition to the loss of life, the economic cost of CVD is considerable; in 2015 the cost of healthcare for CVD alone in the UK amounted to £10.9 billion, with a further £7.7 billion lost from the economy due to productivity loses (Wilkins et al., 2017). The loss of life and financial burden attributable to CVD highlights it as a major public health concern both in the UK and globally.

Interestingly, Bhatnagar et al., using data from the national statistics agencies of the UK and admissions data from the National Health Service (NHS), showed that from 1979-2013, total mortality from CVD in the UK declined by 70% (Figure 1.1) (Bhatnagar et al., 2016). This may be attributed to improvements in cardiovascular care including an increase in pharmacological prescriptions and revascularisation surgeries, highlighted in the same document (Bhatnagar et al., 2016), as well as to reductions in risk factors such as smoking, high blood pressure and high cholesterol levels (Unal, Critchley and Capewell, 2004).

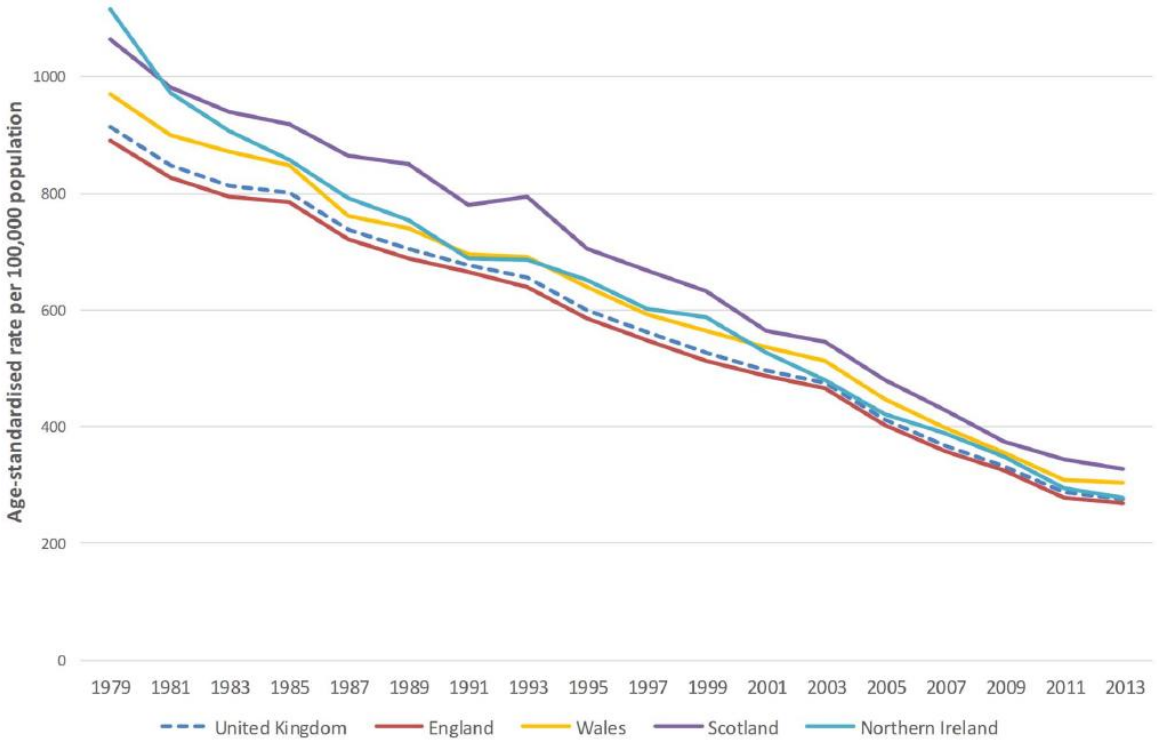

Figure 1.1 Age-standardised death rates per 100,000 from cardiovascular disease, all ages, UK and England, Wales, Scotland, Northern Ireland, 1979–2013 (Bhatnagar et al., 2016). Available at https://heart.bmj.com/content/102/24/1945. Used without changes and licensed under creative commons attribution 4.0

However, despite the impressive decline in mortality, the prevalence of CVD remained stable from 2003-2013 (Figure 1.2), with an increase in CVD-related admissions of over 46,000 between 2010/2011 and 2013/2014 in the UK (Bhatnagar et al., 2016). While these data highlight advances in the successful treatment of CVD for reducing mortality, they also bring to light the ever-increasing burden of CVD on health services in the UK, in the form of increased admissions, prescriptions and surgical procedures.

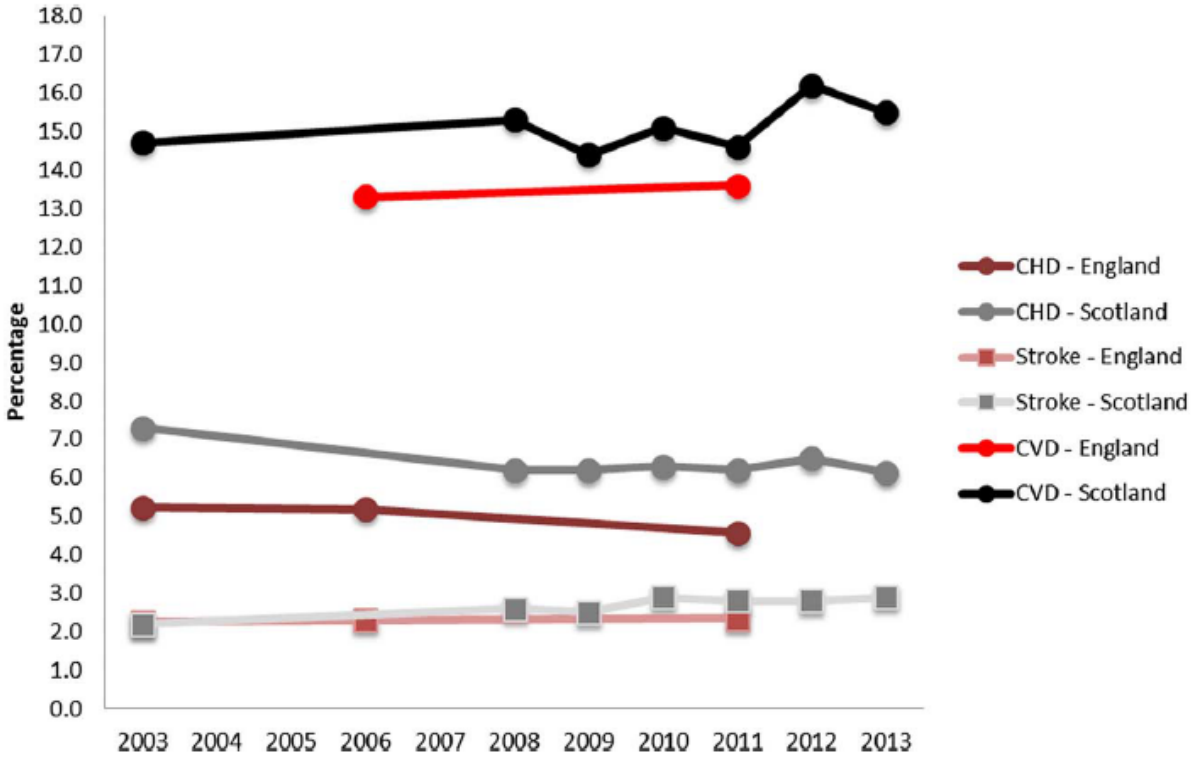

Figure 1.2 Trends in the prevalence of cardiovascular disease (CVD), coronary heart disease (CHD), and stroke, from the health surveys of England and Scotland 2003–2013 (Bhatnagar et al., 2016). Available at https://heart.bmj.com/content/102/24/1945. Used without changes and licensed under creative commons attribution 4.0

The causation of CVD is multifactorial and complex. While genetics play a role in susceptibility to developing CVD (Kessler and Schunkert, 2012), modifiable risk factors contribute to as much as 78% of deaths from heart and circulatory disease, and as much as 93% of deaths from CHD (Foundation, 2019). These modifiable risk factors include but are not limited to poor diet, inactivity, tobacco use, and associated risk markers including dyslipidaemia, high fasting blood glucose, high blood pressure and high body mass index (BMI). These data place lifestyle intervention, focussed on improving the aforementioned risk factors, at the forefront of potential strategies to reduce the risk of developing CVD, thus reducing both the loss of life and economic burden of the condition.

1.1.2 Cardiac Prevention & Rehabilitation

The relevance of modifiable lifestyle factors to the development of CVD has led to the promotion of the practice of cardiac rehabilitation (CR), aimed at those with established CVD and asymptomatic individuals deemed at high risk of developing adverse cardiac events (Anderson et al., 2016; Piepoli et al., 2016). CR has been defined by the British Association for Cardiac Prevention and Rehabilitation (BACPR) as "the coordinated sum of activities required to influence favourably the underlying cause of cardiovascular disease, as well as to provide the best possible physical, mental and social conditions, so that the patients may, by their own efforts, preserve or resume optimal functioning in their community and through improved health behaviour, slow or reverse progression of disease" (British Association for Cardiovascular Prevention and Rehabilitation, 2017). As such, CR may be viewed as having a role in both the prevention of an individual's first adverse cardiac event (primary prevention) and the prevention of adverse cardiac events subsequent to an initial event (secondary prevention).

CR is a multidisciplinary approach and the components which fall within its remit include, amongst others, health behaviour change and education, psychosocial health, medical risk management and lifestyle risk factor management.

The lifestyle factors managed in CR are:

i. physical activity and exercise,
ii. healthy eating and body composition, and
iii. tobacco cessation and relapse prevention (British Association for Cardiovascular Prevention and Rehabilitation, 2017).

CR consists of 4 distinct phases. Briefly, phase I is prior to discharge when patients are identified, phase II is the period after discharge and prior to starting a 6-12 week phase III programme. Phase III typically involves graded exercise, often in a group setting with a lifestyle education component. Phase IV is long-term maintenance of health behaviour change, usually through ongoing exercise in specific facilities provided at leisure centres or gyms in the private sector (Foundation, 2011). It should be noted that not all patients receive all phases and therefore may not have a complete CR experience.

1.1.3 The Role of Exercise

In the UK, according to guidelines set by the National Institute for Health and Care Excellence (NICE), all cardiac patients should be provided with the option of participating in a CR programme with an exercise component (National Institute for Health and Care Excellence, 2013). While there is considerable variation in the standards and formats of CR programmes, both throughout the UK and internationally (Dalal, Doherty and Taylor, 2015; Anderson et al., 2016; Price et al., 2016; Doherty et al., 2017), there is a general focus on aerobic/endurance-style training. While resistance exercise (RE) (any exercise that causes the muscles to contract against an external resistance with the expectation of increasing strength and/or muscle mass, such as weight training) is also recommended, research into the use of RE in cardiac populations is limited (Vanhees et al., 2012; Piepoli et al., 2016; Price et al., 2016; Khadanga, Savage and Ades, 2019; Kirkman, Lee and Carbone, 2022). Exercise-based CR has been shown to reduce the risks of cardiovascular mortality as well as the risk of cardiac-related hospitalisation, while also improving quality of life when compared to non-exercise controls (Lavie and Milani, 2006; Sagar et al., 2015; Anderson et al., 2016). However, it should be noted that CR seems to have little effect on total mortality nor on the risk of mortality or morbidity following myocardial infarction (MI), nor on revascularisation (West, Jones and Henderson, 2012), highlighting some limitations to its benefits, in its current form. Further

research in cardiac populations, focusing on the use of RE or combinations of RE and aerobic exercise are warranted to elucidate their value for improving specific cardiac outcomes, including mortality.

1.1.4 The Role of Diet

While the benefits of CR are associated predominantly with its exercise component, there is considerable evidence showing improvements in markers of cardiovascular risk, such as total and low-density lipoprotein (LDL) cholesterol, serum triglycerides and serum glucose etc., through various dietary strategies (Dansinger et al., 2005; Medina-Remon et al., 2016). Of particular relevance, results from studies on both the primary and secondary prevention of CVD suggest Mediterranean-style diet (MedDiet) based approaches are the most adequate to treat these patients and as such, MedDiet dietary patterns are frequently recommended for cardiac patients, and have been for almost 30 years (de Lorgeril et al., 1994; de Lorgeril et al., 1999; Trichopoulou, Bamia and Trichopoulos, 2005; Iestra et al., 2006; Trichopoulou et al., 2007; National Institute for Health Care Excellence, 2013; Deanfield et al., 2014; Panagiotakos et al., 2016; Estruch et al., 2018). MedDiets are typically high in fruit, vegetables, legumes and whole grains, moderate in seafood, nuts and dairy consumption, and low in red and processed meats, with the majority of dietary fat coming from olive oil (Widmer et al., 2015). Results from the PREDIMED (*Prevención con Dieta Mediterranea*) trial have shown MedDiets to improve certain risk markers of CVD, such as plasma glucose levels, systolic blood pressure, the total cholesterol to high-density lipoprotein (HDL) cholesterol ratio (Estruch et al., 2006) as well as reducing LDL particle number and size (Damasceno et al., 2013). In addition to this, MedDiets have been shown to have significant clinical benefits, by reducing the incidence of primary and secondary cardiac events in long-term dietary intervention studies (de Lorgeril et al., 1999; Estruch et al., 2018).

The observed changes in risk markers and clinical outcomes resulting from dietary pattern interventions may be due to multiple dietary variables. This can make it difficult to determine which specific dietary component results in the observed improvement in outcome measures. Evidence from randomized controlled trials suggests that MedDiets exert their cardiometabolic (CM) benefits through diverse mechanisms (Chiva-Blanch, Badimon and Estruch, 2014), including:

i. reductions in blood pressure, potentially due to higher nitrate intake from vegetables (Domenech et al., 2014; Van der Avoort et al., 2018);
ii. reductions in inflammation which can contribute to the development of arterial plaques, possibly due to increased polyphenol and antioxidant intake (Mena et al., 2009; Llorente-Cortes et al., 2010);
iii. improvement of cholesterol levels as well as reductions in cholesterol atherogenicity due to reduced saturated fat and increased unsaturated fat intake and increased polyphenol intake, respectively (Damasceno et al., 2013; Di Daniele et al., 2013);
iv. reductions in serum triglycerides, which is potentially associated with improvements in insulin sensitivity related to greater fibre and polyphenol intake (Davis et al., 2017; Wade et al., 2018); and
v. modulation of the expression of pro-atherothrombotic genes, possibly due to greater intake of antioxidant nutrients and polyphenols (Llorente-Cortes et al., 2010).

Interestingly, many of these CM benefits and subsequent improvements in CV morbidity and mortality have been observed in the absence of weight loss (Estruch et al., 2018), which itself is associated with improvements in risk markers of CVD (Dansinger et al., 2005; Sierra-Johnson et al., 2008).

1.2 Body Composition & Cardiovascular Risk

1.2.1 Obesity

Obesity is a risk factor for the development of CVD through its effects on the major cardiovascular risk markers, such as increased blood pressure, increased blood glucose and insulin resistance, dyslipidaemia, and increased levels of inflammation, as well as having adverse effects on cardiac structure and function in adults, with effects even observed in children with obesity (Poirier et al., 2006; Bastien et al., 2014; Skinner et al., 2015). Global obesity rates continue to rise (Abarca-Gómez et al., 2017), and in the general population, obesity (BMI ≥ 30 kg/m^2) and particularly severe obesity (BMI ≥ 35 kg/m^2) are strongly correlated with increased risk of CVD incidence and mortality (Ortega, Lavie and Blair, 2016), with BMI being a commonly used tool for easily and rapidly identifying those at risk (Ortega et al., 2016).

The accumulation of visceral adipose tissue (VAT) around internal organs, leading to a more central distribution of body fat and greater waist circumference, is strongly associated with CM risk markers such as hyperinsulinaemia, insulin resistance, elevated free fatty acids and triglycerides, high levels of apolipoprotein B, and small, dense low-density lipoprotein (sdLDL) particles (Despres, 2001; Fox et al., 2007; Ebbert and Jensen, 2013; Medina-Urrutia et al., 2015; Kouli et al., 2017). It is thought that this higher CM risk is due, in part, to the endocrine function of adipose tissue, which, in obesity, undergoes a shift towards a more pro-inflammatory profile, further exacerbated in VAT (Kyrou et al., 2017). This leads to a state of chronic, low-grade inflammation, characterised by increased secretion of pro-inflammatory cytokines and adipokines such as IL-6, TNF-α, leptin and resistin, along with decreased secretion of anti-inflammatory adipokines such as adiponectin and omentin (Gregor and Hotamisligil, 2011; Makki, Froguel and Wolowczuk, 2013).

Higher levels of pro-inflammatory cytokines produced in VAT (Schrager et al., 2007) can lead to dysfunction and damage of the vascular endothelia (Jou et al., 2020). This endothelial dysfunction induces the secretion of further cytokines and growth factors, resulting in pro-coagulant properties in the endothelium as well as allowing higher levels of atherogenic lipoproteins to enter the intima and be taken up by macrophages, a key stage in the development of atherosclerosis (Ross, 1999; Geovanini and Libby, 2018). Eventually, this leads to smooth muscle cell proliferation and increased leukocyte migration, causing further inflammation, a thickening of the artery wall and the development of atherosclerotic plaques, which can eventually result in cardiac events, such as ischemia of the heart and brain (Ross, 1999; Geovanini and Libby, 2018). As such, depending on the distribution of body fat stores, individuals of similar BMI and adiposity may have considerably different risk for CVD, questioning the validity of using only BMI as a screening tool.

1.2.2 Assessing Risk

To add to the complexity of using BMI as a screening tool for CVD, an “obesity paradox” exists amongst individuals with CVD, where increased mortality has been linked to lower BMI (Lavie et al., 2003; Romero-Corral et al., 2006; Lavie et al., 2012a). One potential explanation for this lower BMI is lower lean body mass (LBM) in cardiac patients, rather than fat mass (Lavie et al., 2012b), which may be due to sarcopenia, the progressive loss of muscle mass associated with aging (Fielding et al., 2011). This loss of LBM is predominantly associated with loss of muscle tissue and its causation is multifactorial, including chronic disease, inflammation, insulin resistance, nutritional deficiencies and inactivity (Fielding et al., 2011; Hunter et al., 2019), many of which are also associated with higher levels of fat mass (Antonopoulos and Tousoulis, 2017). Indeed, the chronic inflammation observed in obesity may contribute, through multiple pathways, to a net catabolic environment within muscle tissue which may contribute to the loss of LBM over time (Collins et al., 2018). This reduction in muscle mass

and the subsequent reduction in strength contribute to the greater risk of numerous chronic conditions and overall mortality in sarcopenic individuals (Kim and Choi, 2015; Scott, de Courten and Ebeling, 2016; Addison et al., 2018; Zhang et al., 2019), as well as decreased quality of life due to impaired mobility (Beaudart et al., 2017b; Tsekoura et al., 2017).

Indeed, higher levels of LBM are associated with lower risk of death in heart failure (De Schutter et al., 2014) or stable coronary patients (Lavie et al., 2012b), which may allude to a protective role of LBM in cardiovascular health. Handgrip strength (HGS) is also inversely associated with fatal cardiovascular events (Laukkanen et al., 2020). In contrast to BMI, waist circumference (WC) and waist-to-hip ratio (WHR), which are better indicators of central or visceral adiposity, are strongly associated with the risk of incident CVD events and mortality (de Koning et al., 2007), meaning that the obesity paradox is not observed with these measures, and thus may be limited to the use of BMI as a screening tool.

To further complicate the relation between fat mass and cardiovascular risk, another possible reason for the reduced mortality observed in individuals with higher BMI (Srikanthan, Horwich and Tseng, 2016) is the potentially (counterintuitive) cardioprotective effect of elevated leptin levels due to greater adiposity, which has been shown to protect cardiomyocytes from ischemia/reperfusion (I/R) injury or hypoxia, as observed in MI, stroke, and peripheral vascular disease (Erkasap et al., 2006; Smith et al., 2010). Despite this observed protective effect in the case of acute cardiac events, it should be noted that elevated leptin, from increased adipose tissue, is also associated with a higher risk of MI and stroke, independently of BMI and other cardiometabolic risk markers, due to its pro-inflammatory effects, mentioned previously (Garg, 2006; Sierra-Johnson et al., 2007; Romero-Corral et al., 2008; Makki, Froguel and Wolowczuk, 2013).

1.2.3 Muscle Mass & Cardiovascular Risk

Patients with a combination of low LBM and abdominal distribution of body fat, known as sarcopenic obesity (SO) (Prado et al., 2012), are at greater risk of CVD, which is exacerbated in cardiac populations (Atkins et al., 2014; Kim and Choi, 2015; Gusmao-Sena et al., 2016; Ma et al., 2016). This increased risk of CVD in SO is considered to be, in part, a result of higher levels of pro-inflammatory cytokines produced in VAT (Schrager et al., 2007), which may further contribute to the progression of SO through their association with reduced muscle mass and strength (Visser et al., 2002; Kalinkovich and Livshits, 2015; Rubio-Ruiz et al., 2019), as do other factors associated with dysfunctional adipose tissue, such as ectopic accumulation of fat between muscle fibres, known as intramuscular adipose tissue (IMAT), which has been shown to impose a significant risk of muscle dysfunction in older adults (Addison et al., 2014; Buch et al., 2016). Losses of muscle mass and function leading to difficulty in locomotion may reduce activity levels in older adults (Troiano et al., 2008; Law, Clark and Clark, 2016; Lee et al., 2018), which not only further compounds muscle loss (Derbre et al., 2014; Aggio et al., 2016), but also result in reduced cardiorespiratory fitness (Meier and Lee, 2019), which itself is associated with increased incidence of cardiac events (Henriksson et al., 2019; Sillars et al., 2019; Steell et al., 2019). This vicious cycle results in reduction in muscle mass and quality, leading to decreased activity, further reduction in muscle mass, and subsequent increased risk of CVD (Figure 1.3)

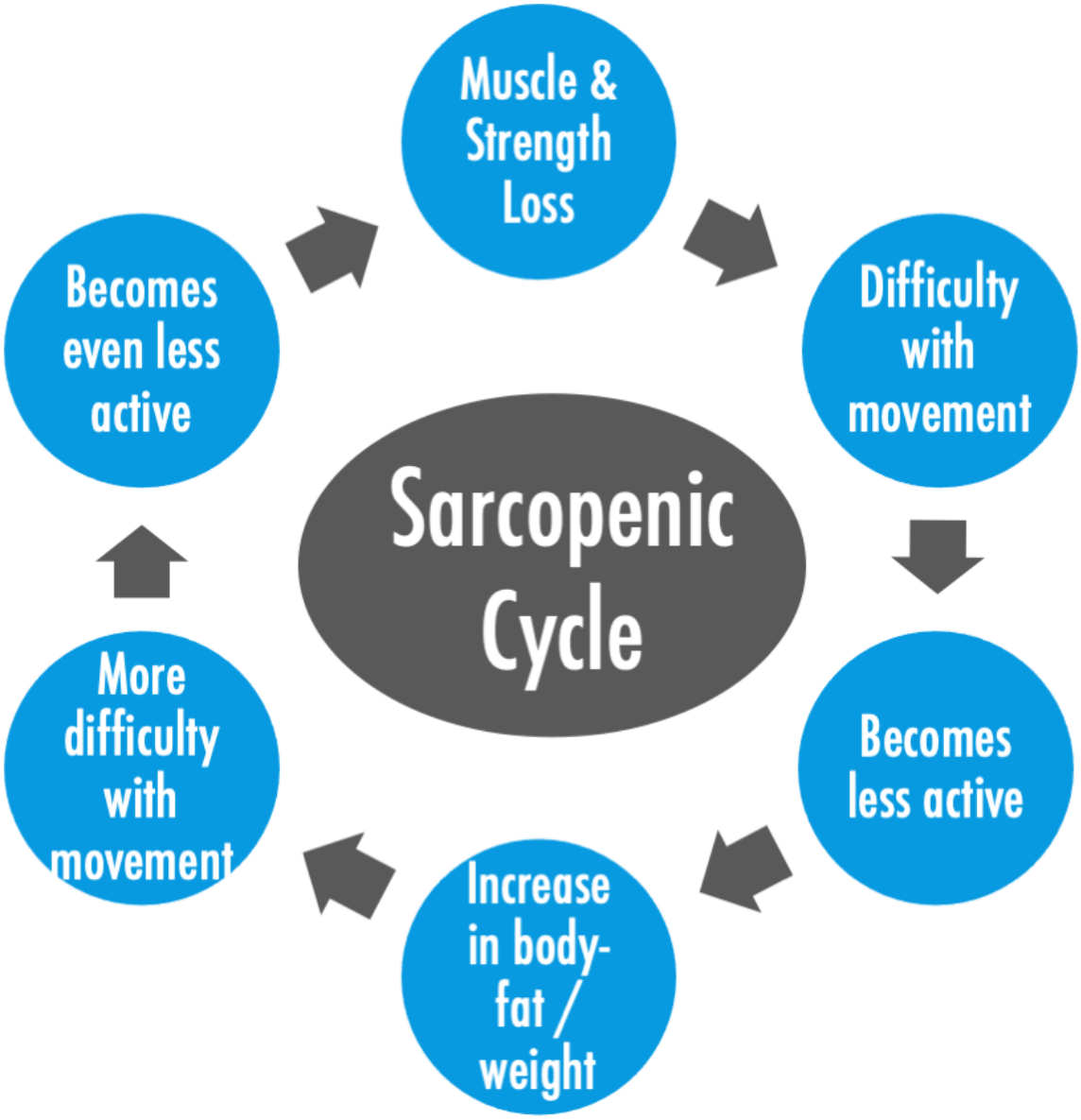

Figure 1.3 Sarcopenic cycle whereby initial muscle loss due to aging can result in reduced mobility, in turn leading to lower energy expenditure and accumulation of adipose tissue. This decreased muscle mass and increased fat mass/body weight may further reduce mobility and levels of physical activity, leading to further loss of muscle mass and strength, propagating the cycle.

1.2.4 Potential Interventions

Increasing relative LBM content, or specifically muscle mass, rather than simply promoting weight loss, may be an appropriate target in CR patients. Maintenance of skeletal muscle mass is determined by the balance of two opposing processes in the body, muscle protein synthesis (MPS) and muscle protein breakdown (MPB). When MPB exceeds MPS there is a net reduction in muscle mass and correspondingly, when MPS exceeds MPB, a net increase in muscle mass is observed (Kumar et al., 2009a; Hodson et al., 2019). Resistance exercise and adequate intakes of protein are known to be potent stimuli of the MPS response, and are widely used in exercise programmes aimed at augmenting LBM in healthy adults (Atherton and Smith, 2012; Morton, McGlory and Phillips, 2015). Such increases in muscle mass might improve cardiorespiratory fitness (Boo et al., 2019; Nichols et al., 2019) and other factors associated with the risk of CVD, such as improving blood glucose control (Lee, Kim and Kim, 2017) and reductions in pro-inflammatory cytokines levels (Nunes et al., 2016; Sardeli et al., 2018).

1.2.5 Anabolic Resistance

One particular barrier to increasing LBM is the presence of anabolic resistance in older adults, which can result in a reduced muscle protein synthetic response to anabolic stimuli such as the ingestion of amino acids or exercise (Welle, Totterman and Thornton, 1996; Katsanos et al., 2005; Morton et al., 2018; Hodson et al., 2019) . Overcoming this anabolic resistance may play a key role in increasing LBM in SO subjects. Resistance exercise, when practiced regularly and of sufficient intensity, has been shown to increase muscle strength and size in older adults (Frontera et al., 1988; Montero-Fernandez and Serra-Rexach, 2013; Miller et al.,

2017; Hodson et al., 2019) while also being regarded as safe to use in cardiac populations (Khadanga, Savage and Ades, 2019).

Currently, global recommendations for protein intake are 0.8 g/kg/body weight (WHO, 2007), but it is thought that these levels may be insufficient for maintaining muscle mass in the elderly (Breen and Phillips, 2011), whereas higher intakes of protein combined with resistance training positively influence muscle mass (Bouchonville and Villareal, 2013; Liao et al., 2017b; Kirwan et al., 2021b), although not all studies support their efficacy (Ten Haaf et al., 2018). Leucine, an amino acid that induces a particularly potent MPS response (Devries et al., 2018), is considered to play a key role in overcoming anabolic resistance, and this is why large doses of total protein (30-40 g/meal), which will contain higher amounts of leucine, may be particularly useful for stimulating MPS in older populations (Breen and Phillips, 2011; Churchward-Venne et al., 2016). This may be especially important in the post-exercised state, due to the increased MPS response to the presence of amino acids after a bout of resistance exercise (Churchward-Venne et al., 2016).

Higher protein intakes have also been shown to promote greater improvement in body composition, due to a reduction in total body fat (Galbreath et al., 2018a), which may result in further improvements in metabolic health (Srikanthan, Horwich and Tseng, 2016). This, combined with the well-established safety of higher protein intakes in individuals without pre-existing kidney disease (Bauer et al., 2013a; Antonio et al., 2015; Antonio et al., 2016), makes higher protein intakes a viable option for overcoming anabolic resistance, stimulating MPS and augmenting LBM in cardiac populations, which may contribute to amelioration of risk markers of CVD.

1.3 Protocol Preparation & Impact of COVID-19

In light of the available evidence, I hypothesized that a CR intervention based on a Mediterranean-style diet with increased protein content, in conjunction with resistance exercise, may be more effective for improving cardiovascular health in CR patients with SO, than the current CR guidelines based around aerobic exercise with dietary change focused on weight management (British Association for Cardiovascular Prevention and Rehabilitation, 2017). As such, I designed a pilot randomised control trial (RCT) consisting of a high-protein Mediterranean-style diet and resistance exercise, with the aim to assess whether it could augment LBM and ameliorate cardiovascular risk.

The protocol for this intervention will be detailed in chapter 7 but briefly, it will consist of a 12-week pilot randomised controlled trial (RCT). Suitable CR patients with SO will be assigned to one of four groups:

i. the control group will receive standard CR lifestyle guidelines (CONT);
ii. the exercise group will be prescribed resistance exercise (EX);
iii. the diet group will be prescribed a modified high-protein (1.2-1.6 g/kg of body weight per day) Mediterranean-style diet (HPMD); and
iv. the exercise and diet group will be prescribed both resistance exercise and the high-protein Mediterranean diet (as previously described) (HPMD + EX).

Baseline measurements of CM risk markers as well as body composition and anthropometric measures will be taken, along with grip strength tests to assess muscle strength. Diet will be monitored using a MEDAS questionnaire which measures adherence to a Mediterranean-style diet (Papadaki et al., 2018), and using validated, four-day food diaries as used in years 1-4 of the UK National Diet and Nutrition Survey (Whitton et al., 2011).

The primary outcome of the study will be the feasibility of conducting a larger, fully powered, multicentre study aimed at determining the effectiveness of a high-protein Mediterranean-style diet and resistance exercise protocol for CR in patients with SO. This will be based on rates or recruitment, adherence to dietary and exercise guidelines using food diaries and participant exercise logs, and feedback from patient focus groups.

Secondary outcomes will be changes in body composition, changes in strength measured by hand-grip dynamometer, and changes to markers of cardiometabolic health, including systolic and diastolic blood pressure, plasma glucose, insulin, dyslipidaemia, HbA1c and CRP

1.3.1 Laying the Groundwork

The initial stage of the development of this intervention focused on the determination of a suitable recruitment and intervention centre. After both phone and face-to-face meetings with staff from a number of CR centres in the Merseyside area, Knowsley Community Cardiac Services (KCCS), based at Liverpool Heart and Chest Hospital's (LHCH) was chosen for the following reasons:

i. Implementation of a well-structured CR programme with strong ties to a community-based CR phase 3 exercise scheme
ii. Use of modern gym equipment, in community gyms with experienced staff, ideal for the proposed progressive resistance exercise programme
iii. An experienced CR team that was both receptive to and interested in the proposed study
iv. Proximity to LJMU to facilitate ease of transport for participants

After considerable consultation with the chosen CR centre, Knowsley Community Cardiac Services and Liverpool Heart and Chest Hospital's (LHCH) Service Users Research Endeavour (SURE) group (a Patient & Public Involvement entity) involving patient focus groups, the protocol was further refined. Participant facing documents were developed, including an official protocol, participant information sheets and consent forms. Furthermore, extensive diet and recipe guides focusing on easy-to-make recipes using economical and easy-to-procure ingredients were developed. In consultation with a BACPR-affiliated exercise physiologist specializing in RE, an easy-to-implement and flexible, progressive RE training plan was developed to provide to participants and supervising exercise trainers.

The use of DXA, which involves ionising radiation, dictated that study ethics be applied for via the NHS ethics application system, the Integrated Research Application System (IRAS). Upon consultation with both a certified medical physics expert (MPE) and clinical radiation expert (CRE), the radiation doses proposed for the study were deemed to be well within safety levels.

A research ethics proposal, totalling 276 pages, was submitted to the LJMU Research Ethics Committee (REC) in September 2019. Approval was given by LJMU to proceed with submission via IRAS and, after one round of minor revisions, full ethical approval for the proposed research intervention was granted by the North West - Greater Manchester East Research Ethics Committee (NHS) (REC reference: 19/NW/0762) on 24/01/2020. Discussions with LHCH began immediately in order to begin recruitment for the intervention in March 2020.

1.3.2 COVID-19

From their beginning in March 2020, regulations in response to the COVID-19 pandemic greatly affected the lives of billions of people and academic research too, which was forced to adapt to these restrictions. While “the best laid plans of mice and men often go awry” may be aptly suited to research and, in particular, the PhD journey, this sentiment has never hit so close to home for many students attempting to complete their PhD research in the last 2 years.

Due to the close links of this research with the NHS, which was continuously overwhelmed, the designation of the CR research population as “at-risk” and the successive lockdowns which temporarily closed down the very CR centres and fitness centres where I had hoped to carry out this research, the methods and indeed the objectives of this research were forced to change. Despite multiple, successful rounds of both non-substantial and substantial ethical amendments to the study protocol, involving changes of recruitment location and the protocol itself, continued caution around COVID-19 restrictions made recruitment impossible for 2 years after the initial lockdown.

Despite my best efforts, the pilot high-protein Mediterranean-style diet and resistance exercise intervention study, which was to be the primary focus of this research, will not be finished in time to form part of this doctoral thesis.

1.4 Aims and Objectives

With the support of my supervisors a new direction for my PhD was decided. In light of the inability to carry out the intervention study or any other related participant-facing research, it was decided to continue this PhD programme as a PhD by publication, focusing on analysis of the current literature and existing databases, as well as novel analytical techniques. As such the aim of this thesis was to explore the available evidence and data to assess the importance

of LBM on cardiovascular health, while further elucidating the impact of dietary approaches, with a focus on protein intake, and resistance exercise on LBM accrual in older adults.

The specific objectives are to:

I. Analyse the impact of protein intake and resistance training on LBM in older adults through a systematic review and meta-analysis.
II. Explore the impact of the COVID-19 pandemic on cardiac rehabilitation participation and desire to continue in the “at-home” format.
III. Study the genetic basis of the associations between nutrition, body composition and cardiovascular risk, through Mendelian randomization analysis.
IV. Develop and assess the feasibility of an RCT intervention for augmenting LBM and improving CM risk markers in a cardiac rehabilitation population.

1.5 Clinical Relevance

The results of this study have the potential to better inform the way CR is currently approached with patients. Current CR practice in the UK focuses on increasing aerobic exercise capacity and body weight management through nutritional practices (National Institute for Health and Care Excellence, 2013), without specific focus on augmenting LBM. Approaches to improve body composition by specifically increasing LBM may offer an advantage for further improvement of cardiometabolic risk markers above that of current CR practices, as well as hindering the progression of sarcopenia and the associated impairments in physical function and quality of life (Tsekoura et al., 2017). Additionally, resistance exercise may offer further benefits beyond improvements in cardiometabolic risk factors, including reduction in the rates of loss of bone mineral density, and a reduction in the risk of falls (Hurley and Roth, 2000). An increased risk of falls and low bone mineral density are additional concerns in populations in

which sarcopenia is prevalent (Cure-Cure et al., 2005; Landi et al., 2012), such as the CR population.

Given the considerable cost to the UK economy associated with treatment of CVD and lost productivity (Wilkins et al., 2017), developing and promoting more efficient strategies of CR could potentially reduce the future incidence of cardiac events amongst CR patients, thus reducing the cost to the health-care system.

1.6 Thesis structure

The following chapters of this thesis are presented as individual manuscripts. The majority of these manuscripts (chapters 2,3,4,6 & 7) have already passed peer-reviewed and been published, with the exception of chapters 5 and 8, which at the time of submission of this thesis are awaiting submission or have been submitted for publication, respectively. All manuscripts that have passed peer review are presented as the final versions, accepted for publication (UK and North American English spelling are used accordingly).

Following these manuscripts, chapter 9 briefly synthesizes the body of research I have worked on over the course of my PhD, explaining its relevance for both muscle and cardiometabolic health, and its importance for the direction of future research.

Finally, additional manuscripts on which I have had the privilege of collaborating on during my PhD journey, as well as documentation used for my original ethics application and participant-facing documents produced for the intervention study, are included in the appendix

2 Chapter 2: Sarcopenia during COVID-19

Sarcopenia during COVID-19 lockdown restrictions: long-term health effects of short-term muscle loss.

Kirwan R, McCullough D, Butler T, Perez de Heredia F, Davies IG, Stewart C.

Geroscience. 2020;42(6):1547-1578. https://doi.org/10.1007/s11357-020-00272-3

2.1 Context within thesis

Chapter 2 presents the first published study of this PhD thesis, a narrative review of sarcopenia and its long-term health effects within the context of the COVID-19 pandemic. Restrictions due to the pandemic resulted in immediate limitations on activity levels, eating habits and number of other lifestyle factors that all had the potential to affect muscle mass, particularly in older populations. These factors are discussed and exercise and nutritional countermeasures to muscle loss, that could be applied within the limitations of pandemic restrictions, are considered

2.2 Abstract

The COVID-19 pandemic is an extraordinary global emergency that has led to the implementation of unprecedented measures in order to stem the spread of the infection. Internationally, governments are enforcing measures such as travel bans, quarantine, isolation and social distancing leading to an extended period of time at home. This has resulted in reductions in physical activity and changes in dietary intakes that have the potential to accelerate sarcopenia, a deterioration of muscle mass and function (more likely in older populations), as well as increases in body fat. These changes in body composition are associated with a number of chronic, lifestyle diseases including cardiovascular disease (CVD), diabetes, osteoporosis, frailty, cognitive decline and depression. Furthermore, CVD, diabetes and elevated body fat are associated with greater risk of COVID-19 infection and more severe symptomology, underscoring the importance of avoiding the development of such morbidities. Here we review mechanisms of sarcopenia and their relation to the current data on the effects of COVID-19 confinement on physical activity, dietary habits, sleep and stress as well as extended bed rest due to COVID-19 hospitalization. The potential of these factors to lead to an increased likelihood of muscle loss and chronic disease will be discussed.

By offering a number of home-based strategies including resistance exercise, higher protein intakes and supplementation, we can potentially guide public health authorities to avoid a lifestyle disease and rehabilitation crisis post-COVID-19. Such strategies may also serve as useful preventative measures for reducing the likelihood of sarcopenia in general and in the event of future periods of isolation.

2.3 Introduction

Sarcopenia is the age associated decline in muscle mass, strength and quality that begins as early as the fourth decade of life and is a major contributor to poor health and disability in older adults (Geisler et al., 2016; Cruz-Jentoft et al., 2019). The progressive loss of muscle mass and the concomitant decline in muscle strength (dynapenia) are associated with a large and diverse group of pathologies including type 2 diabetes mellitus (T2DM) (Scott, de Courten and Ebeling, 2016) cardiovascular disease (CVD) (Bahat and İlhan, 2016), frailty and disability (Malmstrom et al., 2016; Xu et al., 2020), increased risk of falls and fractures (Schaap et al., 2018; Zhang et al., 2018), loss of physical independence (Dos Santos et al., 2017), cognitive decline and depression (Hsu et al., 2014; Hayashi et al., 2019), lower quality of life (Tsekoura et al., 2017) and all-cause mortality (Nichols et al., 2019; Sipers et al., 2019). The aetiology of this muscle loss is known to be multifactorial with reductions in activity levels and inappropriate nutrition playing central roles (Houston et al., 2008a; A et al., 2010; Isanejad et al., 2015; Aggio et al., 2016; Mijnarends et al., 2016; Meier and Lee, 2019).

The COVID-19 pandemic is an extraordinary global emergency with over 26.5 million confirmed cases and more than 870,000 deaths as of September 5th, 2020 (WHO, 2020b), which has led to the implementation of unprecedented measures in order to stem the spread of the infection. Internationally, governments are recommending and/or enforcing such measures as travel bans, quarantine, isolation and social distancing (Parmet and Sinha, 2020;

Sjödin et al., 2020) which in practice have resulted in an extended period of time spent in one's place of residence. This has resulted in reductions in physical activity (PA) and increases in sedentary behavior (Ammar et al., 2020; Sun et al., 2020) which are associated with the loss of muscle mass (Breen et al., 2013). Furthermore, hospitalization from COVID-19 can lead to extended bed rest with some recent reports noting average hospital stays of 11 days (Zhou et al., 2020). More severe presentation of COVID-19 infection can result in admission to intensive care units (ICU) or requirement for invasive mechanical ventilation (IMV) (Caussy et al., 2020; Kalligeros et al., 2020). This can result in further restricted movement with reports of median length of ICU stay as 8 days with an interquartile range (IQR) up to 12 days (Zhou et al., 2020). Such extended periods of bed rest, as a result of COVID-19 isolation/quarantine or hospitalization, pose a further risk to muscle loss, particularly to older individuals (English and Paddon-Jones, 2010). This is of particular relevance given the higher rates of hospitalization reported in older individuals (≥65 years) (Garg et al., 2020).

Access to food has also been affected due to the pandemic with older populations and lower socio-economic groups in particular, experiencing the most relevant disruptions (Dunn et al., 2020; Power et al., 2020). Furthermore, quarantine and social isolation are known to result in increased levels of stress and anxiety (Blendon et al., 2004; Jeong et al., 2016; Lei et al., 2020) the consequence of which may be increased markers of atrophy and elevated loss of muscle mass (Allen et al., 2010). This psychological stress may also lead to poorer dietary choices with a switch to hyperpalatable, convenience foods that are simultaneously high in sugar and/or fat (Gibson, 2006) and which may displace more nutrient dense foods, reducing dietary protein intake (Rauber et al., 2018). Such dietary changes are also associated with poorer markers of cardiometabolic risk including overweight/obesity, hypertension, dyslipidemia and other features of metabolic syndrome (Silva Meneguelli et al., 2020).

In this article we will discuss how this combination of reduced physical activity and poorer diet quality, along with other lifestyle-related factors and the risk of hospitalization, has the potential to accelerate the loss of muscle and physical function. The long-lasting, deleterious effects of this muscle loss on multiple aspects of metabolic, physical and psychological health will be discussed.

2.4 Effects of COVID-19 restrictions on skeletal muscle mass

2.4.1 Inactivity, sedentary behaviour and muscle loss

Prior to this pandemic, World Health Organization recommendations for PA (150 minutes/week of moderate-intensity aerobic PA with muscle strengthening exercises 2 day/week etc.) were not being met, particularly in older populations (WHO, 2020a). COVID-19 presents a number of risks for further reductions in activity levels for the general population. Quarantine, self-isolation, social distancing and other government measures have led to the closure of gyms and leisure centers as well as the suspension of group exercise and rehabilitation programs. It has never been easier to be physically inactive. In addition to and independently of reduced PA, increased sitting time and sedentary behavior, which have been reported to increase during COVID-19 confinement (Ammar et al., 2020), are also associated with multiple adverse health outcomes (Patterson et al., 2018), further compounding the risk to health.

Recently published research in children and adolescents (baseline age range 6-18 years) living through COVID-19 quarantine in Italy has shown a decrease in sports activity of 2.3 hours and an increase in electronic device/screen time of 4.85 hours per day. Similarly, a survey of 1047 participants from Asia, Africa and Europe reported a 33.5% decrease in the number of minutes/day of PA, a decrease in metabolic equivalents of task (MET) values (a

measure of exercise intensity) of 42.7% and an increase in sitting time from 5 to 8 h per day (Ammar et al., 2020). The results of these studies highlight the potentially detrimental effect of quarantine/self-isolation on physical activity and sedentary behavior (Pietrobelli et al., 2020). Hospitalization due to COVID-19, and in particular admission to ICU can result in much lower levels of activity or even complete immobilization (Caussy et al., 2020; Kalligeros et al., 2020; Zhou et al., 2020), which may greatly accelerate the loss of muscle mass and function in those affected (Dirks et al., 2016). Furthermore, some governmental recommendations on social distancing have advised particular stringency in older adults (Government, 2020), who are deemed clinically vulnerable (Wu et al., 2020), meaning physical activity in this group may be even further reduced compared to the general population. Concerns around a "second-wave" of COVID-19 infections that is expected to follow a relaxation of current lockdown restrictions (Vaid et al., 2020) may also result in such at-risk populations enduring significantly decreased physical activity for longer periods of time.

Even short periods of reduced activity (both immobilization, simulating bed rest or hospitalization, and step reduction, which may better model COVID-19 confinement) have been shown to result in the rapid loss of muscle mass and physical function, even in younger adults (Abadi et al., 2009; Breen et al., 2013). As much as 1.7% of muscle volume can be lost after as little as 2 days of immobilization, with greater losses (5.5% of muscle volume) observed after only 7 days (Kilroe et al., 2020). A recent study using smart phone data from 1062 participants in 5 European countries observed that individuals had lower step counts and heart rates and spent more time in sedentary activity such as using their phones during COVID-19 lockdown (Sun et al., 2020). The sudden reduction in activity and increase in sedentarism brought on by COVID-19 measures would closely mirror the "catabolic crisis" model of sarcopenia, proposed by English and Paddon-Jones (English and Paddon-Jones, 2010). In this model, sarcopenia is not simply a gradual process, but is in fact accelerated by periodic occasions of inactivity (such as periods of extended bed rest or hospitalization)

(Figure 2.1). Indeed, in a study of 118 ICU patients (mean age 55 years), muscle thickness measured by ultrasonography was negatively correlated with length of stay in ICU with loss of muscle thickness higher during the first 2–3 weeks of immobilization (Gruther et al., 2008). With ICU durations of up to 12 days being observed in some COVID-19 infected patients (Zhou et al., 2020), the loss of muscle mass is a very likely scenario. The lean tissue lost during these times of inactivity may not be fully regained leading to a progressive loss of muscle mass and function. Highlighting this, in a study of 27 ICU patients (age range 23-78 years), both muscle mass and strength were decreased 7 days after ICU discharge and, while significantly improved after 6 months, did not normalize in the majority of patients (Dos Santos et al., 2016).

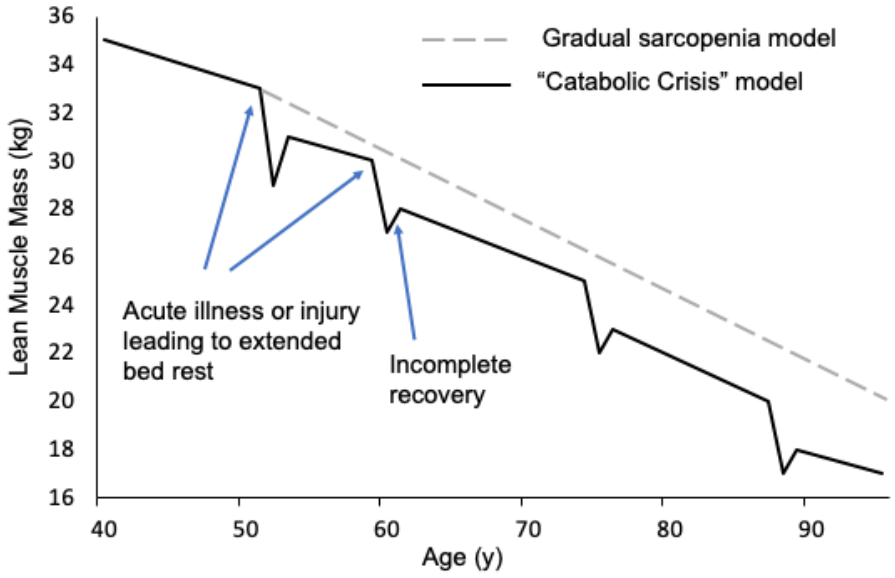

Figure 2.1 Potential model of age-associated muscle loss (sarcopenia) exacerbated by periods of extended bed rest/hospitalization due to acute illness or injury (catabolic crises). Adapted from English and Paddon-Jones (2010) (English and Paddon-Jones, 2010)

The age-related decline in muscle mass is primarily due to the selective atrophy of type II fibers (Deschenes, 2004; Verdijk et al., 2014). This decline may be attributed to neurodegeneration of the skeletal muscle fiber, thereby reducing the potential to recruit type II fibers during RE, resulting in a diminished anabolic response (Deschenes et al., 2010). Indeed, regular RE has been shown to reduce this decline in type II fibers (Deschenes et al., 2010; Verdijk et al., 2014). Additionally the rapid loss of muscle related to inactivity may be due to a number of further mechanisms including induced anabolic resistance, insulin resistance (IR), mitochondrial dysfunction and its associated oxidative stress (Biolo et al., 2008; Gram et al., 2014; Dirks et al., 2016; Rudwill et al., 2018; Rubio-Ruiz et al., 2019). Interestingly, this inactivity-induced reduction in skeletal muscle-associated muscle protein synthesis (MPS) can be rescued with resistance exercise (RE) and sufficient protein ingestion (Areta et al., 2014; Devries et al., 2015) offering practical solutions to overcoming this driver of muscle loss (which will be discussed later in this article). Indeed, older individuals who have engaged in life-long RE/strength training have significantly greater rates of force development and increased muscle size compared with untrained control individuals (Aagaard et al., 2007). This increased muscle size was predominantly attributed to type II muscle fibers, the loss of which is responsible for the decrease in muscle mass seen in sarcopenia (Brunner et al., 2007).

Another potential, although indirect, mechanism by which reduced PA as a consequence of self-isolation may be detrimental to muscle mass is through the role of inactivity in poor appetite control (Blundell et al., 2003), a concept underpinned by recent research into the “gravitostat” model of body weight feedback and control. In this model, osteocytes may be capable of detecting changes in body mass and affecting appetite in order to maintain a set body weight. Reduced physical activity/increased time spent sitting may reduce the effectiveness of this feedback system leading to increased appetite, overconsumption of food and weight gain (Ohlsson et al.; Jansson et al., 2018). In addition to these effects of activity

on appetite control, decreases in muscle mass, as a result of reduced activity, may result in increased appetite as a consequence of the protein leverage model of appetite regulation (Grannell et al., 2019). This model hypothesizes that a lower proportion of protein in the diet, potentially due to overconsumption of ultra-processed foods (UPFs), leads to compensatory increases in energy intake in an attempt to maintain a higher absolute protein intake (Hall, 2019). Thus, a cycle of muscle loss, increased appetite and fat mass gain may be perpetuated. Reduced activity, may also lead to poor sleep duration and quality (Yang et al., 2012a; Tseng et al., 2020) which also has the potential to affect appetite and subsequently weight control (Spiegel et al., 2004; Morselli et al., 2010; Dweck, Jenkins and Nolan, 2014) with the possibility of further loss of muscle mass (Nedeltcheva et al., 2010).The relevance of these concepts will be further discussed later in this article.

2.4.2 Mechanisms of muscle maintenance and loss

As alluded to above, due to the process of ageing which is accelerated by disuse, skeletal muscle displays features of plasticity, enabling growth and decrements over the life course in response to, among other things, the stimulus of physical activity (Timmons, 2011). The driving force behind these changes in mass is the equilibrium between MPS and muscle protein breakdown (MPB) with net increases in MPS resulting in increases in muscle size (Atherton and Smith, 2012). Both weight bearing or RE and, more acutely, the ingestion of high quality protein rich in essential amino acids (EAA) (particularly leucine) are potent stimuli of MPS (Wackerhage and Rennie, 2006).

The mammalian target of rapamycin complex 1 (mTORc1) is a key regulator of MPS and muscle protein turnover. mTORc1 regulates protein synthesis via activation of the eukaryotic initiation factor 4E-binding proteins (4E-BPs) and p70 S6 kinase 1 (S6K1) (Liu and Sabatini, 2020). This results in increased translation efficiency and capacity of mRNA leading to

increased protein synthesis. Resistance exercise activates upstream signaling of mTORc1 to increase MPS and muscle hypertrophy of type II fibers (Egan and Zierath, 2013). Mechanical loading of skeletal muscle may be key in mediating mTORc1 stimulation via mechano-sensing proteins however, exercise-induced muscle damage and metabolic stress may also have a role to play. The direct mechanisms from RE stimulus to mTORc1 activation are yet to be elucidated (Wackerhage et al., 2019). Similarly, leucine activates mTORc1 via an amino acid-sensing pathway, perhaps via dissociation of Sestrin1 from the GATOR2 complex, to synergistically enhance RE induced MPS (Kimball et al., 2016; Xu et al., 2019a). Given the importance of this pathway in enabling MPS, any perturbations in the process (*e.g.* social isolation and reduced PA) may culminate in catastrophic losses of muscle mass.

Furthermore, older adults experience a phenomenon known as anabolic resistance, a diminished response to the MPS-stimulating effects of physical activity and protein ingestion (Moore et al., 2015; Phillips et al., 2017; Morton et al., 2018), which is believed to be a primary contributor to the development of sarcopenia. For example, it has been reported that older compared to younger men (mean 71 years vs 22 years) require approximately twice the amount of high-quality protein (0.60 vs 0.25 g/kg lean body mass) to maximally stimulate MPS (Moore et al., 2015). For the average older adult this may be approximately 40 g of protein per meal (Churchward-Venne et al., 2016). Similarly, exercise-induced MPS rates are attenuated in older compared with younger individuals (Kumar et al., 2009b) meaning that greater durations or intensities of exercise may be needed to maintain muscle in older individuals. In contrast to expectations, rather than a reduction in mTORC1 activation with ageing, rodent studies have illustrated that mTORc1 may actually be hyper-activated in older, sarcopenic individuals (Joseph et al., 2019), suggesting the presence of mTORC1 resistance with age.

Anabolic resistance and the subsequent muscle loss is multifactorial and associated with an often-interrelated decrease in physical inactivity, inadequate dietary quality, increased adiposity, increased inflammation, dysregulated hormones and other comorbidities (Rezuş et al., 2020). The age associated increase in inflammation, or inflamm-aging, is highlighted by chronic elevation of inflammatory biomarkers such as interleukin-6 (IL-6), tumor necrosis factor alpha (TNF-α) and C-reactive protein (CRP) amongst others (Singh and Newman, 2011). Indeed, sarcopenic populations have been shown to have higher levels of CRP compared to age matched controls without sarcopenia (Bano et al., 2017). Inflammation has been shown to upregulate catabolic pathways and downregulate anabolic pathways, thereby reducing net MPS (Dalle, Rossmeislova and Koppo, 2017). For example, in vitro studies have reported that TNF-α inhibits myogenesis and upregulates nuclear factor-kappa beta (NF-κβ), a key transcription factor in skeletal muscle atrophy (Bhatnagar et al., 2010; Jackman et al., 2013). In the context of the current COVID-19 pandemic, the importance of inflammatory cytokines is becoming more apparent with increased levels of pro-inflammatory cytokines such as IFN-α, IL-6, IL-12, IL-17, IL-18, IL-33, TNF-α, CRP and MCP1 (known as the “cytokine storm”) observed in patients with severe COVID-19 (Meftahi et al., 2020). These not only contribute directly to tissue damage (Cao, 2020) but may also contribute to sarcopenia by blunting MPS (Bano et al., 2017) during immobilization and beyond.

Ageing is also associated with a decrease in hormones that regulate muscle mass such as growth hormone, dehydroepiandrosterone, testosterone, insulin-like growth factor-I (IGF-I) and estrogens (Vitale, Cesari and Mari, 2016). Pro-inflammatory states such as those observed in severe COVID-19 infection are also associated with reductions in hormones such as testosterone (Maggio et al., 2005) and IGF-I (Wang et al., 2020b) and are believed to contribute to reductions in muscle mass (Visser et al., 2002; Kalinkovich and Livshits, 2017). Therefore, it is not simply the bed rest, which culminates in wasting, but elevated pro-inflammatory and reduced anabolic agents exerting direct effects on muscle catabolism.

During puberty, these anabolic hormones are increased leading to increased height, muscle mass and sex-specific phenotypes (Holmes and Shalet, 1996). Therefore, the age-related decline in these hormones, particularly testosterone in males, may regulate the decline in muscle mass with age. For example, long term testosterone replacement therapy has been reported to increase lean body mass, muscle strength and power in older men (Storer et al., 2017; De Spiegeleer et al., 2018). Testosterone regulates MPS via the androgen receptor (Cheung and Grossmann, 2018) and its administration has been reported to augment anabolic signaling and MPS in response to RE in older males, suggesting a role in reducing anabolic resistance to RE (Gharahdaghi et al., 2019). However, testosterone administration alone in older adults may be unable to fully reduce the age-related decline in MPS (Henderson et al., 2009).

Low levels of physical activity and poor dietary habits, which may be more prevalent during COVID-19 confinement (Ammar et al., 2020), are associated with obesity and a range of comorbidities including metabolic syndrome (MetS), T2DM and CVD (Pattyn et al., 2013; Sperling et al., 2015; Kolb and Martin, 2017). Obesity, particularly abdominal obesity and the aforementioned comorbidities are also associated with increased levels of inflammation and dysregulated anabolic hormones, which may further exacerbate anabolic resistance (Després and Lemieux, 2006). Indeed, these comorbidities and lifestyle factors are typically associated with low muscle mass and sarcopenia and may also contribute to anabolic resistance (Abete et al., 2019). For example, the PI3K/Akt signaling cascade is a key pathway in regulating growth, with its activation, particularly by the anabolic hormone insulin, inhibiting the atrophy-related protein forkhead Box-O1 (FOXO) while also activating mTORc1 (Léger et al., 2006; Samuel and Shulman, 2016). The diminished response to insulin is a prominent phenotype observed in insulin resistant states such as MetS and T2DM and is also associated with age (Facchini et al., 2001; Sperling et al., 2015). In the context of sarcopenia and the current prevalence of T2DM, this has important implications for muscle mass. Indeed, both

hyperglycemia and IR are important in the declining muscle mass observed in diabetes (O'Neill et al., 2016; Hirata et al., 2019; Hong and Choi, 2020). Insulin resistance can be induced by extended periods of inactivity/bed rest (Dirks et al., 2016) which may not only puts people at greater risk of muscle loss due to social distancing measures but also may lead to greater susceptibility to COVID-19 itself (Kumar et al., 2020).

Indeed, recent reports of confinement during COVID-19 highlight a 33.5% decrease in number of minutes/day of PA and increases in number of main meals and snacking (Ammar et al., 2020) which may make positive energy balance and fat accumulation more likely. Furthermore, increased adiposity in older populations, such as ectopic accumulation of fat between muscle fibers, known as intramuscular adipose tissue (IMAT) has also been shown to impose a significant risk of muscle dysfunction in older adults (Addison et al., 2014; Buch et al., 2016). Alterations in energy status (such as increased lipid metabolites like diacylglycerols and ceramides) in addition to increased inflammation have been shown to activate protein kinase C theta (PKC-θ), c-Jun-N-terminal kinase (JNK) and inhibitor of kappa B kinase (IKK) (Boura-Halfon and Zick, 2009; Marino et al., 2013; Hong and Choi, 2020) resulting in suppressed protein synthesis and increased protein breakdown. Increased adiposity has also been reported as a risk factor for COVID-19 infection and severity such as admission to ICU (adjusted OR 5.39) and the need for IMV (aOR 9.99) (Kalligeros et al., 2020) thus posing a double risk at this time.

Another mechanism which may contribute to anabolic resistance in older populations is reduced capillarization of skeletal muscle, which may blunt the hypertrophic effect of RE. To illustrate this, Moro et al. (Moro et al., 2019) demonstrated that amongst a group of older adults (mean age 71 years) participating in a 12-week RE program, those with lower baseline muscle capillarization did not experience muscle hypertrophy, whereas participants with higher

muscle capillarization did. As mentioned previously, IMAT may also contribute to the reduced hypertrophic response seen in aging muscle as Marcus et al. (Marcus, Addison and LaStayo, 2013) demonstrated that in older adults (mean age 73 years) performing 12 weeks of RE, only those with low IMAT showed improvements in muscle quality (Marcus, Addison and LaStayo, 2013). Sarcopenia is very much a “chicken or egg” scenario as it is unknown if these age-related changes precede sarcopenia and frailty, leading to decreased activity or if chronically reduced activity results in dysregulated anabolic/catabolic signaling (Cesari et al., 2014). However, what is well established is that regular exercise throughout the lifespan reduces the severity of sarcopenia and its associated comorbidities (Rezuş et al., 2020) as well as being associated with improved immune function (Zheng et al., 2015; Duggal et al., 2018; Bartlett and Duggal, 2020). Older individuals are already compromised in terms of muscle mass, compared with younger counterparts and are therefore, relatively speaking, at a significantly elevated risk of muscle loss if unexpected perturbations are encountered. Therefore, a number of factors related to the COVID-19 pandemic may further contribute to this loss of muscle mass and function with ageing and significantly impact on the health span of an ageing population.

2.4.3 Food Access, Dietary Intake and Energy Balance

Changes in access to food, for example due to temporary shortages because of panic buying or due to less frequent visits to grocery stores, as a result of government restrictions and/or fear or anxiety of possible infection (Brooks et al., 2020), may lead to changes in food choices and diet quality. These dietary changes, along with changes in appetite regulation (which will be discussed later) have the potential to take two, opposing directions: that is, scenarios involving positive and negative energy balance are both possible. Indeed, recent research has reported that 30% of respondents to a COVID-19-related survey reported weight gain (mean 3.0 kg) and over 18% reported weight loss (mean -2.9 kg). There was a tendency for

participants with overweight and obesity, and subjects over 36 years to gain weight, whereas underweight participants tended to lose weight (Sidor and Rzymski, 2020). This may indicate that confinement during COIVD-19 may exacerbate over- or undereating in different individuals depending on pre-existing tendencies.

On one hand, positive energy balance may result from an increased reliance on UPFs and convenience foods due to both their longer shelf life, and an increase in emotional/stress eating (Cotter and Kelly, 2018; Brooks et al., 2020). Indeed, an increase in the intake of such foods (specifically, potato chips and sugary drinks) has been observed amongst children living through lockdown in Italy (Pietrobelli et al., 2020). The same study also reported an increase in average number of meals of 1.15 per day. Further research from the Italian lockdown reported that 46.1% of respondents felt they ate more during confinement and in particular, high-calorie "comfort foods" such as chocolate, ice-cream, desserts and salty snacks, which was mostly attributed to higher levels of anxiety (Scarmozzino and Visioli, 2020). This increased frequency of eating and reliance on high-calorie, UPFs can potentially affect muscle mass in two ways. Firstly, diets higher in UPFs tend to be lower in quality, specifically, lower in protein which may reduce the capacity to stimulate muscle growth (Rauber et al., 2018). Secondly, such diets can lead to an increase in calorie intake, leading to a positive energy balance that may result in body fat gain (Rauber et al., 2018; Hall et al., 2019). Excess body fat can contribute to muscle loss by reducing ease of locomotion: an individual with sarcopenia and elevated fat mass (sarcopenic obesity [SO]) will have difficulty in moving due to low muscle strength and the excess weight of the fat mass, resulting in decreases in non-exercise activity thermogenesis (NEAT) and physical activity (Hunter and Byrne, 2005). This can lead to further weight gain, exacerbating the cycle. Excess fat mass is also known to lead to low-grade systemic inflammation which can result in IR (Kim et al., 2013), obesity-related metabolic diseases (Collins et al., 2018) and contribute to sarcopenia (Kalinkovich and Livshits, 2017), as previously discussed. A further potential complication of this confinement-

induced obesity is the increased risk of COVID-19 infection and severity (Caussy et al., 2020; Lighter et al., 2020) with severe obesity being associated with admission to ICU (adjusted OR 5.39) (Kalligeros et al., 2020).

Contrary to the potential for weight gain, there is also a risk of reduced access to and/or means to buy enough food to maintain weight and/or adequate nutrition (Power et al., 2020), which could lead to weight loss as an alternate outcome. As of 2016, 21% of UK adults (16 years and older) were classified as marginally to severely food insecure, with a high proportion of unemployed or those in low-income households reporting difficulties in meeting food needs (FSA, 2016). For older adults, this food insecurity may be amplified by a reluctance to leave home to go grocery shopping, due to their recognition as an “at-risk” population (CDC, 2020; Wu et al., 2020) coupled with a lower use of online/delivery-based grocery services (Knowles and Hanson, 2018). Thus, there is also a risk of reduced food intake which may lead to weight loss. As approximately 25% of body mass lost during weight loss can be attributed to fat-free mass (including muscle mass) in young and healthy individuals (Leidy et al., 2007), undesired weight loss may further contribute to the acute loss of muscle mass in older individuals (Miller and Wolfe, 2008) during the COVID-19 pandemic.

This situation of altered access to food may be further compounded by financial issues due to the pandemic-associated restrictions. The UK Office for National Statistics has reported that almost one quarter (23%) of surveyed adults have admitted that their household finances were affected with the majority being worried about their income (ONS, 2020), although older individuals in receipt of a pension may not be affected.

2.4.4 Impaired Sleep, Stress and Anxiety

While not immediately apparent, psychological factors, sleep and anxiety may play a considerable role in the loss of muscle during a pandemic. This can be due to their effects on health behaviors such as eating habits and physical activity, as well as changes in metabolic pathways related to maintenance of muscle mass. Enforced quarantine and even isolation due to social distancing measures during the COVID-19 pandemic have the potential to cause considerable emotional issues. Indeed, a recent COVID-19-related study from Italy reported poor sleep quality in 57.1%, high anxiety in 32.1% and high distress in 41.8% of participants (Casagrande et al., 2020). Another recently published study has also reported symptoms of COVID-19-related post-traumatic stress disorder (PTSD) in as many as 29.5% of respondents (Forte et al., 2020).

Eating is recognized as a coping mechanism for dealing with stress and emotions (Solomon, 2001; Timmerman and Acton, 2001). Those with the lowest scores for emotional and stress-related eating, as measured by the Eating and Appraisal Due to Emotions and Stress (EADES) Questionnaire, are up to 13.38 times more likely to present with overweight or obesity, compared with those with the highest scores (Ozier et al., 2008). With many people experiencing negative emotions and stress due to lockdown, there is also an increased likelihood of stress eating and overconsumption. Combined with hyperpalatable UPFs (Schulte, Avena and Gearhardt, 2015; David et al., 2018; Hall et al., 2019) frequently purchased in anticipation of times of food shortage (Moran et al., 2019), overeating becomes an even more probable consequence.

Stress is also associated with sleep disturbance, shorter sleep duration, nighttime awakening and insomnia (Haynes, Adams and Franzen, 1981; Hall et al., 2015). Changes in daily schedules due to confinement may also contribute to poor sleep quality due to disruptions in circadian rhythms which may already be disrupted in older adults (Kim and Duffy, 2018).

Indeed, recent data from individuals quarantined during the COVID-19 outbreak in China reported anxiety correlated with stress resulting in reduced sleep quality (Xiao et al., 2020), while an Italian study (Cellini et al., 2020) reported lower sleep quality despite participants spending more time in bed.

Both stress and sleep curtailment can contribute directly to muscle loss through changes in key chemical messengers in metabolic pathways related with muscle mass. Short term, modest sleep curtailment (from 8 to 6h/night) has been shown to increase proinflammatory cytokines such as IL-6 and TNF-α) (Vgontzas et al., 2004) which are associated with muscle loss (Visser et al., 2002). Sleep loss is also associated with dysregulation of hormone secretion, such as elevated cortisol resulting from 2 nights of 4 hours of sleep (Guyon et al., 2014) or reduction in testosterone levels by 10%–15% resulting from 8 nights of 5 hours of sleep (Leproult and Van Cauter, 2011). Hypercortisolemia is reported to increase MPB, which is amplified by inactivity (Ferrando et al., 1999), a potentially likely situation during both COVID-19 confinement and hospitalization.

Reductions in sleep duration and/or quality can also lead to changes in appetite and hunger (Morselli et al., 2010). Recent data from populations during COVID-19 confinement indicates that as many as 57.1% of some cohorts experience poor sleep quality (Casagrande et al., 2020) and other surveys have reported as many as 46.1% of respondents were consuming more high-calorie foods (Scarmozzino and Visioli, 2020). It is believed that at least some of these effects are caused by changes in satiety hormones such as leptin (which reduces appetite) and ghrelin (which increases food intake). For example, sleep deprivation studies have shown that after only 2 nights of 4 hours sleep each, leptin levels can drop by 18% and ghrelin can increase by 28%, resulting in a 23% increase in hunger with a preference for high carbohydrate foods (Spiegel et al., 2004). Similarly, Yang et al. observed that after only one

night of modest sleep curtailment, food cravings, food reward, and selected portion sizes of food increased in healthy women (Yang, Schnepp and Tucker, 2019). Such dysregulation of appetite control coupled with access to hyperpalatable UPFs with low satiety value (Hall et al., 2019), and reduced activity levels creates a perfectly obesogenic storm. As previously discussed, excess adipose tissue can contribute to muscle loss through impaired locomotion and metabolic/hormonal dysregulation such as chronic inflammation and IR (Kim et al., 2013; Kalinkovich and Livshits, 2017; Livshits and Kalinkovich, 2019).

It has been shown that COVID-19 confinement can also result in weight loss in certain individuals and when combined with reduced sleep, may also contribute to muscle loss. Nedeltcheva et al. showed that in a calorie deficit, individuals who slept 5.5 hours lost 55% less fat and 60% more fat-free mass, compared to those who slept 8.5 hours over 2 weeks (Figure 2.2) (Nedeltcheva et al., 2010). Thus the problem of weight loss resulting in lean mass loss in the older individuals (Miller and Wolfe, 2008), may be exacerbated by poor sleep during the pandemic.

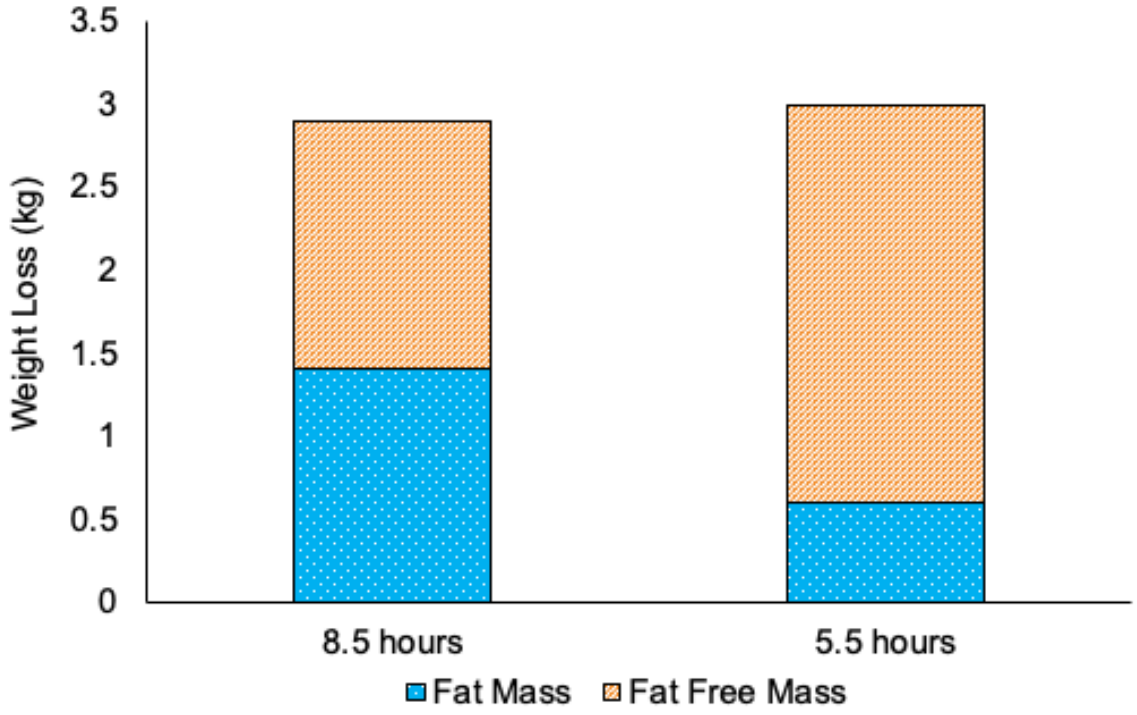

Figure 2.2 Composition of changes in body weight during calorie restriction under normal and restricted sleep conditions. Under conditions of restricted sleep (5.5 h), greater weight was lost as fat-free mass (including muscle) and less body fat was lost, compared to conditions of sufficient sleep (8.5 h). Adapted from Nedeltcheva et al. (2010) (Nedeltcheva et al., 2010)

Another mechanism by which stress, anxiety and impaired sleep may lead to muscle loss is through their effects on health behaviors. A study by Strine et al. (Strine and Chapman, 2005) highlighted that people with frequent sleep insufficiency were significantly more likely to engage in adverse health behaviors including smoking, physical inactivity, and heavy drinking. These results were replicated by Walsh et al. who also reported that those suffering with depression, anxiety and stress were less likely to engage in health-promoting behaviors such as consuming vegetables and eating breakfast (Walsh, Senn and Carey, 2013). It could be speculated that lockdown-induced low mood and stress could make it less likely for people to engage in health behaviors necessary for the maintenance of muscle mass, namely exercise,

and research on how stress impairs efforts to exercise has been reported previously (Stults-Kolehmainen and Sinha, 2014). Poor sleep duration and quality may also result in higher levels of perceived stress and anxiety, thus fueling a vicious cycle of sleep disturbances and stress (Franzen et al., 2011; Minkel et al., 2012)

Critically, even after the lifting of quarantine restrictions, psychological distress may result in some individuals continuing to avoid enclosed places where large groups of people gather or even outdoor public spaces (Brooks et al., 2020). This is particularly relevant in a post-COVID-19 situation as access to gyms and fitness centers along with outdoor recreational spaces such as sports grounds and public parks may be vital to efforts to improve muscle mass, strength and physical fitness as well as for improving social interaction and engagement (Bedimo-Rung, Mowen and Cohen, 2005; Frank and Kavage, 2009; Lee, Lo and Ho, 2018).

2.4.5 Reduced sun exposure and vitamin D

Vitamin D (specifically the active form 1,25-dihydroxycholecalciferol) has historically been linked to bone health. However, there are multiple studies that have shown poor vitamin D status to be associated with multiple chronic diseases (Wang et al., 2017) and reduced muscle mass (Luo et al., 2018). This may be especially important during the current COVID-19 pandemic due to lockdown measures that lead to people experiencing less direct sunlight, thus impacting negatively on vitamin D synthesis (van Schoor and Lips, 2017).

Vitamin D plays an important role in the regulation of muscle contraction, with deficiency altering sarcoplasmic calcium handling leading to prolonged muscle relaxation (Rodman and Baker, 1978). This may also impair mitochondrial energetics and indeed correcting vitamin D status has been shown to improve mitochondrial oxidative function in humans (Sinha et al.,

2013). Similarly, Dzik et al. (Dzik et al., 2018) showed that vitamin D supplementation relieved lower back pain, reduced cytosolic superoxide dismutase (SOD) and glutathione peroxidase (GPx) activities and decreased 8-isoprostanes and protein carbonyls in patients' multifidus muscle.

In vitro studies have shown that vitamin D can enhance insulin signaling via the Akt/mTORc1 pathway, and stimulate protein synthesis to a greater extent than when cells were exposed to insulin plus leucine alone (Salles et al., 2013). Increased phosphorylation of the insulin receptor was also observed, together with an upregulation of the vitamin D receptor (VDR). More recent work in mice has shown that muscle-specific deletion of VDR leads to significant changes in body composition, resulting in greater percentage of fat mass and reduced lean tissue. (Girgis et al., 2019). These physical changes were accompanied by functional alterations, including decreased time spent running, lower speed and lower grip strength (reflecting chronic and acute types of effort) (Girgis et al., 2019). Indeed, it has been shown that vitamin D decreases the expression of myostatin, a negative regulator of muscle mass (Garcia et al., 2011), potentially explaining the negative consequences of vitamin D deficiency on muscle size. These findings offer some potential mechanistic insight into the studies that have shown an association between vitamin D status and muscle mass and strength in older people (Tieland et al., 2013; Owens et al., 2015).

Much of the data regarding vitamin D status and muscle status in humans is derived from observational studies, however, there have been several insightful randomized controlled trials examining the effect of vitamin D repletion on muscle function. Burns patients are at increased risk of hypovitaminosis D and therefore present a novel opportunity to examine restoration of vitamin D status. In 15 adults with thermal burns, quarterly intramuscular injections with 200,000 IU vitamin D and daily oral calcium led to a significant increase in

quadriceps strength when compared to baseline values, showing a direct effect of vitamin D supplementation (Rousseau et al., 2015). While a recent systematic review and meta-analysis suggested a small, non-significant (P=0.06) increase in muscle strength, subgroup analysis showed improvement with doses of >1000 IU/d, >3 month treatment duration, and in participants with a baseline vitamin D concentration of <30 ng/mL (Abshirini et al., 2020). Thus, improvements may not be seen in those individuals who have an adequate vitamin D status. Data presented at the 21st European Congress of Endocrinology also suggests that more substantial benefits from vitamin D on muscle tissue are observed when combined with increased protein supplementation (Gkekas et al., 2019).

Vitamin D deficiency (25(OH)-vitamin D level <20 ng/ml) has also been suggested as a risk factor for COVID-19 infection (Meltzer et al., 2020) and may contribute to its severity through its association with increased proinflammatory cytokines (Weir et al., 2020). The prevalence of vitamin D deficiency amongst older adults may be as high as 65% in some groups in the UK (Jolliffe et al., 2016; van Schoor and Lips, 2017). Additionally, older adults with reduced mobility/muscle function and those who spend most of the day indoors are at a greater risk of deficiency (Webb et al., 1990; Whitmore, 1996). Therefore, deficiency may play a considerable role in not only the etiology of sarcopenia but also the severity of COVID-19 during lockdown, when sun exposure may be further reduced in the self-isolating elderly or those hospitalized due to COVID-19.

2.5 The relationship between muscle loss and chronic lifestyle conditions

Muscle loss is associated with a number of metabolic, physiologic and psychologic/cognitive pathologies. It is likely that the development of these pathologies is related to not only the loss

of muscle mass, but also to an increased prevalence of adipose tissue and particularly visceral adipose tissue (VAT) and IMAT as observed in sarcopenic obesity (Lim et al., 2010; Prado et al., 2012). Visceral adipose tissue is independently associated with the incidence of CVD, even after adjusting for other clinical risk factors such as T2DM, total cholesterol, smoking, hypertension and body mass index (BMI) (Britton et al., 2013), which may be a result of higher levels of pro-inflammatory cytokines produced in VAT (Schrager et al., 2007). These may further contribute to the progression of SO through their association with reduced muscle mass and strength (Visser et al., 2002; Kalinkovich and Livshits, 2015; Rubio-Ruiz et al., 2019). Furthermore, IMAT has also been shown to increase as we age and can cause both a reduction in the physical capacity of skeletal muscle (Delmonico et al., 2009) and an increase in local and systemic inflammation, again through the secretion of proinflammatory cytokines (Addison et al., 2014; De Carvalho et al., 2019). The comorbidities associated with this loss of muscle mass and increase in VAT and IMAT and their potential consequences in relation to COVID-19 infection and severity will be discussed briefly here (Figure 2.3).

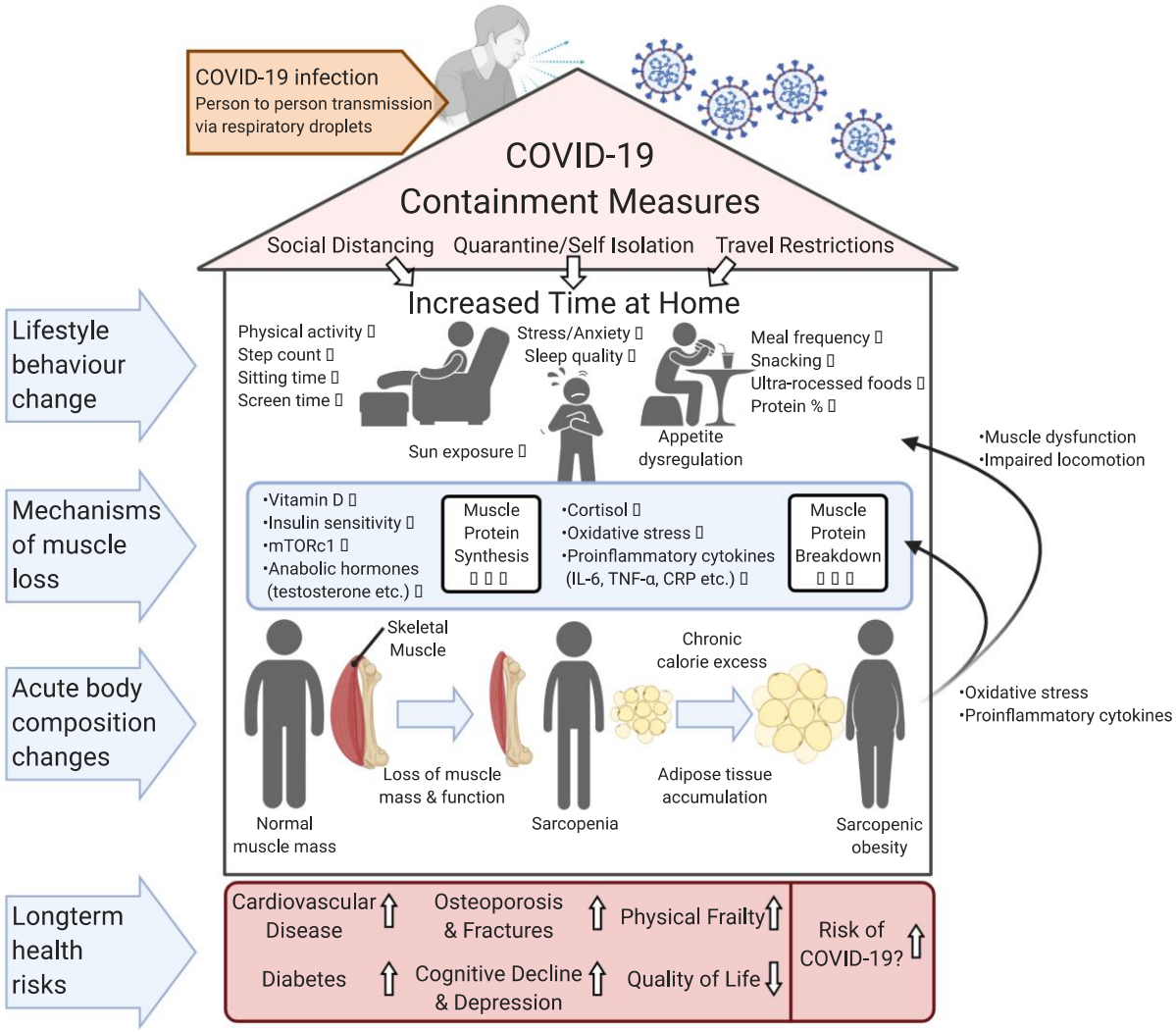

Figure 2.3 Summary of potential effects of government restrictions on lifestyle behaviors and the mechanisms by which they can lead to reduced muscle protein synthesis and increased muscle protein breakdown resulting in muscle loss. The development of sarcopenia, or in the presence of caloric excess, sarcopenic obesity, is associated with a significantly increased risk of multiple comorbidities, some of which may also increase the risk of COVID-19 infection and severity. COVID-19 severe acute respiratory syndrome coronavirus 2, mTORc1 mammalian target of rapamycin complex 1, IL-6 interleukin-6, TNF-α tumor necrosis factor alpha, CRP C-reactive protein

2.5.1 Cardiovascular disease

Low muscle mass is associated with greater risk of and mortality from CVD (Aubertin-Leheudre et al., 2006; Chin et al., 2013). In a population of 6,451 patients with CVD, Srikanthan et al. demonstrated that both disease specific and all-cause mortality were significantly greater in those with lower compared to higher muscle mass, regardless of fat mass, indicating high muscle mass may play a protective role in CVD (Srikanthan, Horwich and Tseng, 2016). Sarcopenia is also independently associated with non-alcoholic fatty liver disease (NAFLD) and T2DM, both of which are risk factors for CVD (Kim et al., 2010; Cruz et al., 2019). A recent systematic review reported that gait speed and handgrip strength, both of which are used in some definitions of sarcopenia (Cruz-Jentoft et al., 2019) and are dependent on muscle function, are associated with CVD mortality and in many of the included studies, this association was independent of traditional risk factors such as smoking and dyslipidemia (Chainani et al., 2016). Potential mechanisms for this elevated risk of CVD in sarcopenia are increased LDL cholesterol, blood pressure, oxidative stress, proinflammatory cytokines and decreased insulin sensitivity associated with sarcopenic changes in muscle tissue (Fisher et al., 2017; Kalinkovich and Livshits, 2017; Bellanti et al., 2018). These factors are known to contribute to the development of atherosclerotic plaques, which is a key process in coronary heart disease (Ross, 1999). Of particular concern is the elevated incidence of COVID-19 in individuals with comorbidities such as hypertension and diagnosed CVD, which were observed in up to 31% and 15% of COVID-19 patients, respectively (Wang et al., 2020a). In the same cohort it was observed that hypertension and CVD are even more prevalent in patients requiring ICU admission, 58% and 25%, respectively. With such significant associations between reduced muscle mass, CVD and the risk of severe COVID-19 infection, public health authorities need to carefully consider measures to reduce the potential declines in muscle mass that can precede CVD. This will be vitally important if individuals are to improve

healthspan and reduce risk of mortality from COVID-19, should second wave predictions become a reality (Vaid et al., 2020).

2.5.2 Diabetes

In the English Longitudinal Study of Ageing, participants with obesity and with handgrip strength below the threshold of weakness (a proxy for sarcopenia/dynapenia) were over3.5 times more likely to develop T2DM over 6 years (Cuthbertson et al., 2016). Skeletal muscle is the largest insulin-sensitive tissue in the body and accounts for 80% of glucose uptake under hyperinsulinemic, euglycemic conditions and IR of this tissue is a key process in the development of T2DM (Thiebaud et al., 1982; Samuel and Shulman, 2016). Thus, lower levels of muscle mass, as observed in sarcopenia may lead to a reduced capacity for glucose disposal in older adults. Older age and sarcopenia are also associated with IMAT accumulation (Buch et al., 2016) which may reduce insulin sensitivity (Goodpaster, Thaete and Kelley, 2000; Yim et al., 2007). IMAT may also contribute to a pro-inflammatory state through elevated levels of cytokines such as IL-6, CRP and adipokines such as leptin as well as reduced levels of anti-inflammatory and insulin-sensitizing adipokines such as adiponectin (Vella and Allison, 2018). It should be highlighted that this may also contribute to further muscle loss due to impairments in regulation of protein metabolism/synthesis, thereby maintaining a vicious cycle of worsening sarcopenia and IR (Guillet et al., 2012). Of further concern, a recently published meta-analysis showed the pooled prevalence of diabetes in COVID-19 was 9.8% and it was significantly associated with both risk of severity and mortality with pooled odds ratios of 2.75 and 1.90, respectively (Kumar et al., 2020). A study of COVID-19 associated mortality in Italy also observed diabetes in 36% of deaths (Onder, Rezza and Brusaferro, 2020). Furthermore, an increased incidence of fasting glycemia and acute-onset diabetes has been reported among patients with COVID-19 leading to the hypothesis that it may cause “new-onset” diabetes in patients without diabetes (Rubino et al., 2020). This further

highlights the links between muscle loss, metabolic perturbations and increased risk of COVID-19 during this pandemic.

2.5.3 Cognitive decline and depression

Sarcopenia is independently associated with cognitive impairment (declines in cognitive functions such as verbal memory, working memory, interference control, and processing speed) and depression (Kim et al., 2011; Hsu et al., 2014; Chang et al., 2016; Ge et al., 2020). Many of the risk factors associated with cognitive impairment such as low levels of exercise, reduced anabolic hormones, malnutrition, and low-grade chronic inflammation are also known causes of sarcopenia (Etgen et al., 2011). Cognitive function is strongly associated with the integrity of the neural connection pathways needed for muscle movement and coordination (Leisman, Moustafa and Shafir, 2016) and this may highlight the importance of including measures of muscle function/strength and not just size in definitions of sarcopenia. While studies have established an association between depressive symptoms and sarcopenia, this seems not to be related directly to muscle mass and instead is related to reduced muscle strength and function (Hayashi et al., 2019). To complicate this relationship further, late-life depression can lead to further declines in cognition (Wilkins, Mathews and Sheline, 2009) and is also associated with reduced physical activity and increased sedentary behavior (Santos et al., 2017) which may further exacerbate sarcopenia. Higher levels of physical activity and lower levels of sedentary time are consistently associated with better mood scores (Harvey et al., 2010; Hoare et al., 2016) but current social distancing and self-isolation measures will likely lead to greater social isolation in some individuals which is associated with lower levels of physical activity (Herbolsheimer, Ungar and Peter, 2018; Schrempft et al., 2019). Thus, social isolation resulting from COVID-19 social distancing measures may have significant implications on physical activity levels, mental health and wellbeing, feelings of isolation, depressed mood and muscle loss.

2.5.4 Osteoporosis and risk of fractures

Low muscle mass and strength are associated with bone mineral density abnormalities and osteoporosis in older men and women (Marin et al., 2010; Pereira, Leite and de Paula, 2015). In a sample of 679 middle-aged and elderly male Europeans, those with sarcopenia were 3 times more likely to have osteoporosis compared with those with normal muscle mass, defined as relative appendicular skeletal muscle mass ≥7.26 kg/m² (Verschueren et al., 2013). As osteoporosis is frequently associated with fracture risk, it is not surprising that sarcopenia is also associated with an elevated risk of fractures (Zhang et al., 2018; Artiaco et al., 2020). The process of bone remodeling is carried out by bone cells such as osteoblast which help with the formation and repair of bone, osteoclasts which break down bone and osteocytes which have a mechano-sensitive function which can detect the mechanical forces of muscle movement (Florencio-Silva et al., 2015; Tarantino et al., 2015). Decreases in the physical stimulus of muscle contraction, such as could be induced by inactivity or hospitalization during the COVID-19 pandemic, leads to a reduction in hormones such as testosterone, estrogen or growth hormone, for example, (Halloran et al., 1995; Orwoll and Nelson, 1999; Martin, 2011) and increases in proinflammatory cytokines, such as interleukin-1 (IL-1), IL-6, and tumor necrosis factor-alpha (TNF-α) (McLean, 2009). Such conditions have been shown to lead to reduced osteoblast and enhanced osteoclast activity which can result in osteoporosis (Cederholm, Cruz-Jentoft and Maggi, 2013). Thus, bone mass, size and density are influenced by exercise, similarly to how muscle size and quality can be affected by regular activity (Frost, 2000). With the significant decreases in PA during the COVID-19 there is an increased risk of falls-related fractures with associated morbidity and early mortality as a consequence (Katsoulis et al., 2017). A program of prehabilitation and rehabilitation for older adults may therefore be prudent.

2.5.5 Frailty risk of falls and quality of life

While there is no consensus definition of frailty (Fried et al., 2001), it is considered to be responsible for disability independently of clinical and subclinical disease. The central features of frailty include weakness, decreased endurance, and slowed performance resulting from a cumulative decline across multiple physiologic systems with advancing age (Fried et al., 2001). The loss of muscle size, and more importantly, loss of strength and function associated with sarcopenia may contribute to the development of frailty (Bernabei et al., 2014; Landi et al., 2015) and as such the diagnosis of sarcopenia may be a useful predictor of frailty (Vanitallie, 2003; Hirani et al., 2017). An additional consequence of the physical decline resulting from sarcopenia/frailty is an increased risk of falls (Beaudart et al., 2015; Gadelha et al., 2018; Xu, Ebeling and Scott, 2019) and it should be noted that falls are the leading cause of fatal and non-fatal injuries in older individuals (Bergen, Stevens and Burns, 2016). With older individuals being more susceptible to severe COVID-19 and more likely to require admission to ICU (Guan et al., 2020; Liu et al., 2020) they may be at a greater risk of suffering further muscle loss due to hospitalization, further compounding their degree of frailty.

As illustrated here, sarcopenia is associated with multiple other debilitating pathologies which can greatly add to the disease burden of older adults and reduce their quality of life (QoL). In a population of over 500 community dwelling older adults, Beaudart et al. (Beaudart et al., 2015) reported that even after adjustment for multiple confounders such as age, BMI and number of comorbidities, participants with sarcopenia had a worse physical health-related QoL, were more frail, were at higher risk of falls, had more difficulty with achievement of activities of daily living and were also more dependent on others for household than those without sarcopenia. As autonomy in activities of daily living plays a role in multiple bio-psycho-social factors of life in the elderly, reduced autonomy can contribute to reduced quality of life and well-being (Hughes, 2005; Muszalik et al., 2012). COVID-19 may contribute to this

reduced autonomy by imposed isolation measures and reduced time spent outdoors as well as through sarcopenia, induced by inactivity and/or hospitalization.

2.5.6 Mortality

While the association between reduced muscle mass and multiple other comorbidities is apparent, it should also be highlighted that sarcopenia is itself associated with greater risk of death in multiple elderly populations. Of particular concern is the potential risk of mortality that sarcopenia may confer on older patients in acute hospital care, potentially as a result of COVID-19 infection. Sipers et al. reported that in a hospitalized geriatric population, the presence of sarcopenia was significantly associated with up to 4.3 times greater 2-year mortality compared to patients without (Dam et al., 2014). In fact, the detrimental effects of reduced muscle mass and strength may be further augmented by elevated fat mass as seen in SO which is also associated with greater all-cause mortality (Atkins et al., 2014; Farmer et al., 2019). While there is no consensus definition of SO, the use of measurements of visceral fat area seems to be particularly strongly associated with increased mortality risk compared with those without SO (HR = 2.54) further highlighting the detrimental health effects of this pattern of fat distribution (Zhang et al., 2019). Similarly, lower rates of all-cause mortality have been observed in older individuals with high muscle mass and low fat mass (Srikanthan, Horwich and Tseng, 2016).

2.5.7 Immune function and risk of COVID-19 infection

While we have briefly described some of the long-term risks of muscle loss and other body compositional changes here, it should also be highlighted that these changes may also result in a more immediate problem, that being susceptibility to, and risk of more extreme presentation of, COVID-19. Early reports from multiple centers worldwide have highlighted

that individuals with cardiometabolic comorbidities including T2DM, CVD and also obesity are at greater risk of COVID-19 infection (Hu et al., 2020; Lighter et al., 2020; Simonnet et al., 2020), more likely to require acute care such as IMV (Caussy et al., 2020; Kalligeros et al., 2020; Simonnet et al., 2020) and at a greater risk of death (Docherty et al., 2020; Li et al., 2020). This has led government bodies such as the Center for Disease Control and Prevention (CDC) to advise that individuals with these conditions (all of which have been associated with sarcopenia), are amongst those at greatest risk from COVID-19 (CDC, 2020).

Skeletal muscle is recognized as an endocrine organ (Pedersen and Febbraio, 2012) which secretes cytokines (known as myokines) such as IL-6 (Fischer, 2006), IL-7 (Haugen et al., 2010) and IL-15 (Rinnov et al., 2014) in response to physical activity. Changes in circulating levels of these myokines, resulting from the various aspects of the aging process including increased inflammation and sarcopenia, are believed to play a role in the age-associated impairment of the immune response (immunosenescence) (Nelke et al., 2019). This highlights another mechanism by which sarcopenia may impact the health of older adults. Accordingly, lower levels of activity are associated with reduced immune function. For example, in a sample of older adults (60-79 years), sedentary individuals (2,000-4,500 steps/day) showed lower frequency of naive T cells and a higher frequency of memory T cells which is indicative of impairments in immune responses or immunosenescence, compared with physically active individuals (10,500-15,000 steps/day) (Bartlett and Duggal, 2020). Higher levels of physical activity may therefore be useful for maintaining immune function in older adults. In a sample of older men (65-85 years) those who regularly engaged in moderate or intense exercise demonstrated superior antibody responses to the influenza vaccine, resulting in higher percentages of seroprotected individuals, compared with age-matched, sedentary controls (de Araújo et al., 2015). Similarly, a 10-month, moderate intensity exercise intervention was reported to increase the antibody titer in response to influenza immunization in adults over 65 (Kohut et al., 2004). Additionally, the relationship between muscle mass and myokines may

be bi-directional and changes in myokine secretion due to aging may contribute to anabolic resistance and sarcopenia. For example, reduced secretion of IL-15 may contribute to inflammation-related muscle loss in older adults (O'Leary et al., 2017). The global decrease in PA during COVID-19, as evidenced by reductions in step counts and increases in sedentary activity (Sun et al., 2020) may contribute to a decline in muscle mass and subsequently immune function. This impaired immune function and pro-inflammatory status may at least partially explain the higher risk of mortality from COVID-19 experienced by older adults (Bonanad et al., 2020).

In light of these data, prevention of the development and progression of these conditions in the general population and already at-risk older individuals (Wu et al., 2020) should be (and in some cases already is) considered amongst government strategies for the management of the COVID-19 pandemic (England, 2020). Such countermeasures are discussed below.

2.6 Countermeasures to prevent sarcopenia during COVID-19

2.6.1 Resistance Exercise

Exercise should be considered of prime importance in attempting to halt and even reverse the progression of sarcopenia. Multiple studies have shown that RE alone (without any dietary, supplementary or pharmaceutical assistance) can improve muscle size and strength in older individuals (Frontera et al., 1988; Kosek et al., 2006; Stewart, Saunders and Greig, 2014; del Campo Cervantes, Macías Cervantes and Monroy Torres, 2019). This hypertrophic response may be further augmented by the addition of supplementary protein, amino acids or high-protein diets (Morton, McGlory and Phillips, 2015; Liao et al., 2017b), the use of nutritional supplements such as creatine (Chilibeck et al., 2017) or by the use of therapeutic doses of

androgenic hormones such as testosterone, which may be used in some clinical settings (SULLIVAN et al., 2005).

Resistance exercise has also been shown to improve markers of cardiovascular health (e.g. LDL cholesterol and blood pressure) (Kim and Kim, 2013; Nascimento et al., 2018), glycemic control (lower HbA1c and improved insulin sensitivity) (Egger et al., 2013; Acosta-Manzano et al., 2020), functional capacity (Liao et al., 2017a; Liao et al., 2020), bone mineral density (Hong and Kim, 2018; Souza et al., 2020), body composition (Liao et al., 2017a; Yan et al., 2019), sleep (Yoon et al., 2019) and cognitive performance (Macaulay, Fisher and Schroeder, 2020). It should also be noted that regular exercise is known to improve immune function, a faculty that is particularly important in times of pandemic (Zheng et al., 2015; Duggal et al., 2018; Bartlett and Duggal, 2020). Thus, the potential benefits of encouraging exercise, and in particular, RE, at all times and especially during a pandemic, cannot be overstated.

While there are many different ways of implementing RE protocols (Schoenfeld, 2010), a meta regression of data from 25 studies in the older men and women (mean age of 70.4 years, age range 60-90 years)) (Borde, Hortobagyi and Granacher, 2015) reported that RE to improve muscle size seems to be effective using the following independently computed training variables (Figure 2.4):

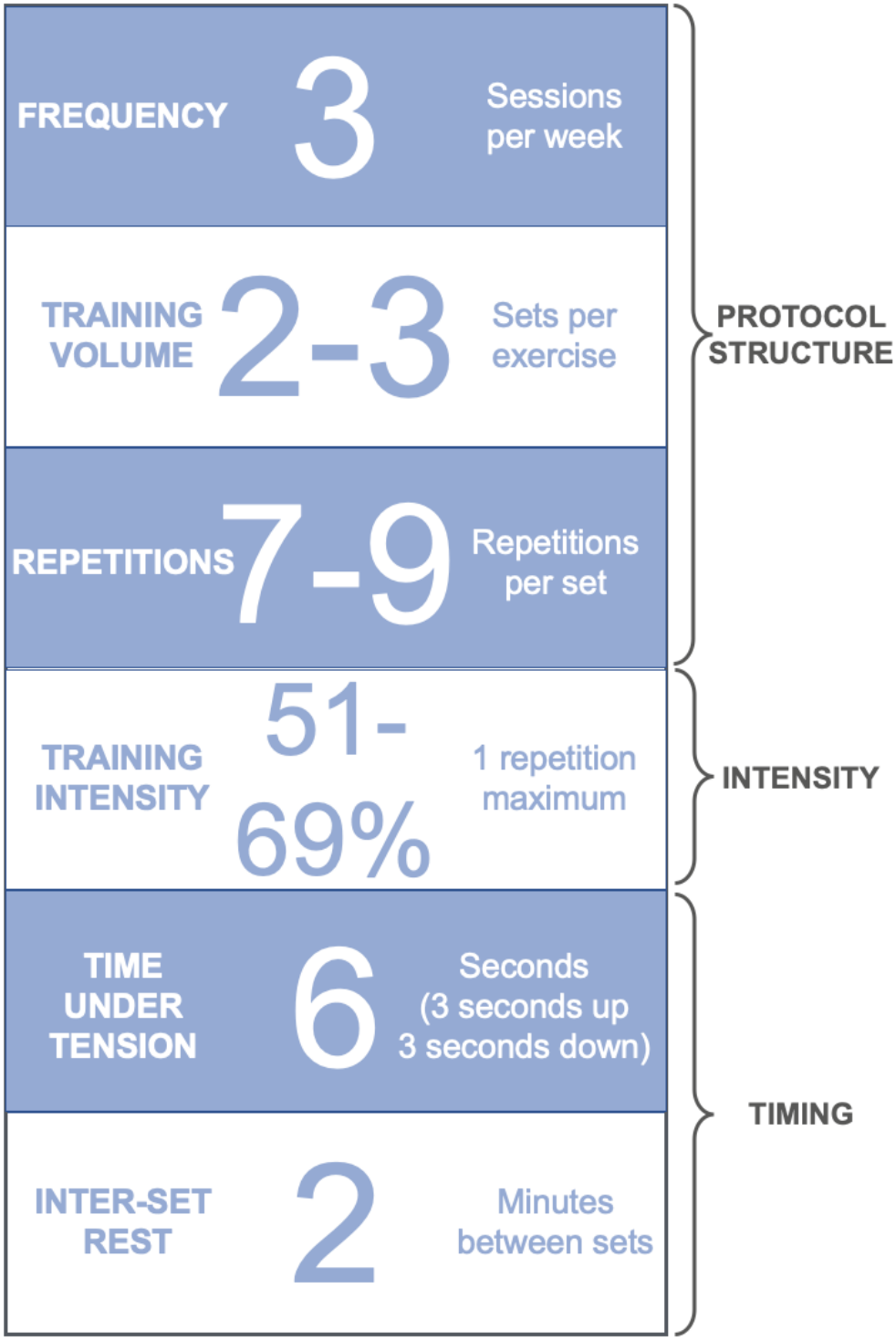

Figure 2.4 Summary of evidence-based resistance exercise variables reported to improve muscle size in older adults. These figures were calculated using data from a meta-regression of 25 randomized controlled trials. As many variations of training protocols are feasible for muscle gain, this collection of variables should be considered as guidelines only and not as a defined training program. Adapted from Borde et al. (2015) (Borde, Hortobagyi and Granacher, 2015)

While implementation of all of these variables may not be feasible during a pandemic, they may act as a useful set of guidelines for developing RE protocols for older adults. Should subsequent waves of COVID-19 enforce future bouts of self-isolation, home-based exercise programs with clear guidance on how to undertake them should be considered, in order to circumvent further periods of inactivity.

Interestingly, recent qualitative research with trainers and older participants in physical activity programs in France highlighted that attendance had fallen even before quarantine restrictions were in place because participants "no longer wanted to have close contact" with the other participants and "no longer wanted to touch the equipment." However, these same older participants also expressed a need to perform exercise at home (Goethals et al., 2020), therefore, recommendations for suitable home-based research strategies should be given priority. Even for those who prefer a gym setting, both during and in the aftermath of the COVID-19 pandemic, access to gyms or gym equipment is/will be limited. This may be due to continued social distancing measures and/or measures to protect at-risk groups such as those in older age categories and/or with underlying co-morbidities (CDC, 2020). Therefore, as detailed above, alternatives to free-weights and RE machines must be considered and indeed the pandemic may provide an opportunity to engage older age groups in sustainable home-based exercise interventions.

The use of resistance bands is a cost effective and widely available option that has been proven to be equally effective to conventional (free-weights and machines) RE for improving strength and physical function in older individuals (Martins et al., 2013; Lima et al., 2018). Band-based and bodyweight training regimes may not offer the resistance offered by adjustable free-weights and RE machines, thus not allowing for the use of training intensities

in the 50-70% of 1RM range mentioned previously. However, lower intensity, higher repetition exercise is effective for inducing muscle hypertrophy, as long as momentary muscle failure is achieved (Van Roie et al., 2013; Schoenfeld et al., 2017). Indeed, at-home training protocols are being developed to maintain physical activity levels and prevent physical decline using minimal equipment, during the COVID-19 pandemic (Aung et al., 2020; Guadalupe-Grau et al., 2020) and these should be scrutinized and translated safely from the academic to the home environment. As reduced daily step counts contribute to the loss of lean mass and strength, reductions in insulin sensitivity and increases in systemic inflammation (Oikawa, Holloway and Phillips, 2019), enabling older people to be more physically active in their own homes will be an essential health measure as we navigate through the pandemic and beyond. Encouraging older adults to walk more, even within their homes and reminding them that physical chores such as cleaning and gardening are relevant and important forms of PA, may be a useful and free initial strategy.

Barriers to participation in RE, including a fear of looking too muscular or a fear of a heart attack or stroke during exercise have been reported (Burton et al., 2017). Given the importance of encouraging engagement in PA, addressing any possible barriers and tailoring progressive PA interventions to ability must be considered. Such barriers can likely be overcome by providing clear information and detailed guidelines to reduce fears and to clarify the health benefits, including: preventing muscle deterioration, delaying the disability threshold, reducing risk of falls, building function, feeling more alert and improving concentration (Burton et al., 2017). This is essentially the promotion of PA as a method for maintaining health and wellbeing into older age, regardless of the climate in which we find ourselves. Furthermore, focusing on modifications in training protocols to improve enjoyment may also be a useful technique for encouraging those at risk of COVID-19-exacerbated sarcopenia to participate in RE, as people are more likely to engage in activities that are enjoyable and avoid activities that are disagreeable (Ekkekakis and Dafermos, 2012). For

example, beginning an exercise session with a heavy load and ending with a lighter load has been shown to increase the enjoyment, post-exercise pleasure and remembered pleasure of a bout of RE (Hutchinson et al., 2020). Similarly, in directed exercise settings, focusing on enjoyment in the sessions and using some of the following guiding principles has been shown to encourage affective states and promote exercise adherence over 8 weeks:

- involving participants in exercise selection and program design
- providing positive feedback
- regulating intensity according to participants abilities and wishes
- being transparent about the contents of future training sessions
- increasing training diversity (Jekauc, 2015)

In addition to principles outlined in the paper above, the following guidelines may also prove useful:

- setting goals and highlighting achievements and progress
- enabling safe, virtual exercise and social domains for those who are motivated by group training
- empowering individuals in the cohorts who are exercising to motivate and to recruit others

While the benefits of RE have been discussed extensively in relation to its ability to improve lean muscle mass and strength, aerobic exercise (AE) should not be overlooked as a potential strategy for the maintenance of healthy muscle mass and function during COVID-19. Chambers et al (Chambers et al., 2020) analyzed muscle size and adiposity in a population of older individuals (mean age 74 years) who performed, on average, 7 hours/week of AE over the previous 52 years. Lifelong AE was shown to attenuate the decline in quadriceps muscle size and isometric strength by ~50% in men, compared with non-exercising controls, and higher intensities of exercise were reported to reduce lower body IMAT by ~30%. Similarly,

Aagaard et al reported that older individuals (68-78 years) who have engaged in either life-long RE or endurance training have significantly greater maximal muscle strength compared with untrained, control individuals (Aagaard et al., 2007) although only strength trained participants demonstrated increased muscle mass. This superiority of RE in comparison to AE is reflected in the widespread use of RE as a key strategy for improving muscle mass and strength in older individuals (Frontera et al., 1988; Kosek et al., 2006; Stewart, Saunders and Greig, 2014; del Campo Cervantes, Macías Cervantes and Monroy Torres, 2019).

While AE in isolation may not be as effective as RE in helping to improve or maintain muscle mass and strength in older adults, it may be useful in addition to RE as it can reduce total body fat and IMAT (Timmons et al., 2018; Chambers et al., 2020), thereby improving muscle function relative to body weight. Such concurrent training strategies have been shown to be more effective than RE or AE alone for increasing gait speed and lower limb strength, and reducing body fat in community dwelling older adults (mean age 69 years) (Timmons et al., 2018). Similarly, in a population of untrained, older adults (60-80 years) with abdominal obesity, concurrent training was reported to be more effective for reducing functional limitations and IR than either RE or AE alone (Davidson et al., 2009). As has been discussed, IR can contribute to anabolic resistance and sarcopenia (Hong and Choi, 2020) therefore exercise strategies to further reduce IR may be optimal for improving muscle health in the long term. Engagement in AE may offer further benefits by helping to modulate immune response. In an older, sedentary population (61-66 years), 6 months of both AE and RE resulted in increased circulating levels of anti-inflammatory IL-10 and reduced levels of IL-6, CRP and TNF-α (Abd El-Kader and Al-Shreef, 2018), which are all involved in the cytokine storm observed in severe cases of COVID-19 (Cao, 2020). Interestingly, these improvements were observed to be greater in the AE group.

Current UK exercise guidelines for older adults recommend to accumulate 150 minutes of moderate intensity aerobic activity, such as brisk walking, per week (Government, 2019). Indeed, with the closure of gyms or suspension of group physical activity programs that may occur in response to a pandemic, walking may be a useful, low cost and easily implementable strategy for increasing PA levels. The promotion of walking as physical activity amongst older adults has been shown to be highly feasible and effective for improving physical function, even in those who are functionally limited (Nicklas et al., 2020). Increasing daily steps has also been reported to lead to improved health related quality of life, better immune function and improvements in metabolic syndrome and weight maintenance (Tudor-Locke et al., 2011). Walking interventions with a frequency of only 3 days per week have been shown to reduce depression indices in older women (Bernard et al., 2015), which may be especially important considering the increased risk of poorer mental health status during social isolation due to COVID-19. A further benefit to the promotion of walking may also be the increased exposure to sunlight which may help improve vitamin D status (Webb et al., 1990; Whitmore, 1996) and musculoskeletal health.

To facilitate these changes in exercise behavior, telehealth services aimed at increasing physical activity, which may involve the use of instructional videos or on-screen interaction with an exercise trainer, should be implemented where possible. There are numerous examples of home-based exercise programs administered through telehealth services that have been beneficial for maintaining physical activity levels and improving health markers such as waist circumference, HOMA index of insulin resistance and total/HDL cholesterol ratio (Vroege et al., 2014; Avila et al., 2020). A trial of telehealth services aimed specifically at people with sarcopenia and using remote one-on-one instruction to each participant via video conferencing over 12 weeks, resulted in improvements in muscle mass as well as improvements in functional parameters (Hong et al., 2017). Preliminary studies have also highlighted the cost-effectiveness of such telehealth services, and costs may be further

reduced if provided as interactive group classes instead of private and if the participant already has their own device (smart phone, tablet, laptop etc.) (Middleton et al., 2020). Indeed, group classes may be preferred, particularly in times of social isolation. These results highlight the potential utility of telehealth services for combatting sedentarism and sarcopenia both during and in the aftermath of this pandemic. A list of COVID-19-applicable countermeasures to the loss of muscle mass and function is summarized below (Figure 2.5) and described in further detail, below.

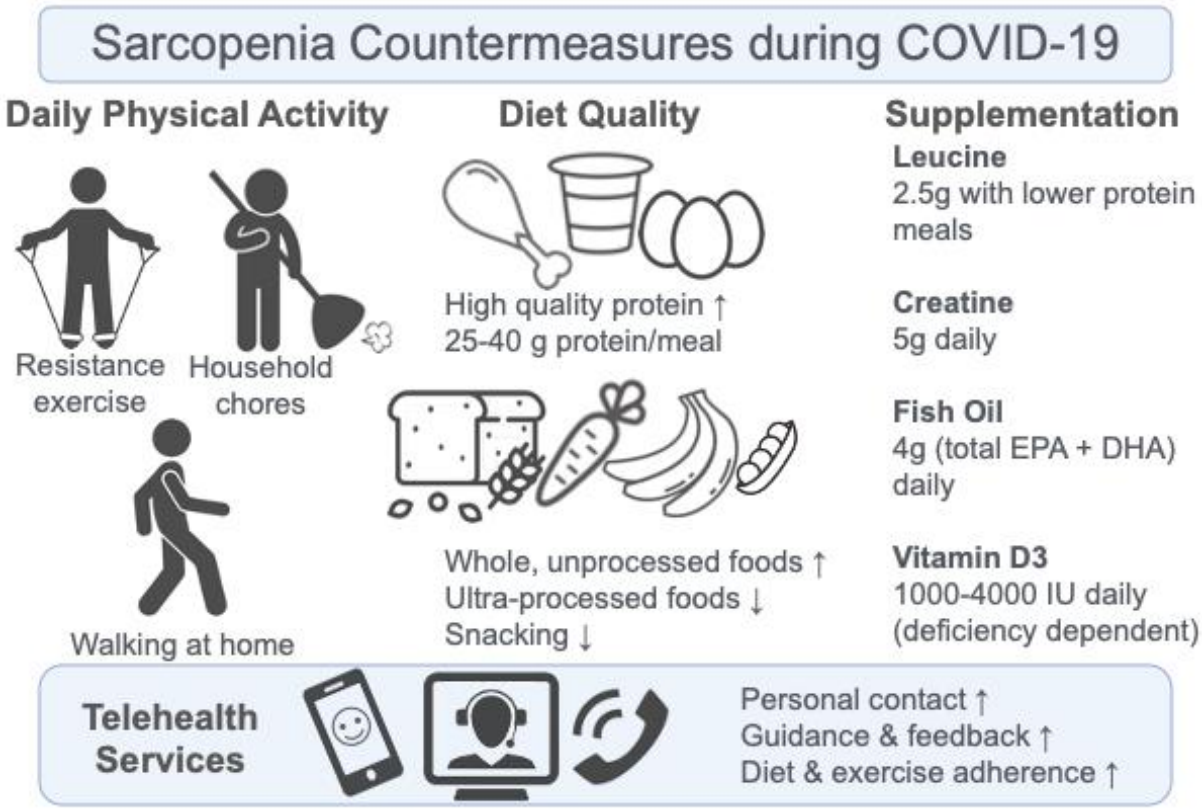

Figure 2.5 A summary of the physical activity, dietary and supplement countermeasures that may be useful for preventing the loss of muscle mass and function in both younger and older adults. The inclusion of telehealth services offering regular contact, guidance and support to such countermeasures may result in greater adherence and positive outcomes

2.6.2 Protein Intake

Higher protein intakes can augment the muscle hypertrophic response to RE (Morton, McGlory and Phillips, 2015). The current UK reference nutrient intake (RNI) for protein is 0.75 g of protein per kilogram of body weight per day (g/kg/d) (Department of Health, 1991). However, this number does not take into consideration the age-related changes in hormone levels, progression of sarcopenia, or anabolic resistance previously discussed in this article. More recent research indicates that older adults may need 1.2-1.5 g/kg/d of protein to maintain optimal health and physical function (Bauer et al., 2013b; Traylor, Gorissen and Phillips, 2018). These articles also highlight the importance of focusing on high-quality proteins i.e. those that are high in the amino acid, leucine, which is a determinant of both short and long-term MPS responses in older adults (Devries et al., 2018). Encouraging higher protein intakes amongst older adults should be further prioritized as this group has been shown to have protein intakes below the already inadequate RNI with a recent study showing that 35% of participants fail to consume ≥ 0.75 g/kg/day and fewer than 15% consume ≥1.2 g/kg/day (Morris et al., 2020). Protein intakes may be even lower in older individuals hospitalized due to COVID-19, those with disabilities (Covinsky et al., 1999) or as previously mentioned, amongst those whose diets may depend more on lower quality, lower protein UPFs as a result of food insecurity due to current government sanctions (Rauber et al., 2018; Moran et al., 2019). The importance of encouraging protein intake in older adults in order to prevent muscle loss is further highlighted in the Health, Aging, and Body Composition (Health ABC) Study. Over 3 years it was observed that community-dwelling adults in the highest quintile of protein intake lost approximately 40% less lean mass (LM) and appendicular LM than those in the lowest quintile of protein intake (Houston et al., 2008b).

Higher meal frequency and higher per meal protein dose are associated with greater lean mass and strength (Loenneke et al., 2016) and a more even distribution of protein amongst the main meals is also recommended to maintain muscle mass (Bauer et al., 2013b). For older adults, this even distribution of protein could range from 25-40 g of protein, three times a day, focusing on higher quality (leucine-rich) proteins such as meat, fish, dairy and eggs (Bauer et al., 2013b). As breakfast is traditionally one of the lowest protein meals in the UK, with a mean intake of 12g in adults over 65 years (Gaal et al., 2018), encouraging inclusion of protein/leucine-rich, lower-calorie foods at breakfast such as low-fat dairy products (Greek yoghurt, quark, cottage cheese etc.) may be beneficial. Indeed, the use of protein-rich dairy products (both whole food and as protein supplements) has been effective for improving LM and function in multiple RCTs (Tieland et al., 2012b; Aleman-Mateo et al., 2014; ten Haaf et al., 2019). Pre-bed protein ingestion is also thought to be a viable strategy to enhance muscle mass accretion (Snijders et al., 2019). Therefore, encouraging the addition of a small, high-protein meal before bedtime may further help prevent sarcopenia. Research has also shown that such late-night protein meals (specifically, 48g of casein protein powder) do not negatively affect sleep (Morehen et al., 2020) thus eliminating the potential catabolic effects of sleep reduction on muscle mass (Nedeltcheva et al., 2010).

While protein powders/shakes are frequently used to augment lean mass in scientific research (Chale et al., 2013), there may be issues with the acceptability of such products or even protein enriched foods in older populations. Investigations have found that older people are skeptical about such protein-enriched functional foods and barriers to their use in this population can include confusion, distrust and a perceived lack of personal relevance (van der Zanden et al., 2014; Banovic et al., 2018). A further issue is that older individuals regularly cite price as affecting their food purchasing decisions (Falk, Bisogni and Sobal, 1996) the price of protein supplements could result in them being used as meal replacements. This could be speculated to reduce the intake of more nutrient dense, whole foods and reduce overall diet quality (Lee,

Ralston and Truby, 2011). Thus, focusing on education relating to high-protein, familiar food options (meat, fish, dairy, eggs, legumes etc.) may be more acceptable. Alternatively, where budget allows, education around high-protein functional foods as well as their relevance for older people may improve their acceptability and use (van der Zanden et al., 2014) which may be of particular importance for improving muscle mass, at this time.

Finally, it should be noted that higher protein diets are frequently cited as being problematic for kidney health, a concept that likely developed from the use of controlled protein diets (0.8g/kg/d) in patients with existing chronic kidney disease or reduced glomerular filtration rates (Levey et al., 1996). This perception may be common amongst older individuals and may pose a further barrier to the use of higher protein diets to prevent sarcopenia. In individuals with healthy kidney function, however, higher protein intakes do not pose a risk to kidney function (Ramel et al., 2013; Beasley et al., 2014). A clinical trial comparing lower with higher protein intakes in individuals with T2DM and nephropathy showed no benefit on glomerular filtration rates from following the lower protein diet, which was also difficult to adhere to (Koya et al., 2009). Education about this common misconception may be useful in promoting higher protein intakes.

2.6.3 Supplementation

There is a broad range of supplements that may be potentially beneficial for improving or at least maintaining muscle mass during the COVID-19 quarantine/social distancing measures. However, a full discussion of their mechanisms of action is beyond the scope of this review, and we will only briefly mention those supplements with the most promise of utility in the current situation.

2.6.3.1 Leucine

The presence of the amino acid leucine in protein sources is a key determinant of the MPS response (Devries et al., 2018). As the protein recommendations in this review are considerably higher (up to 40 g per meal, post exercise (Churchward-Venne et al., 2016)) than current intakes of protein in the older population, they may be difficult to achieve. Older people may not want to make large changes to their normal eating habits (Morris et al., 2020) and the satiating effect of protein may make consuming sufficient protein more difficult (Tremblay and Bellisle, 2015). Furthermore, high-quality protein sources (meat, fish, dairy etc.) can be more expensive than other, lower-protein foods, adding another barrier to higher intakes (Brooks, Simpson and Raubenheimer, 2010). However, the addition of leucine (2.5 g) to a smaller dose (20 g) of high-quality protein has been shown to enhance MPS under resting conditions in older men (Wall et al., 2013) and it has also been shown to partially protect against muscle loss during prolonged periods of inactivity (English et al., 2016). The use of leucine to supplement meals with insufficient protein content to maximally stimulate MPS may be a useful, cost-effective and acceptable strategy to maintain muscle mass during lockdown.

2.6.3.2 Creatine

Creatine (Cr) is a non-protein amino acid found in red meat and seafood (Harris, 2011) and it is widely used as an ergogenic aid for athletes (Kreider et al., 2017). In the body, Cr combines with a phosphoryl group to form phosphocreatine (PCr). Elevated muscle levels of PCr help to maintain ATP availability through recycling of ADP to ATP, a process essential for maintaining energy availability, particularly during maximal effort anaerobic sprint-type exercise (Schlattner et al., 2016). Creatine supplementation has been shown to be particularly beneficial for strength and power athletes (Buford et al., 2007) and a number of its ergogenic effects may be useful for countering the muscle mass and functional losses associated with sarcopenia/aging, namely:

- Improved performance in sets of high intensity muscle contractions
- Increased muscle mass and strength adaptations from training
- Enhanced recovery
- Greater training tolerance (Kreider and Jung, 2011)

Creatine supplementation has been shown to be safe and effective for improving accrual of LM and improving strength in older people (Chilibeck et al., 2017). A study by Aguiar et al. (Aguiar et al., 2013) in healthy women (mean age 65 years) undergoing a 12 week RE program reported that those supplementing with 5 g of creatine per day experienced a greater improvement in bench press, knee extension and bicep curl 1RM strength, improvements in functional performance, as well as a greater increase in muscle mass (+2.8 kg) than the control group. It has also been observed that plasma and muscle creatine levels are lower in those who eat vegetarian/low-meat diets (Blancquaert et al., 2018) and older populations (Möller et al., 1980), thus, highlighting the importance of supplementing creatine in these groups. Creatine's safety and cost effectiveness make it a potentially useful supplement, to take in conjunction with a (home based) RE protocol, for the prevention of muscle atrophy and sarcopenia (Dolan et al., 2019).

2.6.3.3 Long-chain, omega-3 fatty acids

Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are omega-3 fatty acids of marine origin that are widely investigated for their potential health benefits in conditions such as CVD, cognitive decline, chronic inflammation and depression (Du et al., 2016; Zhang et al., 2016; Bäck and Hansson, 2019; Innes and Calder, 2020). Long-chain, marine, omega-3 fatty acid supplementation has also been shown to augment the MPS response to protein ingestion in younger and older adults (Smith et al., 2011b; Smith et al., 2011a). There is evidence that this effect is partially mediated via activation of the mTORc1- S6K1 signaling pathway which

is essential in the process of protein synthesis and muscle growth (Smith et al., 2011a; Liu and Sabatini, 2020). This indicates that sufficient omega-3 supplementation may, at least partially, be able to counter the anabolic resistance typical of the aging process. Clinical trials have added evidence for this possibility. After a 3 month RE program with 45 healthy women (mean age 64 years), the two groups supplementing with 2 g of fish oil per day experienced greater improvements in muscle strength and functional capacity compared to a control group (Rodacki et al., 2012). Similarly, high-dose fish oil supplementation (4 g per day) has been shown to increase thigh muscle volume and grip strength in older men and women (mean age 68 years) despite no RE protocol being included in the trial (Smith et al., 2015). Thus, long-chain, marine, omega-3 fatty acids may be a useful adjunct strategy to overcoming the anabolic resistance-induced losses in muscle mass that are observed in ageing. It should be noted however, that a number of the trials mentioned here used a particularly high-grade and high-dose (4 g per day) omega-3 supplement known as Lovaza (Smith et al., 2011b; Smith et al., 2011a; Smith et al., 2015) and accordingly, such high doses may be necessary to achieve a physiologically significant effect. Due to their anti-inflammatory effects, omega-3 supplementation may offer the further benefit of managing the “cytokine storm” observed in severe COVID-19 infections (Calder, 2013) and has already been suggested as an adjuvant therapy (Torrinhas et al., 2020). Furthermore, the use of EPA or EPA/DHA combinations are recommended in the treatment of mood disorders, which may be more common during COVID-19 confinement (Guu et al., 2019).

2.6.3.4 Vitamin D

The relevance of vitamin for muscle health has already been discussed but we will briefly mention the results of trials investigating the effects of vitamin D supplementation on muscle mass and function. In a 6-month intervention in institutionalized older adults (≥ 60 years) with vitamin D deficiency, those receiving vitamin D improved hip flexor strength by 16.4% and

knee extensor strength by 24.6% without any RE protocol (Moreira-Pfrimer et al., 2009). This was in contrast to the control group which received no vitamin D and reported no improvement in strength. The dosage in this trial averaged approximately 3,666 IU of oral vitamin D3/day. Similarly, in a 9 month study of an older population (≥ 70 years), vitamin D supplementation (400 IU vitamin D3/day) was reported to improve timed up and go performance and gait speed compared to controls (Bunout et al., 2006). As older adults may require higher doses of vitamin D3 to achieve adequate serum levels (30 ng/mL) (Whiting and Calvo, 2010) and supplementation is safe up to 10,000 IU/day (upper limit of safety), an intake of 1000-4000 IU/day may be suitable, based on current evidence. As low vitamin D status is a potential risk factor for COVID-19 infection (Meltzer et al., 2020), supplementation may be a pragmatic strategy for reducing risk of both sarcopenia and COVID-19.

2.6.4 Energy Balance

In addition to encouraging higher intakes of protein, older individuals may need to reduce total calorie intake in order to avoid excess accumulation of body fat due to the potential reduction in activity levels caused by social distancing and quarantine measures (Goethals et al., 2020; Pietrobelli et al., 2020). Reducing total calorie intake through a reduction in portion sizes and snacking occasions may be effective methods for maintaining energy balance in the elderly (Hall et al., 2012). Maintaining higher protein intakes may be particularly beneficial for avoiding the loss of lean mass during such calorie restriction (Gordon et al., 2008; Westerterp-Plantenga et al., 2009), especially when combined with (home-based) RE which is known to help preserve LM (Wycherley et al., 2010; Galbreath et al., 2018b). Where possible, focusing on more whole foods such as fruit, vegetables, wholegrains and legumes, has been shown to help reduce ad libitum food intake, while also benefiting cardiometabolic health (K. et al., 2020). Higher protein intakes (lean meats, fish, low-fat dairy etc.) and higher fiber foods (vegetables, fruit, wholegrains, legumes etc.) can also help reduce feelings of hunger that may

arise from reduced caloric intake, improving adherence and helping to avoid body fat gain (Weigle et al., 2005; Tremblay and Bellisle, 2015). Similarly, reducing UPFs may be a useful strategy to reduce excessive consumption of food and weight gain (Hall et al., 2019).

Telehealth services aimed at promoting improved dietary habits may also be beneficial as the addition of supervision and behavioral support is known to enhance the effectiveness of dietary advice (Kelly et al., 2016; Lemstra et al., 2016). While there is evidence to suggest that these dietary telehealth strategies are effective, there is also evidence to suggest that certain individuals may find “no-contact” approaches to be more effective (Hellerstedt and Jeffery, 1997; Churchward-Venne et al., 2016). This should be considered when providing older people with appropriate support and guidance, in order to better tailor advice to their needs and circumstances.

2.7 Conclusions

The COVID-19 pandemic has and will continue to have wide-reaching repercussions on all aspects of society. While social distancing and isolation measures implemented by governments are necessary for the greater societal good, governments also have a responsibility to provide some form of care for those that are quarantined or isolated and in particular, those at greatest risk of infection (Giubilini et al., 2018). Reductions in physical activity, disruption to normal eating habits, stress and altered sleeping patterns will put older people at greater risk of sarcopenia which, along with its own implications for quality of life and mobility, can lead to the progression of multiple lifestyle-related diseases. Many of those hospitalized by COVID-19 will also suffer from some degree of muscle loss and will likely require some form of rehabilitation to regain that lost muscle mass and function (Barker-Davies et al., 2020). In this review we have highlighted some of the primary causes of muscle loss and sarcopenia. Their relevance to both short- and long-term health burden, as well as their

relevance to the risk of contracting COVID-19, or experiencing worsened outcomes post-infection, should be recognized and considered carefully by governmental and public health bodies. We have also suggested some of the most useful and practical, evidence-based counter measures that can be safely implemented to reduce the progression of sarcopenia, improve physical function and wellbeing and potentially reduce the risk and severity of infection. Physical activity will play a key role and tailoring such programs to the needs and abilities of the participants will be vital. This highlights the importance of online and phone-based virtual care and telehealth services, which have become common place in standard medical care during this pandemic (Jones et al., 2020). This digital health framework can be leveraged to provide older adults with the remote supervision and guidance needed to encourage the adoption of the exercise habits and dietary practices necessary for musculoskeletal health. Subsidization or out-right provision of such online support services as well as their promotion amongst those that need it most should be considered by governments and local authorities, as should subsidization of low-cost equipment that may improve uptake of said services. The potential for under-, over- and malnutrition during COVID-19 lockdown is also very real, especially amongst disadvantaged groups and governments must consider policies to ensure that people have access to sufficient, reasonably priced, high protein, predominantly whole foods in order to maintain muscle mass and avoid energy imbalances leading to either excess fat accumulation or unnecessary body weight loss. Like many difficult global health problems, the solutions may be apparent but the logistics of implementing them may be lacking. Success in counteracting the risk of muscle loss caused by the pandemic will be determined by our capability to develop efficient strategies that can protect vulnerable populations and maintain or improve the health status of the populace at large.

3 Chapter 3: Meta-analysis of protein's effects on lean mass

Protein interventions augment the effect of resistance exercise on appendicular lean mass and handgrip strength in older adults: a systematic review and meta-analysis of randomized controlled trials.

Kirwan RP, Mazidi M, García CR, Lane KE, Jafari A, Butler T, Perez de Heredia F, Davies IG.

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3.1 Context within thesis

Chapter 3 presents the second published study of this PhD thesis, a systematic review and meta-analysis of the effects of protein supplementation, with and without resistance exercise, on lean mass and strength in older adults. As the ultimate aim of our research is to investigate the effects of muscle mass on cardiometabolic risk markers, an understanding of optimal strategies to attain increased lean mass in older adults is essential. We use this meta-analysis to discuss the utility of protein supplementation and the relevance of resistance exercise for augmenting muscle mass and strength in an older population.

3.2 Abstract

Background: Increased protein intake is suggested as a strategy to slow or reverse the loss of muscle mass and strength observed in sarcopenia, but results from studies that directly tested this possibility have been inconsistent.

Objectives: We assessed the evidence on the effects of whole protein supplementation or higher-protein diets, without the use of amino acids or supplements known to stimulate hypertrophy, alone or in combination with resistance exercise (RE) interventions, on lean body mass (LBM) and strength in older adults.

Design: A systematic search was conducted using PubMed, Medline, Web of Science and Cochrane CENTRAL databases from January 1990 up to July 2021. Randomized controlled trials that assessed the effects of protein supplementation and/or higher-protein dietary interventions in older adults (mean age ≥ 50 years), on total LBM, appendicular lean mass (ALM), handgrip (HG) and knee extension strength (KE) were included.

Results: 28 studies were identified. In pooled analysis, compared with lower protein controls, protein supplementation did not have a significant positive effect on total LBM [weighted mean

difference in change (WMD):0.34, 95% CI:-0.21,0.89, I^2:90.01%], ALM [WMD:0.4, 95% CI:-0.01,0.81, I^2:90.38%], HG [WMD:0.69, 95% CI:-0.69,2.06, I^2:94.52%] or KE [WMD:1.88, 95% CI:-0.6,4.35, I^2:95.35%]. However, in interventions that used also RE, statistically significant positive effects of protein were observed for ALM [WMD:0.54, 95% CI:0.03,1.05, I^2:89.76%] and HG [WMD:1.71, 95% CI:0.12,3.30, I^2:88.71%]. Meta-regression revealed no significant association between age, per-meal protein dose, duration, and baseline protein intake with change in any outcome. Sub-group analysis revealed the statistically significant effects on ALM only occurred in sarcopenic/frail populations (WMD:0.88, 95% CI:0.51,1.25, I^2:79.0%). Most studies (n=22) had some risk of bias.

Conclusions: In older adults performing RE, increased protein intake leads to greater ALM and HG, compared with lower protein controls. Without RE, protein has no additional benefit on changes in total LBM, ALM or HG.

Registry: PROSPERO ID: CRD42019142045 https://www.crd.york.ac.uk/prospero/

3.3 Introduction

By 2050, more than 1 in 5 people, worldwide, will be over 60 years old (Nations, 2014), but while life expectancy is increasing, health span (years free of disease and disability) is not keeping pace (Salomon et al., 2012). A major contributor to poor health and disability in later life is sarcopenia, the age-associated decline in muscle size, strength and quality (Cruz-Jentoft et al., 2019) which accelerates considerably in one's fifties (Keller and Engelhardt, 2013). Sarcopenia is positively associated with a great variety of non-communicable diseases including cardiovascular disease and type 2 diabetes mellitus, as well as lower quality of life and mortality (Bahat and İlhan, 2016; Tsekoura et al., 2017; Sipers et al., 2019). Decreased muscle strength (dynapenia) precedes a decrease in muscle size (Manini and Clark, 2012)

and muscle strength is used as a principal determinant of sarcopenia in clinical diagnosis (Alley et al., 2014; Cruz-Jentoft et al., 2019).

Reduced activity as we age plays a central role in muscle loss (A et al., 2010; Mijnarends et al., 2016), which itself leads to lower activity levels in older adults (Troiano et al., 2008; Law, Clark and Clark, 2016; Lee et al., 2018) further compounding muscle loss (Derbre et al., 2014; Aggio et al., 2016). Additionally, unfavourable hormonal changes (Gray et al., 1991; Baulieu, 2002; Deschenes, 2004), increases in oxidative stress (Bellanti et al., 2018), and inflammation (Visser et al., 2002; Kalinkovich and Livshits, 2015; Livshits and Kalinkovich, 2019) all contribute to anabolic resistance (the reduced muscle protein synthesis (MPS) response to anabolic stimuli) (Morton et al., 2018). As part of the normal aging process, muscle loss is considered primary sarcopenia but when associated with other pathologies, such as diabetes, the muscle loss is considered secondary sarcopenia (Collins et al., 2018; Rubio-Ruiz et al., 2019).

Observational studies have identified that higher protein intakes (1.2 *vs.* the recommended 0.8 g/kg body weight/day) may help counteract reduced muscle mass and function associated with aging (Houston et al., 2008b; Isanejad et al., 2015). Dietary protein stimulates muscle protein synthesis and inhibits muscle protein break down, leading to the maintenance or even accretion of lean body mass (LBM) over time (Atherton and Smith, 2012). This effect is further enhanced when protein is consumed following resistance exercise (RE) (Atherton and Smith, 2012; Trommelen, Betz and van Loon, 2019), thus strengthening the rationale for the benefits of higher protein intakes when combined with exercise.

Alongside whole protein, interventions to augment LBM in older adults may also use amino acids, vitamins, creatine and essential fatty acids (Kirwan et al., 2020). Previous meta-

analyses on the effect of protein on LBM have not excluded such substances, rendering it impossible to determine the effect of protein in isolation (Tieland et al., 2017; Hou et al., 2019). Therefore an analysis of the effects of RCTs using protein-only interventions, with or without RE is needed. Furthermore, the accrual of LBM is believed to be influenced by numerous factors including per-meal-protein-dose, protein frequency and duration of intervention (Bauer et al., 2013a), leading to considerable heterogeneity in interventions aimed at increasing LBM. Therefore, further investigation of the effects of these variables is warranted.

To investigate the role of increased protein in increasing LBM we completed a systematic review and meta-analysis of RCTs assessing the effect of protein supplementation or higher-protein diets, without the use of EAAs or supplements known to stimulate hypertrophy, with or without concomitant RE interventions, on LBM, appendicular lean mass (ALM) and strength in older adults.

3.4 Methods

The systematic review protocol was performed in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) statement guidelines (Liberati et al., 2009). The meta-analysis was carried out following the criteria outlined in the Cochrane Handbook for Systematic Reviews of Interventions (Higgins et al., 2019b). The protocol was registered with PROSPERO (ID: CRD42019142045).

3.4.1 Search strategy

PubMed, Medline, Web of Science and Cochrane CENTRAL databases were searched from January 1990 until July 17th, 2021, limiting searches to human RCTs in English language. The following search strategy and keywords were used, as presented, in each database: (diet OR

dietary) AND (protein OR whey OR soy OR egg OR casein OR pea) AND (strength OR "lean mass" OR muscle OR "muscle mass" OR hypertrophy OR "body composition") AND (adult* OR "older adult*" OR elder*).

3.4.2 Study selection criteria

Two independent investigators (RPK and CRG) screened titles and abstracts for relevant studies. We included only RCTs that assessed the effects of protein supplementation and/or higher protein dietary interventions in older adults (mean age ≥ 50 years) (Keller and Engelhardt, 2013) on LBM or ALM (primary outcome), and if available, strength (secondary outcome) (Table 3.1). Acceptable measures of LBM were limited to dual-energy X-ray absorptiometry (DXA), bioimpedance analysis (BIA), hydrostatic weighing, air-displacement plethysmography and/or magnetic resonance imaging (MRI). Acceptable measures of strength included handgrip strength (HG) or any reproducible test of 1 repetition maximum (1RM) strength, measured in kilograms. Studies were required to specify duration and only those with a dietary intervention of a minimum of 6 weeks duration were included. Interventions with an energy intake restriction were excluded.

Studies involving supplementation with amino acids, vitamins, performance enhancing drugs and other supplements known to stimulate hypertrophy (such as creatine or *n-3* fatty acids), or studies which did not have at least one intervention group without these substances, were excluded. Studies in populations suffering from pathologies other than sarcopenia and frailty (*e.g.,* cancer, cardiovascular disease, diabetes etc.) were also excluded.

Table 3.1 Inclusion and exclusion criteria

Inclusion Criteria	Exclusion Criteria
Population	**Population**
・Mean age >50 years ・Male and/or female ・Healthy, frail or sarcopenic	・Individuals with pathologies including cardiovascular disease, type 2 diabetes, cancer, cachexia, chronic kidney disease, immunodeficiency disease etc
Intervention	**Intervention**
・Randomized controlled trial ・Supplementary protein, high-protein food or high-protein dietary intervention ・Non-supplemented control or protein intake lower than intervention group ・With or without resistance exercise ・Minimum duration of 6 weeks	・Isolated amino acids ・Anabolic steroids, hormones, vitamins or supplements known to induce hypertrophy
Primary outcomes ・Lean body mass or fat-free mass (kg) ・Appendicular lean mass or skeletal muscle mass (kg) ・Measured using dual-energy X-ray absorptiometry (DXA), bioimpedance analysis (BIA), hydrostatic weighing, air-displacement plethysmography and/or magnetic resonance imaging (MRI)	
Secondary outcomes ・Hand grip strength or 1-repetition maximum strength test (kg)	
Other	**Other**
・Full paper ・English language	・Protocol papers ・Abstract only

3.4.3 Data extraction

Two investigators (RPK and CRG) independently extracted data from the original publications. Data on sex, age, health status, baseline protein intake (where available), protein amount, protein source, intervention duration and baseline and endpoint measurements of LBM and/or strength measures were extracted. Where available, data on ALM was extracted. Strength measures for handgrip, knee extension (KE) or leg press were extracted only if absolute measures were available in kilograms. Information on adverse events was also extracted. Any differences in extracted data were resolved by consultation (RPK and CRG) and if necessary, with a third author (MM) until consensus was reached. In order to avoid double counting of control arms, where multiple treatment arms were used with only one control group, priority was given to treatment arms with: RE; dairy proteins; or post-exercise protein. Where data was available in graph form, numerical data was extracted using WebPlotDigitizer (Version 4.3, 2020; https://automeris.io/WebPlotDigitizer). Where necessary data was not available in the original publication, corresponding authors were contacted and asked to provide said data. Where data was not forthcoming, the article was not included in the specific meta-analysis.

3.4.4 Risk of Bias Assessment

Risk of bias of RCTs was evaluated independently by two investigators (RPK and CRG). The assessment was performed at the study level with the revised Cochrane risk of bias tool which grades the risk of selection, performance, attrition, detection, and reporting biases (Sterne et al., 2019). This tool assesses whether a study has a low, unclear, or high risk of bias. Differences in opinion were resolved by group consultation (RPK, CRG and KL) until consensus was reached.

3.4.5 Statistical analysis

Following the recommendation of the Cochrane Handbook, to calculate the effect size, we used the mean change from baseline to end point in the measures and standard deviation (SD) of the variables of interest for both control and intervention groups (Higgins et al., 2019a). For RCTs, the net changes in measurements (change scores) were calculated as: Net change score = (FT – BT) − (FC – BC) (where FT and BT are the measures at the end of follow-up and at baseline, respectively, in the treatment group, and FC and BC are the corresponding measures in the control group). The net changes in SD of measurements were calculated as: square root $[(SD_{BT})^2 + (SD_{FT})^2 - (2\times R\times SD_{BT}\times SD_{FT})]$ used a correlation coefficient (R) as 0.9 (Borenstein et al., 2011) (where SD_{FT} and SD_{BT} are the SD of measures at the end of follow-up and at baseline, respectively, in the treatment group, and SD_{FC} and SD_{BC} are the corresponding SD of the measures in the control group). Studies reported median with interquartile ranges or 95% CIs converted to mean and SD (Wan et al., 2014). Standard errors (SEs) were converted to SDs using the following formula: SD = SEM × √n, where n is the number of participants.

A random-effects model (using the DerSimonian-Laird method) and the generic inverse variance method were used to derive pooled estimates across studies (Huedo-Medina et al., 2006). Data were expressed as weighted mean differences (WMDs) with 95 % confidence intervals (CIs). Random effects meta-regression was performed using the unrestricted maximum likelihood method to evaluate the association between exposure and primary outcome of interest with potential moderators when sufficient data was available. Analyses included pooled analysis of total LBM, ALM, HG and KE along with sub-group analysis of each outcome according to inclusion or exclusion of RE intervention. A further sub-group analysis by health status (healthy or sarcopenic/sarcopenic obese/frail) was performed for the primary outcomes of lean mass (total LBM and ALM). Meta-regression was performed for all outcomes

based on baseline outcome measure (Total LBM, ALM, HG and KE, respectively) and, baseline protein intake, per-meal protein dose and intervention duration, and age. Heterogeneity was quantitatively assessed using the I^2 index and Cochrane Q statistic, which measures the extent of true heterogeneity (Huedo-Medina et al., 2006). It can be interpreted as the percentage of the total variability in a set of effect sizes due to true heterogeneity, that is, due to between-study variability. Low, moderate, and high I^2 values are 25%, 50%, and 75%, respectively. However, heterogeneity is to be expected in meta-analyses involving different study designs and as such should be quantified with values such as tau-squared (τ^2) (Higgins, 2008). Additionally, subgroup analysis according to the exercise status was performed to detect potential sources of heterogeneity. A leave-one-out sensitivity analysis was performed by iteratively removing 1 study at a time to confirm that our findings were not driven by any single study.

We visually inspected the Begg's funnel plot asymmetry and Egger's weighted regression tests to evaluate the potential publication bias when at least 10 studies were involved (Duval and Tweedie, 2000). If publication bias was suspected, this step was followed by adjusting the analysis for the effects of publication bias using the Duval & Tweedie 'trim and fill' methods (Duval and Tweedie, 2000). All analyses were conducted using STATA software, version 16 (StataCorp, College Station, TX). The statistically significant was considered as P values < 0.05.

3.5 Results

3.5.1 Flow and characteristics of included studies

Figure 3.1 shows the flowchart of studies in the review process. After removal of duplicates, 5,680 records were identified by the initial literature search. Through review of titles and

abstracts, 99 potentially relevant articles were selected for full-text evaluation. Subsequently, 28 eligible randomized controlled studies met the inclusion criteria (Campbell et al., 1994; Iglay et al., 2009; Verdijk et al., 2009; Carlsson et al., 2011; Aleman-Mateo et al., 2012; Bjorkman, Finne-Soveri and Tilvis, 2012; Tieland et al., 2012a; Tieland et al., 2012b; Arnarson et al., 2013; Chale et al., 2013; Leenders et al., 2013; Shahar et al., 2013; Aleman-Mateo et al., 2014; Gryson et al., 2014; Zdzieblik et al., 2015; Zhu et al., 2015; Thomson et al., 2016; Dirks et al., 2017; Mitchell et al., 2017; Ottestad et al., 2017; Rossato et al., 2017; Nabuco et al., 2018; Sugihara Junior et al., 2018; Nabuco et al., 2019a; Nabuco et al., 2019b; Nahas et al., 2019; ten Haaf et al., 2019; Li et al., 2021). Due to lack of primary data, 6 of the 28 retrieved papers were not included in the meta-analyses (Iglay et al., 2009; Carlsson et al., 2011; Bjorkman, Finne-Soveri and Tilvis, 2012; Arnarson et al., 2013; Zhu et al., 2015; Ottestad et al., 2017) (Table 3.2)

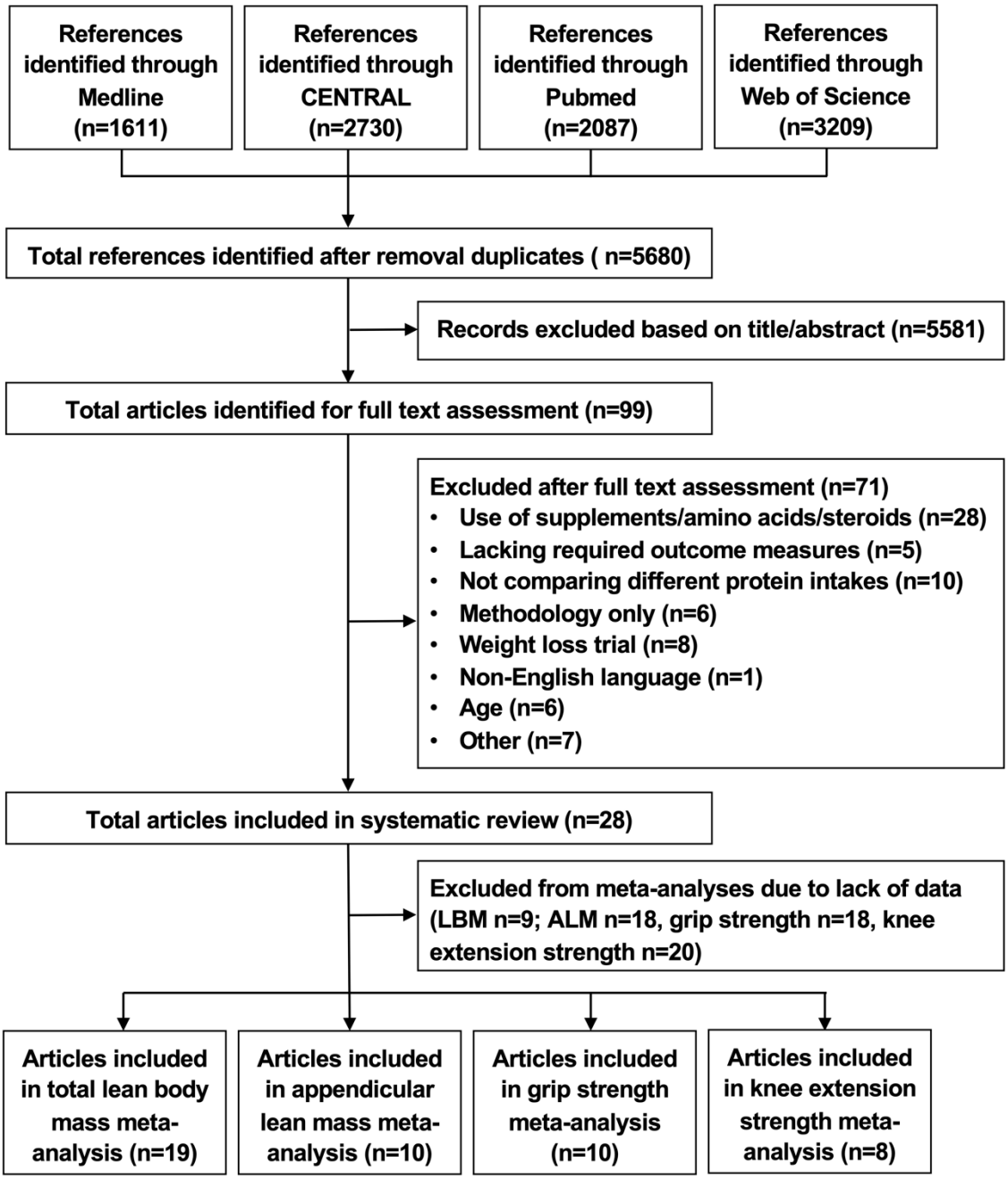


Figure 3.1 Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA) flowchart of studies through systematic review process. ALM, appendicular lean mass; LBM, lean body mass.

The characteristics of the studies included in the systematic review are presented in Table 3.2. Briefly, studies ranged in size from 12 to 196 participants per study, with mean ages of participants ranging from 61 to 85 years. Of the included study populations, 17 were healthy (Campbell et al., 1994; Iglay et al., 2009; Verdijk et al., 2009; Bjorkman, Finne-Soveri and Tilvis, 2012; Arnarson et al., 2013; Leenders et al., 2013; Aleman-Mateo et al., 2014; Gryson et al., 2014; Zhu et al., 2015; Thomson et al., 2016; Mitchell et al., 2017; Rossato et al., 2017; Nabuco et al., 2018; Sugihara Junior et al., 2018; Nabuco et al., 2019b; Nahas et al., 2019; ten Haaf et al., 2019) while 6 were considered frail (Carlsson et al., 2011; Tieland et al., 2012a; Tieland et al., 2012b; Chale et al., 2013; Dirks et al., 2017; Ottestad et al., 2017), 4 were sarcopenic (Aleman-Mateo et al., 2012; Shahar et al., 2013; Zdzieblik et al., 2015; Li et al., 2021) and 1 included participants with sarcopenic obesity (Nabuco et al., 2019a). Study durations ranged from 10 weeks (3 studies) (Mitchell et al., 2017; Rossato et al., 2017; Nahas et al., 2019) to 12 weeks (15 studies) (Campbell et al., 1994; Iglay et al., 2009; Verdijk et al., 2009; Aleman-Mateo et al., 2012; Arnarson et al., 2013; Shahar et al., 2013; Aleman-Mateo et al., 2014; Zdzieblik et al., 2015; Thomson et al., 2016; Ottestad et al., 2017; Nabuco et al., 2018; Sugihara Junior et al., 2018; Nabuco et al., 2019a; Nabuco et al., 2019b; ten Haaf et al., 2019), 13 weeks (1 study) (Carlsson et al., 2011), 16 weeks (1 study) (Gryson et al., 2014), 24 weeks (7 studies) (Bjorkman, Finne-Soveri and Tilvis, 2012; Tieland et al., 2012a; Tieland et al., 2012b; Chale et al., 2013; Leenders et al., 2013; Dirks et al., 2017; Li et al., 2021) and 104 weeks (1 study) (Zhu et al., 2015).

Table 3.2. Participant characteristics and intervention details of the 25 included studies.

Author		n	Mean age (years)	Health Status	Baseline protein intake (g/kg/d)[1]	Intervention/ Control	Frequency of protein intervention (per week)	Added protein dose (g/dose)	Total added protein (g/day)	Resistance Exercise Protocol	Duration (weeks)	Main finding, Intervention *vs* Control	Included in meta-analysis
Aleman-Mateo *et al* (2012)	Intervention	20	76	Sarcopenic	N/A	Ricotta cheese	21	5.2	16	No	12	ALM, LBM in arms and muscle strength ↑ in men only	Yes
	Control	20			N/A	Habitual diet							
Aleman-Mateo *et al* (2014)	Intervention	50	70.2	Healthy	N/A	Ricotta cheese	21	6	18	No	12	ALM and physical performance ↑	Yes
	Control	50			N/A	Habitual diet							
Arnarson *et al* (2013)	Intervention	75	74	Healthy	1.0 ± 0.3	Whey protein	3	20	20	Yes	12	No greater gains in lean mass, strength, or physical function	No
	Control	66			0.9 ± 0.3	CHO[2]							
Bjorkman *et al* (2012)	Intervention	46	83.6	Healthy	N/A	Whey protein in juice	21	6.7	20	No	24	Body weight ↑ and maintenance of skeletal muscle mass	No
	Control	51			N/A	Juice							
Campbell *et al* (1994)	Intervention	6	65	Healthy	N/A	Higher protein diet plan	N/A	N/A	63	Yes	12	No greater increase in LBM	Yes
	Control	6			N/A	Lower protein diet plan							
Carlsson *et al* (2011)	Intervention	89	84.5	Frail	N/A	Milk protein	2.5	7.4	7	Yes	13	No greater increase in LBM	No
	Control	88			N/A	Placebo							
Chale *et al* (2013)	Intervention	42	77.7	Frail	0.91[3]	Whey protein	14	20	40	Yes	24	No greater increases in LBM, strength, power, or physical function	Yes
	Control	38			0.93[3]	Isocaloric control							
Dirks *et al* (2017)	Intervention	17	76.5	Frail	N/A	Milk protein	14	15	30	Yes	24	Type I and type II muscle fiber hypertrophy ↑	Yes
	Control	17			N/A	Placebo							
Gryson *et al* (2014)	Intervention	27	60.8	Healthy	N/A	Milk protein or whey protein	7	10	10	Yes	16	Muscle mass and strength ↑	Yes
	Control	18			N/A	Placebo							

												and muscle fatigue ↓	
Iglay *et al* (2009)	Intervention	18	61	Healthy	N/A	Higher protein diet plan	N/A		6	Yes	12	No greater increase in LBM	No
	Control	18			N/A	Lower protein diet plan							
Leenders *et al* (2013)	Intervention	27	70	Healthy	1.2[3]	Milk protein	7	15	15	Yes	24	No greater increases in LBM, strength, or functional capacity	Yes
	Control	26			1.2[3]	CHO placebo							
Li *et al* (2021)	Intervention	31	71	Sarcopenic	N/A	Whey protein	14	7.9	15.8	No	24	No greater gains in LBM, ALM or grip strength	Yes
	Control	30			N/A	Habitual diet							
Mitchell *et al* (2017)	Intervention	15	74.2	Healthy	1.1 ± 0.3	Higher protein diet plan	N/A	N/A	48	No	10	LBM and knee-extension power output ↑	Yes
	Control	16			1.2 ± 0.4	Lower protein diet plan							
Nabuco *et al* (2018	Intervention	43	66.7	Healthy	0.93 ± 0.36	Whey protein	3	35	35	Yes	12	ALM, muscular strength, and functional capacity ↑	Yes
	Control	23			0.97 ± 0.28	CHO placebo							
Nabuco *et al* (2019 A)	Intervention	13	69.1	Sarcopenic obese	0.93[3]	Whey protein	3	35	35	Yes	12	ALM ↑ and trunk fat mass ↓	Yes
	Control	13			0.95 ± 0.27	CHO placebo							
Nabuco *et al* (2019 B)	Intervention	15	69.2	Healthy	0.94 ±0.3	Whey protein	3	35	35	Yes	12	LBM ↑ and waist circumference and body fat ↓	Yes
	Control	15			0.94 ±0.3	CHO placebo							
Nahas *et al* (2019)	Intervention	22	63.4	Healthy	0.76 ± 0.05	Higher protein diet plan	N/A		23	Yes	10	Functional capacity but no additional increase in strength and LBM ↑	Yes
	Control	25			0.76 ± 0.06	Lower protein diet plan							
Ottestad *et al* (2016)	Intervention	17	77	Frail	1.0 ± 0.3	Milk protein	14	20	40	No	12	No greater increase in LBM or strength	No
	Control	19			1.0 ± 0.3	Isocaloric control							
Rossato *et al* (2017)	Intervention	11	63.2	Healthy	0.79[3]	Higher protein diet plan	N/A	20-30	24	Yes	10	No greater increase in LBM	Yes
	Control	12			0.75[3]	Lower protein diet plan							

Shahar *et al* (2013)	Intervention	30	67.1	Sarcopenic	0.83[3]	Soy protein	7	20 (M), 40 (W)	20 (M), 40 (W)	Yes	12	Upper body strength ↑ but no greater increase in LBM	Yes
	Control	35			0.91[3]	Habitual diet							
Sugihara Junior *et al* (2018)	Intervention	15	67.6	Healthy	0.85 ± 0.1	Whey protein	3	27	35	Yes	12	ALM and strength ↑	Yes
	Control	16			0.81 ± 0.1	Isocaloric CHO control							
ten Haaf *et al* (2019)	Intervention	58	69	Healthy	0.86 ± 0.23	Milk protein	14	15	31	No	12	LBM ↑ and fat mass ↓	Yes
	Control	56			0.92 ± 0.24	Isocaloric control							
Thomson *et al* (2016	Intervention	118	61.5	Healthy	N/A	Dairy protein or soy protein	7	27	27	Yes	12	No greater increases in LBM, strength, or physical function	Yes
	Control	61			N/A	Habitual diet							
Tieland *et al* (2012 A)	Intervention	34	79.5	Frail	1.0 ± 0.0	Milk protein	14	15	30	No	24	Physical performance ↑ no greater increase in ALM	Yes
	Control	31			1.0 ± 0.0	Placebo							
Tieland *et al* (2012 B)	Intervention	31	78	Frail	1.0 ± 0.1	Milk protein	14	15	30	Yes	24	LBM and ALM ↑, no greater increase in strength	Yes
	Control	31			1.0 ± 0.1	Placebo							
Verdijk *et al* (2009)	Intervention	13	72	Healthy	1.1 ± 0.1	Casein protein	3	10 (pre-exercise), 10 (post-exercise)	20	Yes	12	No greater increase in LBM or strength	Yes
	Control	13			1.1 ± 0.1	Placebo							
Zdzieblik *et al* (2015)	Intervention	26	74.3	Sarcopenic	N/A	Collagen protein	7	15	15	Yes	12	LBM and strength ↑ fat mass ↓	Yes
	Control	27			N/A	Silica placebo							
Zhu *et al* (2015)	Intervention	101	74.3	Healthy	1.2 ± 0.3	Milk plus whey protein	7	30	30	No	104	No greater increase in LBM or physical function	No
	Control	95			1.1 ± 0.3	Isocaloric skim milk placebo							

[1]Mean ± SD where available, or calculated means from available data; [2]CHO: carbohydrate; [3]Value calculated from available data; M: men; W: women

3.5.1.1 Protein interventions

Protein intake was increased in intervention groups using supplementary protein drinks (21 studies) (Verdijk et al., 2009; Carlsson et al., 2011; Bjorkman, Finne-Soveri and Tilvis, 2012; Tieland et al., 2012a; Tieland et al., 2012b; Arnarson et al., 2013; Chale et al., 2013; Leenders et al., 2013; Shahar et al., 2013; Gryson et al., 2014; Zdzieblik et al., 2015; Zhu et al., 2015; Thomson et al., 2016; Dirks et al., 2017; Ottestad et al., 2017; Nabuco et al., 2018; Sugihara Junior et al., 2018; Nabuco et al., 2019a; Nabuco et al., 2019b; ten Haaf et al., 2019; Li et al., 2021), higher protein diet plans (5 studies) (Campbell et al., 1994; Iglay et al., 2009; Mitchell et al., 2017; Rossato et al., 2017; Nahas et al., 2019) and supplementary protein foods (2 studies) (Aleman-Mateo et al., 2012; Aleman-Mateo et al., 2014). Frequency of supplementary protein intake (excluding studies using high-protein diet plans) ranged from 2 times per week (1 study) (Carlsson et al., 2011) to 3 times per week (6 studies) (Verdijk et al., 2009; Arnarson et al., 2013; Nabuco et al., 2018; Sugihara Junior et al., 2018; Nabuco et al., 2019a; Nabuco et al., 2019b), 7 times per week (once daily) (6 studies) (Leenders et al., 2013; Shahar et al., 2013; Gryson et al., 2014; Zdzieblik et al., 2015; Zhu et al., 2015; Thomson et al., 2016), 14 times per week (twice daily) (7 studies) (Tieland et al., 2012a; Tieland et al., 2012b; Chale et al., 2013; Dirks et al., 2017; Ottestad et al., 2017; ten Haaf et al., 2019; Li et al., 2021) and 21 times per week (3 times daily) (3 studies) (Aleman-Mateo et al., 2012; Bjorkman, Finne-Soveri and Tilvis, 2012; Aleman-Mateo et al., 2014). Per-meal supplementary protein dose also varied with ranges of 5-9 g (5 studies) (Carlsson et al., 2011; Aleman-Mateo et al., 2012; Bjorkman, Finne-Soveri and Tilvis, 2012; Aleman-Mateo et al., 2014; Li et al., 2021), 10-19 g (8 studies) (Verdijk et al., 2009; Tieland et al., 2012a; Tieland et al., 2012b; Leenders et al., 2013; Gryson et al., 2014; Zdzieblik et al., 2015; Dirks et al., 2017; ten Haaf et al., 2019), 20-29 g (6 studies) (Arnarson et al., 2013; Chale et al., 2013; Shahar et al., 2013; Thomson et al., 2016; Ottestad et al., 2017; Rossato et al., 2017; Sugihara Junior et al., 2018) and ≥30 g (5 studies) (Shahar et al., 2013; Zhu et al., 2015; Nabuco et al., 2018; Nabuco et al., 2019a;

Nabuco et al., 2019b). Sources of supplementary protein included whey protein (10 studies) (Bjorkman, Finne-Soveri and Tilvis, 2012; Arnarson et al., 2013; Chale et al., 2013; Gryson et al., 2014; Zhu et al., 2015; Nabuco et al., 2018; Sugihara Junior et al., 2018; Nabuco et al., 2019a; Nabuco et al., 2019b; Li et al., 2021), mixed milk protein (10 studies) (Carlsson et al., 2011; Tieland et al., 2012a; Tieland et al., 2012b; Leenders et al., 2013; Gryson et al., 2014; Zhu et al., 2015; Thomson et al., 2016; Dirks et al., 2017; Ottestad et al., 2017; ten Haaf et al., 2019), ricotta cheese (2 studies) (Aleman-Mateo et al., 2012; Aleman-Mateo et al., 2014), soy protein (1 study) (Shahar et al., 2013), casein (1 study) (Verdijk et al., 2009) and collagen (1 study) (Zdzieblik et al., 2015).

3.5.1.2 Exercise interventions

Of the 28 articles included in this review, 19 made use of RE in at least one arm of their intervention (Campbell et al., 1994; Iglay et al., 2009; Verdijk et al., 2009; Carlsson et al., 2011; Tieland et al., 2012a; Arnarson et al., 2013; Chale et al., 2013; Leenders et al., 2013; Shahar et al., 2013; Gryson et al., 2014; Zdzieblik et al., 2015; Thomson et al., 2016; Dirks et al., 2017; Rossato et al., 2017; Nabuco et al., 2018; Sugihara Junior et al., 2018; Nabuco et al., 2019a; Nabuco et al., 2019b; Nahas et al., 2019). The frequency of RE was relatively consistent, ranging from twice per week (5 studies) (Carlsson et al., 2011; Tieland et al., 2012a; Shahar et al., 2013; Dirks et al., 2017; Nabuco et al., 2019b) to 3 times per week (14 studies) (Campbell et al., 1994; Iglay et al., 2009; Verdijk et al., 2009; Arnarson et al., 2013; Chale et al., 2013; Leenders et al., 2013; Gryson et al., 2014; Zdzieblik et al., 2015; Thomson et al., 2016; Rossato et al., 2017; Nabuco et al., 2018; Sugihara Junior et al., 2018; Nabuco et al., 2019a; Nahas et al., 2019). The RE involved numerous different protocols including resistance machines only (9 studies) (Campbell et al., 1994; Iglay et al., 2009; Verdijk et al., 2009; Tieland et al., 2012a; Arnarson et al., 2013; Chale et al., 2013; Leenders et al., 2013; Thomson et al., 2016; Dirks et al., 2017), machines and free-weights (8 studies) (Gryson et al., 2014; Zdzieblik

et al., 2015; Rossato et al., 2017; Nabuco et al., 2018; Sugihara Junior et al., 2018; Nabuco et al., 2019a; Nabuco et al., 2019b; Nahas et al., 2019), elastic resistance bands (Shahar et al., 2013) (1 study) and high intensity functional exercise (Carlsson et al., 2011) (1 study). The number of repetitions used in all but one study ranged from 6 to15. The remaining study, which used elastic resistance bands (Shahar et al., 2013), did not provide data on the number of repetitions used. All 19 studies which made use of RE incorporated some form of progressive resistance *i.e.*, the intensity, resistance, or volume of the exercises performed were increased over the course of the intervention period.

3.5.1.3 Outcome measures

The majority of articles included measured body composition using DXA (23 studies) (Iglay et al., 2009; Verdijk et al., 2009; Aleman-Mateo et al., 2012; Tieland et al., 2012a; Tieland et al., 2012b; Arnarson et al., 2013; Chale et al., 2013; Leenders et al., 2013; Aleman-Mateo et al., 2014; Gryson et al., 2014; Zdzieblik et al., 2015; Zhu et al., 2015; Thomson et al., 2016; Dirks et al., 2017; Mitchell et al., 2017; Ottestad et al., 2017; Rossato et al., 2017; Nabuco et al., 2018; Sugihara Junior et al., 2018; Nabuco et al., 2019a; Nahas et al., 2019; ten Haaf et al., 2019; Li et al., 2021) with the remaining articles using BIA (4 studies) (Carlsson et al., 2011; Bjorkman, Finne-Soveri and Tilvis, 2012; Shahar et al., 2013; Nabuco et al., 2019b) and hydrostatic weighing (1 study) (Campbell et al., 1994). Data was extracted for LBM and ALM, where available. Strength and muscle function measures varied greatly amongst the included studies and two strength measures were selected for meta-analysis due to their frequency of use and the availability of data: handgrip (10 studies) (Aleman-Mateo et al., 2012; Tieland et al., 2012a; Tieland et al., 2012b; Shahar et al., 2013; Aleman-Mateo et al., 2014; Thomson et al., 2016; Dirks et al., 2017; Mitchell et al., 2017; ten Haaf et al., 2019; Li et al., 2021); and 1 RM knee extension (8 studies) (Verdijk et al., 2009; Tieland et al., 2012a; Tieland et al., 2012b;

Dirks et al., 2017; Nabuco et al., 2018; Sugihara Junior et al., 2018; Nabuco et al., 2019a; Nahas et al., 2019).

Amongst the 28 studies included in the systematic review, only 13 reported on whether intention-to-treat (ITT) or per-protocol (PP) analysis was used (Carlsson et al., 2011; Aleman-Mateo et al., 2012; Tieland et al., 2012a; Tieland et al., 2012b; Chale et al., 2013; Aleman-Mateo et al., 2014; Zdzieblik et al., 2015; Zhu et al., 2015; Thomson et al., 2016; Mitchell et al., 2017; Nahas et al., 2019; ten Haaf et al., 2019; Li et al., 2021). Of these, 9 studies published data from intention-to-treat (ITT) analyses (Carlsson et al., 2011; Aleman-Mateo et al., 2012; Tieland et al., 2012a; Tieland et al., 2012b; Chale et al., 2013; Aleman-Mateo et al., 2014; Zhu et al., 2015; Nahas et al., 2019; Li et al., 2021) and 4 published data from per-protocol (PP) analyses (Zdzieblik et al., 2015; Thomson et al., 2016; Mitchell et al., 2017; ten Haaf et al., 2019). As only one set of data was available from each of the studies (ITT or PP), no particular set of data was prioritized in the data extraction for our study. Five studies (Tieland et al., 2012b; Chale et al., 2013; Aleman-Mateo et al., 2014; Zhu et al., 2015; Thomson et al., 2016) completed both analyses, but published results from only one, and in all cases, it was specified that the results were similar in both analyses.

3.5.2 Adverse Events

Information on adverse events, where available (8 studies) (Aleman-Mateo et al., 2012; Chale et al., 2013; Aleman-Mateo et al., 2014; Zdzieblik et al., 2015; Zhu et al., 2015; Thomson et al., 2016; Mitchell et al., 2017; ten Haaf et al., 2019), is reported in Supplementary Table 3.1.

3.5.3 Risk of Bias Assessment

Risk of bias of RCTs was evaluated with the revised Cochrane risk of bias tool. This tool determined 6 studies had low risk of bias (Bjorkman, Finne-Soveri and Tilvis, 2012; Tieland et al., 2012a; Tieland et al., 2012b; Chale et al., 2013; Zdzieblik et al., 2015; Dirks et al., 2017), 18 studies had some concerns of bias (Campbell et al., 1994; Iglay et al., 2009; Verdijk et al., 2009; Carlsson et al., 2011; Aleman-Mateo et al., 2012; Leenders et al., 2013; Aleman-Mateo et al., 2014; Gryson et al., 2014; Zhu et al., 2015; Thomson et al., 2016; Mitchell et al., 2017; Ottestad et al., 2017; Rossato et al., 2017; Nabuco et al., 2018; Sugihara Junior et al., 2018; Nabuco et al., 2019b; ten Haaf et al., 2019; Li et al., 2021) and 4 studies had high risk of bias (Arnarson et al., 2013; Shahar et al., 2013; Nabuco et al., 2019a; Nahas et al., 2019) (Supplementary Figure 3.1). Regarding dietary protocol adherence, only 11 studies provided details on how this was monitored and included: collection of used supplement containers (Tieland et al., 2012b; Chale et al., 2013; Shahar et al., 2013; Zdzieblik et al., 2015; Zhu et al., 2015; Ottestad et al., 2017; ten Haaf et al., 2019; Li et al., 2021); observation by research staff (Tieland et al., 2012a); adherence phone calls from research staff (Nahas et al., 2019); and dietary counselling with provision of key foods (Thomson et al., 2016).

3.5.4 Meta-analysis

3.5.4.1 Total lean body mass

A pooled estimate of the effect of protein on total LBM using 21 intervention groups involving 967 participants revealed the change in total LBM was not statistically significantly different between the protein intervention and lower protein control groups (weighted mean difference in change (WMD): 0.34, 95% CI: -0.21, 0.89, I^2: 90.01%) (Figure 3.2). Sub-group analysis of those interventions that did not use a RE arm (7 intervention groups) revealed that additional

protein did not result in a change in total LBM compared to the lower protein control group (WMD: 0.18, 95% CI: -0.14, 0.51, I^2: 0.00%) (Figure 3.2). In interventions that did use RE (14 interventions), sub-group analysis revealed the change in LBM was not statistically significantly greater in protein interventions compared with lower protein control groups (WMD: 0.29, 95% CI: -0.45, 1.04, I^2: 93.33%) (Figure 3.2). Results of tests for heterogeneity for no-RE group, RE group, between group and overall were p=0.78, p<0.001, p=0.79 and p<0.001, respectively.

Meta-regression analysis revealed that changes in total LBM were not significantly associated with any of the tested mediators including: baseline total LBM (β = 0.04, 95% CI: 0.0, 0.08, ρ = 0.054, I^2residual = 77.47%); age (β = 0.07, 95% CI: -0.01, 0.14, ρ = 0.1, I^2residual = 75.93%); per-meal protein dose (β = -0.05, 95% CI: -0.11, 0.02, ρ = 0.13, I^2residual = 64.78%); intervention duration (β = 0.07, 95% CI: 0.0, 0.13, ρ = 0.06, I^2residual = 74.47%); baseline protein intake (β = 3.21, 95% CI: -0.53, 6.94, ρ = 0.09, I^2residual = 81.68%); and frequency of protein intervention (β = 0.04, 95% CI: -0.04, 0.12, ρ = 0.28, I^2residual = 63.87%).

Pooled sub-group analysis of studies by health status revealed that neither healthy nor unhealthy (sarcopenic, sarcopenic obese & frail) populations experienced greater increases in total lean body mass compared with lower protein control groups (n=11, WMD: 0.23, 95% CI: -0.49,0.96, p=0.53 and n=10, WMD: 0.5, 95% CI: -0.17,1.17, p=0.15, respectively).

A sensitivity analysis was performed, comparing RCTs that used double blinded, placebo-controlled methodology with those that did not. Sensitivity analysis revealed there was no significant difference in change in total LBM between protein groups and lower protein control groups in double blinded nor non-double blinded studies (n=12, WMD: 0.42, 95% CI: -0.31,1.16, p=0.26 and n=9, WMD: 0.16, 95% CI: -0.26,0.58, p=0.45, respectively).

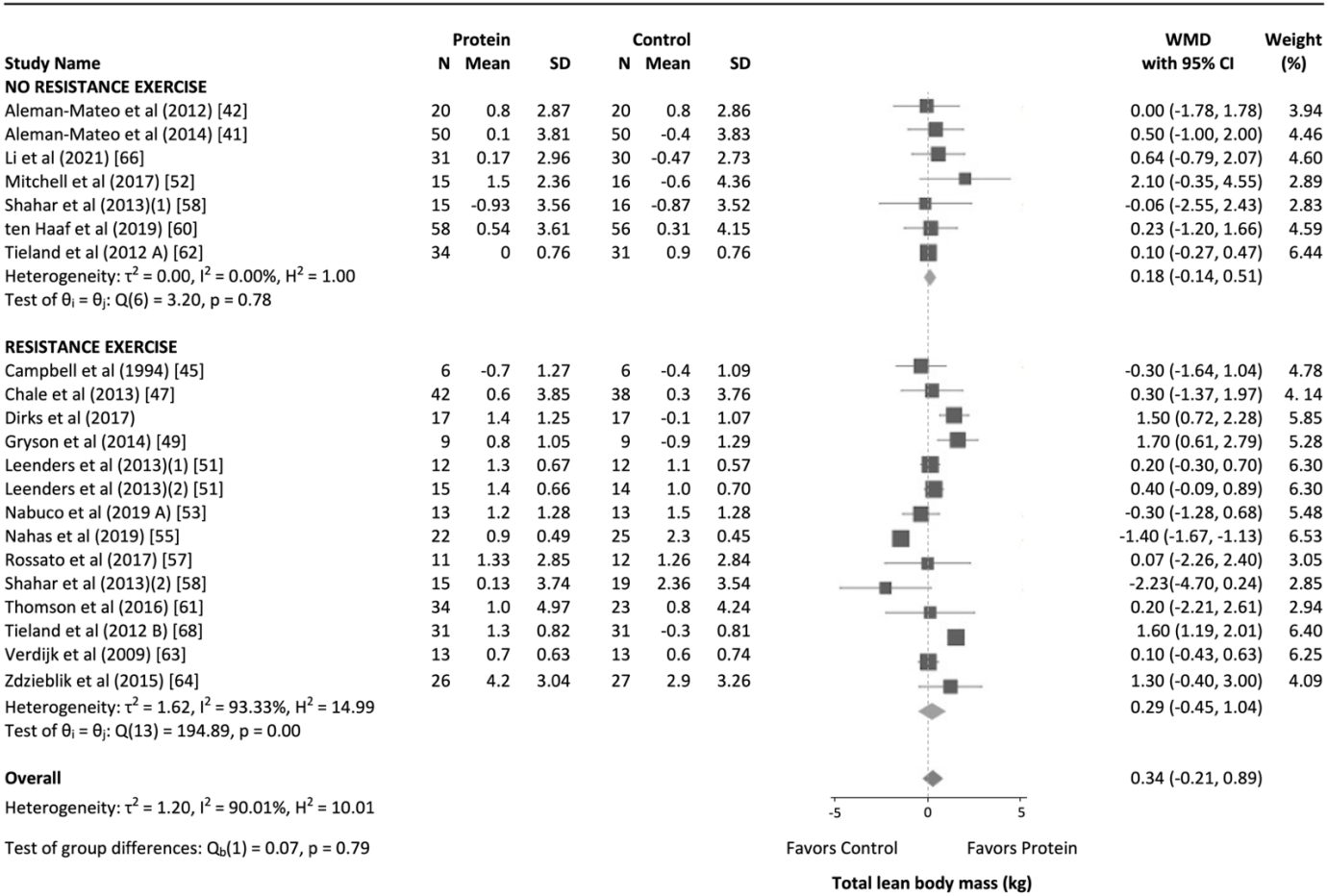


Figure 3.2 Forest plot of standard difference between lower protein control and protein groups on lean body mass of 21 intervention arms organized by trials that did not, and did, use RE. A random effects model (using the DerSimonian-Laird method) and the generic inverse variance method were used to derive pooled estimates across studies. Squares indicate the point estimate for each trial, with the size of the square proportional to the contribution of the study to the overall estimate. The overall estimate and 95% confidence interval are indicated by the diamonds. Where two versions of the same study are mentioned, results from two different intervention arms were reported: Shahar 2013: (1) no resistance exercise, (2) resistance exercise; Leenders 2013: (1) females, (2) males. Nabuco (2019 A & B) and Tieland (2012 A & B) are different studies published in the same year.

3.5.4.2 Appendicular lean mass

A pooled estimate of the effect of protein on ALM using 10 intervention groups involving 470 participants revealed the change in ALM was not statistically significantly different between the protein intervention and lower protein control groups (WMD: 0.4, 95% CI: -0.01, 0.81, I^2: 90.38%) (Figure 3.3). Sub-group analysis of those interventions that did not use a RE arm revealed that additional protein did not result in a change in ALM compared to the lower protein control group (WMD: 0.05, 95% CI: -0.12, 0.21, I^2: 0.00%) (Figure 3.3). However, in interventions that did use RE, sub-group analysis revealed the change in ALM was statistically significantly greater in protein interventions compared with lower protein control groups (WMD: 0.54, 95% CI: 0.03, 1.05, I^2: 89.76%) (Figure 3.3). Results of tests for heterogeneity for no-RE group, RE group, between group and overall were p=0.61, p<0.001, p=0.07 and p<0.001, respectively.

Sub-group analysis of studies by health status and use of RE revealed that only unhealthy (sarcopenic, sarcopenic obese & frail) populations that performed RE experienced greater increases in total lean body mass compared with lower protein control groups (WMD: 0.88, 95% CI: 0.51, 1.25, I^2: 79.0%). No effect was observed in frail populations without RE (WMD: 0.3, 95% CI: -0.13, 0.2, I^2: 0.0%) nor healthy populations with or without RE (WMD: -0.08, 95% CI: -0.96, 0.80, I^2: 75.0% and WMD: 0.26, 95% CI: -0.42, 0.95, I^2: 0.0%, respectively).

Meta-regression analysis revealed that changes in ALM were not significantly associated with any of the tested mediators including: baseline ALM (β = -0.36, 95% CI: -0.16, 0.09, ρ = 0.52, I^2residual = 86.88%); age (β = 0.05, 95% CI: -0.02, 0.11, ρ = 0.16, I^2residual = 83.36%); per-meal protein dose (β = 0.01, 95% CI: -0.03, 0.05, ρ = 0.59, I^2residual = 88.91%); intervention

duration (β = 0.03, 95% CI: -0.03, 0.1, ρ = 0.33, I^2residual = 85.23%); and baseline protein intake (β = 1.7, 95% CI: -18.1, 21.49, ρ = 0.8, I^2residual = 91.88%).

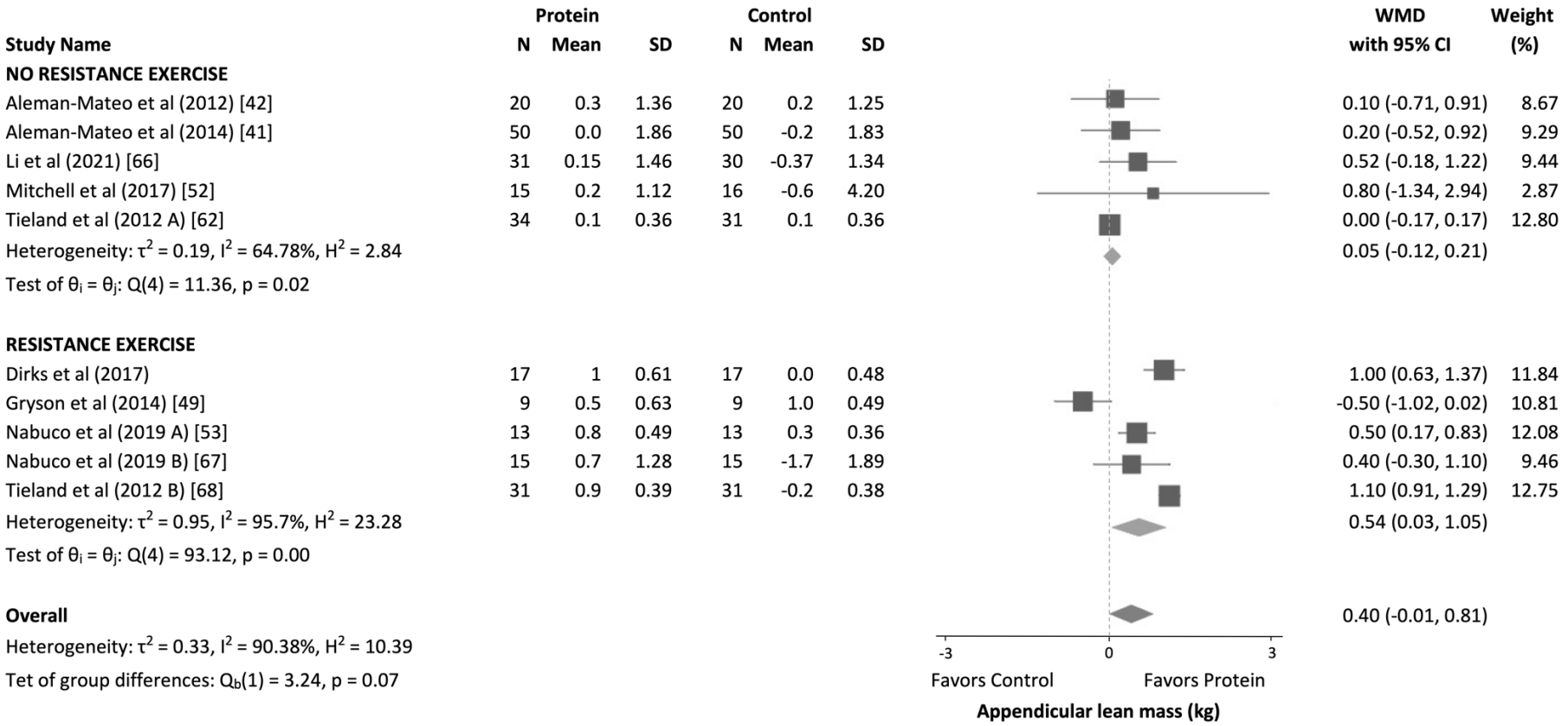


Figure 3.3 Forest plot of standard difference between control and protein groups on appendicular lean mass of 10 intervention arms organized by trials that did not, and did, use RE. A random effects model (using the DerSimonian-Laird method) and the generic inverse variance method were used to derive pooled estimates across studies. Squares indicate the point estimate for each trial, with the size of the square proportional to the contribution of the study to the overall estimate. The overall estimate and 95% confidence interval are indicated by the diamonds. Nabuco (2019 A & B) and Tieland (2012 A & B) are different studies published in the same year

3.5.4.3 Handgrip strength

A pooled estimate of the effect of protein on HG using 11 intervention groups involving 629 participants revealed the change in HG was not statistically significantly different between the protein intervention and lower protein control groups (WMD: 0.69, 95% CI: -0.69, 2.06, I^2: 94.52%). Sub-group analysis of those interventions that did not use a RE arm revealed that additional protein did not result in a change in HG compared to the lower protein control group (WMD: -0.01, 95% CI: -0.39, 0.38, I^2: 0.00%) (Figure 3.4). However, in interventions that did use RE, sub-group analysis revealed the change in HG was statistically significantly greater in protein interventions compared with lower protein control groups (WMD: 1.71, 95% CI: 0.12, 3.3, I^2: 88.71%) (Figure 3.4). Results of tests for heterogeneity for no-RE group, RE group, between group and overall were p=0.55, p<0.001, p=0.04 and p<0.001, respectively.

Sub-group analysis of studies by health status and use of RE revealed that only unhealthy (sarcopenic, sarcopenic obese & frail) populations that performed RE experienced greater increases in HG compared with lower protein control groups (WMD: 2.06, 95% CI: 0.66, 3.47, I^2: 84.3%). No effect was observed in frail populations without RE (WMD: 0.0, 95% CI: -0.41, 0.41, I^2: 0.0%) nor healthy populations with (n=1) or without RE (WMD: -1.0, 95% CI: -3.35, 1.35 and WMD: 0.1, 95% CI: -1.59, 1.79, I^2: 42.99%, respectively).

Meta-regression analysis revealed that changes in HG were not significantly associated with any of the tested mediators including: baseline handgrip strength (β = 0.02, 95% CI: -0.13, 0.17, ρ = 0.79, I^2residual = 91.62%); age (β = 0.19, 95% CI: -0.02, 0.39, ρ = 0.07, I^2residual = 87.04%); per-meal protein dose (β = -0.04, 95% CI: -0.26, 0.18, ρ = 0.69, I^2residual =

92.68%); intervention duration (β = 0.12, 95% CI: -0.07, 0.31, ρ = 0.19, I^2residual = 88.99%); and baseline protein intake (β = 14.36, 95% CI: -10.75, 39.47, ρ = 0.19, I^2residual = 93.88%).

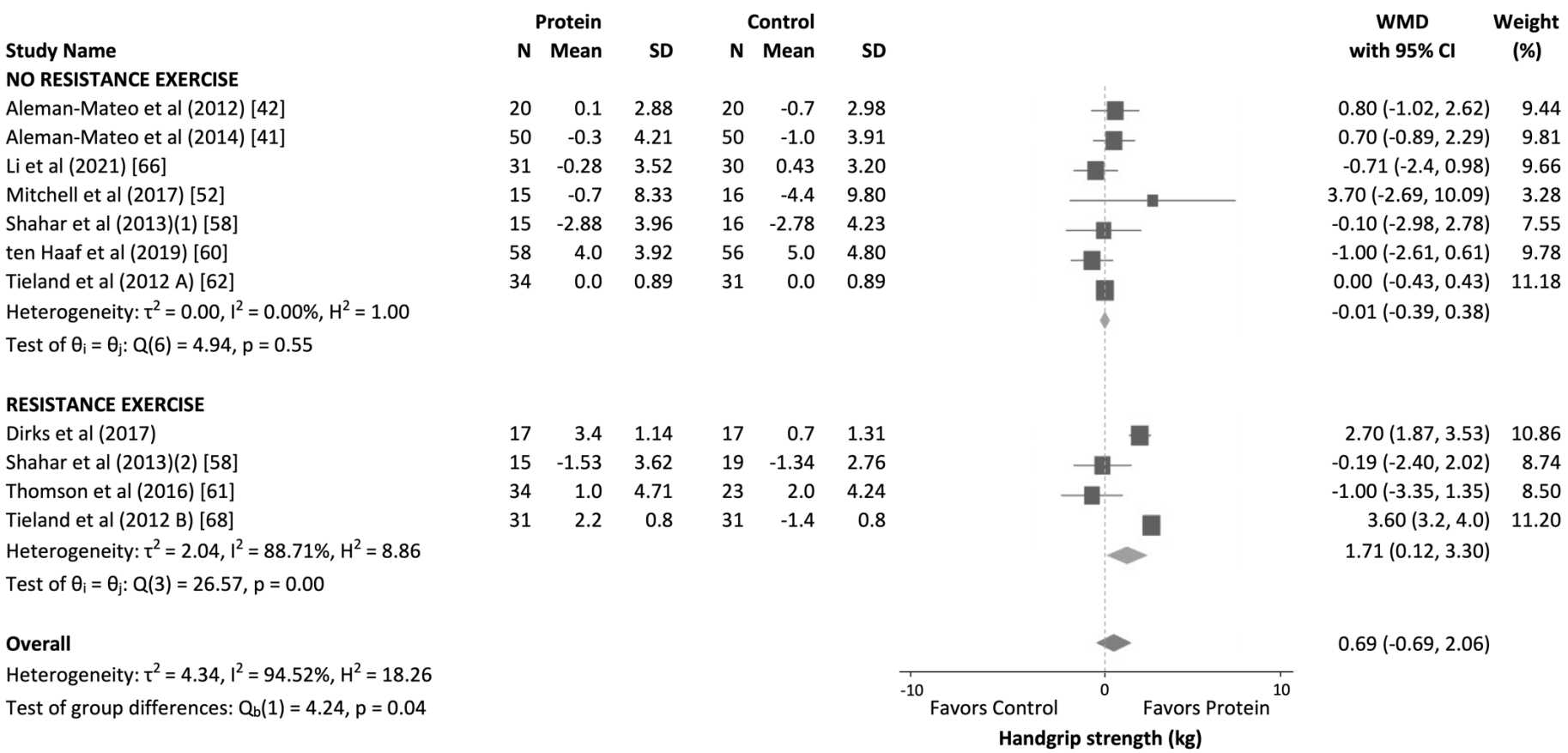


Figure 3.4 Forest plot of standard difference between control and protein groups on handgrip strength of 11 intervention arms organized by trials that did not, and did, use RE. A random effects model (using the DerSimonian-Laird method) and the generic inverse variance method were used to derive pooled estimates across studies. Squares indicate the point estimate for each trial, with the size of the square proportional to the contribution of the study to the overall estimate. The overall estimate and 95% confidence interval are indicated by the diamonds. Where two versions of the same study are mentioned, results from two different interventions were reported: Shahar 2013: (1) no resistance exercise, (2) resistance exercise. Tieland (2012 A & B) are different studies published in the same year

3.5.4.4 Knee extension strength

A pooled analysis of 8 intervention groups involving 335 participants revealed the change in KE was not statistically significantly different between the protein intervention and lower protein control groups (WMD: 1.88, 95% CI: -0.6, 4.35, I^2: 95.35%) (Figure 3.5). Sub-group analysis by use of RE revealed that in the RE sub-group, KE was not statistically significantly greater in protein interventions compared with lower protein control groups (WMD: 1.37, 95% CI: -1.01, 3.76, I^2: 93.06%) (Figure 3.5). Only one study not including RE, which used frail participants, was available for analysis. This study reported the change in KE was statistically significantly greater in protein interventions compared with lower protein control groups (WMD: 5.0, 95% CI: 3.91, 6.09). Results of tests for heterogeneity for RE group, between group and overall were p<0.001, p<0.001, p=0.007 and p<0.001, respectively.

Meta-regression analysis revealed that changes in knee extension strength were not significantly associated with: baseline knee extension strength (β = 0.16, 95% CI: -0.09, 0.4, ρ = 0.17, I^2residual = 93.49%), age (β = 0.21, 95% CI: -0.31, 0.73, ρ = 0.35, I^2residual = 93.19%); per-meal protein dose (β = -0.11, 95% CI: -0.5, 0.29, ρ = 0.52, I^2residual = 93.53%); and intervention duration (β = 0.04, 95% CI: -0.48, 0.55, ρ = 0.87, I^2residual = 94.3%). However, a trend was observed for an association with baseline protein intake (β = 23.61, 95% CI: -0.47, 47.68, ρ = 0.053, I^2residual = 89.59%). A summary diagram of the main results from these meta-analyses can be seen in Figure 3.6.

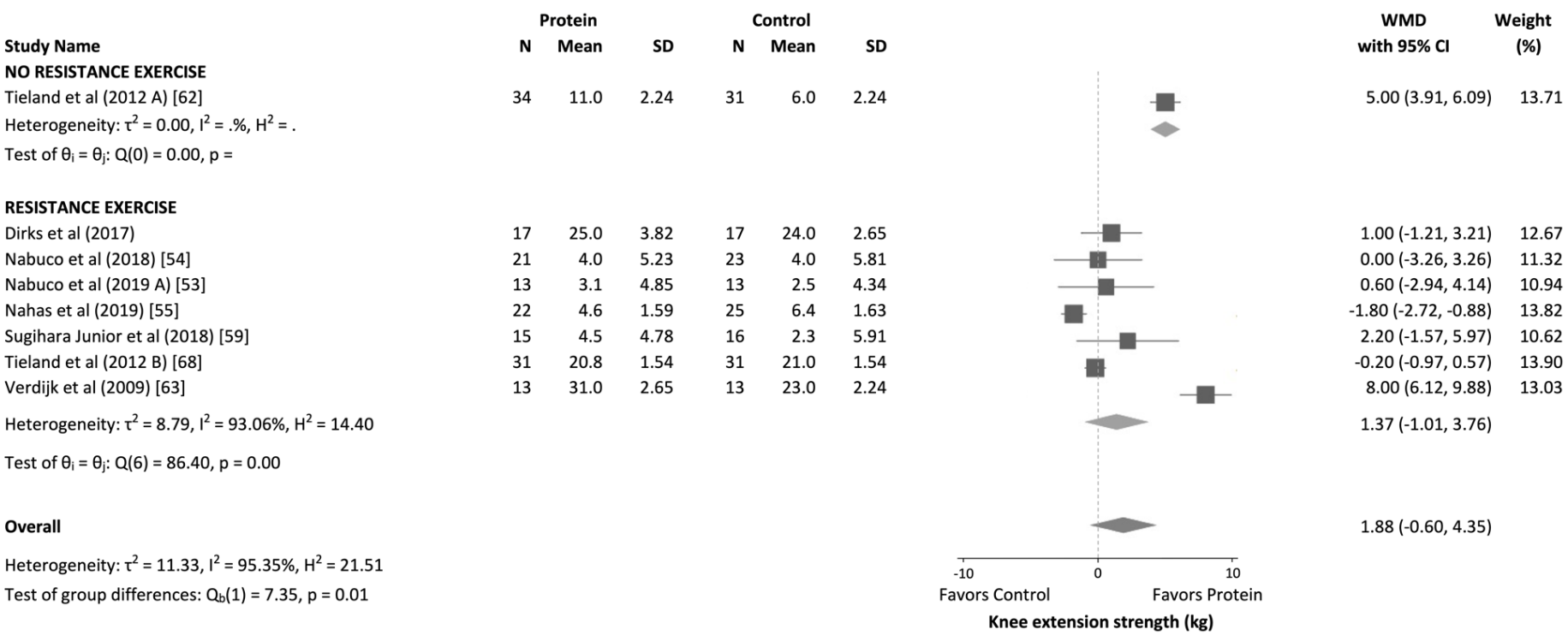


Figure 3.5 Forest plot of standard difference between control and protein groups on knee extension strength of 8 intervention arms organized by trials that did not, and did, use RE. A random effects model (using the DerSimonian-Laird method) and the generic inverse variance method were used to derive pooled estimates across studies. Squares indicate the point estimate for each trial, with the size of the square proportional to the contribution of the study to the overall estimate. The overall estimate and 95% confidence interval are indicated by the diamond. Nabuco (2019 A & B) and Tieland (2012 A & B) are different studies published in the same year

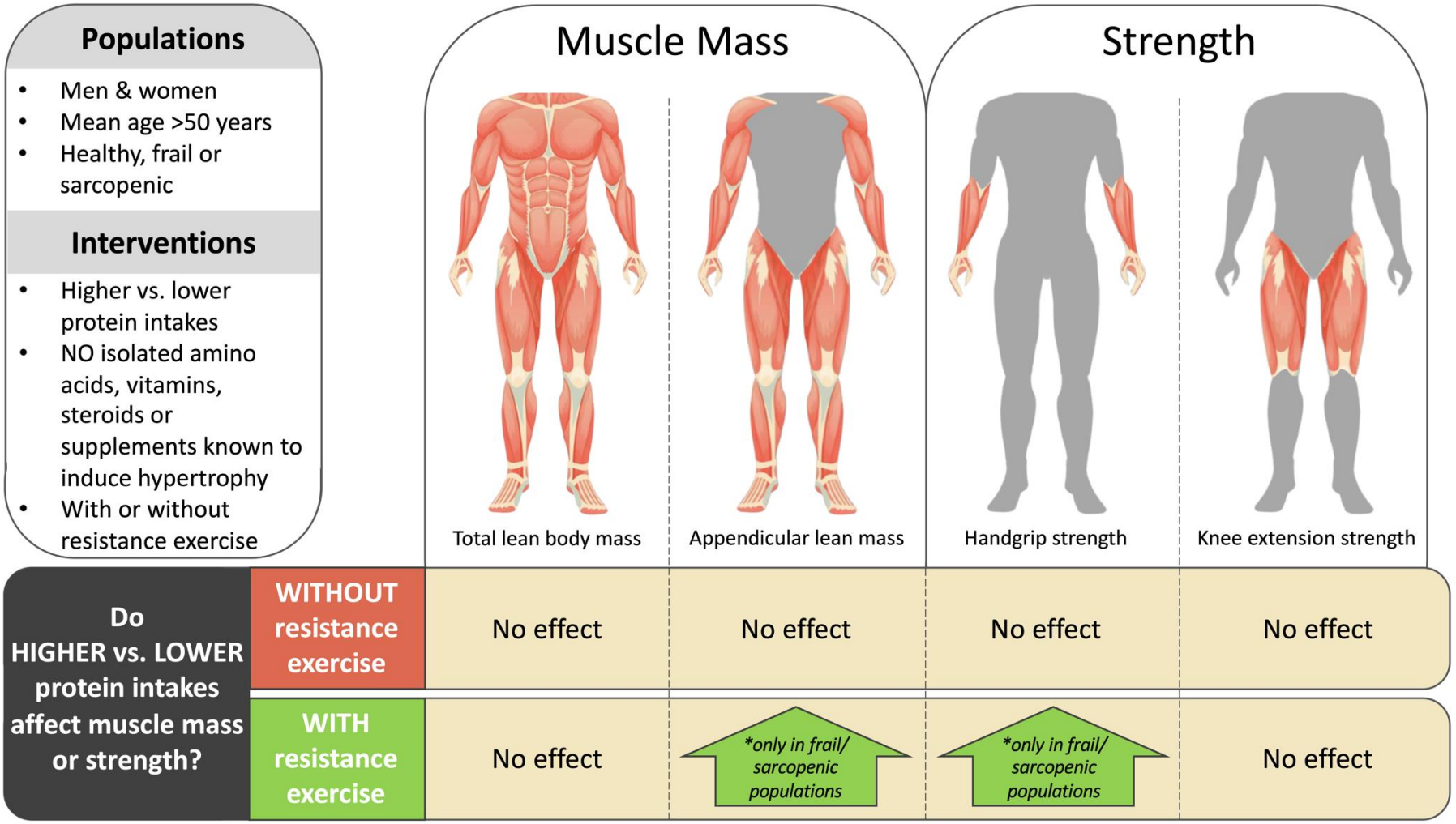


Figure 3.6 Summary diagram of the effects of higher vs. lower protein intakes on total lean body mass, appendicular lean mass, handgrip strength and knee extension strength in older adults

3.5.4.5 Sensitivity analysis

In leave-1-out sensitivity analyses, the pooled effect estimates remained similar across all studies and their subgroups, which confirmed that the statistically significant difference between the studied groups is the overall effect of all included studies.

3.5.4.6 Publication bias

No evidence for funnel plot asymmetry was found, and Eggers test showed no evidence of small study effect for LBM (p=0.969), ALM (p=0.863), HG (p=0.767), or KE (p=0.985)

3.6 Discussion

In the present study, we systematically reviewed RCTs investigating the effect of increased protein intake on muscle mass and strength, with or without exercise interventions, in older adults. To our knowledge, this is the first meta-analysis to show that whole protein interventions, without the use of EAAs or supplements known to stimulate hypertrophy, lead to superior gains in appendicular lean mass and handgrip strength in frail older adults, only when combined with an RE intervention.

Analysis of all applicable studies revealed that protein interventions increased ALM and HG but only in interventions that included an RE component. This highlights that the benefits of RE on ALM accrual and HG are augmented by higher protein intakes in older adults. As such, RE interventions to improve ALM and strength in the elderly may benefit from protein supplementation. This increase in ALM may be of clinical significance as Brown et al. (Brown, Harhay and Harhay, 2017), using data from older adults (mean age 74.9 y) participating in the

Third National Health and Nutrition Examination Survey, 1988–1994, observed that each 5.5 kg increase in ALM was robustly associated with a 50% lower risk of mortality [HR: 0.5 (95% CI: 0.27,0.92); p=0.03]. The combination of protein with resistance exercise was determined in this meta-analysis to result in an increase in ALM (WMD: 0.54, 95% CI: 0.03, 1.05, I^2: 89.76%) which may be viewed as clinically important.

The results of our analysis are partially in agreement with previous meta-analyses by Hou et al. (Hou et al., 2019) and Liao et al. (Liao et al., 2017b), which investigated the effects of protein or amino acid supplementation together with RE on muscle mass and physical function. Hou et al. (Hou et al., 2019) reported that protein increased fat-free mass, appendicular skeletal muscle mass, HG, KE and leg press strength, while Liao et al. (Liao et al., 2017b) reported greater lean mass and leg strength gains. One key metric in which our results differ with the meta-analyses by Hou et al. and Liao et al. (Liao et al., 2017b; Hou et al., 2019) is that we did not observe an increase in KE. While sub-group analysis by use of RE revealed no positive effect of protein in the RE sub-group, only one study not including RE, which used frail participants, was available for analysis. This study reported the change in KE was statistically significantly greater in protein interventions compared with lower protein control groups (WMD: 5.0, 95% CI: 3.91, 6.09). The reason for this result in a study which did not incorporate RE is not clear and as this result from one single study does not constitute a meta-analysis, it should not be considered representative of similar interventions.

However, our results contrast with meta-analyses by Tieland et al. (Tieland et al., 2017) and Ten Haaf et al. (Ten Haaf et al., 2018) which found no additional improvements in LBM or strength with increased protein. A potential reason for this discrepancy is that both meta-analyses included studies that used EAAs for the intervention, whereas our meta-analysis only included studies which used whole protein. The ingestion of whole protein (whey) has been

observed to result in greater skeletal muscle protein accrual than ingesting the equivalent content of constituent EAAs alone (Katsanos et al., 2008). However, when whey (15 g) is compared with an isocaloric quantity of EAA (15 g), the EAA-induced rate of MPS is greater, although other studies have reported that similar doses of EAA (15 g) have not resulted in increased muscle mass after 24 weeks (Markofski et al., 2019). It is possible that the inclusion of multiple studies using EAAs with overall low amounts of protein (≤ 15 g per dose) may have led to the non-statistically significant results of the findings of the aforementioned papers. For example, of 6 studies which used supplementary amino acids in the meta-analysis by Ten Haaf et al., only 2 reported improvements in LBM (Dillon et al., 2009; Ispoglou et al., 2016) with no statistically significant improvements in total LBM reported in the remaining 4 studies (Godard, Williamson and Trappe, 2002; Scognamiglio et al., 2005; Kawada et al., 2013; Markofski et al., 2019).

It is also important to highlight that the meta-analysis by Tieland et al. (Tieland et al., 2017) did not include studies that used exercise interventions, which augment the MPS-stimulating effect of acute protein ingestion (Atherton and Smith, 2012). As our meta-analysis revealed that protein-induced improvements in ALM were only observed in interventions which included RE, it is reasonable that a meta-analysis of studies without RE would show no benefit of added protein. A further difference from our study is that the meta-analysis by Ten Haaf et al. (Ten Haaf et al., 2018) only used interventions in non-frail, community-dwelling older adults. One might speculate that those suffering from frailty may have lower muscle mass than healthy older adults and might be more likely to benefit from interventions aimed at increasing muscle mass. Indeed, our sub-group analysis revealed that sarcopenic/frail populations performing RE did experience significant increases in ALM. As such, the results of our study lend support to the concept that populations at greatest risk of muscle and strength loss may increase ALM through RE with protein supplementation.

Anabolic resistance to protein ingestion is one potential explanation for the lack of effect of protein intervention in some of the studies included in our systematic review (Morton et al., 2018). Twenty grams of protein may be sufficient to maximally stimulate MPS in young people (Moore et al., 2009; Witard et al., 2014) however, bolus doses of 40 g of protein have been shown to stimulate the MPS response more robustly in older adults (Yang et al., 2012b). As such, larger per-meal doses of total protein (30-40 g/meal) may be useful for stimulating MPS in older populations (Breen and Phillips, 2011; Churchward-Venne et al., 2016). In our review, of the 25 studies included, only six used interventions involving per-meal-protein-boluses of 30 g or more (Shahar et al., 2013; Zhu et al., 2015; Rossato et al., 2017; Nabuco et al., 2018; Nabuco et al., 2019a; Nabuco et al., 2019b). Therefore, the majority of studies included in our systematic review may have been using protein doses which sub-maximally stimulate MPS and lean mass accrual. However, our meta-regression revealed that higher per-meal protein doses were not associated with greater increases in total LBM or ALM accrual.

The anabolic action of protein intake may be especially relevant in the post-exercised state, as the MPS response to the presence of amino acids is known to be augmented after a bout of RE (Churchward-Venne et al., 2016) for more than 24 hours post-exercise (MacDougall et al., 1995). Therefore, frequent stimulation of MPS via protein ingestion in this anabolically sensitive period may further benefit the accrual of muscle mass (Biolo et al., 1997). This may partially explain why we only detected a statistically significant effect of protein supplementation on ALM in interventions using RE.

Data on baseline protein intake was only available for 17 of the included studies. In these studies, the average protein intake was 0.91 g/kg of body weight per day, which is higher than the protein reference nutrient intake (RNI) of 0.75 g/kg/d (Department of Health, 1991). It may be speculated that many of the populations included in our meta-analysis might not benefit

from further protein supplementation, as would be expected in those with lower baseline protein intakes below the RNI (Bauer et al., 2013a). While our meta-regression revealed that baseline protein intake tended to be positively associated with increases in KE, the effect was not statistically significant (p=0.053). Further information on baseline protein intake would have allowed for a more thorough analysis of its specific effects on LBM accrual.

Our meta-analysis revealed that the effect of protein on HG strength was only statistically significant in interventions that used RE, specifically in frail/sarcopenic populations. Handgrip strength is frequently used as an indicator of strength, physical function and health in older adults (Stenholm et al., 2014; Rijk et al., 2016; Oksuzyan et al., 2017; Kim et al., 2019) and is also used as part of the diagnostic criteria for sarcopenia itself (Cruz-Jentoft et al., 2019). However, research by Tieland et al. indicates that handgrip strength may not be an ideal outcome measure to evaluate the efficacy of RE interventions in elderly individuals (Tieland et al., 2015). In contrast to our results, Tieland et al. (Tieland et al., 2012b) observed no difference in handgrip strength between intervention and placebo groups, despite improvements in leg muscle strength and physical performance in the intervention group. One explanation for this is that the strength and size adaptations of muscle to RE are specific to the muscle trained (West et al., 2010). As handgrip-specific training is not a frequent modality in RE programs, improvements in handgrip strength may not be expected. As such, our finding supports the use of handgrip strength as a useful measure of efficacy of interventions aimed at improving lean mass and strength.

A particular strength of our study is that it includes meta-analyses of both total LBM and ALM. Animal studies have shown that high protein intakes can result in visceral organ hypertrophy, which can contribute to increases in total LBM (Fluharty and McClure, 1997). As such, ALM may be a more appropriate measure of skeletal muscle hypertrophy, and thus the efficacy of

protein and exercise interventions, than total LBM. We may speculate that increases in ALM, rather than total LBM, are more desirable for improving muscle strength and function in older people. Indeed, appendicular skeletal muscle mass (a specific measure of ALM) is used as part of the diagnostic criteria for sarcopenia, as specified by the European Working Group on Sarcopenia in Older People (EWGSOP) (Cruz-Jentoft et al., 2019). In our analysis, only ALM was found to be positively influenced by protein intake but only in frail/sarcopenic populations and only when combined with RE (not without) and may be more clinically significant than total LBM.

There are also some limitations to this study. Firstly, this was an aggregate data analysis as opposed to an individual participant data (IPD) analysis due to poor response to requests for IPD from authors. IPD analysis can overcome some issues of aggregate analysis such as selective reporting, publication bias and low power to detect interactions at the individual level (Burke, Ensor and Riley, 2017). Secondly, and in line with this first point, it was not possible to investigate sex-specific effects of protein or RE in these studies, which may be of interest due to potential differences in MPS between sexes (Smith et al., 2008).

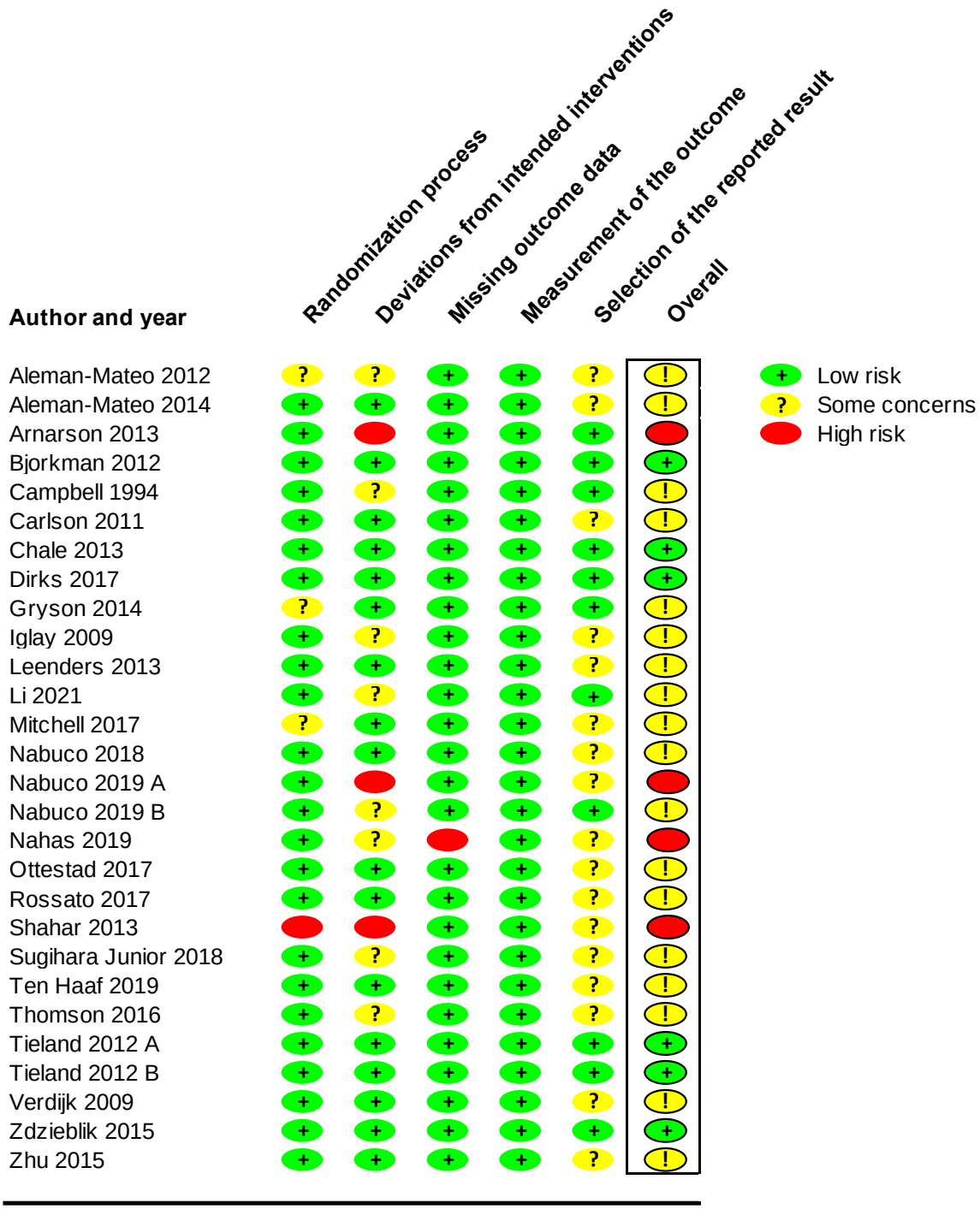
3.6.1 Conclusion

In conclusion, compared with lower protein controls, protein supplementation leads to increases in appendicular lean mass and handgrip strength in older adults, but only when combined with resistance exercise. With 22 of 28 studies presenting some risk of bias, caution should be exercised in the interpretation of these results

3.7 Supplementary material

Author	Adverse events / reported safety issues in protein interventions
Aleman-Mateo *et al* (2012)	25% of participants in intervention group reported early satiety
Aleman-Mateo *et al* (2014)	13.1±23.4% relative increase in GFR (significantly lower than the increase observed in the control group [27.6±16.1%])
Arnarson *et al* (2013)	Not reported
Bjorkman *et al* (2012)	Not reported
Campbell *et al* (1994)	Not reported
Carlsson *et al* (2011)	Not reported
Chale *et al* (2013)	14.3% of participants in intervention group experienced serious adverse events (CVD/ falls/ other) 33.3% of participants in intervention group experienced non-serious adverse events (GI distress/ near fall/ musculoskeletal pain etc) These were not signifcantly different from the control groups
Dirks *et al* (2017)	Not reported
Gryson *et al* (2014)	Not reported
Iglay *et al* (2009)	Not reported
Leenders *et al* (2013)	Not reported
Li *et al* (2021)	Not reported
Mitchell *et al* (2017)	No adverse events related to the diets were reported during the study
Nabuco *et al* (2018)	Not reported
Nabuco *et al* (2019 A)	Not reported
Nabuco *et al* (2019 B)	Not reported
Nahas *et al* (2019)	Not reported
Ottestad *et al* (2016)	Not reported
Rossato *et al* (2017)	Not reported
Shahar *et al* (2013)	Not reported
Sugihara Junior *et al* (2018)	Not reported
ten Haaf *et al* (2019)	There were no serious adverse events reported during the supplementation period
Thomson *et al* (2016)	No serious injuries or adverse events were associated with the diet or exercise program
Tieland *et al* (2012 A)	Not reported
Tieland *et al* (2012 B)	Not reported
Verdijk *et al* (2009)	Not reported
Zdzieblik *et al* (2015)	No serious adverse events were noted and, especially, no pathological findings could be observed in the routine blood tests
Zhu *et al* (2015)	There were no significant differences between the protein group and the placebo group in the rate of incident cancer (protein, 5.0% and control, 5.3%), type 2 diabetes (protein, 3.0% and control, 1.1%), diarrhea (protein, 4.0% and control, 1.1%), esophageal reflux (protein 2.0%, control 5.3%) or fracture (protein 3.0%, control 3.2%). Two participants in the protein group reported constipation

Supplementary table 3.1. Adverse events reported for 28 included studies



Supplementary figure 3.1. Risk of bias assessment of included studies based on Cochrane risk of bias tool

4 Chapter 4: Serum vitamin D and muscle mass

Genetically determined serum 25-hydroxyvitamin D is associated with total, trunk, and arm fat-free mass: a Mendelian randomization study

Kirwan R, Isanejad M, Davies IG, Mazidi M.

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4.1 Context within thesis

Chapter 4 presents an analysis of the relevance of serum vitamin D levels for muscle size. As mentioned in chapter 2, vitamin D supplementation has shown some promise for augmenting muscle size and strength in elderly individuals who are deficient in this nutrient. This chapter utilizes the technique of Mendelian randomisation to determine whether the association between serum vitamin D and muscle mass may be causal. We use this analysis to further discuss the potential of vitamin D supplementation for increasing muscle mass, in the context of our aims to ultimately combat sarcopenia and improve cardiometabolic health.

4.2 Abstract

Background: Low serum vitamin D status has been associated with reduced muscle mass in observational studies although the relationship is controversial and a causal association cannot be determined from such observations. Two-sample Mendelian randomization (MR) was applied to assess the association between serum vitamin D (25(OH)D) and total, trunk, arm and leg fat-free mass (FFM).

Methods: MR was implemented using summary-level data from the largest genome-wide association studies (GWAS) on vitamin D (n=73,699) and total, trunk, arm and leg FFM. Inverse variance weighted method (IVW) was used to estimate the causal estimates. Weighted median (WM)-based method, and MR-Egger, leave-one-out were applied as sensitivity analysis.

Results: Genetically higher serum 25(OH)D levels had a positive effect on total (IVW = Beta: 0.042, p = 0.038), trunk (IVW = Beta: 0.045, p = 0.023) and arm (right arm IVW = Beta: 0.044, p = 0.002; left arm IVW = Beta: 0.05, p = 0.005) FFM. However, the association with leg FFM was not significant (right leg IVW = Beta: 0.03, p = 0.238; left leg IVW = Beta: 0.039, p = 0.100). The likelihood of heterogeneity and pleiotropy was determined to be low (statistically

non-significant), and the observed associations were not driven by single SNPs. Furthermore, MR pleiotropy residual sum and outlier test did not highlight any outliers.

Conclusions: Our results illustrate the potentially causal, positive effect of serum 25(OH)D concentration on total, trunk and upper body appendicular fat-free mass.

4.3 Introduction

Vitamin D is an essential nutrient for human health with roles in multiple biological pathways and low vitamin D status is associated with multiple chronic diseases (Wang et al., 2017) as well as being associated with musculoskeletal health (Tieland et al., 2013; Girgis, 2020) highlighting this nutrient's significance in the global burden of disease. However, up to 40% of the European population may suffer from vitamin D insufficiency (serum 25-hydroxy vitamin D [25(OH)D] concentration <50 nmol/L) (Cashman et al., 2016) and vitamin D deficiency (25(OH)D concentration <30 nmol/L) is widespread enough to be considered a global health issue (Del Valle et al., 2011; Cashman et al., 2016; Cashman, 2020).

Loss of muscle mass directly affects muscle strength and physical function and as such, sarcopenia, the progressive loss of muscle mass and strength in aging, and frailty (Cruz-Jentoft et al., 2019; Xu et al., 2020). Furthermore, muscle mass loss has been associated with a multitude of chronic conditions including cardiovascular disease (CVD) (Bahat and İlhan, 2016), type 2 diabetes mellitus (T2DM) (Scott, de Courten and Ebeling, 2016), increased risk of falls and fractures (Schaap et al., 2018), cognitive decline and depression (Hsu et al., 2014; Hayashi et al., 2019), and all-cause mortality (Abramowitz et al., 2018). Older adults may spend more time indoors due to poor mobility/reduced muscle function which can further lead to an elevated risk of vitamin D inadequacy (Webb et al., 1990; Whitmore, 1996), leading to a vicious cycle of vitamin D deficiency and loss of muscle mass.

Epidemiological studies suggest an association between low vitamin D status and reduced muscle mass (Tieland et al., 2013; Wierzbicka et al., 2016; Luo et al., 2018) although some studies have found no such association (Ceglia et al., 2011; De Pergola et al., 2019). However, such studies are limited as observational data cannot determine whether an association is causal. Mendelian randomization (MR) analysis uses functional polymorphisms (single nucleotide polymorphisms (SNPs)) associated with specific changes in exposures (in this case, serum 25(OH)D) as genetic instruments to determine whether the risk factor is a cause of the disease (Larsson, 2021). A major advantage of MR analysis is that they are considerably less prone to confounding, residual bias, and reverse causation than conventional risk-factor epidemiology (Smith and Ebrahim, 2003). MR analysis may also circumvent the financial, logistical and ethical limitations of randomised controlled trials (RCTs) and additionally, the data from such studies can inform the design of pilot RCTs and clinical trials by providing information for the potential magnitude of effect of nutrients on a given outcome in specific populations (Plotnikov and Guggenheim, 2019).

In the present study, we used MR analysis to determine whether a potential causal relationship exists between serum 25(OH)D concentration and total, trunk, arm and leg fat-free mass (FFM).

4.4 Methods

4.4.1 Study design

A two-sample MR study design was used. In a 2-sample MR, the summary statistics are provided from various studies for the association of the genetic instruments with the exposure and outcome. In our study, we obtained the summary statistics from the largest genome wide

association studies (GWAS) on serum 25(OH)D (exposure (Jiang et al., 2018)) and FFM (outcome). We applied methods to estimate the unbiased effect of serum 25(OH)D on FFM (total, trunk, arms and legs,).

4.4.2 Genetic predictors of exposures

We used six SNPs identified to be associated with circulating 25(OH)D concentration by the SUNLIGHT meta-GWAS, which are samples of European ancestry (79,366 discovery samples and 42,757 replication samples) (**Table 1**). GWAS were performed within each cohort according to a uniform analysis plan. Additive genetic models using linear regression on natural-log-transformed 25(OH)D were fitted and a fixed-effects inverse variance weighted (IVW) meta-analysis across the contributing cohorts was performed (Jiang et al., 2018).

4.4.3 Association of genetic instruments with outcome

SNPs associated with bioelectrical-impedance-measured fat mass and total, trunk, arm and leg FFM were obtained from analyses by Neale Lab (http://www.nealelab.is). We retrieved the association of the six genetic instruments with SNPs associated with bioelectrical impedance measured FFM using data obtained from UK Biobank. Detailed descriptions of the methods used to measure body composition is available on the UK Biobank website (*UK Biobank-Body Composition Measurement*, 2011). Briefly, whole body as well as site-specific (trunk, leg, arm) fat-free mass/fat mass were evaluated with bioelectrical-impedance analysis (Tanita BC418MA body composition analyser). Body composition of a subset of participants was also assessed using dual-energy X-ray absorptiometry (DXA) which showed high correlation with bio-impedance values (fat-free mass: r = 0.96) (*UK Biobank-Body Composition Measurement*, 2011). The UK Biobank is a population-based cohort of approximately 500,000 individuals; 54% are female, the average age is 57 (range 37–73),

while 94% report as being White British. Further details on the rationale, design and methodology for UK Biobank can be found elsewhere (Sudlow et al., 2015).

4.4.4 Mendelian Randomisation analysis

We combined the effect of six instruments using inverse variance weighted (IVW) method as implemented in Two Sample MR package of the statistical software, R (R Core Team, Vienna, Austria. https://www.R-project.org/). We assessed the heterogeneity using Q value for IVW. To address the potential effect of pleiotropic variants on the final effect estimate, we conducted sensitivity analysis including weighted median (WM) and MR-Egger. Sensitivity analysis was conducted using the leave-one-out method. The weighted median (WM) estimate, as the weighted median of the SNP-specific estimates, provides correct estimates as long as SNPs accounting for ≥50% of the weight are valid instruments. WM MR allows some variants to be invalid instruments provided at least half are valid instruments. It uses inverse variance weights and bootstrapping to estimate confidence intervals (CIs) (Bowden et al., 2016). MR-Egger has an ability to make estimates by assumption of all SNPs are invalid instruments as long as the assumption of instrument strength independent of direct effect (InSIDE) is satisfied (Bowden et al., 2016). MR-Egger allows free estimation of the intercept, although further assumptions, such as the independence between instrument strength and direct effects, cannot be easily verified. Average directional pleiotropy across genetic variants was assessed from the *p*-value of the intercept term from MR-Egger (Bowden et al., 2016). Causal estimates in MR Egger are less precise than those obtained by using IVW MR (Bowden, Davey Smith and Burgess, 2015). Analysis using MR-Egger has a lower false positive rate but a higher false negative rate than IVW (Burgess et al., 2017).

Further, to assess heterogeneity between individual genetic variant estimates, we used the Q′ heterogeneity statistic (Bowden et al., 2017) and the MR pleiotropy residual sum and outlier

(MR-PRESSO) test (Bowden et al., 2017). The Q′ statistic uses modified 2nd order weights that are a derivation of a Taylor series expansion and take into account uncertainty in both numerator and denominator of the instrumental variable ratio (this eases the no-measurement-error [NOME] assumption) (Bowden et al., 2017). The MR-PRESSO framework relies on the regression of variant-outcome associations on variant-exposure associations and implements a global heterogeneity test by comparing the observed distance (residual sums of squares) of all variants to the regression line with the distance expected under the null hypothesis of no pleiotropy (Verbanck et al., 2018). In case of evidence of horizontal pleiotropy, the test compares individual variants expected and observed distributions to identify outlier variants. Further we applied on MR-Robust Adjusted Profile Score (RAPS) this method is able to correct for pleiotropy using robust adjusted profile scores. We consider as results, causal estimates that agreed in direction and magnitude across MR methods, pass nominal significance in IVW MR, and did not show evidence of bias from horizontal pleiotropy using heterogeneity tests. We used R version 3.4.2 (R Core Development Team 2017).

The MR studies assume that the SNPs (instrumental variables) are associated with the outcome only *via* the exposure (Lawlor et al., 2008), so we performed sensitivity analysis excluding SNPs with potentially pleiotropic effects. To assess the instrumental variable analysis "exclusion-restriction" assumption we used Ensembl release (http://useast.ensembl.org/index.html). Ensembl contains a base of SNP phenotypes.

4.4.5 Ethics

This investigation uses published or publicly available summary data with no involvement of participants in the study. No original data were collected for this manuscript. Ethical approval for each of the studies included in the investigation can be found in the original publications (including informed consent from each subject).

4.5 Results

In total, 6 SNPs were identified as instrumental variables for serum 25(OH)D, none of which were significantly associated with FFM. A list of all SNP associations is shown in Table 4.1. The results of MR analysis, displayed as beta-coefficient for interested outcomes per increase in serum 25(OH)D, demonstrate a positive and statistically significant effect on total FFM (MR Egger= β:0.019, p= 0.657 and IVW= β: 0.042, p= 0.038; respectively, Table 4.2 and Figure 4.1), trunk (MR Egger= β:0.037, p= 0.406 and IVW= β: 0.045, p=0.023, respectively, Table 4.2 and Figure 4.1) FFM. This data suggests that each 25 nmol/L increase in serum 25(OH)D is associated with an increase of 0.042 kg of total FFM. Serum 25(OH)D also demonstrated a positive and statistically significant effect on arm FFM (Right arm: MR Egger= β:0.043, p= 0.225 and IVW= β: 0.044, p=0.002; Left arm: MR Egger= β:0.033, p= 0.398 and IVW= β: 0.05, p=0.005, respectively, Table 4.2. However, results for leg FFM did not demonstrate a statistically significant effect (Right leg: MR Egger= β: -0.025, SE: 0.04, p= 0.561 and IVW= β: 0.03, SE: 0.026, p=0.238; Left leg: MR Egger= β: -0.008, SE: 0.038, p= 0.838 and IVW= β: 0.039, SE: 0.023, p=0.1, respectively, Table 4.2).

Table 4.1 Summary results of the 6 genetic loci associated with serum vitamin D

SNP	Nearest gene	GX	GX SE	EA	OA	EAF	p-value
rs3755967	GC	-0.089	0.0023	T	C	0.28	4.74E-343
rs10741657	CYP2R1	0.031	0.0022	A	G	0.4	2.05E-46
rs12785878	NADSYN1/DHCR7	0.036	0.0022	T	G	0.75	3.80E-62
rs10745742	AMDHD1	0.019	0.002	T	C	0.4	2.10E-20
rs8018720	SEC23A	-0.019	0.0027	C	G	0.82	1.11E-11
rs17216707	CYP24A1	0.026	0.0027	T	C	0.79	8.14E-23

All serum vitamin D markers were associated at genome-wide significance ($p < 5 \times 10^{-8}$).

EA: effect allele; OA: other allele, EAF: effect allele frequency; GX: the per-allele effect on standard deviation units of the telomere length; GX SE: standard error of GX.

Table 4.2 Results of the Mendelian Randomization (MR) analysis for effects of serum vitamin D on total, trunk, arm and leg fat-free mass

Exposure	Outcome	MR				Heterogeneity			Pleiotropy		
		Method	beta	SE	p	Method	Q	p	Intercept	SE	p
Vitamin D (Serum 25(OH)D)	Total fat-free mass	MR-Egger	0.019	0.039	0.657	MR-Egger	11.018	0.026	0.001	0.002	0.503
		WM	0.031	0.015	0.029						
		IVW	0.042	0.02	0.038	IVW	12.506	0.029			
		RAPS	0.036	0.016	0.03						
	Trunk fat-free mass	MR-Egger	0.037	0.039	0.406	MR-Egger	11.479	0.022	0.0004	0.002	0.817
		WM	0.039	0.015	0.008						
		IVW	0.045	0.02	0.023	IVW	11.655	0.04			
		RAPS	0.039	0.017	0.019						
	Arm fat-free mass (right)	MR-Egger	0.042	0.029	0.225	MR-Egger	6.415	0.17	0.0001	0.001	0.94
		WM	0.042	0.014	0.003						
		IVW	0.044	0.015	0.002	IVW	6.425	0.267			
		RAPS	0.042	0.014	0.002						
	Arm fat-free mass (left)	MR-Egger	0.033	0.0349	0.398	MR-Egger	8.746	0.068	0.001	0.002	0.589
		WM	0.041	0.015	0.007						
		IVW	0.05	0.018	0.005	IVW	9.499	0.091			
		RAPS	0.046	0.016	0.005						
	Leg fat-free mass (right)	MR-Egger	-0.025	0.0401	0.561	MR-Egger	10.775	0.029	0.003	0.002	0.171
		WM	0.015	0.0152	0.334						
		IVW	0.03	0.0258	0.238	IVW	18.269	0.003			
		RAPS	0.022	0.021	0.281						
	Leg fat-free mass (left)	MR-Egger	-0.008	0.0382	0.838	MR-Egger	9.807	0.044	0.003	0.002	0.215
		WM	0.022	0.0148	0.143						
		IVW	0.039	0.0234	0.1	IVW	15.108	0.009			
		RAPS	0.031	0.019	0.098						

25(OH)D: 25-hydroxy vitamin D; WM: weighted median; IVW: inverse variance weighted; SE: standard error; beta: beta-coefficients; MR: Mendelian randomization; RAPS: robust adjusted profile score

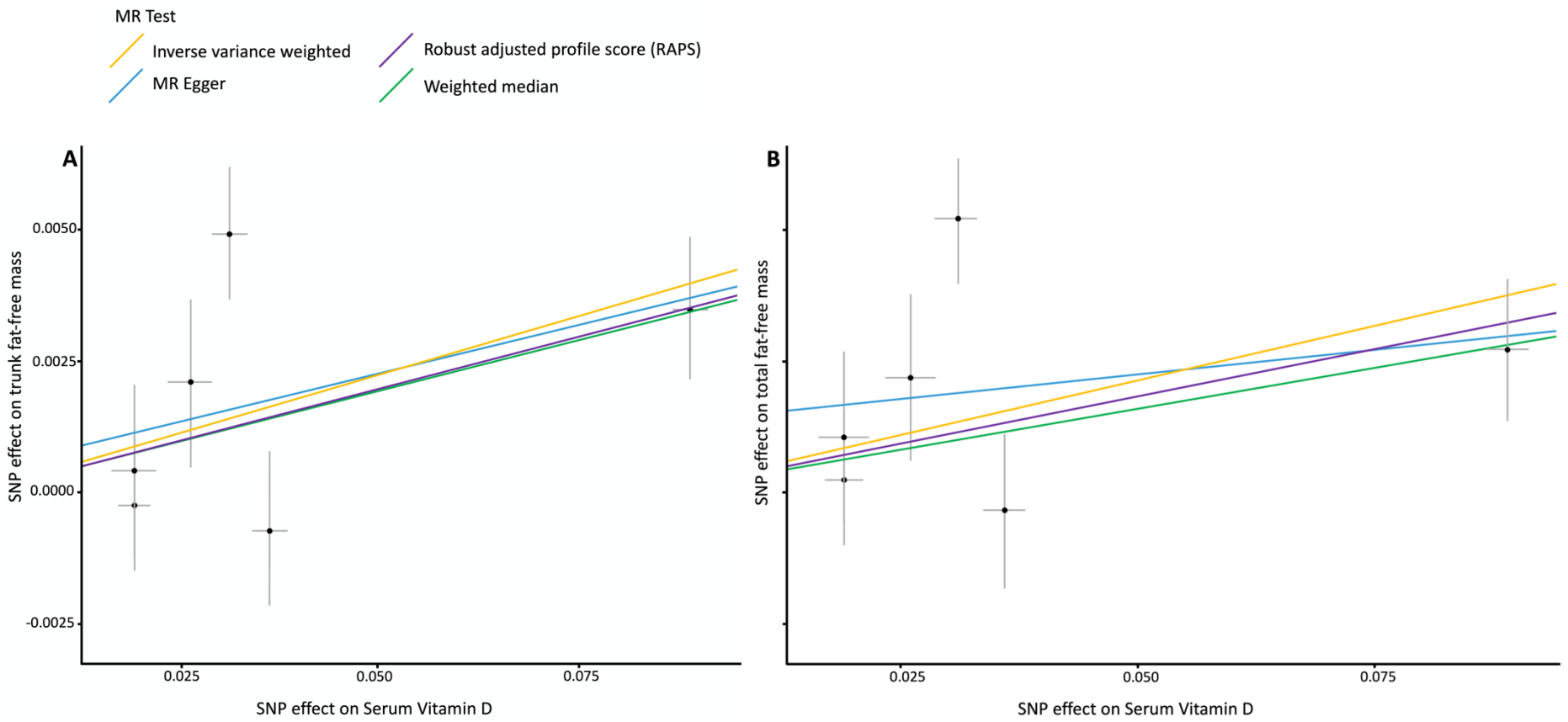

Figure 4.1 Scatter plots of the association of the effect of SNP-determined serum 25(OH)D on trunk (A) and total (B) fat-free mass. Each black point represents an SNP, plotted by the estimate of SNP on serum 25(OH)D level (x-axis, nmol/L) and the estimate of SNP on fat-free mass (y-axis, kg). The slopes of each line represent the potential causal associations for each method.

The horizontal pleiotropy test, with very negligible Egger regression intercept, also showed a low likelihood of pleiotropy for all our estimations (all $p > 0.171$, Table 4.2). Further the result of the MR-RAPS was identical with the IVW prediction, which again indicated a statistically low chance of pleiotropy. Heterogeneity tests highlighted no trace of heterogeneity (Table 4.2). Furthermore, MR-PRESSO analysis did not indicate any outliers for all estimates. Results of leave-one-out method demonstrated that the links are not driven by any single SNP.

4.6 Discussion

Our results illustrate the potentially causal, positive effect of lifetime serum 25(OH)D concentration on total, trunk and arm FFM. These findings are in agreement with a number of cross-sectional, population-based studies, which have shown a positive relationship between serum 25(OH)D status and FFM in a wide range of age groups and clinical populations (Tieland et al., 2013; Wierzbicka et al., 2016; Luo et al., 2018). In a study of 100 adolescents (15.1 ± 1.9 y), serum 25(OH)D was positively associated with lean body mass and inversely with fat mass (Wierzbicka et al., 2016). In a cross-sectional study of 127 pre-frail and frail elderly people (79.0 ± 7.8 y) in The Netherlands, Tieland et al. (Tieland et al., 2013) reported that low 25(OH)D status was associated with reduced muscle mass and poorer physical performance (Tieland et al., 2013). Additionally, a meta-analysis of 12 studies with data from 22,590 individuals (mean range 50 – 88 yrs) reported that sarcopenic individuals had lower blood 25(OH)D concentrations than non-sarcopenic controls (Luo et al., 2018). Conversely, some studies have reported no such association between 25(OH)D and lean body mass (LBM) or FFM (Ceglia et al., 2011; De Pergola et al., 2019).

Mechanistically, vitamin D is known to exert its effects of muscle tissue both by regulating expression of target genes via the vitamin D receptor (VDR) and by non-genomic regulation of skeletal muscle intracellular signaling pathways (Boland, 2011). In animal models, vitamin

D supplementation has been demonstrated to activate the mammalian target of rapamycin/S6 kinase (mTOR/S6K) pathway, which leads to increased muscle protein synthesis (MPS) (Vignale et al., 2015) essential for increases in muscle protein accrual and size (Atherton and Smith, 2012). Cell culture models have also reported that vitamin D enhances the stimulating effect of leucine and insulin on muscle protein synthesis rates (Salles et al., 2013) and promotes myogenic differentiation and reduces the expression of myostatin, a known negative regulator of muscle size (Garcia et al., 2011). Vitamin D has also been reported to stimulate the expression of genes involved in the control of cellular growth (Neary, 1997; Boland, 2011). These varied mechanisms may partly explain the adverse effects of low vitamin D status on muscle mass and function.

The present study did not find a statistically significant relationship between genetically determined serum 25(OH)D concentration and leg FFM. This is not the first study to identify a discrepancy in the relationship between 25(OH)D status with upper and lower body appendicular lean mass. In a study of frail elderly Dutch people (n = 127; mean 79 y) 25(OH)D status was associated with appendicular lean mass (ALM) (β=0.012 [P=0.05]) but was not significantly associated with leg lean mass (β=0.008 [P=0.08]) (Tieland et al., 2013). Furthermore, a cross-sectional study of the association of 25(OH)D status with muscle strength (n = 419; healthy men and women; 20-76 y) has also reported a stronger association between 25(OH)D and muscle strength in the arms compared to the legs (Grimaldi et al., 2013). One potential explanation for this discrepancy is the reported greater distribution of VDR in type 2 muscle fibres (Srikuea, Hirunsai and Charoenphandhu, 2020) which make up a greater proportion of upper body skeletal muscle (Johnson et al., 1973; Travnik, Pernus and Erzen, 1995; Klein et al., 2003; Ørtenblad et al., 2018). Vitamin D affects both the diameter and the number of type 2 muscle fibres, which are important for not only young athletes but also the elderly, due to their capacity to reduce the risk of falls, for example (Koundourakis et al., 2016; Remelli et al., 2019). Greater expression of VDR has been reported to stimulate

muscle hypertrophy through a number of potential mechanisms including increased protein synthesis (Bass et al., 2020). Furthermore, the greater daily utilization of lower extremities, for example, due to locomotion and bearing the individuals body weight during movement, may provide a superior stimulus for muscle hypertrophy. Further research is clearly needed to elucidate the mechanisms by which vitamin D differentially affects lower and upper body appendicular muscle physiology.

This study highlights the importance of serum vitamin D concentrations in accruing and maintaining FFM, which itself is associated with lower risk of frailty and mortality (Abramowitz et al., 2018; Schaap et al., 2018; Xu et al., 2020). Addressing vitamin D insufficiency is challenging as the main source of vitamin D in humans is sun exposure (Engelsen, 2010) which is unlikely to become a widely accepted and implemented strategy. Furthermore, dietary intakes of vitamin D are typically low (Kiely and Black, 2012) due to low levels in common foodstuffs (Schmid and Walther, 2013). Therefore, at a population level, food fortification with vitamin D, and at an individual level, supplementation may be the most effective methods to increase 25(OH)D status to sufficient levels (Cashman, 2020).

A major strength of our study was the large sample population study with access to individual participant data of high validity and with the relevant SNPs available for both 25(OH)D serum concentration and FFM. Furthermore, the use of the Mendelian randomisation approach allowed us to examine the potential causal effects of serum 25(OH)D, largely without the disadvantages of confounding or reverse causation.

A potential limitation of this study is the use of segmental bioelectrical impedance analysis (BIA) as the method for determining FFM in the UK Biobank cohort. The accuracy of BIA measurement is known to be affected by hydration status; however the UK Biobank protocol

did not specify any procedures to standardise some determinants of hydration before the assessment. This could potentially lead to inaccuracies in the values attained for FFM (Kyle et al., 2004). Furthermore, evidence suggests that BIA is less accurate at high BMI levels (Neovius et al., 2006) and considering the range of BMI included in the UK Biobank cohort, this should be taken into consideration with these results.

4.6.1 Conclusion

Evidence for a potentially causal association of serum 25(OH)D with total, trunk and arm FFM was found. However, the relationship between serum 25(OH)D and leg FFM was not statistically significant. This finding highlights the importance of maintaining sufficient 25(OH)D status throughout the life course in order to maintain adequate lean mass, a factor associated with multiple chronic disease. Future research should address the causal role and potential mechanisms of serum 25(OH)D on FFM accrual and maintenance as well as the apparent lack of effect on leg FFM.

5 Chapter 5: Lean mass and serum lipoprotein particle size

The association of appendicular lean mass and grip strength with LDL, VLDL and HDL particle diameter: a Mendelian randomization study of the UK Biobank cohort

Kirwan R, Mazidi M, Butler T, Perez de Heredia F, Davies IG,

Awaiting submission

5.1 Context within thesis

Chapter 5 investigates the potentially causal relationship of lean mass, specifically appendicular lean mass with the size of HDL, VLDL and LDL particle size. As lipoprotein particle size is considered to be relevant for the development and progression of atherosclerosis, an investigation of how muscle mass may influence particle size is warranted. As muscle mass is associated with lower risk of heart disease, its relationship with lipoprotein particle size may, at least partially, explain a mechanism by which muscle mass may exert its cardio-protective effects.

5.2 Abstract

Background: Reduced muscle mass and strength is frequently associated with both alterations in blood lipids and poorer cardiometabolic outcomes in epidemiological studies, however, a causal association cannot be determined from such observations. Two-sample Mendelian randomization (MR) was applied to assess the association of genetically determined appendicular lean mass (ALM) and handgrip strength (HGS) with serum lipid particle diameter.

Methods: MR was implemented using summary-level data from the largest genome-wide association studies (GWAS) on ALM (n=450,243), HGS (n=461,089) and LDL, VLDL and HDL particle diameters, (n=115,078). Inverse variance weighted method (IVW) was used to estimate the causal estimates. Weighted median (WM)-based method, and MR-Egger, leave-one-out were applied as sensitivity analysis.

Results: Increased ALM had a statistically significant positive effect on HDL particle diameter (MR Egger= β:0.055, p=0.081 and IVW= β: 0.068, $p=6.15x10^{-7}$; respectively), and a negative and statistically significant effect on VLDL particle diameter (MR Egger= β:-0.114, p=0.003 and IVW= β: -0.081, $p=1.57x10^{-6}$, respectively). Increased HGS, had a statistically significant

positive effect on HDL particle diameter (MR Egger= β: 0.433, SE: 0.184, p=0.019 and IVW= β: 0.121, SE: 0.052, p=0.021), and a negative and statistically significant effect on VLDL particle diameter (MR Egger= β: -0.416, SE: 0.163, p=0.011 and IVW= β: -0.122, SE: 0.046, p=0.009). There was no statistically significant effect of either ALM or HGS on LDL particle diameter.

Conclusions: Evidence for a potentially causal association of both increasing ALM and HGS, with both increasing HDL particle size and decreasing VLDL particle size was found, highlighting their potential for improving CVD risk profile.

5.3 Introduction

Cardiovascular diseases (CVD) are the leading cause of mortality globally, resulting in 18.6 million deaths in 2019 alone (Roth et al., 2020) and both reduced muscle mass and reduced grip strength have been associated with increased risk of CVD (Srikanthan, Horwich and Tseng, 2016; Tyrovolas et al., 2020) and CVD mortality (Chainani et al., 2016; Kim et al., 2019). Muscle mass is known to decline progressively from the fifth decade of life, a process known as sarcopenia (Cruz-Jentoft et al., 2019), a condition that is also associated with cardiovascular disease (CVD) (Bahat and İlhan, 2016). Greater muscle mass and function are also known to be associated with favourable levels of a number of relevant risk factors for CVD including, plasma triglycerides (Amarante do Nascimento et al., 2022) and high-density lipoprotein cholesterol (HDL-C) (Abe and Fukunaga, 1994; Landi et al., 2007), although unfavourable associations have also been observed (Pietrobelli et al., 1999). However, the relationships between muscle mass/strength with low density lipoprotein (LDL), very low-density lipoprotein (VLDL) and HDL particle diameter, remain to be investigated.

Elevated LDL levels are causally associated with risk of CVD and particularly, coronary heart disease (CHD) (Ference et al., 2017). Furthermore, LDL and other lipoprotein particle sizes

can also be measured using techniques such as nuclear magnetic resonance (NMR) spectroscopy and sub-fractionation. Accordingly, LDL is known to have particle subclasses, commonly divided into small, medium and large according to their diameter (Williams et al., 2014). Small dense LDL (sdLDL) particles, despite their lower cholesterol load may contribute equally to CVD risk due to their greater propensity to enter and become trapped in the subintimal space of the arterial wall, contributing to the development of atherosclerosis (Rizzo and Berneis, 2006; Pichler et al., 2018; Sniderman et al., 2019). VLDL is another subclass of lipoprotein that is considered to be atherogenic, with larger VLDL particles linked to a greater risk of the development of CVD in healthy populations (Freedman et al., 1998; Colhoun et al., 2002). Conversely, lower levels of HDL-C are associated with increased CVD risk (Rader and Hovingh, 2014) while smaller HDL particle size is associated with an adverse cardiometabolic risk profile (Freedman et al., 1998; Arsenault et al., 2009). However, the relationship between muscle mass and the size of various serum lipid particles is unknown.

Despite the epidemiological associations of lower muscle mass and strength with poorer cardiometabolic risk markers and outcomes (Chainani et al., 2016; Srikanthan, Horwich and Tseng, 2016; Kim et al., 2019; Tyrovolas et al., 2020), a causal association cannot be determined from such observations. Mendelian randomization (MR) analysis uses genetic polymorphisms, known to be associated with distinct alterations in phenotypes (for example, genetically determined appendicular lean mass), as statistical instruments (Larsson, 2021). This allows the determination of whether a particular physiological trait is a probable cause of a known risk factor or specific condition (Larsson, 2021). This means MR analysis is capable of determining both unbiased and robust evidence of the mechanisms of disease pathogenesis. A further advantage of MR analysis is that it is considerably less prone to confounding, residual bias and reverse causation than conventional risk-factor epidemiology (Smith and Ebrahim, 2003). As such, data from MR analysis can inform the design of pilot

RCTs and clinical trials by identifying potential treatment targets and even the magnitude of effect of targeted treatments in specific populations (Plotnikov and Guggenheim, 2019).

In the present study, we used MR analysis to determine the relationship between genetically determined appendicular lean mass (ALM) and handgrip strength (HGS) with LDL, VLDL and HDL particle sizes.

5.4 Methods

5.4.1 Study design

A two-sample MR study design was used whereby the summary statistics are provided from various studies for the association of the genetic instruments with the exposure and outcome. In our study, we obtained the summary statistics from the largest genome wide association studies (GWAS) on ALM (n = 450,243) (Pei et al., 2020) and HGS (n = 223,315) (Tikkanen et al., 2018) (exposures) and lipoprotein particle size (outcome). We applied methods to estimate the unbiased effects of ALM and HGS on LDL, HDL and VLDL particle size.

5.4.2 Genetic predictors of exposures

We used single nucleotide polymorphisms (SNPs) identified to be associated with ALM from the UK Biobank (Pei et al., 2020) with samples of self-reported white ancestry (n=450,243) and partial replication in a smaller population of South-Asian ancestry (n=7,452). We used SNPs identified to be associated with HGS, also from the UK biobank (Tikkanen et al., 2018), also with self-reported white British or European Caucasian ancestry (n=223,315). The UK Biobank is a population-based cohort of approximately 500,000 individuals; 54% are female, the average age is 57 (range 37–73), and 94% report being White British. Further details on

the rationale, design and methodology for UK Biobank can be found elsewhere (Sudlow et al., 2015).

GWAS were performed within each cohort according to uniform analysis plans. Additive genetic models using linear regression on natural-log-transformed ALM or HGS, respectively, were fitted and a fixed-effects inverse variance weighted (IVW) meta-analysis across the contributing cohorts was performed (Tikkanen et al., 2018; Pei et al., 2020) .

5.4.3 Association of genetic instruments with outcome

We retrieved the association of genetic instruments with SNPs associated with NMR-determined lipoprotein particle size using data obtained from the Medical Research Council Integrative Epidemiology Unit (MRC-IEU) Open GWAS project (Bristol, 2021). Detailed descriptions of the methods used to measure body composition are available on the UK Biobank website (UK Biobank-Body Composition Measurement, 2011). Briefly, whole body as well as site-specific (trunk, leg, arm) fat-free mass and fat mass were evaluated with bioelectrical-impedance analysis (BIA) (Tanita BC418MA body composition analyser). Body composition of a subset of participants was also assessed using dual-energy X-ray absorptiometry (DXA) which showed high correlation with BIA values (fat-free mass: r = 0.96) (UK Biobank-Body Composition Measurement, 2011).

5.4.4 Mendelian Randomisation analysis

We combined the effect of genetic instruments using inverse variance weighted (IVW) method as implemented in two-sample MR package of the statistical software R (R Core Team, Vienna, Austria. https://www.R-project.org/). We assessed the heterogeneity using the Q value for IVW. To address the potential effect of pleiotropic variants on the final effect estimate,

we conducted sensitivity analysis including weighted median (WM) and MR-Egger. Sensitivity analysis was conducted using the leave-one-out method. The WM estimate, as the weighted median of the SNP-specific estimates, provides correct estimates as long as SNPs accounting for ≥50% of the weight are valid instruments. It uses IVW and bootstrapping to estimate confidence intervals (CIs) (Bowden et al., 2016). MR-Egger has the ability to make estimates under the assumption that all SNPs are invalid instruments as long as the assumption of instrument strength independent of direct effect (InSIDE) is satisfied (Bowden et al., 2016). MR-Egger allows free estimation of the intercept, although further assumptions, such as the independence between instrument strength and direct effects, cannot be easily verified. Average directional pleiotropy across genetic variants was assessed from the p-value of the intercept term from MR-Egger (Bowden et al., 2016). Causal estimates in MR-Egger are less precise than those obtained by using IVW MR (Bowden, Davey Smith and Burgess, 2015). Analysis using MR-Egger has a lower false positive rate but a higher false negative rate than IVW (Burgess et al., 2017).

To assess heterogeneity between individual genetic variant estimates, we used the Q′ heterogeneity statistic (Bowden et al., 2017) and the MR pleiotropy residual sum and outlier (MR-PRESSO) test (Bowden et al., 2017). The Q′ statistic uses modified 2nd order weights that are a derivation of a Taylor series expansion and take into account uncertainty in both numerator and denominator of the instrumental variable ratio (this eases the no-measurement-error [NOME] assumption) (Bowden et al., 2017). The MR-PRESSO framework relies on the regression of variant-outcome associations on variant-exposure associations and implements a global heterogeneity test by comparing the observed distance (residual sums of squares) of all variants to the regression line with the distance expected under the null hypothesis of no pleiotropy (Verbanck et al., 2018). In case of evidence of horizontal pleiotropy, the test compares individual variants expected and observed distributions to identify outlier variants. Further we applied on MR-Robust Adjusted Profile Score (RAPS): this

method is able to correct for pleiotropy using robust adjusted profile scores. We considered as results, causal estimates that agreed in direction and magnitude across MR methods, passed nominal significance in IVW MR, and did not show evidence of bias from horizontal pleiotropy using heterogeneity tests. We used R version 3.4.2 (R Core Development Team 2017).

The MR studies assume that the SNPs (instrumental variables) are associated with the outcome only via the exposure (Lawlor et al., 2008), so we performed sensitivity analysis excluding SNPs with potentially pleiotropic effects. To assess the instrumental variable analysis "exclusion-restriction" assumption we used Ensembl release (http://useast.ensembl.org/index.html). Ensembl contains a base of SNP phenotypes (http://useast.ensembl.org/index.html/).

5.4.5 Ethics

This investigation used published or publicly available summary data with no involvement of participants in the study. No original data were collected for this manuscript. Ethical approval for each of the studies included in the investigation can be found in the original publications (including informed consent from each subject).

5.5 Results

In total, 608 SNPs and 169 SNPs were identified as instrumental variables for ALM, and right HGS, respectively, none of which were significantly associated with LDL, VLDL or HDL particle diameter, indicating a low risk of SNPs affecting multiple phenotypes via independent biological pathways. The results of MR analysis, displayed as beta-coefficient for interested outcomes per increase in ALM, demonstrated a positive and statistically significant effect on

HDL particle diameter (MR Egger: β= 0.055, SE = 0.031, p = 0.081 and IVW: β = 0.068, SE = 0.014, p = $6.15x10^{-7}$; respectively, Table 5.1 and Figure 5.1), and a negative and statistically significant effect on VLDL particle diameter (MR Egger: β = -0.114, SE = 0.039, p=0.003 and IVW: β = -0.081, SE = 0.017, p=$1.57x10^{-6}$, respectively, Table 5.1 and Figure 5.2). These data suggest that each unit (kg) increase in ALM is associated with an increase of 0.07 nm in HDL particle diameter and a decrease of 0.08 nm in VLDL particle diameter. However, no statistically significant effect of ALM was observed for LDL particle diameter (MR Egger: β = 0.035, SE = 0.03, p = 0.178 and IVW: β = -0.006, SE = 0.011, p = 0.575, Table 5.1 and Figure 5.3).

Table 5.1 Results of the Mendelian Randomization analysis for effects of genetically determined appendicular lean mass and handgrip strength on LDL, VLDL and HDL particle size

Exposure	Outcome	MR				Heterogeneity			Pleiotropy		
		Method	beta	SE	p-value	Method	Q	p-value	Intercept	SE	p-value
Appendicular lean mass	**LDL**	**MR Egger**	0.03541	0.02624	0.1777	**MR-Egger**	987.3	$9.8x10^{-23}$	-0.001	0.0006	0.078
		WM	-0.002108	0.0154	0.8911						
		IVW	-0.006395	0.01139	0.5745	**IVW**	992.6	$4.3x10^{-23}$			
		RAPS	-0.02011	0.03798	0.5967						
	VLDL	**MR Egger**	-0.1137	0.0387	0.003427	**MR-Egger**	2359.7	$3.9x10^{-210}$	0.0008	0.0009	0.341
		WM	-0.05141	0.01483	0.0005289						
		IVW	-0.08051	0.01677	$1.57x10^{-6}$	**IVW**	2363.3	$1.9x10^{-210}$			
		RAPS	-0.04956	0.02853	0.0829						
	HDL	**MR Egger**	0.05491	0.03144	0.08124	**MR-Egger**	1723.9	$5.7x10^{-112}$	0.0003	0.0007	0.647
		WM	0.06454	0.01457	$9.42\ x10^{-6}$						
		IVW	0.06788	0.01361	$6.15\ x10^{-7}$	**IVW**	1724.5	$8.02x10^{-112}$			
		RAPS	0.07245	0.03312	0.02911						
Handgrip strength	**LDL**	**MR Egger**	0.1764	0.1397	0.2085	**MR-Egger**	260.9	$8.1x10^{-07}$	-0.0012	0.0017	0.463
		WM	0.06296	0.04887	0.1976						
		IVW	0.07787	0.03945	0.04839	**IVW**	261.8	$8.7x10^{-07}$			
		RAPS	0.1377	0.1824	0.4515						
	VLDL	**MR Egger**	-0.4159	0.1625	0.01142	**MR-Egger**	388.3	$5.1x10^{-21}$	0.0037	0.0019	0.061
		WM	-0.1884	0.04841	$9.96\ x10^{-5}$						
		IVW	-0.1219	0.04634	0.008504	**IVW**	396.9	$6.1x10^{-22}$			
		RAPS	-0.2736	0.1171	0.02068						
	HDL	**MR Egger**	0.4328	0.1841	0.01997	**MR-Egger**	551.8	$1.6x10e^{-44}$	-0.0039	0.0022	0.079
		WM	0.03131	0.04646	0.5003						
		IVW	0.1211	0.05242	0.0209	**IVW**	562.5	$6.2x10^{-46}$			
		RAPS	-0.03558	0.1262	0.7783						

WM: weighted median; IVW: inverse variance weighted; RAPS: robust-adjusted profile score; SE: standard error; beta: beta-coefficients; Q: Cochran's Q statistic; MR: Mendelian randomization; LDL: low-density lipoprotein; VLDL: very low-density lipoprotein; HDL: high-density lipoprotein

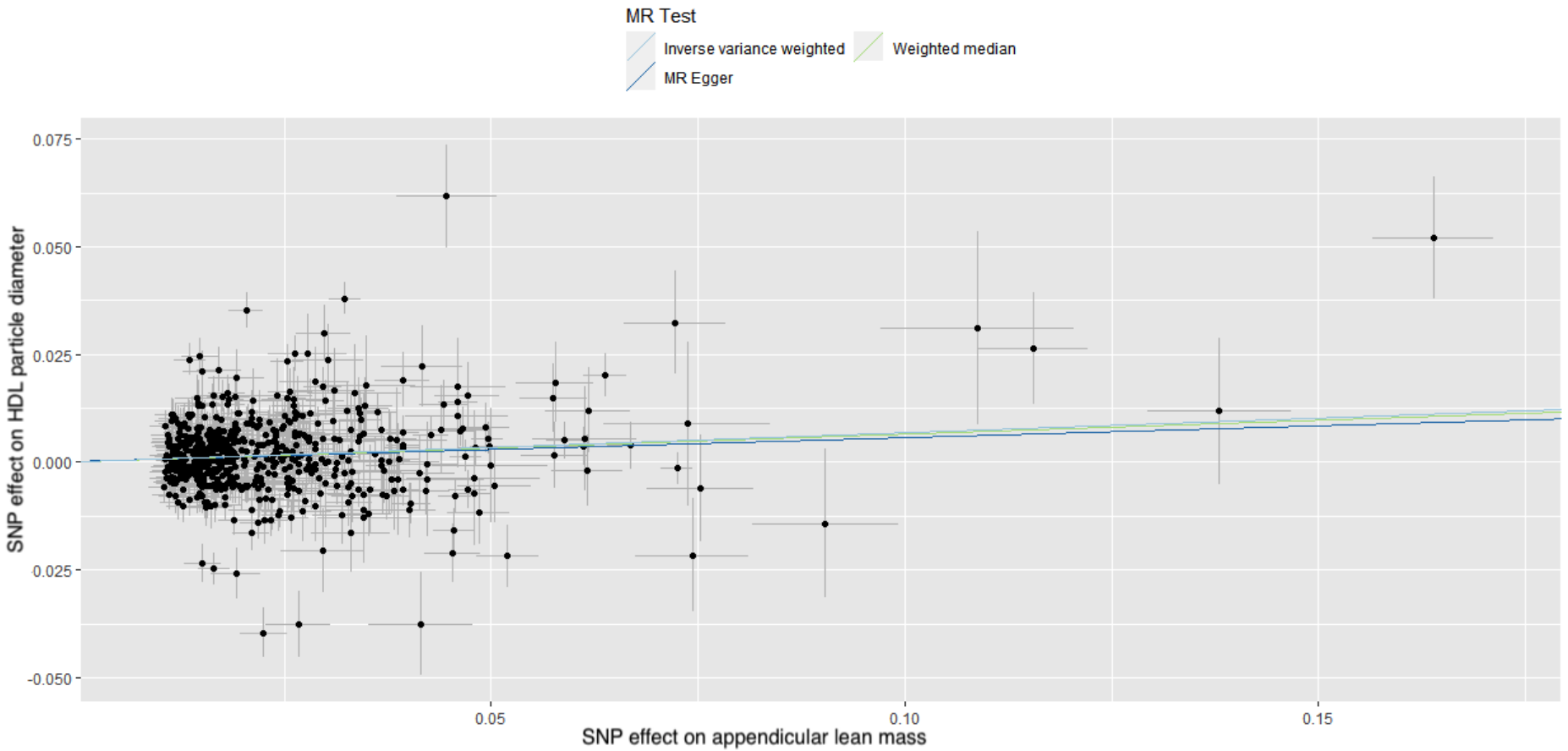

Figure 5.1 Scatter plot of the association of the effect of SNP-determined serum appendicular lean mass on HDL particle diameter. Each black point represents an SNP, plotted by the estimate of SNP on appendicular lean mass (X-axis, kg) and the estimate of SNP on HDL particle diameter (Y-axis, nm). The slopes of each line represent the potential causal associations for each method.

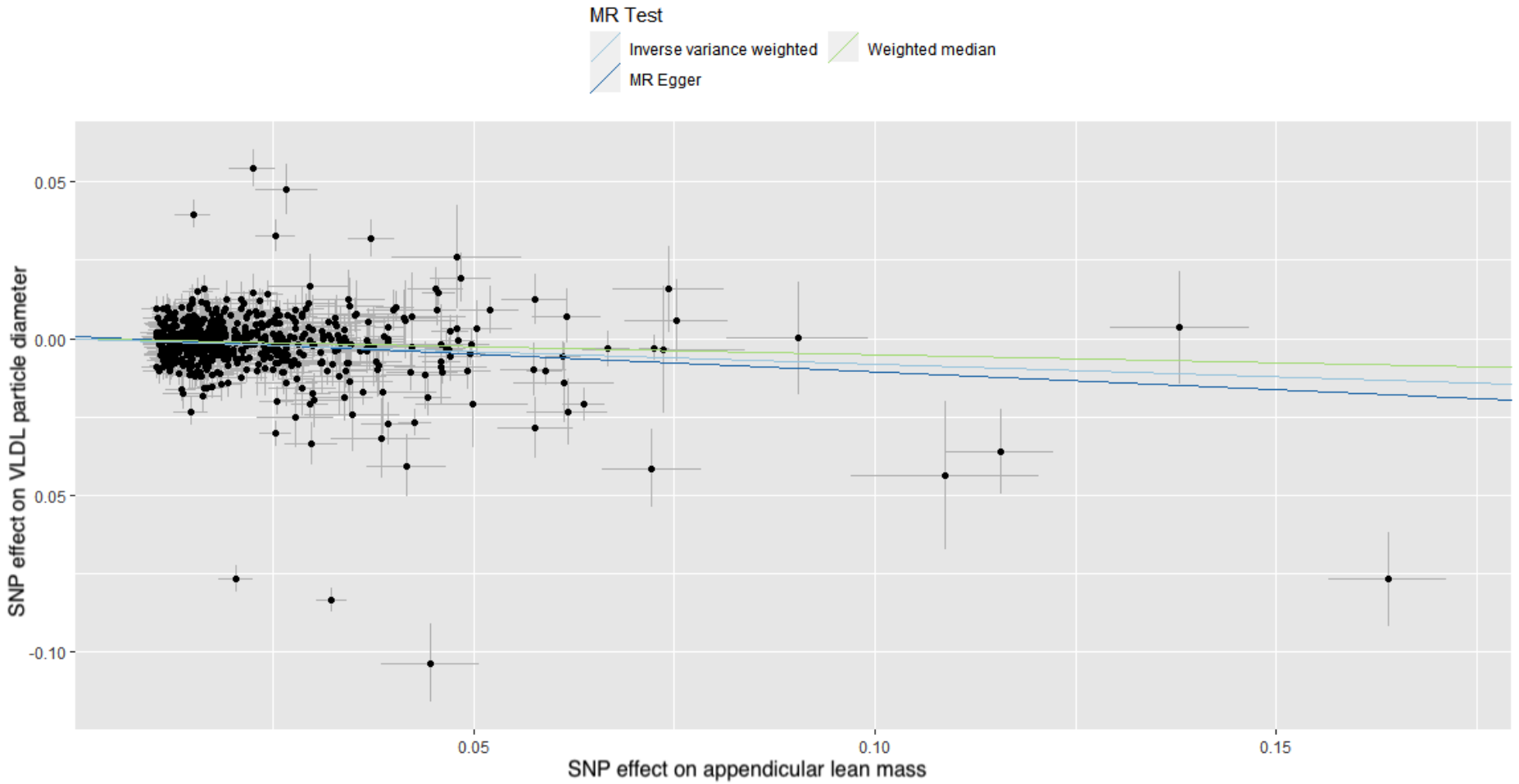

Figure 5.2 Scatter plot of the association of the effect of SNP-determined serum appendicular lean mass on VLDL particle diameter. Each black point represents an SNP, plotted by the estimate of SNP on appendicular lean mass (X-axis, kg) and the estimate of SNP on VLDL particle diameter (Y-axis, nm). The slopes of each line represent the potential causal associations for each method.

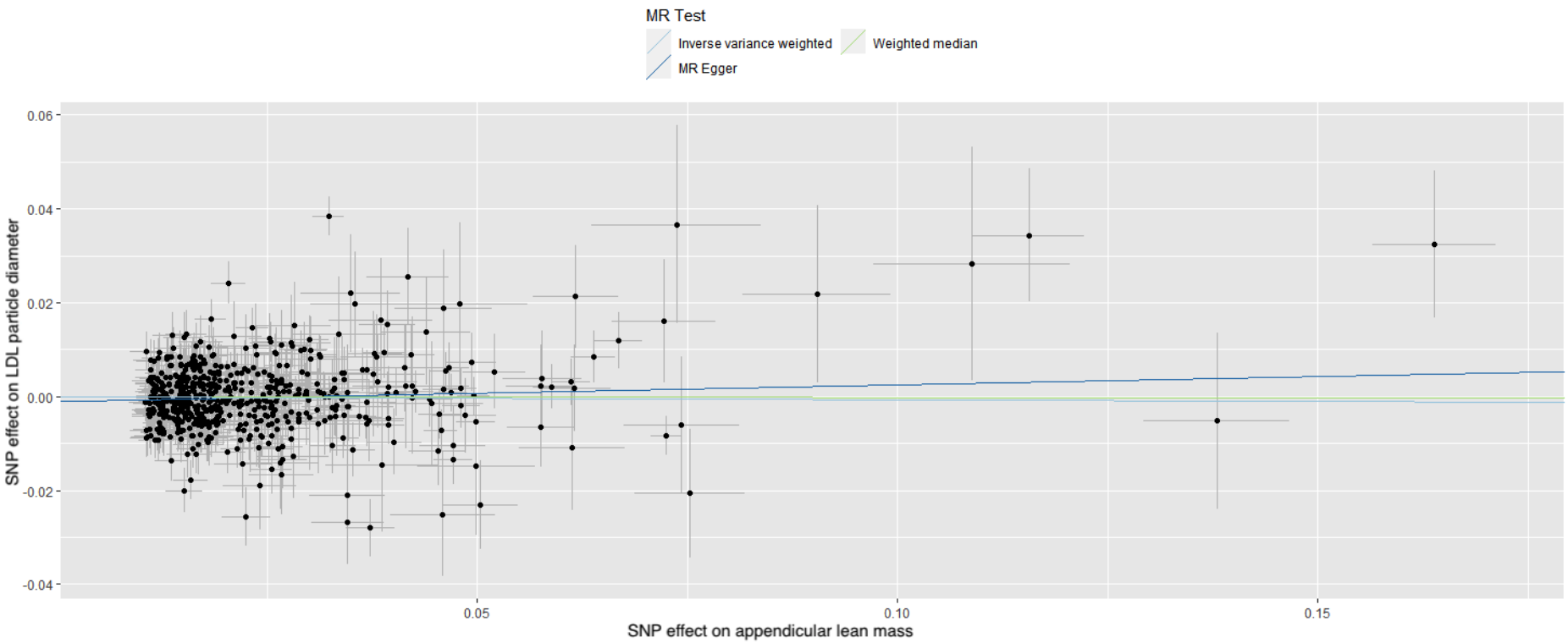

Figure 5.3 Scatter plot of the association of the effect of SNP-determined serum appendicular lean mass on LDL particle diameter. Each black point represents an SNP, plotted by the estimate of SNP on appendicular lean mass (x-axis, kg) and the estimate of SNP on LDL particle diameter (y-axis, nm). The slopes of each line represent the potential causal associations for each method.

The MR analysis of handgrip strength, displayed as beta-coefficient for interested outcomes per increase in HGS, a positive and statistically significant effect on HDL particle diameter (MR Egger: β = 0.433, SE = 0.184, p = 0.019 and IVW: β = 0.121, SE = 0.052, p = 0.021, Table 5.1), and a negative and statistically significant effect on VLDL particle diameter (MR Egger: β = -0.416, SE = 0.163, p = 0.011 and IVW: β = -0.122, SE = 0.046, p = 0.009, Table 5.1), was observed. These data suggest that each unit (kg) increase in handgrip strength is associated with an increase of 0.12 nm in HDL particle diameter and a decrease of 0.12 in VLDL particle diameter. No statistically significant effect of HGS was observed for LDL particle diameter (MR Egger: β = 0.176, SE = 0.139, p = 0.209 and IVW: β = 0.078, SE = 0.039, p = 0.048, Table 5.1).

The horizontal pleiotropy test, with very negligible Egger regression intercept, also showed a low likelihood of pleiotropy for all our estimations (all p > 0.05, Table 5.1), indicating a low risk of SNPs affecting multiple phenotypes via independent biological pathways. Furthermore, the result of the MR-RAPS was identical with the IVW prediction, which again indicated a statistically low chance of pleiotropy. Heterogeneity tests highlighted no trace of heterogeneity (Table 5.1). Furthermore, MR-PRESSO analysis did not indicate any outliers for any estimates. Results of leave-one-out method demonstrated that the links are not driven by any single SNP.

5.6 Discussion

To our knowledge, this is the first paper to reveal a potentially causal link between both genetically determined ALM and HGS with increased HDL particle diameter and decreased VLDL diameter. Due to the relative novelty of the relationship of lipid particle diameter with muscle mass and strength, especially in terms of CVD risk, we cannot compare our results

directly with the results of other studies. However, there are a number of observational studies and randomised controlled trials that have shown relationships between HDL-C and VLDL-C concentrations and either muscle mass or strength. In a population of Japanese men and women (n=991, age range 35-77 y), greater muscle thickness in the abdomen and thigh (quadriceps and hamstring muscles), relative to BMI, was significantly and positively associated with HDL-C concentrations in both sexes (Abe and Fukunaga, 1994). Comparing a group of healthy men (n=72, mean age 41 y) and men with CHD (n=20, mean age 48 y), Tikkanen and colleagues observed that a greater percentage of slow twitch muscle fibres was associated with higher concentrations of HDL-C (Tikkanen et al., 1998). In a cross-sectional study, Wu and colleagues assessed the HGS of 17,703 Chinese men and women aged 40 years and older (median 45.2, IQR = 51.3-59.2) and determined that reduced HGS was associated with reduced HDL-C, as well as other components of metabolic syndrome, including elevated triglycerides, blood pressure and fasting glucose levels (Wu et al., 2019).

Intervention trials have also revealed a relationship between increases in muscle mass with improved HDL-C levels. Ullrich and colleagues (Ullrich, Reid and Yeater, 1987) enrolled 25 young men (18-35 yr) in an 8-week resistance exercise (RE) program and reported that while body weight did not change significantly, muscle mass was observed to increase and was accompanied by a 14% increase in HDL-C concentrations (38.8 to 44.1 mg/dl (1-1.14 mmol/L), $p < 0.001$). It should be noted that the increase in muscle occurred with a simultaneous decrease of body fat percentage from 14-12.7%, which may independently affect HDL-C levels (Ullrich, Reid and Yeater, 1987). Similarly, acute bouts of RE, known to elicit increases in muscle size and strength (Lysenko, Vinogradova and Popov, 2021), have also been shown to reduce plasma VLDL triglyceride levels (Magkos et al., 2008). However, it is important to note that, to our knowledge there are no interventions assessing the effects of RE on HDL or VLDL particle size.

Clinically, CVD risk is associated inversely with plasma concentrations of HDL-C, and positively with those of VLDL-C (Nordestgaard and Tybjaerg-Hansen, 1992; Baigent et al., 2005). VLDL is an apolipoprotein B (apoB)-containing lipoprotein, which, along with LDL and intermediate-density lipoprotein (IDL), plays a significant role in the development of atherogenic plaques (Ross, 1999; Sniderman et al., 2019). The diameter of these apoB-containing lipoproteins is small enough for them to pass freely into the endothelial intima of blood vessels where, in the presence of endothelial damage or dysfunction, they may be taken up by macrophages (Ross, 1999). This leads to further inflammation and endothelial smooth muscle cell proliferation, and the development of atherosclerotic plaques typical of CHD (Ross, 1999). In contrast to this direct effect, a larger VLDL diameter is associated with greater CVD risk (Freedman et al., 1998; Colhoun et al., 2002), potentially via modification to other lipoproteins. Large VLDL particles, rich in triglycerides, may potentially play a role in the development of CHD through mechanisms such as increased formation of highly atherogenic sdLDL (Packard, 2003) and increased catabolism of HDL (Rashid et al., 2003; Adiels et al., 2008).

Conversely, HDL is the key particle involved in reverse cholesterol transport, which transports excess cholesterol from peripheral body tissues to the liver for recycling or eventual excretion (Hill and McQueen, 1997). It is via this mechanism, as well as through its antioxidant and anti-inflammatory actions, that HDL is thought to reduce the progression of atherosclerosis and the risk of CVD such as coronary artery disease (CAD) (Hill and McQueen, 1997; Navab et al., 2011; Soran, Schofield and Durrington, 2015). Furthermore, smaller HDL particle size has been associated with an adverse cardiometabolic risk profile, with larger particle size associated with more favourable risk profile in the EPIC-Norfolk prospective population study (Arsenault et al., 2009). However, upon adjustment for other markers of CAD such as ApoB and triglyceride levels, smaller HDL particle size was deemed to not contribute directly to CAD risk and may instead reflect a state of metabolic syndrome (El Harchaoui et al., 2009). In

contrast, in a dietary study of extra virgin olive oil (EVOO) supplementation, older participants were found to have both lower cholesterol efflux capacity (CEC) and a predominance of smaller HDL particles compared to younger participants. After 12 weeks of supplementation with EVOO, the CEC of HDL was found to be improved through an increase in larger HDL and a decrease in smaller HDL particles, highlighting the role of particle size in HDL function (Otrante et al., 2021). Further study is required to fully assess the role of HDL particle size in CVD development and risk.

It should be noted that our study did not reveal an effect of increased ALM or HGS on LDL particle diameter. Due to LDL being the primary apoB containing lipoprotein in circulation, it plays a major causal role in the development of atherosclerosis (Ference et al., 2017). LDL particle size is known to contribute to CAD risk, with smaller particles having a longer plasma residence time, greater propensity to oxidation, and potentially infiltrating the endothelial intima more readily than larger particles and initiating an atherosclerotic cascade (Ross, 1999; Thongtang et al., 2017). Previous research has indicated that exercise training may lead to increases in LDL particle size (Houmard et al., 1994; Sarzynski et al., 2015) but it should be noted that exercise may exert effects on lipoprotein patterns through multiple mechanisms and as such may not be directly comparable with our results which focus on the effects of muscle size and strength.

Both low skeletal muscle mass and low HGS are important risk factors for the development of CVD and indeed mortality from CVD and all-causes (Chainani et al., 2016; Srikanthan, Horwich and Tseng, 2016; Kim et al., 2019; Tyrovolas et al., 2020). More specifically, low skeletal muscle and low HGS may be independent risk factors for greater carotid intima-media thickness and high plaque score (Shin and Lim, 2021), highlighting their relevance in the development of atherosclerosis. However, the mechanisms by which muscle mass and

strength may affect atherosclerosis are poorly understood. For example, muscle cells have been observed to efflux cholesterol to apoA1 during reverse cholesterol transport, which may contribute to elevations in circulating HDL-C (Muscat et al., 2002), and greater muscle mass and strength are known to be associated with increased circulating HDL-C (Abe and Fukunaga, 1994; Wu et al., 2019). The results of our study highlight a possible causal link between both greater ALM and HGS and increased HDL particle size, which may partially explain the mechanism by which muscle mass and strength contribute to reduced CVD risk.

Similarly, VLDL concentration is known to be acutely influenced by exercise and particularly RE (Magkos et al., 2008) although, to our knowledge no studies have associated muscle mass with VLDL-C concentration. However, the results of our study indicate that greater ALM and HGS are potentially causally associated with smaller VLDL particle size. On a per-particle basis, triglyceride-rich apoB-containing lipoproteins, such as large VLDL, are considered to exert a greater risk of myocardial infarction than other apoB-containing lipoproteins (Balling et al., 2020; Johansen et al., 2021), highlighting their relevance in CVD. Our results suggest another potentially clinically significant benefit of increased muscle mass through potentially reducing VLDL particle diameter and leading to a possible reduction in its atherogenic potential.

Exercise, in particular RE, is known to be the key driving force for increases in muscle mass and strength (Lysenko, Vinogradova and Popov, 2021) and chronic exercise is associated with greater muscle mass and function in older adults (Aagaard et al., 2007). As such, it may be hypothesised that the deliberate use of exercise to improve muscle mass and function may lead to the changes in HDL and VLDL particle diameter that were determined in this study. These changes in lipoprotein properties may confer an improved risk profile for CVD. Further

research is required to fully elucidate the effect of interventions to increase ALM and muscle strength on HDL and VLDL particle size and their relation to risk of CVD.

A major strength of our study was the large sample population study with access to individual participant data of high validity from the UK Biobank cohort, and with the relevant SNPs available for both ALM and HGS. The use of ALM instead of fat-free mass (FFM) is also of importance. ALM consists predominantly of skeletal muscle, while FFM is composed of skeletal, smooth and cardiac muscle as well as bone and other non-fat tissues (Kyle et al., 2001). Sarcopenia and the chronic conditions associated with it are defined by decreases in skeletal muscle mass and strength (Cruz-Jentoft et al., 2019) and these deficiencies in muscle size and function can be ameliorated with appropriate exercise and nutrition interventions (Hou et al., 2019), highlighting the clinical relevance of ALM. The agreement of our results for the similar effects of both greater ALM and strength on HDL and VLDL particle diameter further strengthen our findings. Additionally, the use of the Mendelian randomisation approach allowed us to examine the potential causal effects of genetically determined ALM and HGS on lipoprotein particle size, largely without the disadvantages of confounding or reverse causation. The use of segmental bioelectrical impedance analysis (BIA) for determining ALM in the UK Biobank cohort is a potential limitation of this study. BIA measurement accuracy is known to be affected by hydration status; however, the UK Biobank protocol did not specify any procedures to standardise hydration status before assessment. There exists the potential for such variation in hydration status to lead to inaccuracies in the ALM values attained (Kyle et al., 2004). Evidence suggests that BIA may be less accurate at high BMI levels, which may be relevant considering the range of BMI included in the UK Biobank cohort (Neovius et al., 2006).

5.6.1 Conclusion

In conclusion, we found evidence for a potentially causal association of both genetically-determined, elevated ALM and HGS, with both increasing HDL particle size and decreasing VLDL particle size. The relationship between both ALM and HGS with LDL particle size was not statistically significant. Our findings highlight the potential importance of maintaining higher levels of ALM and HGS in order to improve one's CVD risk profile. Further research is required to investigate the mechanisms of greater ALM and HGS on lipoprotein particle size.

6 Chapter 6: Impact of COVID-19 on cardiac rehabilitation

Impact of COVID-19 lockdown restrictions on cardiac rehabilitation participation and behaviours in the United Kingdom

Kirwan R, Perez de Heredia F, McCullough D, Butler T, Davies IG.

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6.1 Context within thesis

While the COVID-19 pandemic led to considerable limitations on research and society in general, it also presented an opportunity to study the effects of a major societal change on participation in the very programme being investigated in this PhD *i.e.* cardiac rehabilitation. As such chapter 6 will discuss a cross-sectional analysis with the aim of determining the impact of COVID-19 lockdown restrictions on CR behaviours and perceptions of effectiveness, motivation and intent to continue. Analysis of such data may potentially lead to the development of more efficacious CR practices for those who are unable to participate in centre-based CR and may also be useful for rapid and effective implementation of CR in potential future scenarios that limit the availability of CR.

6.2 Abstract

Background: COVID-19 lockdown measures led to the suspension of centre-based cardiac rehabilitation (CR). We aimed to describe the impact of lockdown on CR behaviours and perceptions of efficacy in a sample of CR participants.

Methods: An online survey was conducted amongst CR participants from May to October 2020, COVID-19-related lockdown restrictions. Anthropometric data, participant-determined levels of motivation and self-perceived efficacy, CR practices etc., pre- and post-lockdown, were collected.

Results: The probability of practicing CR in public gyms and hospitals decreased 15-fold (47.2% pre-, 5.6% post-lockdown; OR [95% CI]: 0.065 [0.013; 0.318], p<0.001), and 34-fold (47.2% pre, 2.8% post; OR [95% CI]: 0.029 [0.004; 0.223], p<0.001), respectively. Amongst participants, 79.5% indicated that their CR goals had changed and were 78% less likely to engage in CR for socialization after lockdown (47.2% pre, 16.7% post; OR [95% CI]: 0.220 [0.087; 0.555]; p=0.002). The probability of receiving in-person supervision decreased by 90%

(94.4% pre, 16.7% post; OR [95% CI]: 0.011 [0.002; 0.056]), while participants were almost 7 times more likely to use online supervision (11.1% pre, 44.4% post; OR [95% CI]: 6.824 [2.450; 19.002]) (both p<0.001). Fifty percent indicated that their enjoyment of CR was lower than before lockdown and 27.8% reported they would be less likely to continue with CR in the newer format.

Conclusions: Lockdown was associated with considerable changes in how CR was practiced, motivation levels and willingness to continue with CR. Further research is warranted to develop and improve strategies to implement in times when individuals cannot attend CR in person and not only during pandemics.

6.3 Introduction

Cardiovascular disease (CVD) is responsible for 1 in 4 deaths in the United Kingdom (UK) or over 170,000 deaths/year (Foundation, 2019). Cardiac rehabilitation (CR) is a primarily exercise-based intervention for those with established CVD or those at high risk of developing adverse cardiac events, aimed at reducing the risk of said events (Anderson et al., 2016; Piepoli et al., 2016). Exercise-based CR is known to reduce the risk of mortality from CVD as well as the risk of cardiac-related hospitalisation, and also improving quality of life compared to non-exercise controls (Lavie and Milani, 2006; Sagar et al., 2015; Anderson et al., 2016). As such, exercise-based CR can be considered a key practice in maintaining the health of those at risk of CVD.

The COVID-19 pandemic developed into an unparalleled crisis leading to the execution of diverse measures aimed at reducing the spread of the virus. In the UK, travel bans, quarantine, isolation, and social distancing measures were enforced to varying degrees (Parmet and Sinha, 2020; Sjödin et al., 2020). This led to the suspension of group-based CR programmes, which are frequently hosted in hospitals, community centres and public gyms (Dawkes et al.,

2020; Papathanasiou et al., 2020) with up to 72% of Phase IV (long-term, community-based) CR programmes being suspended (O'Doherty et al., 2021). Long-term maintenance of physical activity, as observed in CR Phase IV, is known to lead to greater benefits to cardiac health (Giuliano et al., 2017). Furthermore, these restrictions were observed to lead to decreases in physical activity (PA) and increases in sedentary behaviour (Ammar et al., 2020; Sun et al., 2020). This extended period of reduced physical activity has the potential to impact the health of the general population (Hall et al., 2020; Kirwan et al., 2020; Akbari et al., 2021) and especially those at high risk of CVD (Peçanha et al., 2020). Furthermore, those with CVD are at greater risk of COVID-19 mortality (Noor and Islam, 2020), and in turn severe infection may lead to cardiovascular complications and myocardial injury, putting this population at even greater risk (Babapoor-Farrokhran et al., 2020; Seecheran et al., 2020).

Accordingly, health professionals and physical and rehabilitation medicine (PRM) specialists who play a vital role in the management of CR programmes (Papathanasiou et al., 2016) were recommended to provide home-based CR resources, in order to maintain this essential health service (Dawkes et al., 2020). This resulted in the increased use of formats such as telephone and online-video to facilitate at-home CR (O'Doherty et al., 2021). It has been demonstrated that home-based CR can be at least as effective as centre-based CR in terms of outcomes such as blood pressure, total cholesterol, psychological status and exercise capacity (Jolly et al., 2009; Buckingham et al., 2016). However, there is little data relating to the use of the particular modalities of at-home CR employed during the COVID-19 pandemic. As such, it is unknown whether these particular methods of at-home CR could negatively impact home-based CR, or result in reduced uptake, inadequate implementation and/or reduced efficacy. This may result from a number of obstacles including but not limited to lack of appropriate exercise equipment/facilities, inadequate supervision and poor motivation due to exercising alone (Manaf, 2013).

Determining the impact of COVID-19 lockdown restrictions on CR-related exercise behaviours and PA, as well as motivation to continue with CR, may provide valuable information for improving current home-based CR recommendations. This may lead to more efficacious CR for those who are unable to or do not wish to participate in centre-based CR and may also be useful for rapid and effective implementation of CR in potential future waves of COVID-19 or other pandemics (Grech, Cuschieri and Gauci, 2020). The aim of this study was to determine the impact of COVID-19 lockdown restrictions on CR behaviours and perceptions of effectiveness, motivation and intent to continue.

6.4 Methods

6.4.1 Design

A cross-sectional, online questionnaire was conducted amongst CR participants, from May to October 2020, while the UK experienced varying degrees of COVID-19-related lockdown restrictions. Briefly, in March 2020, schools and non-essential businesses were closed, unnecessary travel was discouraged, time outside of the home was limited and social distancing guidelines were enforced. This included suspension of “non-essential” healthcare services such as CR. From May 2020, restrictions began to be gradually eased, while still encouraging social distancing measures. In October 2020, due to high rates of COVID cases, a “three-tier” COVID system was implemented based on area-specific infection rates (Government, 2021).

6.4.2 Recruitment

The questionnaire was distributed via a variety of methods including the authors’ interpersonal and professional networks, social media (Instagram), and through direct contact with centre-based CR services in the UK. Initial contact was with CR service providers, *i.e.*, CR-certified

exercise physiologists and exercise instructors or related professional bodies such as British Association of Cardiac Prevention and Rehabilitation (BACPR) and British Heart Foundation (BHF). Instructors were asked to sign a gatekeeper consent form and then contact their CR participants directly using email and/or text messages including a link to the online questionnaire. The link included a participant information sheet with information on the study as well as a consent form and screening questionnaire. A recruitment flow diagram can be seen in Figure 6.1.

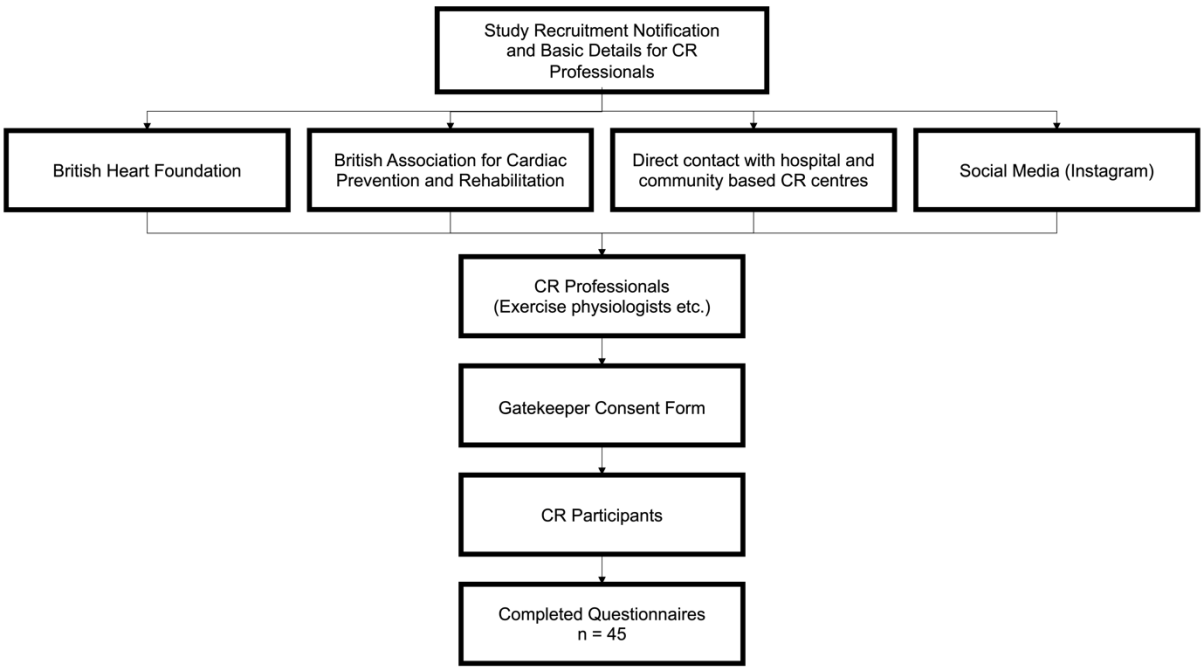


Figure 6.1. Recruitment flow diagram. CR: cardiac rehabilitation

6.4.3 Ethics

This study received ethical approval from the University Research Ethics Committee at Liverpool John Moores University (UREC reference: 20/NSP/026). The study was conducted according to the ethical principles of the Declaration of Helsinki (Assembly, 2018) and online informed consent was obtained from all participants before participation.

6.4.4 Participants

A screening questionnaire was used to ensure participants fulfilled the following requirements: 1) they were participating in phase IV CR regularly at least twice per week before the UK Government implemented lockdown measures to limit the spread of COVID-19; 2) participant's face-to-face CR was suspended due to Government regulations in response to the COVID-19 pandemic; 3) participant's cardiac condition was not congenital or due to drug or alcohol abuse; 4) participants were older than 18 years; 5) participants were not pregnant. Phase IV CR was selected due to the significant impact of COVID-19 restrictions on the suspension of such programmes and the importance of long-term physical activity maintenance for improving cardiac health (Giuliano et al., 2017; O'Doherty et al., 2021).

6.4.5 Implementation and assessments

The questionnaires were administered through JISC Online Surveys (Bristol, UK) and took approximately 40 minutes to complete. An ad hoc online questionnaire was developed to gather the following information: age, gender, area of residence, ethnicity, height and weight, as well as questions relating to CR practices and perceptions, pre- and post-lockdown. These included questions on CR location, exercise types, CR modality (group, online etc.), duration, frequency, perceived effort, perceived efficacy, goals, motivation etc.

The survey also included further, validated questionnaires relating to physical activity levels, quality of life, psychological-wellbeing, food access and perceptions around healthy eating. Data from these questionnaires will be discussed in separate publications.

6.4.6 Data analysis

The demographic characteristics and the length of engagement with CR were compared between participants still actively engaged in their programmes and those who had quit CR after lockdown, by means of non-parametric Mann-Whitney tests. For those participants still conducting some form of CR, we compared their behaviours, attitudes, and conditions of CR practice before and after lockdown. Changes after lockdown in location of CR practice, goals, mode of CR practice/supervision, purchase of equipment, and moment of the day for CR practice were analysed by binomial regression, using a generalised linear model with repeated measures and fixed effects for time (post vs. pre-lockdown) and sex (women vs. men). Duration and frequency of the sessions were compared before and after lockdown by McNemar-Bowker tests (as these were categorical variables). Due to low counts, the initial categories for duration of exercise (*i.e.*, 0-20 min, 20-40 min, 40-60 min, and >60 min/session) were pooled into two categories: 0-40 min and >40 min/session, and frequencies were compared between before and after lockdown with the McNemar test. Number of exercises per session and participant's change in perceived effort were analysed by repeated measures Wilcoxon signed ranked tests. All analyses were conducted with IBM SPSS software v. 26. Statistical significance was set at $p<0.05$, and Bonferroni corrections were applied to account for multiple testing in the binomial regressions, according to the number of tests conducted for each variable (namely the number of different locations, goals, mode of practice, etc.).

6.5 Results

6.5.1 Demographics

A total of 45 people participated in the questionnaire. Demographic details of the sample are presented in Table 6.1. Thirty-six participants (80%) were still participating in some form of CR at the time of the study. There were trends for this subsample to have been engaged in their programme for longer and to be older, although these differences were not significant (U=101.5, p=0.086 and U=103.5, p=0.097, respectively). Similarly, other demographic variables (sex, ethnicity, or body mass index) were not significantly associated with the likelihood of continuing with or ceasing CR.

Table 6.1. Demographic characteristics of the sample.

	All (n=45)	Still engaging in CR (n=36)	No longer engaging in CR (n=9)
Age (years)	70 (63, 74)	71 (63.8, 74.8)	67 (61.5, 70.5)
Male (%)	88.9	91.7	77.8
White ethnicity (%)	91.1	88.9	100.0
Height (cm)	175.0 (169.0, 182.0)	176.5 (169.8, 182.3)	172.0 (162.5, 177.5)
Weight (kg)	79.3 (73.6, 92.6)	79.4 (73.0, 93.0)	77.1 (70.2, 92.4)
Body mass index (kg/m2)	25.4 (23.9, 29.4)	25.2 (23.8, 29.3)	28.5 (25.0, 29.7)
Time in CR before lockdown (weeks)	70.0 (22.5, 229.0)	102.0 (50.5, 257.5)	22.0 (8.5, 130.0)
Engaging in CR at time of study (%)	80	-	-

Values are presented as percentages, or as median (Q1, Q3). Participants still actively engaged in their CR programmes and those who had quit CR were compared by Mann-Whitney tests, significance set at $p<0.05$; n for multiple testing. CR = Cardiac rehabilitation.

6.5.2 CR location before and after lockdown

Compared to before lockdown, the probability of participants attending public gyms decreased 15-fold (47.2% pre- vs. 5.6% post-lockdown; OR [95% CI]: 0.065 [0.013; 0.318]), and the probability of performing CR at hospitals fell by 34-fold (47.2% pre- vs. 2.8% post-lockdown; OR [95% CI]: 0.029 [0.004; 0.223]) (both $p<0.001$). In contrast, participants were 59 times more likely to engage in CR at home (11.1% pre- vs. 86.1% post-lockdown; OR [95% CI]: 59.2

[14.4; 244.0]) (p<0.001) (Figure 6.2 and Supplementary Table 6.1). In general, women were significantly less likely to conduct CR at hospitals than men (OR [95% CI]: 5.4×10−5 [2.7×10−5; 0.0]; p<0.001) (Supplementary Table 6.1).

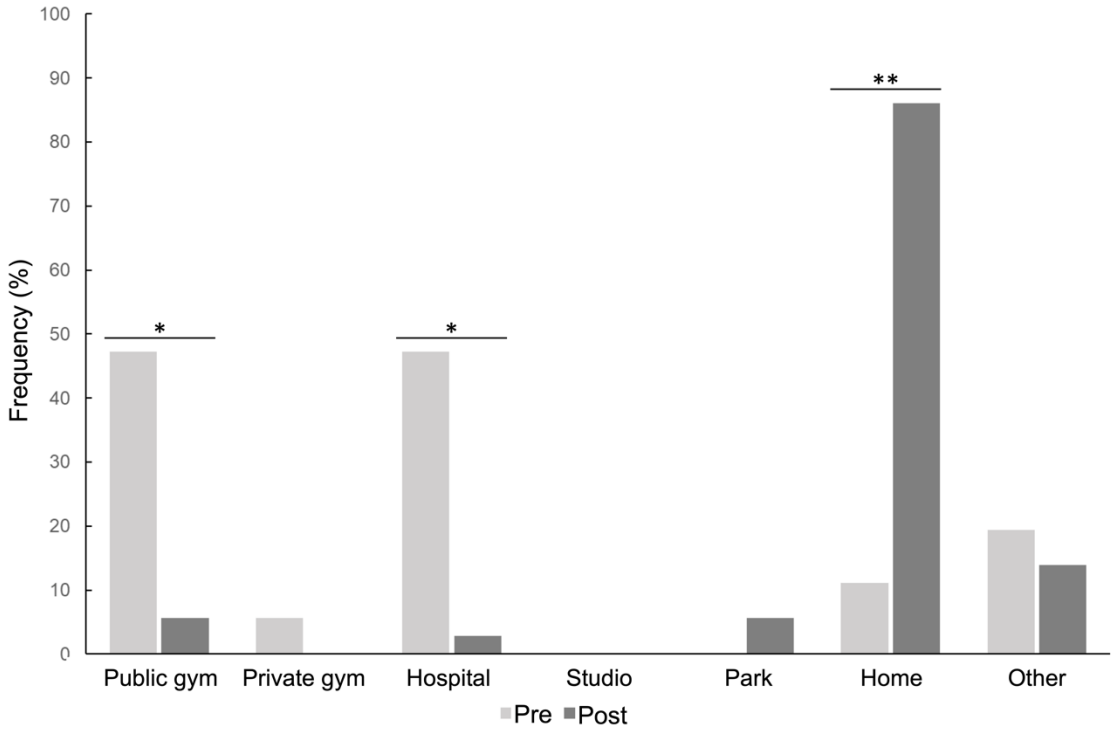


Figure 6.2. Influence of lockdown on location of cardiac rehabilitation practice. Bars represent the proportion (%) of participants who responded “Yes” to practising CR in each location. The change in the probability of practicing CR in a given location post-lockdown vs. pre-lockdown was analysed by mixed model GLM with repeated measures. (*, **) Significant differences after post-hoc correction for multiple testing, *p<0.0083, **p<0.001.

6.5.3 Goals

During lockdown, 79.5% of participants indicated that their CR goals had changed compared with pre-lockdown. Of note, participants were 78% less likely to engage in CR for socialization after lockdown (47.2% pre- vs. 16.7% post-lockdown; OR [95% CI]: 0.220 [0.087; 0.555];

p=0.002) (Figure 6.3 and Supplementary Table 6.2). Regardless of lockdown, women were significantly more likely to conduct CR for increasing muscle mass (OR [95% CI]: 1.7×107 [1.0×106; 2.7×108]; p<0.001) (Supplementary Table 6.2).

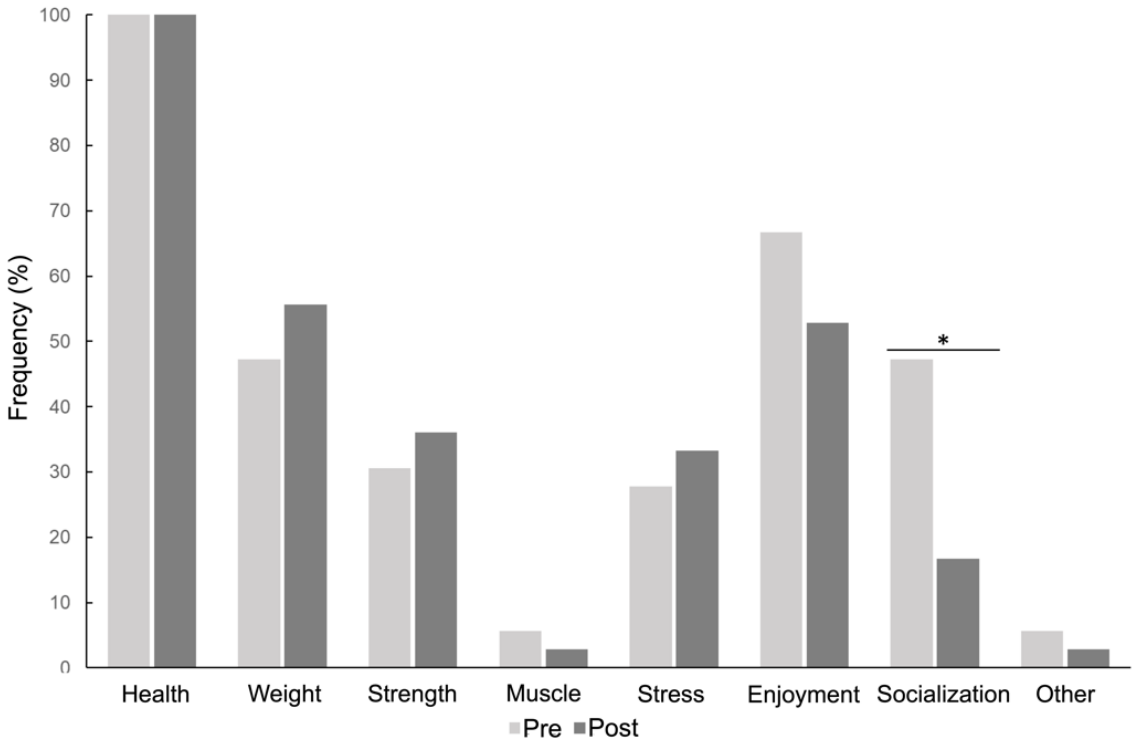


Figure 6.3. Influence of lockdown on cardiac rehabilitation-related goals. Bars represent the proportion (%) of participants who responded "Yes" to practising CR in each location. The change in the probability of practicing CR for a given goal post-lockdown vs. pre-lockdown was analysed by mixed model GLM with repeated measures. (*) Significant differences after post-hoc correction for multiple testing, *p<0.0071.

6.5.4 Mode of practice/supervision and purchase of equipment

There were significant changes in the supervisory arrangements after lockdown. The probability of receiving in-person supervision decreased by 90% (94.4% pre- vs. 16.7% post-lockdown; OR [95% CI]: 0.011 [0.002; 0.056]), while participants were almost 7 times more likely to use online supervision (11.1% pre- vs. 44.4% post-lockdown; OR [95% CI]: 6.824 [2.450; 19.002]) (both $p<0.001$), compared with pre-lockdown (Figure 6.4). Women were significantly less likely to engage in online and on-demand/video supervision (online OR [95% CI]: 2.76×10^{-5} [1.41×10^{-5}; 5.40×10^{-5}]; on demand/video OR [95% CI]: 0.00 [6.56×10^{-5}; 0.00]; both $p<0.001$) (Supplementary Table 6.3).

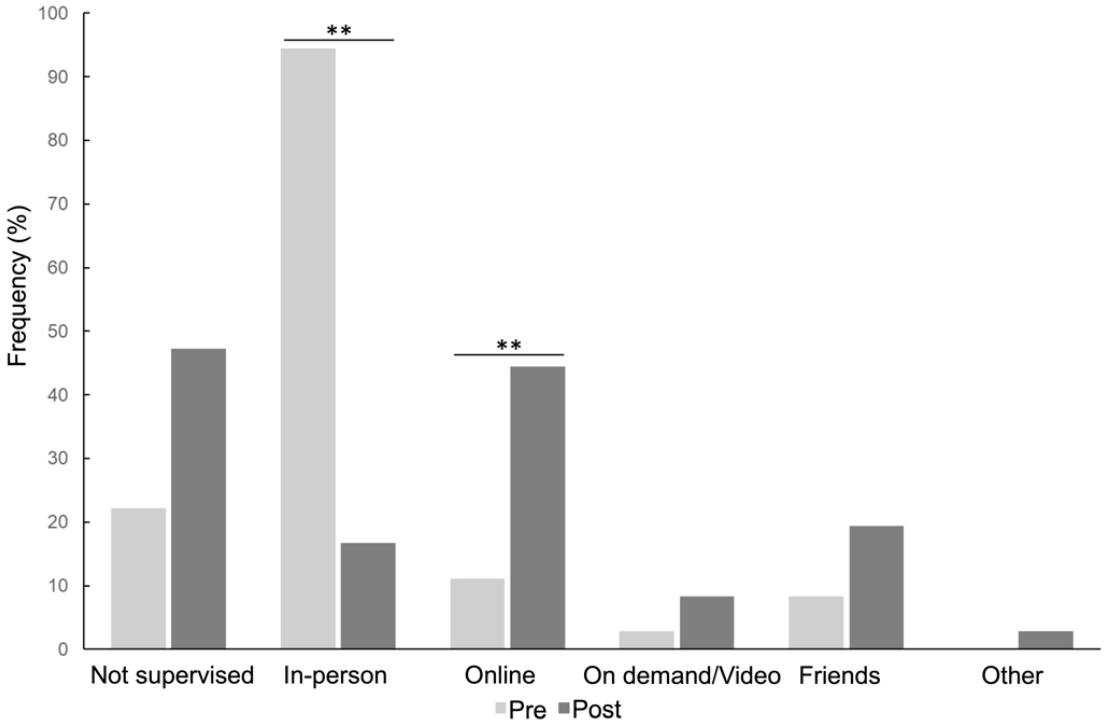


Figure 6.4. Influence of lockdown on cardiac rehabilitation mode of practice/supervision. Bars represent the proportion (%) of participants who responded “Yes” to practising CR in each location. The change in the probability of practicing CR in a given mode of supervision post-lockdown vs. pre-lockdown was analysed by mixed model GLM with repeated measures. (*, **) Significant differences after post-hoc correction for multiple testing, *p<0.0083, **p<0.001.

Prior to lockdown, 25% of participants had purchased specific equipment (including weights, machines, bands and/or other devices) to support their CR, while 36.1% participants indicated they had purchased specific equipment since the implementation of lockdown measures. From the latter, 30.8% had also answered “Yes” to purchasing equipment prior to lockdown. Half of the participants did not purchase equipment either before or after lockdown. COVID-19 restrictions did not seem to have an influence on the purchase of exercise equipment (Supplementary Table 6.3).

6.5.5 Time of day

The time of day when participants conducted CR changed significantly after the lockdown, with participants shifting from evening practice (4.7 times less likely compared to pre-lockdown: 58.3% pre- vs. 25.0% post-lockdown; OR [95% CI]: 0.214 [0.098; 0.467]; p<0.001) towards morning practice (3.7 times more likely: 30.6% pre- vs. 61.1% post-lockdown; OR [95% CI]: 3.663 [1.542; 8.700]; p=0.004) (Supplementary Figure 3.1 and Supplementary Table 3.4). Regardless of restrictions, women were significantly less likely to conduct CR later in the day (evening and night) than men (evening OR [95% CI]: 1.13×10−5 [5.77×10−6; 2.20×10−5; night OR [95% CI]: 0.002 [0.000; 0.016]; both p<0.001) (Supplementary Table 3.4).

6.5.6 Number, duration, and frequency of CR exercises

There was no difference in the number of exercises per session after lockdown (W=80.0, p=0.074) (Supplementary Figure 6.2), but the duration of the sessions increased significantly: prior to lockdown, only 22% of participants reported to exercise for more than 40 min/session, and this increased to 72% after lockdown (X2=13.136, p<0.001) (Supplementary Figure 6.2).

Finally, there was a trend for participants to shift towards the lowest (once per week) or the highest exercise frequencies (more than 3 times/week) but this was not statistically significant (X2=9.200, p=0.056) (Supplementary Figure 6.3).

6.5.7 Perceived effort, motivation, enjoyment, and willingness to continue

Overall participants' perception of effort increased significantly during lockdown (median [Q1; Q3]: 5 [4; 7], vs. pre-lockdown 6 [5; 6]; W=335.5; p<0.001) (Figure 6.5A), although with considerable heterogeneity, with 66.7% participants reporting an increase, 25% reporting a decrease and 9% reporting similar levels of perceived effort (Figure 6.5B).

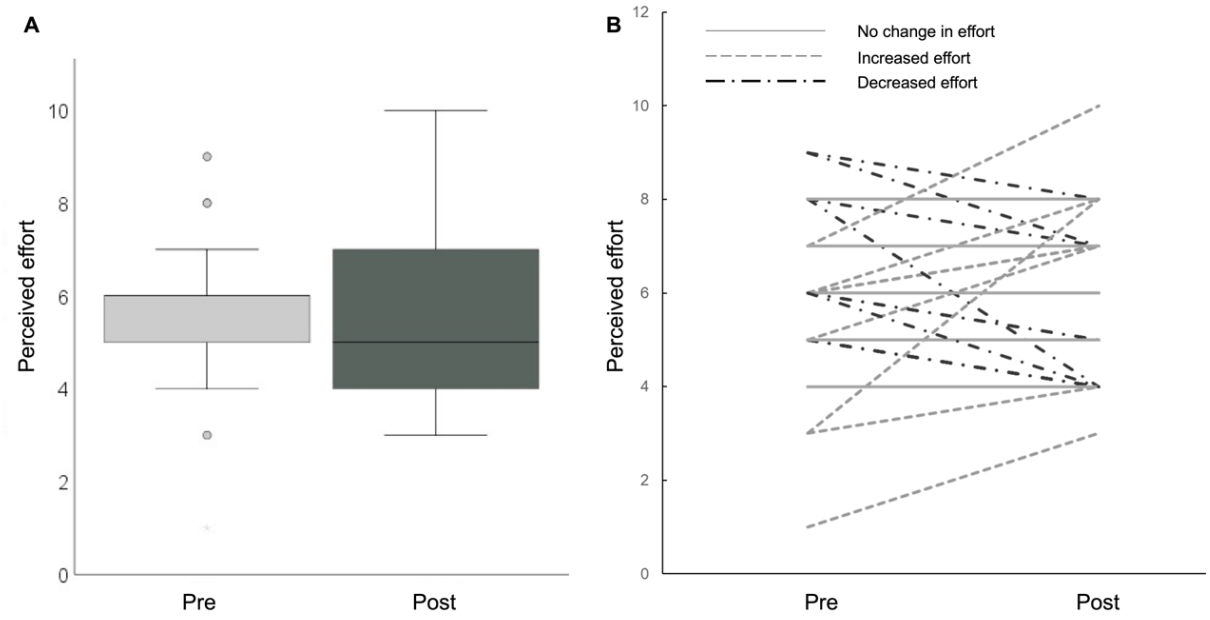


Figure 6.5. A) Influence of lockdown on perceived effort of cardiac rehabilitation in our sample. Differences between pre- and post-lockdown were analysed by repeated measures Wilcoxon signed ranked tests, $p<0.05$. B) Individual changes in perception of effort. Simple dashed lines indicate increased perceived effort post-lockdown; dot-dashed lines indicate decreased perceived effort post-lockdown; solid lines indicate no-change in perceived effort post-lockdown

Changes in motivation levels were symmetrical, with most participants (44.4%) experiencing similar levels of motivation pre- and post-lockdown, and comparable proportions of participants experiencing either more or less motivation (Figure 6.6). However, 50% of participants indicated that their enjoyment of CR was either lower or far lower than before lockdown, and only 16.7% indicated they enjoyed CR more after lockdown (Figure 6.6). Despite this, compared with pre-lockdown, only 27.8% of participants reported they would be less likely to continue with CR in the post-lockdown format, 55.6% admitted they would be similarly likely to continue with CR, and 16.7% were more likely or much more likely to continue (Figure 6.6).

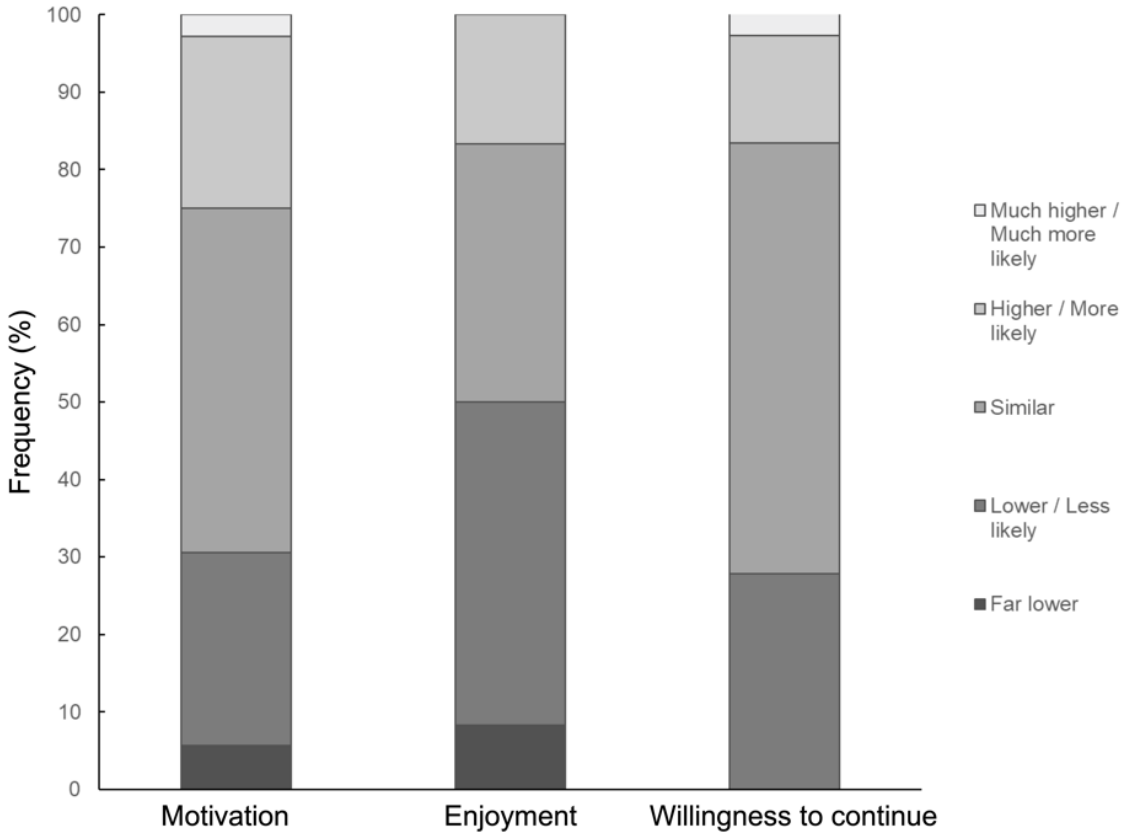


Figure 6.6. Motivation to practice, enjoyment of and willingness to continue with cardiac rehabilitation in comparison with pre-lockdown levels

6.6 Discussion

In this study we aimed to determine the impact of COVID-19 lockdown restrictions on CR behaviours and perceptions of effectiveness, motivation and intent to continue. Our results indicate that COVID-19 lockdown restrictions were associated with decreased participation in CR, changes in CR location, goals, supervision, duration and enjoyment, and increased perceived effort. While most participants were willing to continue with CR in its COVID-modified form, almost 30% indicated they were less likely to do so.

Physical activity is an essential component of a healthy lifestyle with higher levels of physical activity associated with reduced risk of CVD, type-2 diabetes mellitus (T2DM), sarcopenia, osteoporosis, cognitive decline and depression (Van Gelder et al., 2004; Morseth, Emaus and Jørgensen, 2011; Breen et al., 2013; Pattyn et al., 2013; Kolb and Martin, 2017; Santos et al., 2017). In cardiac populations, physical activity in the form of CR has been shown to reduce the risk of future cardiac events and mortality (Lavie and Milani, 2006; Sagar et al., 2015; Anderson et al., 2016). As such, the importance of maintaining CR has been highlighted during the COVID-19 pandemic due to suspension of some CR services (Yeo, Wang and Low, 2020). It is understood that the benefits of exercise for cardiac health are most significant when exercise is performed continuously and in the long-term (Giuliano et al., 2017) as research has indicated that the benefits of CR may be lost within as little as 3 months of CR cessation (Volaklis et al., 2006). While there are no established guidelines for the frequency or duration of CR, current UK guidelines recommend individuals accumulate 150 minutes of moderate intensity activity or 75 minutes of vigorous activity per week, while minimizing sedentary behaviour (Gibson-Moore, 2019). Highlighting a potential reduction in physical activity levels,

data from the present study revealed there was a trend for an increase in the number of individuals participating in CR only once per week during lockdown (p=0.056).

The establishment and maintenance of regular routines is regarded as a key determinant of whether individuals maintain a health-oriented behaviour, such as exercise (Arlinghaus and Johnston, 2019). While poorly studied, interference with regular routines and habits may reduce the likelihood of those exercise habits being maintained in the long-term, thus reducing the likelihood of health benefits being attained and maintained (Marcus et al., 2000). Accordingly, the occurrence of the COVID-19 pandemic and the associated restrictions on movement and social gatherings may potentially have detrimental effects on maintenance of effective CR exercise habits or routines. Indeed, a recently published BACPR survey of healthcare professionals revealed that as many as 72% of Phase IV CR services were suspended during the COVID-19 pandemic, with almost half of respondents indicating they were no longer providing services for high-risk patients (O'Doherty et al., 2021). Furthermore, the onset of COVID-19 lockdown restrictions were reported to reduce activity levels, in the form of step counts, by 16% in a population of heart failure patients (Vetrovsky et al., 2020). Furthermore, research from CR participants in Japan revealed that COVID-19 related interruptions in CR practice were negatively associated with both activity levels and hemodynamic response, relative perceived exertion during exercise as well as body weight (Ogura et al., 2021). Older patients in particular (≥ 75 years), were also at greater risk of frailty (Ogura et al., 2021).

The maintenance of motivation for CR activities should be considered vitally important for their long-term success. While initial behaviour change motivations amongst CR participants may be rooted in the fear of uncertainty regarding their long term health, it has been hypothesized that motivation for maintaining such behaviours may be different (Kwasnicka et al., 2016).

Temporal self-regulation theory suggests that the enjoyment of such new behaviours may encourage individuals to maintain their practice in the long-term (Hall and Fong, 2007). Apart from enjoyment, the satisfaction of other psychological needs is known to encourage behaviour maintenance and has been reported to be predictive of future home based exercise in CR populations (Russell and Bray, 2009). Accordingly, promoting the fulfilment of psychological needs and enjoyment of CR should be viewed as important for its own maintenance. In this study, 50% of participants indicated that their enjoyment of CR was either lower or much lower than pre-lockdown levels. While only 27.8% of participants reported they would be less likely to continue with CR in its current format, these are still concerning results and highlight the potential importance of guidance aimed at making home-based CR more enjoyable.

CR participants may also be influenced by the perception of attaining results from their exercise efforts (Kwasnicka et al., 2016). Self-efficacy theory posits that positive perception of one's results may reinforce motivation and encourage individuals to maintain their efforts, a theory that has support in the field of exercise maintenance (Desharnais, Bouillon and Godin, 1986). While personal accomplishments are important for self-efficacy, external factors including vicarious experience (observing peers achieve success with an endeavour) and verbal persuasion (verbal cues and feedback, leading to a belief in one's ability to succeed) also play a major role (Bandura, 1977). These vicarious experiences and verbal persuasion are often key features of in-person exercise classes and as such, contact with peers and CR exercise providers/trainers can help encourage self-efficacy (McAuley et al., 2003; Jackson, 2010) and potentially exercise maintenance.

Of particular concern, a recent BACPR survey of CR healthcare professionals highlighted that the three most widely used "technologies" for delivery of CR during the pandemic were

telephone, pre-recorded video, and email (O'Doherty et al., 2021). While these technologies played an important role in allowing CR services to continue operating (Stefanakis et al., 2021), little is known about their efficacy, particularly in encouraging maintenance of CR through the fulfilment of psychological needs and enjoyment. It also remains to be seen how these methods compare in efficacy to other less frequently employed technologies such as live-video conferencing and smart device applications, also identified in the BACPR survey (O'Doherty et al., 2021). It should also be mentioned that the same survey highlighted multiple barriers to the adoption of new technologies amongst both healthcare professionals and patients including lack of patient confidence, lack of patient access to internet and suitable devices such as computers, and professionals lack of confidence in using technology to deliver CR services (O'Doherty et al., 2021). Further research into the potential efficacy of different technologies for delivering CR, as well as research into overcoming the barriers that may inhibit its use by healthcare professionals and patients is warranted.

The results of this study highlight the importance of preparing and implementing strategies to provide not only adequate but also engaging CR when individuals cannot attend CR sessions in person. Such strategies may be of benefit not only to those who cannot attend CR due to pandemic restrictions but also due to issues such as, logistical difficulties in attending CR sessions, geographical isolation, unwillingness to attend group CR etc. This may help augment the uptake of CR which currently has global uptakes of 10-60% (Bjarnason-Wehrens et al., 2010; Dalal, Doherty and Taylor, 2015), and which has been designated as a goal of the UK Department of Health (Britain, 2013).

If non-centre-based CR is to be promoted and developed, a pertinent question is how effective are such home-based CR interventions? Centre-based CR has been shown to be more effective (improved physical endurance and lower serum risk factors such as total and low-

density lipoprotein cholesterol) compared to control groups which only received recommendations to exercise (Aronov et al., 2019). However, a Cochrane systematic review and meta-analysis comparing centre-based with home-based CR found them both to be equally effective in terms of mortality risk, risk of cardiac events, exercise capacity, modifiable risk factors and health related quality of life (Buckingham et al., 2016). This discrepancy may be explained by many of the home-based interventions incorporating clearly planned exercise routines as well as regular support in the form of telephone calls or visits from CR staff (Dalal et al., 2010). Promising strategies to incorporate into home-based CR include distance services such as telehealth which can include both education and supervised exercise delivered via telephone, video-conferencing and mobile apps (Thomas, Gallagher and Grace; Jin et al., 2019). Indeed the use of mobile apps has been shown by a recent meta-analysis to improve CR adherence by up to 1.4 times that of controls (Xu et al., 2019b), while CR approaches incorporating virtual reality and videogames can improve motivation and adherence, and increase physical activity (García-Bravo et al., 2021).

6.6.1 Limitations

There are a number of limitations to our present study. Firstly, recruitment proved difficult and resulted in a low sample size (n=45). The low recruitment rates were potentially due to two factors: i) the multiple steps required to contact participants *i.e.*, initially contacting CR-organizations in order to disseminate news of the study to CR-certified exercise physiologists and exercise instructors, requiring gate-keeper consent, and subsequent gatekeeper requirements to contact potential participants by asking them to forward an email with the questionnaire link. The complexity and multiple steps of this process may have resulted in lower likelihood of uptake; ii) the exclusion criteria, which formed part of the initial screener questionnaire were possibly too strict as they were intended to recruit suitable participants for a longitudinal investigation. This resulted in the exclusion of participants that, for example,

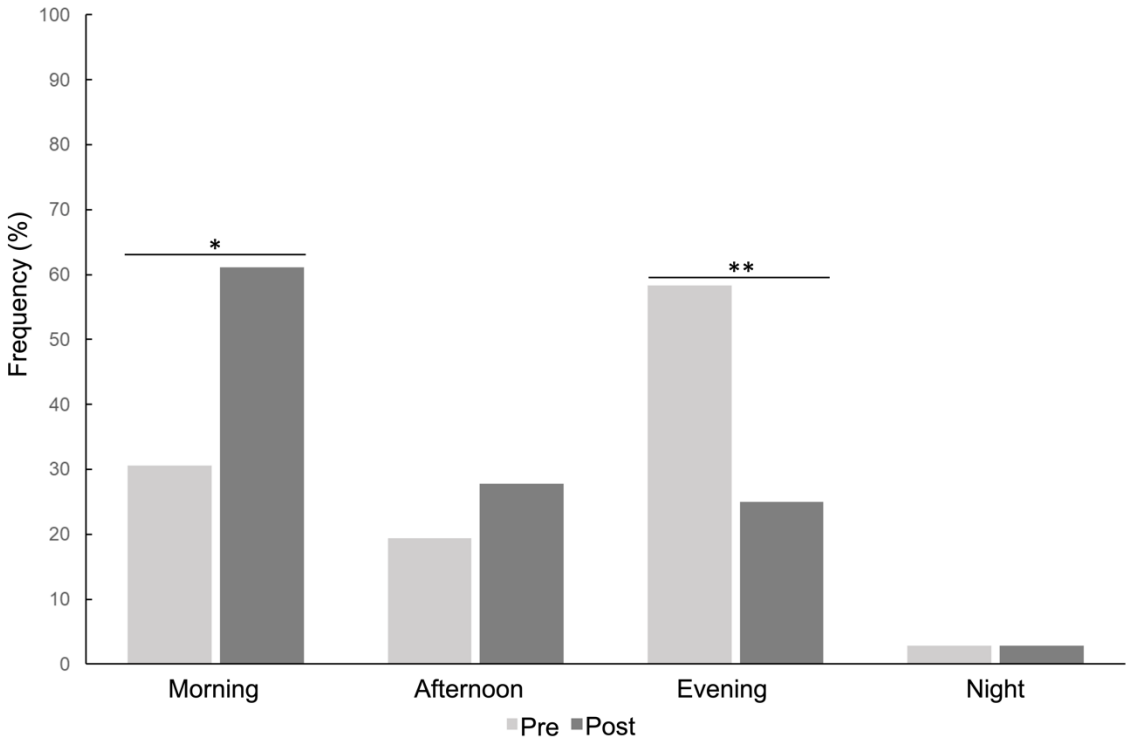
were in CR Phase 3, were participating in CR less than 2x per week, had their face-to-face CR suspended in response to the COVID-19 pandemic and/or whose cardiac condition was congenital or due to drug or alcohol abuse. As such, the population used in our study cannot be considered representative of the UK CR population in general. Future, cross-sectional investigations of CR could benefit from including such individuals in their recruitment efforts and screen-out later, if necessary.

Furthermore, the number of women recruited to this study was low at 11.1%. This number is considerably lower than the average percentage of female CR participants in England, Wales and Northern Ireland, which ranges from 27.8-31.3% (Rehabilitation, 2019). We cannot speculate as to the reasons for the low level of female participation in our study but as our population was predominantly male, the results may not be applicable to female CR participants. The ethnicity of our study population was predominantly white (British/Irish/Other white) at 91.1%, which is similar to a recently published report of CR demographics that reported 83.8% of participants as white (Rehabilitation, 2019). As such, the findings of this report may not be applicable to ethnic minorities participating in CR and clearly more efforts are needed to capture insights from these groups.

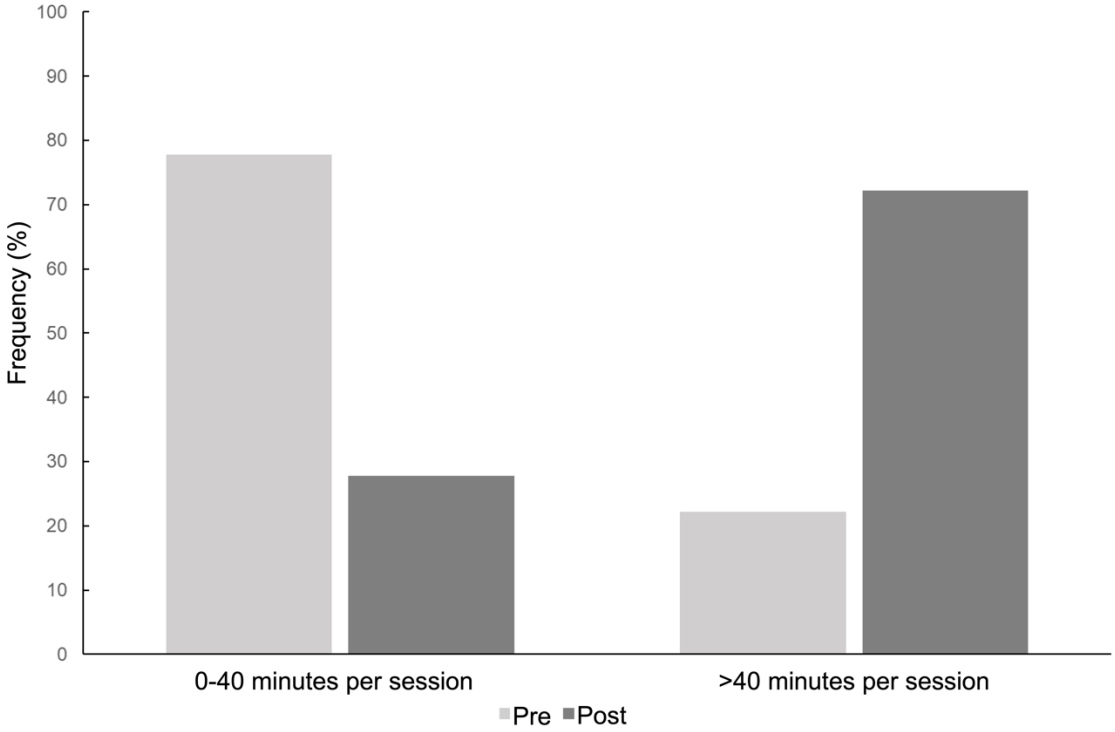
6.6.2 Conclusions

Lockdown was associated with considerable changes in how CR was practiced, levels of motivation and importantly, willingness to continue with this activity. Further research is warranted to develop and improve strategies to implement in times when individuals cannot attend CR in person and not only during pandemics.

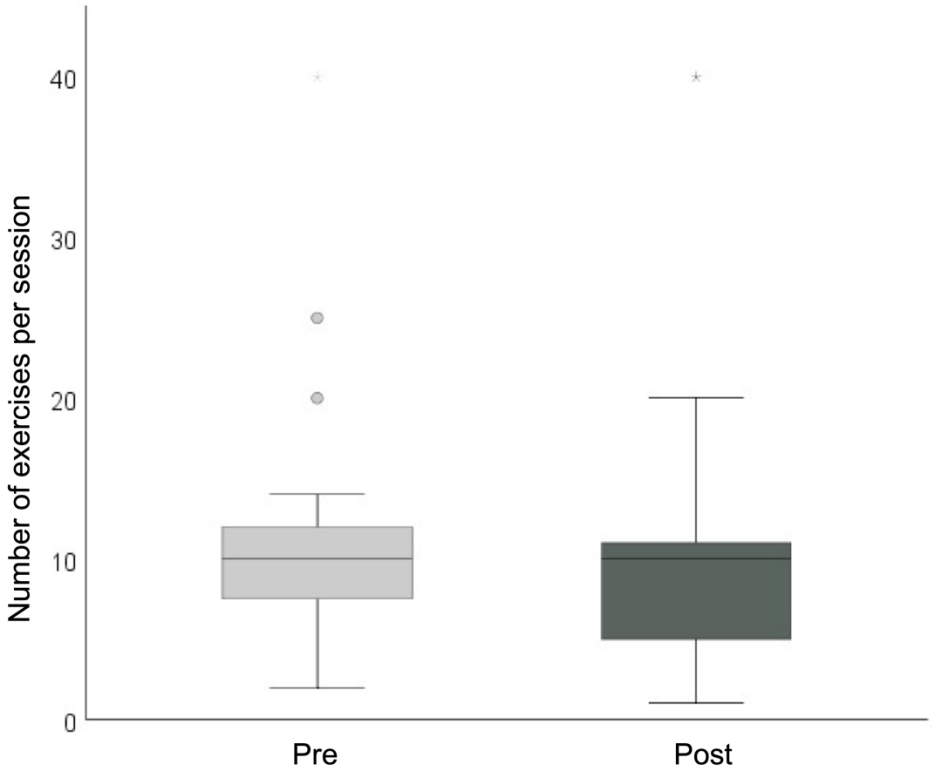
6.7 Supplementary material



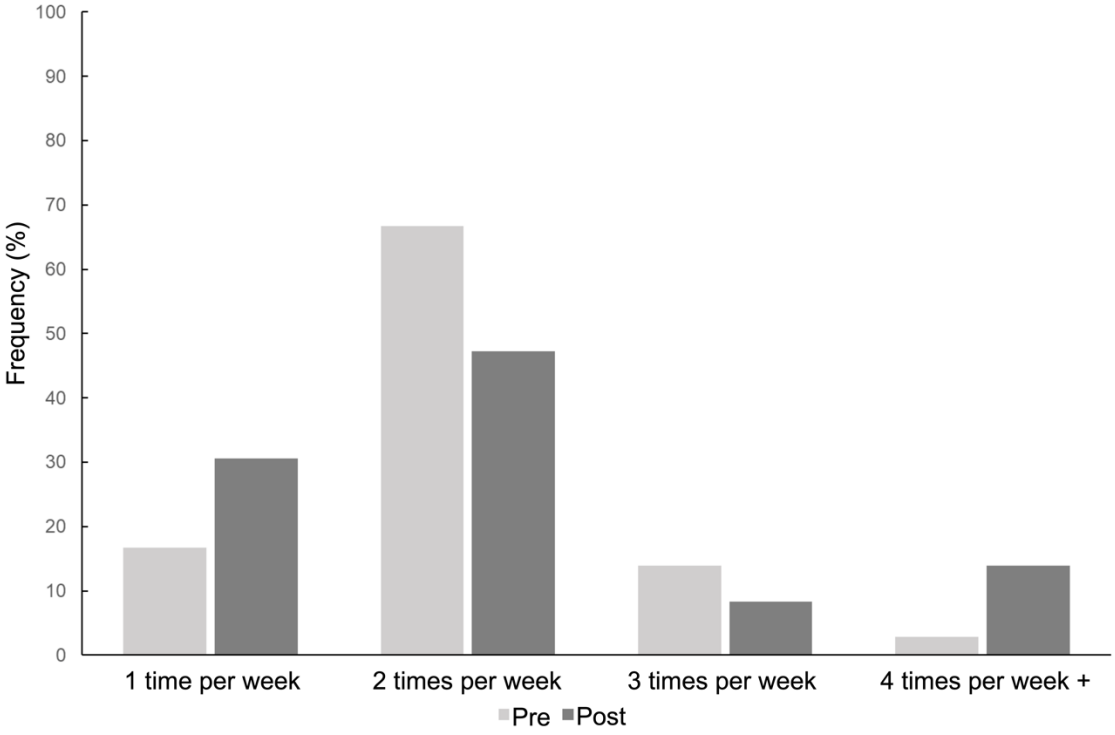
Supplementary Figure 6.1. Influence of lockdown on cardiac rehabilitation practice time. The change in the probability of practicing CR at a given time of day post-lockdown vs. pre-lockdown was analysed by mixed model GLM with repeated measures. (*, **) Significant differences after post-hoc correction for multiple testing, *p<0.0125, **p<0.001.



Supplementary Figure 6.2. Influence of lockdown on cardiac rehabilitation duration. The proportion of participants who reported to exercise for more than 40 min/session increased from 22% to 72% after lockdown (X2=13.136, p<0.001, assessed by McNemar test).



Supplementary Figure 6.3. Influence of lockdown on number of cardiac rehabilitation exercises used per session. There was no difference in the number of exercises per session before and after lockdown (W=80.0, p=0.074, assessed by Wilcoxon signed ranked test).



Supplementary Figure 6.4. Influence of lockdown on weekly frequency of cardiac rehabilitation. Most participants exercised twice per week, with a trend to shift towards lower (once per week) or higher exercise frequencies (more than 3 times/week), but this change was not statistically significant (X2=9.200, p=0.056, assessed by McNemar-Bowker test).

Supplementary Table 6.1. Influence of lockdown on CR location.

	Post v Pre-lockdown			Women v Men			Constant		Model	
	B (SE)	**OR (95% CI)**	**P**	**B (SE)**	**OR (95% CI)**	**P**	**B (SE)**	**P**	**% Correct classification**	**P**
Public gym	**-2.736 (0.7971)**	**0.065 (0.013; 0.318)**	**0.001**	-0.779 (1.1732)	0.459 (0.044; 4.764)	0.509	**3.565 (1.2875)**	**0.007**	**73.6**	**0.003**
Private gym	-0.843 (0.6340)	0.430 (0.122; 1.525)	0.188	-4.533×10-7 (6.2127×10-7)	1.000 (1.000; 1.000)	0.468	3.520 (5.7103×10-7)	<0.001	97.2	0.188
Hospital	**-3.526 (1.0155)**	**0.029 (0.004; 0.223)**	**0.001**	**-9.828 (0.3445)**	**5.395×10-5 (2.714×10-5; 0.000)**	**<0.001**	**13.293 (0.9857)**	**<0.001**	**76.4**	**<0.001**
Park	0.843 (0.6340)	2.323 (0.656; 8.230)	0.188	-4.533×10-7 (6.2127×10-7)	1.000 (1.000; 1.000)	0.468	2.677 (0.6340)	<0.001	97.2	0.188
Home	**4.081 (0.7096)**	**59.224 (14.377; 243.961)**	**<0.001**	1.786 (1.1468)	5.965 (0.605; 58.766)	0.124	**-3.529 (1.1679)**	**0.004**	**87.5**	**<0.001**
Other	-0.414 (0.7230)	0.661 (0.156; 2.797)	0.569	1.191 (0.7313)	3.291 (0.765; 14.155)	0.108	0.775	0.266	83.3	0.268

Mixed model: generalised linear model with repeated measures and fixed effects (time [post v pre-lockdown] and gender [women v men]). Values in bold are significant predictors for p<0.0083, after post-hoc correction for multiple testing.

Supplementary Table 6.2. Influence of lockdown on CR goals.

	Post v Pre			Women v Men			Constant		Model	
	B (SE)	**OR (95% CI)**	**P**	**B (SE)**	**OR (95% CI)**	**P**	**B (SE)**	**P**	**% Correct classification**	**P**
Weight management	0.334 (0.2441)	1.397 (0.859; 2.273)	0.175	-0.062 (1.0047)	0.940 (0.127; 6.977)	0.951	-0.167 (0.9572)	0.862	54.2	0.395
Strength	0.281 (0.3413)	1.324 (0.670; 2.617)	0.413	2.635 (1.0183)	13.946 (1.829; 106.338)	0.012	-1.860 (0.9258)	0.048	72.2	0.014
Muscle growth	-1.386 (3.0000)	0.250 (0.001; 99.335)	0.645	**16.634 (1.4007)**	**1.676×107 (1.025×106; 2.740×108)**	**<0.001**	**0.693 (1.2247)**	**0.573**	**91.7**	**<0.001**
Stress	0.262 (0.2595)	1.300 (0.775; 2.182)	0.315	0.141 (1.2788)	1.151 (0.090; 14.755)	0.913	0.565 (1.2312)	0.648	69.4	0.597
Enjoyment	-0.583 (0.2457)	0.558 (0.342; 0.911)	0.020	0.333 (1.2916)	1.395 (0.106; 18.346)	0.797	-0.417 (1.2442)	0.739	59.7	0.066
Socialization	**-1.515 (0.4643)**	**0.220 (0.087; 0.555)**	**0.002**	-1.030 (1.0792)	0.357 (0.041; 3.075)	0.343	**2.578 (1.0455)**	**0.016**	**68.1**	**0.003**
Other goals	-0.725 (0.7295)	0.484 (0.113; 2.076)	0.324	**-8.435 (0.7608)**	**0.000 (4.758×10-5; 0.001)**	**<0.001**	**11.901 (0.489)**	**<0.001**	**95.8**	**<0.001**

Mixed model: generalised linear model with repeated measures and fixed effects (time [post v pre-lockdown] and gender [women v men]). Values in bold are significant predictors, for p<0.0071, after post-hoc correction for multiple testing

Supplementary Table 6.3. Influence of lockdown on mode of practice and supervision of CR, and on purchase of equipment.

	Post v Pre			Women v Men			Constant		Model	
	B (SE)	**OR (95% CI)**	**P**	**B (SE)**	**OR (95% CI)**	**P**	**B (SE)**	**P**	**% Correct classification**	**P**
Not supervised	1.154 (0.4440)	3.171 (1.307; 7.688)	0.011	0.750 (1.0695)	2.117 (0.251; 17.881)	0.485	-0.577 (1.0472)	0.583	66.7	0.035
In person	**-4.511 (0.8183)**	**0.011 (0.002; 0.056)**	**<0.001**	1.140 (1.1707)	3.126 (0.302; 32.305)	0.334	0.598 (1.0954)	0.587	88.9	<0.001
Online	**1.920 (0.5134)**	**6.824 (2.450; 19.002)**	**<0.001**	**-10.497 (0.3357)**	**2.76×10-5 (1.41×10-5; 5.40×10-5)**	**<0.001**	**10.558 (0.0669)**	**<0.001**	**72.2**	**<0.001**
On demand/video	1.163 (1.2110)	3.200 (0.286; 35.841)	0.340	**-8.618 (0.5085)**	**0.000 (6.556×10-5; 0.000)**	**<0.001**	**10.921 (0.2917)**	**<0.001**	**94.4**	**<0.001**
Friends	1.018 (0.6525)	2.766 (0.753; 10.168)	0.123	1.502 (0.8617)	4.492 (0.805; 25.060)	0.086	0.092 (0.8003)	0.909	86.1	0.160
Equipment purchased	0.533 (0.5385)	1.705 (0.582; 4.994)	0.326	-0.955 (1.1931)	0.385 (0.036; 4.168)	0.426	1.458 (1.1790)	0.221	69.4	0.455

Mixed model: generalised linear model with repeated measures and fixed effects (time [post v pre-lockdown] and gender [women v men]). Values in bold are significant predictors, for p<0.0083, after post-hoc correction for multiple testing.

Supplementary Table 6.4. Influence of lockdown on moment of the day to practice CR.

	Post v Pre			Women v Men			Constant		Model	
	B (SE)	**OR (95% CI)**	**P**	**B (SE)**	**OR (95% CI)**	**P**	**B (SE)**	**P**	**% Correct classification**	**P**
Morning	**1.298 (0.4337)**	**3.663 (1.542; 8.700)**	**0.004**	1.056 (1.3768)	2.874 (0.184; 44.807)	0.446	-1.433 (1.3939)	0.307	66.7	0.014
Afternoon	0.468 (0.4085)	1.597 (0.707; 3.608)	0.256	0.539 (1.2880)	1.714 (0.131; 22.377)	0.677	0.466 (1.2448)	0.709	76.4	0.473
Evening	**-1.540 (0.3909)**	**0.214 (0.098; 0.467)**	**<0.001**	**-11.395 (0.3352)**	**1.126×10-5 (5.767×10-6; 2.197×10-5)**	**<0.001**	**12.375 (0.3208)**	**<0.001**	**70.8**	**<0.001**
Night	-5.297×10-15 (1.095×10-14)	1.000 (1.000; 1.000)	0.630	**-6.100 (1.0155)**	**0.002 (0.000; 0.017)**	**<0.001**	**9.566 (0.0000)**	**-**	**97.2**	**<0.001**

Mixed model: generalised linear model with repeated measures and fixed effects (time [post v pre-lockdown] and gender [women v men]). Values in bold are significant predictors, for p<0.0125, after post-hoc correction for multiple testing

7 Chapter 7: PRiMER: Protocol for a diet and exercise intervention in cardiac rehab

Feasibility of a high-PRotein Mediterranean-style diet and resistance Exercise in cardiac Rehabilitation patients with sarcopenic obesity (PRiMER): Study protocol for a randomised control trial.

McCullough D*, **Kirwan R***, Butler T, Perez de Heredia F, Thijssen D, Lip GYH, Mills J, Davies IG

** Co-first authors*

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7.1 Context within thesis

The publication presented in this chapter is a dietary and exercise protocol for the augmenting muscle mass and strength in a cardiac rehabilitation population and investigating the effects on cardiometabolic risk markers, designed with reference to the previous chapters presented in this thesis. The published protocol that is presented here has been designed in collaboration with cardiac rehabilitation providers (Knowsley Community Cardiac Services) and with Patient Public Involvement (PPI) (Liverpool Heart and Chest Hospital Service Users Research Endeavour). The protocol has been approved by both the Liverpool John Moores University Research Ethics Committee and The NHS Health Research Authority Northwest - Greater Manchester East Research Ethics Committee (19/NW/0762). This protocol will undergo further refinement through PPI involving cardiac rehabilitation participants (end users), before being used to test the feasibility of the proposed intervention that has resulted from this PhD thesis.

7.2 Abstract

Background: Cardiac rehabilitation (CR) is an essential component of long-term recovery following a cardiac event. Typical CR may not be optimal for patients presenting with sarcopenic obesity (SO) who present with reduced muscle mass and elevated adipose tissue, and may indicate greater cardiovascular disease (CVD) risk. Resistance exercise and high-protein diets are known to increase muscle mass, while Mediterranean-style diets have been shown to reduce CVD risk. A high-protein Mediterranean-style diet combined with resistance exercise intervention is yet to be trialled in cardiac rehabilitation populations.

Objectives. Primary outcome: to determine the feasibility of such an intervention by investigating the perceptions, acceptance and adherence to a resistance exercise protocol and high-protein Mediterranean style diet in a UK cardiac rehabilitation population with SO. Secondary outcome: to trial this protocol ahead of a fully powered clinical study.

Methods. Eligible cardiac rehabilitation patients will be randomised to one of the following: 1) a control group (standard CR), 2) high-protein Mediterranean-style diet, 3) resistance exercise group, or 4) both high-protein Mediterranean-style diet and resistance exercise group. The pilot study will last 12 weeks. Measures of body composition (dual energy x-ray absorptiometry) grip strength, CVD risk (*e.g.*, fasting triglycerides, glucose, cholesterol) and dietary adherence will be assessed at baseline and after 12 weeks. To compare groups, a mixed model ANOVA (time x intervention) will be performed. Patient participant involvement throughout the development of this project will be used to determine the feasibility of a future, fully powered, randomised control trial. A feasibility questionnaire will help establish the proportion of eligible participants, their willingness to be randomised, response rates, and ethical considerations. Furthermore, focus groups, food tasting and telephone interviews will be conducted to assess the acceptability of recipes and exercise protocols provided.

Discussion. This pilot trial will determine whether a fully powered, multi-centred randomised control trial in CR patients with SO can be implemented. The information received from patient involvement will be invaluable for identifying possible barriers to participation and tailoring interventions to participant needs, helping to increase the likelihood of long-term compliance to health-promoting lifestyle changes.

Registration

This study is registered at clinicaltrials.gov (NCT04272073), registered on 17/02/2020, https://clinicaltrials.gov/ct2/show/NCT04272073

7.3 Introduction

Currently 7.4 million people in the UK are living with cardiovascular disease (CVD), which is responsible for more than 1 in 4 deaths in the UK per year (British Heart Foundation, 2019) and leads to a considerable economic cost (Wilkins et al., 2017). Obesity, which is commonly associated with risk markers for cardiometabolic disease (CMD), is a key risk factor in the development of CVD (Han and Lean, 2016; Ortega, Lavie and Blair, 2016). Conversely, in adults free of CVD, skeletal muscle mass is shown to have an inverse association with future CVD incidence independent of CVD risk factors such as smoking habits, hypertension, hypercholesterolaemia and diabetes (Tyrovolas et al., 2020). Furthermore, in individuals with CVD, those who have high muscle mass and high fat mass have been shown to have significantly lower rates of mortality compared with individuals with low muscle mass and low fat mass (Srikanthan, Horwich and Tseng, 2016). This highlights the importance of skeletal muscle mass preservation for decreasing CVD risk.

Reduced muscle mass observed in individuals with CVD/CMD is likely due to sarcopenia, the age-associated decline in skeletal muscle mass and function which may begin as early as one's fifth decade of life (Keller and Engelhardt, 2013). Indeed, sarcopenia determined by appendicular skeletal muscle mass (ASM)/height^2 (kg/m^2) has been associated with higher 5-year all-cause mortality in individuals with CHD (Nichols et al., 2019). Sarcopenia can result from a myriad of factors, including sedentary lifestyle, increased inflammation and age-related endocrine alterations (Rezuş et al., 2020). Sarcopenia contributes to CVD risk, and in turn, CVD may also exacerbate sarcopenia severity via increased inflammation, reduced muscle blood flow and dysregulated endocrine function (Curcio et al., 2020). Both skeletal muscle and adipose tissue are metabolically and hormonally active tissues, which contribute to the levels of inflammatory modulators (Li et al., 2017). A shift towards decreasing skeletal muscle mass

and increasing visceral adipose tissue - a combination termed sarcopenic obesity (SO) - can lead to elevated levels of pro-inflammatory cytokines and decreases in anti-inflammatory cytokines, thereby increasing risk of CVD (Ouchi et al., 2016; Kalinkovich and Livshits, 2017). These pro-inflammatory cytokines may also contribute to the progression of SO through their association with reduced muscle mass and strength (Kalinkovich and Livshits, 2015). Consequently, methods of augmenting skeletal muscle tissue, while diminishing VAT and associated markers of CMD may be useful for reducing CVD risk.

Clinically, following a cardiac event, patients are referred to a supervised exercise and weight management intervention termed cardiac rehabilitation (CR), which has been shown to be a cost-effective strategy for decreasing morbidity, mortality and improving quality of life (Dalal, Doherty and Taylor, 2015; British Association for Cardiovascular Prevention and Rehabilitation, 2017; Shields et al., 2018). Although current CR guidelines do encourage resistance exercise (RE), they primarily focus on aerobic exercise with an overall aim of increasing physical fitness and energy expenditure in order to reduce CVD risk (Price et al., 2016; British Association for Cardiovascular Prevention and Rehabilitation, 2017). Aerobic exercise has smaller effects on muscle strength or muscle hypertrophy compared to RE, which has been shown to effectively improve muscle mass and strength in a dose-response manner (Egan and Zierath, 2013; Borde, Hortobagyi and Granacher, 2015; Schoenfeld, Ogborn and Krieger, 2017). In older adults with and without CVD, RE has been shown to effectively diminish the risk of developing and exacerbating CVD symptoms, while also being safe for CR (Williams et al., 2007; Khadanga, Savage and Ades, 2019). To maximise the benefits of RE on muscle hypertrophy, protein intakes greater than the recommended daily allowance may be required (Department of Health, 1991; Hudson et al., 2019). Increased protein intakes may be especially important considering ageing individuals are shown to have a reduced capacity to increase muscle mass with protein intakes similar to young individuals, a phenomenon referred to as anabolic resistance (Breen and Phillips, 2013).

Diet plays a key role in the development of obesity, inflammation and CVD risk. Western-type diets, for example, have shown unfavourable relationships with these conditions whereas Mediterranean-style diets (MedDiets) present inverse relationships with obesity, inflammation, CMD and CVD risk (Medina-Remón et al., 2018; Norde et al., 2021) and mortality (Michaëlsson et al., 2020). Results from studies on secondary prevention of CVD suggest MedDiet-based approaches are effective and are frequently recommended during CR (dos Reis Padilha et al., 2018; Butler et al., 2020). However, MedDiets are not necessarily high in protein content, which may not be optimal for increasing muscle mass in CR patients, who are likely to present with SO. Therefore, consumption of a higher protein (1.3-1.5 g/kg per day) Mediterranean-style diet to support a RE programme may be of greater benefit in CR, due to a greater capacity to enhance skeletal muscle hypertrophy and reduce markers of CMD (Bauer et al., 2013a).

It is worth noting that an intervention such as that proposed here has never been trialled in CR patients with SO, and there may be barriers that lead to poor adherence. Therefore, an aim of this protocol paper is to describe the design of a feasibility study for a high-PRotein Mediterranean diet and Resistance Exercise in cardiac Rehabilitation patients with SO (PRiMER). The study will also assess the effect of a pilot intervention on skeletal muscle mass and function, in addition to markers of cardiometabolic health in CR patients. It is hypothesized that a CR intervention focused on RE and a modified-MedDiet with increased protein (HPMD) content will be feasible and more effective for improving cardiometabolic health in CR patients with SO, than the current CR guidelines based around aerobic exercise with dietary change focused on weight management (British Association for Cardiovascular Prevention and Rehabilitation, 2017).

7.4 Methods

The study is designed as a pilot, 12-week, four-arm, randomised control trial in CR patients with SO (Fig 7.1) and conforms with the SPIRIT 2013 statement (Chan et al., 2013).

7.4.1 Patient participant involvement and feasibility of study design

Prior to beginning recruitment, patient participant involvement (PPI) will be used as a tool to inform future research design and implementation of a fully powered research study using questionnaires, focus groups and interviews, which are described below. Use of a feasibility questionnaire will help establish the number of eligible participants, their willingness to be randomised to different protocol arms, response rates to questionnaires, acceptability of nutritional and exercise protocols, and ethical considerations. A trial oversight committee will be formed, consisting of the academic investigators, Liverpool Heart and Chest Hospital clinical leads, and community centre leads. Equally, there will be a trial steering group (TSG), consisting of patients engaging with CR (phase III and phase IV), the academic investigators, and staff employed on the trial. The TSG will be responsible for reviewing the protocol design and ethical considerations such as participant information, and valuable feedback on patient friendliness and acceptability. Any changes made to the protocol will be reported and documented.

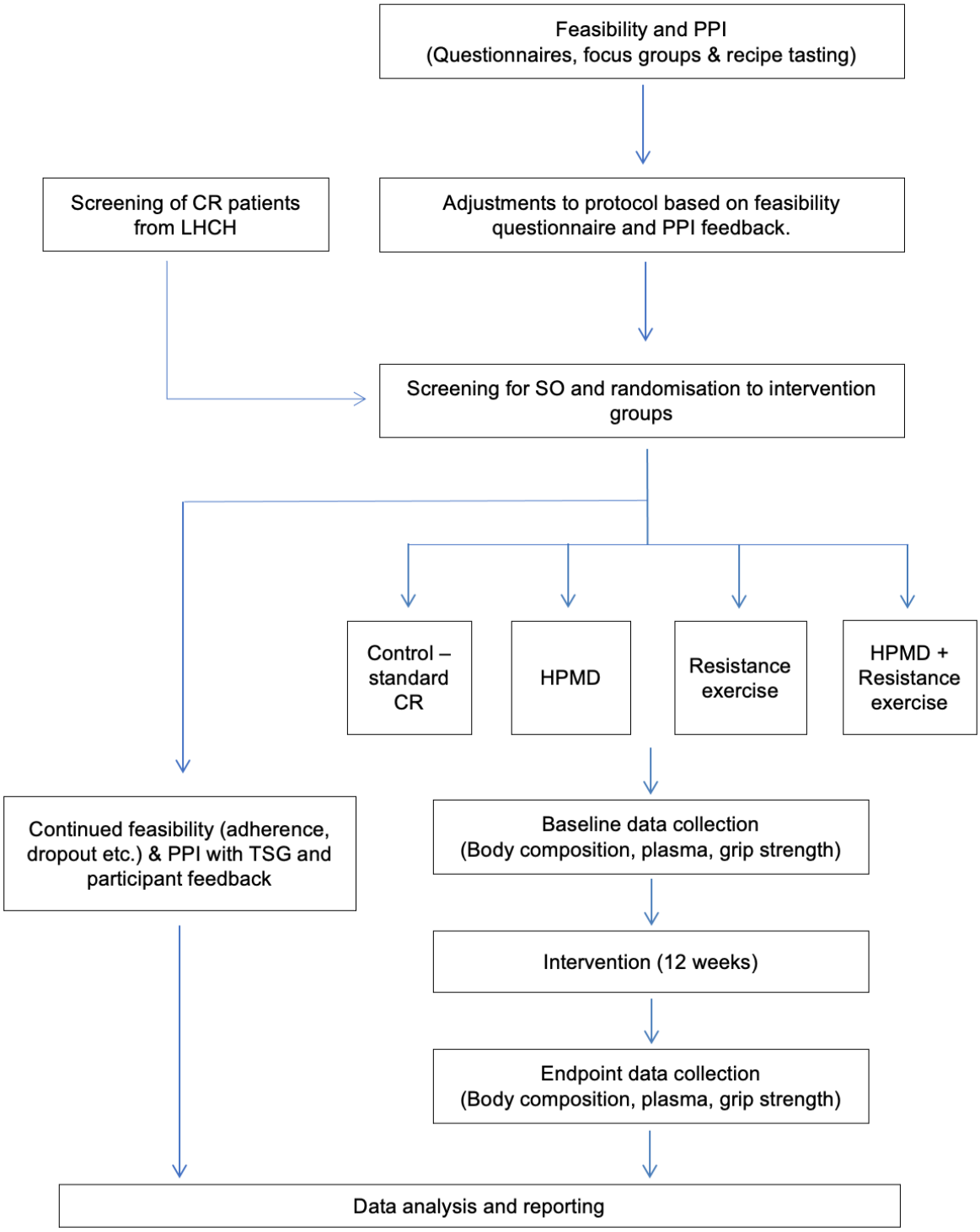

Figure 7.1 Study flow-chart. CR; cardiac rehabilitation, HPMD; high-protein Mediterranean-style diet, LHCH; Liverpool heart and chest hospital, PPI; patient participant involvement, SO; sarcopenic obesity, TSG; trial steering group.

As the study design involves dietary and exercise changes, a recipe booklet and the RE training protocol will be presented to a focus group of CR patients (n = 10-12) who are eligible for, but not necessarily taking part, in the study. Recipes have been designed based on MedDiet criteria as described in the validated English version of the 14-item Mediterranean Diet Adherence Screener (MEDAS) used in the PREDIMED study (Papadaki et al., 2018) but have been adapted to include high-protein dishes typical to England in order to aid adherence in this UK-based population. Focus group questions will be based on the food guidelines and exercises provided in the recipe book (Table 7.1) and training protocol (Table 7.2), respectively. Additionally, CR patients will be asked to prepare and taste recipes from the recipe book and complete a hedonic food scales questionnaire. Exercise videos will also be used to demonstrate the RE training program, and participants will be asked about its suitability for CR. Focus group participants will receive all materials (recipe booklet, training protocol, exercise videos) digitally. Upon review of these materials, participants will participate in recorded phone or web-based focus groups, conducted by one member of the research team. Focus groups will be transcribed and analysed using thematic analysis.

Table 7.1 Sample focus group questions at the recipe tasting session.
1. What do you think about the research study we proposed?
2. What were your thoughts of the recipe book you received?
3. Describe your experiences of the dishes you made at home from the recipe books.
4. Why did you choose to make the dishes you made?
5. Which dishes did you dislike and why?
6. How do you think the foods can be improved?
7. What other types of recipes would you like to see in the recipe book?
8. Describe the pros and cons of making these dishes at home
9. What is your opinion on preparing more food at home?

Table 7.2 Sample focus group questions on the RE programme.
1. What is your opinion on doing this type of exercise programme for your CR?
2. What do you think about the resistance exercise you've seen today?
3. Are there any exercises you think aren't appropriate and why?
4. What would you expect to be the main advantages of the resistance exercise you've seen here?
5. What would you expect to be the main disadvantages of resistance exercise?
6. Are the number of gym sessions per week reasonable for CR patients, considering work and other life commitments and why? If not, do you have alternative suggestions?
7. What do you think about travelling regularly to your local gym for CR?
8. Would you prefer group exercise sessions or private sessions and why?

This PPI will also be continued throughout the intervention to inform participant adherence/compliance rates and attrition, in order to determine the feasibility of the study (Figure 7.1). Qualitative (thematic) analysis will be used to assess responses from focus groups, relating to the dietary and exercise interventions and associated materials/guides, as well as to assess all participant feedback throughout the study. Commonly occurring themes which emerge from this qualitative analysis will be considered for protocol changes in the development of the fully powered study.

7.4.2 Eligibility and recruitment

In a North-West England setting, participants (male and female) will be recruited from Liverpool Heart and Chest Hospital Cardiac Rehabilitation unit. Individuals will have recently completed phase III CR, be deemed as cardiac stable, and referred to phase IV CR. Phase III CR may begin 2 to 6 weeks after a cardiac event and can last between 4 weeks to 6 months depending on the patient and the specific CR centre (Bethell, Lewin and Dalal, 2009). It primarily consists of a graduated exercise programme and is supplemented by education on heart disease and leading a healthier lifestyle (Bethell, Lewin and Dalal, 2009). There is no “one size fits all” approach, and as such a range of physical activities are encouraged such as group exercises, walking, swimming or cycling. When the risk level of the patient is deemed low, patients can proceed to phase IV of CR, which is primarily a continuation of the new lifestyle habits (Bethell, Lewin and Dalal, 2009).

Inclusion criteria include the following: minimum age 40 years old, cardiac function deemed stable after phase III CR, referral to a CR programme and meeting the criteria for defining sarcopenic obesity. Eligible participants will be assessed by whole body dual-energy X-ray

absorptiometry (DXA) (Hologic, Manchester, UK) to determine body composition; as no consensus definition of sarcopenic obesity exists we will use the following definition based on the criteria for sarcopenia as defined by the EWGSOP2 (Cruz-Jentoft et al., 2019) and including a measure of abdominal adiposity:

i) Grip strength <27kg in men or <16kg in women

ii) Appendicular skeletal muscle (ASM)/height^2 <7 kg/m^2 in men or <6 kg/m^2 in women

iii) Waist circumference ≥ 94 cm in men or ≥ 80 cm in women (Alberti et al., 2009)

Exclusion criteria include the following (as determined by medical records): individuals with electric implants (*i.e.* pacemakers), inability to perform RE (determined by primary care team), presenting with chronic kidney disease stage 3-5 (eGFR <60 mmol/L) due to concerns with high-protein intake (Ikizler et al., 2020), inability/unwillingness to digest/consume dairy products, admission to CR due to congenital or drug/alcohol-abuse-induced cardiac events, and pregnancy. Potential participants will receive written study information before being asked to provide informed consent.

7.4.3 Sample size estimation

This pilot investigation will aim to recruit a sample size of 10-15 participants/group (a total of 40-60 participants). Data collected from this pilot study will help determine the correct sample size for a fully powered study. For the future fully powered randomised control trial, power calculations will be calculated by G*Power 3 (Faul et al., 2007) based on the effect size of change in lean body mass.

7.4.4 Ethics approval and data management

The NHS Health Research Authority North West - Greater Manchester East Research Ethics Committee (IRAS: 256927) and Liverpool John Moores University Research Ethics Committee (19/NW/0762) reviewed and granted approval for the trial. This study is also registered at ClinicalTrials.gov (NCT04272073). All data will be collected by a trained researcher and pseudo anonymised by providing each participant with a unique ID which will be used on all questionnaires and biological samples. All biological samples and data collected will comply with the Human Tissue Act (2004) and General Data Protection Regulation guidelines. Only authorised researchers will have access to samples and collected data.

7.4.5 Randomisation and blinding

Eligible participants will be randomised by a computerised stratified randomisation programme for allocation to one of the following arms: standard CR (control group, CON); high-protein Mediterranean-style diet (HPMD group); resistance exercise with standard CR (RE group); or high-protein Mediterranean-style diet and resistance exercise (HPMD+RE group). Participants will be stratified based on body composition and their allocation will be sealed in envelopes until assigned to intervention group by the lead researcher. Due to the nature of the interventions, participants cannot be blinded to their allocation. Data analysis will be carried out by a member of the research team blinded to participant allocation.

7.4.6 Intervention

7.4.6.1 Exercise prescription

If allocated to groups RE or HPMD+RE, participants will be asked to perform RE using weights or weight machines, with the aim of building muscle size and strength. Participants will be shown how to perform the exercises by British Association for Cardiovascular Prevention and Rehabilitation (BACPR) qualified instructors in the community centre where they carry out their current phase III CR. All exercises have been deemed safe for CR patients and will be supervised by the BACPR instructor at all exercise sessions. Participants will be required to attend 3 sessions per week and each session is expected to last approximately 45 minutes.

If allocated to groups CON or HPMD, participants will be asked to continue with the standard, aerobic-style exercise (treadmills, rowing machines, elliptical trainers) they have used in phase III of CR. This will also require 3 sessions per week and be supervised by qualified BACPR instructors. Participants will be excluded if they do not achieve a 90% attendance rate at exercise sessions, based on previous studies reporting similar adherence in this population (Palevo et al., 2009; Pourhabib et al., 2018; Kambič et al., 2019; Petersen et al., 2020).

7.4.6.2 Diet

Participants allocated to groups HPMD or HPMD+RE will be asked to make changes to their eating habits to adapt to a HPMD adapted from the PREDIMED (*Prevención con Dieta Mediterránea*) study, by applying the following guidelines:

- increasing fruit and vegetables
- reducing intake of commercial pastries
- replacing refined carbohydrate foods (white bread, white rice, white pasta) with wholegrains (wholegrain bread, rice and pasta)
- replacing butter and margarine with olive oil as the main culinary fat
- reducing fatty meat and replacing with other high-protein, low-fat foods, such as lean meat, fish, legumes (peas, beans, lentils), low-fat dairy products (Trichopoulou et al., 2014; D'Alessandro and De Pergola, 2018).

The diet will be supplemented by provision of 2 high-protein yoghurts (providing approximately 20g of milk protein each) per day in order to increase per-meal protein intake.

Participants will receive personalised guidance to help follow the new diet during baseline data collection appointments, along with guidebooks and a recipe guide (Appendix 3). All foods included will be designed to be affordable, with ingredients that are easy to find in local supermarkets (shopping guides will be provided).

If allocated to groups CON or RE, participants will be asked to follow the dietary recommendations given during phase III of CR.

Researchers will be in contact with all participants weekly, regardless of intervention allocation, by phone to encourage adherence. Any adverse events will be recorded via this phone contact and reported to the ethics committee.

7.4.7 Outcome assessments

7.4.7.1 Primary outcome

The primary outcome of the study will be the feasibility of conducting a larger, fully powered, multicentre study aimed at determining the effectiveness of a HPMD and RE protocol for CR patients. This will be based on rates of recruitment, adherence to dietary and exercise guidelines, and feedback from patient focus groups prior to and throughout the intervention.

Compliance with the dietary recommendations over the course of the intervention will be assessed via 3-day food diaries (on two weekdays plus one weekend day) being completed prior to starting the intervention, midpoint and at the end of the 12-weeks. Analysis of food diaries will be completed with Dietplan 7 (Forestfield Software Ltd, UK). Consistent with the National Audit of Cardiac rehabilitation, participants will also complete a brief 14-question questionnaire regarding the frequency of their intake of certain foods to determine their MedDiet score (Papadaki et al., 2018).

Adherence to the exercise guidelines will be assessed through participant exercise logs, completed at the same time points of the food questionnaires.

7.4.7.2 Secondary outcomes

Secondary outcomes will be measured at baseline and at 12 weeks of intervention and will include the following:

Anthropometrics will be collected including; weight which will be measured using a digital scale (Seca 704, Birmingham, UK), height will be recorded using a stadiometer (Seca 213, Birmingham, UK) and waist circumference will be recorded 3 times to the nearest 0.1 cm at

the approximate midpoint between the lower margin of the last palpable rib and the top of the iliac crest (Bonita et al., 2003). Blood pressure will be measured 3 times consecutively using a digital sphygmomanometer (Dianamap; GE Pro 300V2, Tampa, FL, USA) with systolic and diastolic measures being recorded. For waist circumference and blood pressure, the average of all 3 measurements will be used for data analysis. Changes in body composition will be determined by a whole body DXA system (Hologic, Manchester, UK) (Visser et al., 1999; Salamone et al., 2000). Participants will be instructed to lay supine on the DXA table with arms adequately separated from the trunk and instructed to remain still throughout the scanning procedure.

To assess changes in muscle strength, participants will be instructed to perform isometric contractions using a hand-held grip dynamometer (Takei Kiki Kogyo, Tokyo, Japan) (Schaubert and Bohannon, 2005; Labott et al., 2019). The hand grip strength test will be done seated with their elbow by their side and flexed to right angles, and a neutral wrist position for 3 s. The maximum value of 3 consecutive measurements in the non-dominant arm will be registered.

To assess changes in markers of cardiometabolic health, fasted (12 hours) venous blood samples will be collected from the antecubital vein. Markers including plasma glucose, insulin, triglycerides, total cholesterol, low density lipoprotein cholesterol, high density lipoprotein cholesterol, C-reactive protein and whole blood HbA1c will be determined by an automated random-access clinical chemistry analyser (Daytona, Randox Laboratories Ltd, UK).

To assess changes in quality of life due to sarcopenia, participants will also complete the validated sarcopenia quality of life questionnaire (SarQol) (Beaudart et al., 2017a).

7.4.8 Statistical analysis

Comparisons between intervention groups for all outcome measures will be performed by means of mixed model ANOVA, to account for inter-subject (differences between treatments) and intra-subject (differences between baseline and endpoint) variability. The non-parametric equivalent will be used if data are not normally distributed as determined by Shapiro Wilks test. Since a sample size calculation will not be conducted for this component, interpretation of the results will be largely descriptive and focused on confidence limits around parameter estimates. In the case of non-compliance or participant dropout, intention to treat analysis will be carried out to maintain the effect of randomisation and avoid selection bias (Tripepi et al., 2020). Moreover, per protocol analysis will also be carried out to investigate the effect of treatments on subpopulations who fully adhere to the protocol. Statistical significance will be set at $p < 0.05$, and all analyses will be conducted using IBM SPSS Statistics v25 (SPSS Inc., Chicago, IL).

7.5 Discussion

This study aims to determine if it is feasible to implement a RE and high-protein modified-MedDiet in phase IV CR patients with SO in the North-West of England. If the primary outcome measures deem the intervention to be feasible, and if the pilot intervention shows improvements in the secondary measures (body composition, strength and cardiovascular health), we will seek to implement a fully powered, multi-centred, randomised control trial in CR patients, in order to confirm the findings of the pilot study and inform future CR exercise and dietary recommendations. CR is an essential component of long-term recovery following a cardiac event; however, an overall aim of increasing physical fitness and energy expenditure primarily through aerobic exercise, may not be optimal for the secondary prevention of CVD in individuals presenting with SO (Price et al., 2016; British Association for Cardiovascular

Prevention and Rehabilitation, 2017). Preservation of muscle mass via RE, supplemented with a high-protein diet may be of greater benefit. For example, individuals with CHD and low skeletal mass were associated with reduced physical fitness and increased 5-year all-cause mortality risk (Nichols et al., 2019). Furthermore, following a Mediterranean-style diet may also reduce CVD risk, as MedDiets show an inverse relationship with obesity, inflammation, CMD and CVD risk (Medina-Remón et al., 2018; Norde et al., 2021) and are recommended for secondary prevention of CVD (dos Reis Padilha et al., 2018; Butler et al., 2020).

In addition, the potential information received from PPI will be invaluable for identifying possible barriers in CR patients and allow for future interventions to be tailored to their needs to help increase the likelihood of long-term compliance to health-promoting lifestyles. The results will also be compared with similar research investigating potential barriers of implementing exercise program in CR patients (Conraads et al., 2012), and Mediterranean-style diets in northern European populations (Moore et al., 2018). Previous barriers reported by CR patients on engaging with exercise consisted of lack of education on benefits, lack of resources, and motivation (Conraads et al., 2012). Furthermore, barriers reported on following a MedDiet were lack of knowledge on MedDiets, lack of cooking skills and resistance to dietary change (Moore et al., 2018). This pilot study has been developed with some of these barriers in mind: dietary guides have been created to facilitate dietary changes using local-style dishes which have been designed to follow Mediterranean-diet guidelines; educational materials regarding the benefits of Mediterranean and high-protein diets will be included; easy recipe booklets focused on convenience and recipes familiar to this population will be provided; and weekly phone calls to maintain motivation will be carried out.

In conclusion, results of a feasibility study on the implementation of RE and high-protein modified-MedDiet in phase IV CR patients with SO will allow potential barriers to be addressed

and allow for a fully powered randomised control trial to be performed. A fully powered study of a RE and high-protein modified-MedDiet in phase IV CR patients will determine if such a protocol is of greater benefit for improving muscle mass and strength while also improving CVD risk compared to current aerobic style CR recommendations

8 Chapter 8: Acceptability of the diet and exercise protocol

Acceptability of a high-protein Mediterranean-style diet and resistance exercise protocol for cardiac rehabilitation patients: Involving service users in intervention design using a mixed-methods participatory approach

Kirwan R, Newson L, McCullough D, Butler T, Davies IG, Perez de Heredia F.

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8.1 Context within thesis

Building on the research findings from the previous chapters, chapter 8 will discuss research regarding the acceptability of the diet and exercise protocol designed to increase muscle mass and strength in a cardiac rehabilitation population. Patient and public involvement (PPI) was used to investigate the acceptability of the protocol to the end user, and the feasibility of implementing an intervention for implementation and achievement of both primary and secondary outcomes, *i.e.*, muscle mass and strength augmentation and improvement of cardiometabolic risk markers, respectively.

8.2 Abstract

Background: Current cardiac rehabilitation (CR) practices focus on aerobic-style exercise with minimal nutrition advice. This approach may not be optimal for CR patients with reduced muscle mass and elevated fat mass. Higher protein, Mediterranean-style diets combined with resistance exercise (RE) may improve muscle mass and reduce the risk of future cardiovascular events, although such an approach is yet to be trialed in a CR population.

Objective: We explored patient perspectives on the proposed design of a feasibility study. Patients reflected on the acceptability of a proposed high-protein Mediterranean-style diet and RE protocol, emphasizing research methodology and the acceptability of the proposed recipes and exercises.

Design: We applied quantitative and qualitative (mixed methods) approaches. The quantitative approach involved an online questionnaire (n=40) regarding the proposed study methodology and relevance. A subset of participants (n=12) received proposed recipe guides and were asked to prepare several dishes and complete an online questionnaire regarding their experience. Another subset (n=18) received links to videos of the proposed RE and completed a questionnaire regarding their impressions of them. Finally, semi-structured

interviews (n=7) were carried out to explore participants' impressions of the proposed diet and exercise intervention.

Results: Quantitative data indicated a high level of understanding of the intervention protocol and its importance within the context of this research. There was a high degree of willingness to participate in all aspects of the proposed study (>90%). The trialed recipes were enjoyed and found to be easy to make by a majority of participants (79% and 92.1%, respectively). For the proposed exercises 96.5% of responses agreed they would be willing to perform them and, 75.8% of responses agreed they would enjoy them. Qualitative analysis revealed that participants viewed the research proposal, diet and exercise protocol in a positive light. The research materials were considered appropriate and well explained. Participants suggested practical recommendations for improving recipe guides and requested more individual-focused exercise recommendations, and more information on the specific health benefits of the diet and exercise protocols.

Conclusions: The study methodology and the specific dietary intervention and exercise protocol were found to be generally acceptable with some suggested refinements.

8.3 Introduction

Cardiovascular disease (CVD) is the leading cause of death worldwide with almost 19.1 million deaths reported in 2020 (Tsao et al., 2022). Individuals who experience or are at high risk of, a cardiac event can be referred to cardiac rehabilitation (CR) (National Institute for Health and Care Excellence, 2013), a lifestyle intervention program predominantly focusing on aerobic exercise, and may involve other components such as advice on diet quality and weight management, smoking cessation, and stress reduction (British Association for Cardiovascular Prevention and Rehabilitation, 2017). The objective of CR is to reduce the risk of future cardiac events and improve quality of life, and considerable evidence points to its efficacy (Lavie and Milani, 2006; Sagar et al., 2015; Anderson et al., 2016).

Sarcopenic obesity (SO), the combination of reduced muscle mass and function associated with aging (sarcopenia) and excessively elevated body fat (obesity) (Prado et al., 2012), has been observed to contribute to a greater risk of CVD (Atkins et al., 2014; Gusmao-Sena et al., 2016). Indeed, SO may at least partially explain the phenomenon known as the obesity paradox, whereby lower body mass index (BMI) in cardiac populations is associated with increased mortality (Lavie et al., 2003; Romero-Corral et al., 2006; Lavie et al., 2012a). The increased risk of CVD in those with SO may result from higher levels of pro-inflammatory cytokines produced in visceral adipose tissue (VAT), known to be elevated in individuals with SO (Schrager et al., 2007). These cytokines can contribute to detrimental changes in cardiometabolic (CM) risk factors such as insulin resistance and dyslipidaemia (Fox et al., 2007; Ebbert and Jensen, 2013; Medina-Urrutia et al., 2015). These pro-inflammatory molecules may further contribute to the progression of SO through their association with reduced muscle mass and strength (Rubio-Ruiz et al., 2019). Reduced muscle mass and function may also contribute to reductions in physical activity levels in adults (Lee et al., 2018), which can reduce cardiorespiratory fitness and lead to an increased risk of CVD (Sillars et al., 2019; Steell et al., 2019).

Accordingly, increasing muscle mass may be an appropriate target in CR patients presenting with SO. Resistance exercise (RE) and increased protein intake are widely used strategies for increasing muscle mass and strength in older adults (Hou et al., 2019). However, while RE may be incorporated into some CR programmes, there is a clear emphasis on aerobic exercise-based CR (Vanhees et al., 2012; Piepoli et al., 2016; Price et al., 2016; Khadanga, Savage and Ades, 2019). Similarly, while Mediterranean diets are recommended to reduce CVD risk (de Lorgeril et al., 1999; Trichopoulou, Bamia and Trichopoulos, 2005; Iestra et al., 2006; Trichopoulou et al., 2007; National Institute for Health Care Excellence, 2013; Deanfield et al., 2014; Panagiotakos et al., 2016), there is little evidence for adherence to such dietary

advice in current CR practices, particularly in non-Mediterranean populations (Linan Pinto et al., 2021; Vanzella et al., 2021). A randomized controlled trial (RCT) trialing a high-protein, Mediterranean-style diet combined with RE in a CR population would contribute evidence for the efficacy of such an approach.

Patient and public involvement (PPI) in the early stages of developing a feasibility study for such an intervention is recognized as good practice and can greatly contribute to the acceptability of such an intervention (Campbell et al., 2007). Both quantitative and qualitative methods can be employed to identify potential barriers to change, determine understanding of the relevance of specific interventions, and help to refine the proposed methodology, thereby potentially improving engagement and adherence prior to the implementation of an RCT (Campbell et al., 2007; Yardley et al., 2015).

The current study was designed to assess and refine the proposed methodology for a high-PRotein Mediterranean-style diet and Resistance Exercise in cardiac Rehabilitation (PRiMER) (McCullough et al., 2021). To this end, we conducted a mixed methods study in which qualitative and quantitative strands contribute to a more comprehensive understanding of CVD patient impressions of the proposed intervention (Creswell and Clark, 2017), particularly on proposed exercises and recipe acceptability.

8.4 Methods

8.4.1 Study Design

The study consisted of i) a cross-sectional, online questionnaire and ii) a phone interview, conducted amongst individuals with a diagnosis, history or elevated risk of CVD, with questions focused on a proposed high-protein Mediterranean-style diet and RE intervention

for CR patients (McCullough et al., 2021). This methodology was used due to COVID-19-related social distancing restrictions implemented in the UK at the time of data collection.

The principles of a “person-based” approach were used to help refine and evaluate the proposed intervention. Such a person-based approach may help those designing the intervention to better understand how potential participants, as individuals, react to the proposed methodology and identify which aspects may need to be refined for a more feasible implementation (Yardley et al., 2015). Core-elements of such an approach include i) intervention planning, ii) design and iii) evaluation of acceptability (Yardley et al., 2015). The initial planning and design of the proposed intervention were carried out in 2019 with the assistance of the Liverpool Heart and Chest Hospital (LHCH) Service Users Research Endeavour (SURE) group (https://www.lhch.nhs.uk/research/sure-group/), a PPI group, and CR staff from LHCH Knowsley Community Cardiovascular Service (KCCS).

8.4.2 Recruitment

Individuals registered as having a diagnosis or history of CVD or type 2 diabetes (T2D) in the Research for the Future (RftF) database were presented with a research survey link via a combination of email and announcements on the RftF website, newsletter, Facebook and Twitter accounts in June and July of 2020. Research for the Future (https://www.researchforthefuture.org/) is an initiative of the National Institute for Health and Care Research Clinical Research Network (NIHR CRN) (https://www.nihr.ac.uk/explore-nihr/support/clinical-research-network.htm) to facilitate recruitment to NIHR and other health research studies. Individuals with T2D were included due to the elevated risk of CVD in this population (Einarson et al., 2018). The link included a participant information sheet with information on the study as well as a consent form. Inclusion criteria were: i) a diagnosis of a

cardiovascular condition or diagnosis as high risk for a cardiac condition, and ii) previous referral to CR.

8.4.3 Ethical approval

Ethical approval for the intervention study was granted by the National Health Service North West - Greater Manchester East Research Ethics Committee (IRAS ID: 256927, REC reference: 19/NW/0762). The study was conducted according to the ethical principles of the Declaration of Helsinki (Assembly, 2018), and online informed consent was obtained from all participants before participation.

8.4.4 Online and telephone questionnaires

The questionnaires were administered through JISC Online Surveys (Bristol, UK. https://www.onlinesurveys.ac.uk/) and took approximately 30 min to complete. For the initial online questionnaire, after completing questions on demographics, participants were asked to read the proposed research plan (Supplementary Figure 8.1) and were told that all following questions would relate to this plan. Briefly, the research plan included information on the relevance of the research in relation to heart disease, muscle mass and CR. The plan also included a brief description of the proposed research intervention including:

- Inclusion/exclusion criteria
- Proposed intervention arms
- Proposed dietary requirements
- Proposed exercise modality
- A study flow diagram (Supplementary Figure 8.1)

Participants then completed questions related to their understanding and thoughts regarding the proposed protocol. The two final questions asked participants if they would be willing to participate in further evaluation. The first asked participants to refer to a digital recipe book to try the healthy recipes from the study and to reflect on their use, acceptability and feasibility. Those who agreed were provided with a PDF containing recipes for the proposed intervention (Appendix 3). Participants were asked to try as many recipes as they liked and reply to an online questionnaire regarding the preparation of the recipes and their impression of the finished meals. These participants were also invited to engage in a semi-structured interview concerning their thoughts, opinions and recommendations regarding the proposed intervention and recipes.

The final question asked participants to complete a follow-up questionnaire related to the proposed RE protocol (Appendix 3). This questionnaire explored how the proposed exercise protocol would be carried out and linked to videos of the RE to be included in the proposed intervention. Participants were asked to answer questions related to the protocol, as well as their willingness to perform, and impressions of, each of the exercises.

8.4.5 Quantitative analysis

Descriptive statistics were analysed in R (Version 1.4.1717, R Core Team 2021).

8.4.6 Qualitative analysis

For the convenience of the participants, interviews were conducted by telephone. Interviews were digitally audio recorded and later transcribed verbatim by the interviewer and first author (RK). Reflective notes were made post-interview, and interview data and analytical notes were discussed between analytical authors (RK, LN) during the analytical process. The interview

transcripts were analyzed based on the interpretive-descriptive method (Thorne, Kirkham and MacDonald-Emes, 1997) to enable the development of themes. The analysis was approached by asking, “what is important to the participants here?” and “what are we learning about the participants’ experiences?” The main themes of the collected data were developed via a constant comparative method of data analysis (Corbin and Strauss, 2014).

We acknowledge that using interpretative-descriptive method, the analysis and subsequent themes were influenced by the research team’s subjective interpretations of the data. However, throughout the analytical process, researcher reflexivity and audited discussions (Noble and Smith, 2015) occurred aiding researcher triangulation (Heale and Forbes, 2013) which ensured rigor in the quality of qualitative analysis conducted (Reynolds et al., 2011). The qualitative data collection and analysis was conducted by the lead author a white male, early career researcher with specific interests in nutrition in cardiac rehabilitation (RK). The qualitative analysis was led by a female Reader in Applied Health Psychology and a Registered Health Psychologist with expertise in qualitative methodology and long-term conditions (LN). The final version of the qualitative analysis was discussed further with the research team: a white female Senior Lecturer in Physiology with expertise in adipose tissue physiology (FPdH), a white male Reader in Nutrition with expertise in nutrition and lipidology (IGD), a white male Senior Lecturer in Nutrition and Dietetics and a Registered Dietitian with expertise in cardiac rehabilitation (TB), and a white male, early career researcher with expertise in nutrition and exercise physiology (DM).

Direct quotes from a range of participants, which we felt would be transparent in context (Reynolds et al., 2011), acted as evidence to support commentary. The authors confirm that the raw data examples supporting this study's findings are available within the article (see Tables 3-5). Due to the nature of this qualitative research, in line with legal and ethical

processes, participants of this study did not agree for their full transcripts to be shared publicly, so supporting data beyond the sample quotation extracts is not feasible. Post quotes, P1-7 indicates from which participant the verbatim quote has been selected. In addition, written feedback from the open-response selection of the questionnaires has also been incorporated into the analysis, to support the validation of findings for each theme; these quotes display (open response) afterwards.

8.5 Results

8.5.1 Demographics

A total of 40 people with a history of CVD, T2D or both, participated in the quantitative questionnaire. Demographic details of the sample are presented in Table 8.1. The majority of participants were obese (class 1) and of White British ethnicity; over half of the participants reported high blood pressure, and 43% reported high cholesterol.

Table 8.1. Demographic characteristics of participants

Characteristic	All samples (n=40)
Female (%)	20 (50%)
Age (years)	64.7 ± 13.5
Height (m)	1.68 ± 0.1
Weight (kg)	85.1 ± 21.9
Body mass index (kg/m^2)	30.0 ± 6.7
White ethnicity (%)	36 (90%)
Do you have high blood pressure? (yes %)	23 (58%)
Do you have high cholesterol? (yes %)	17 (43%)

8.5.2 Quantitative Results

8.5.2.1 Proposed protocol

Importance and willingness to participate in the proposed intervention

Data related to participants' views on the importance of the intervention, the understanding of its relevance and their willingness to participate in such an intervention is displayed in Figure 8.1. All participants stated that the objectives of the study were clear, that they thought that the intervention was important and that they would be willing to participate in it. Furthermore, 85% of participants replied that they thought the intervention design could to improve muscle strength and blood markers to help reduce heart disease risk.

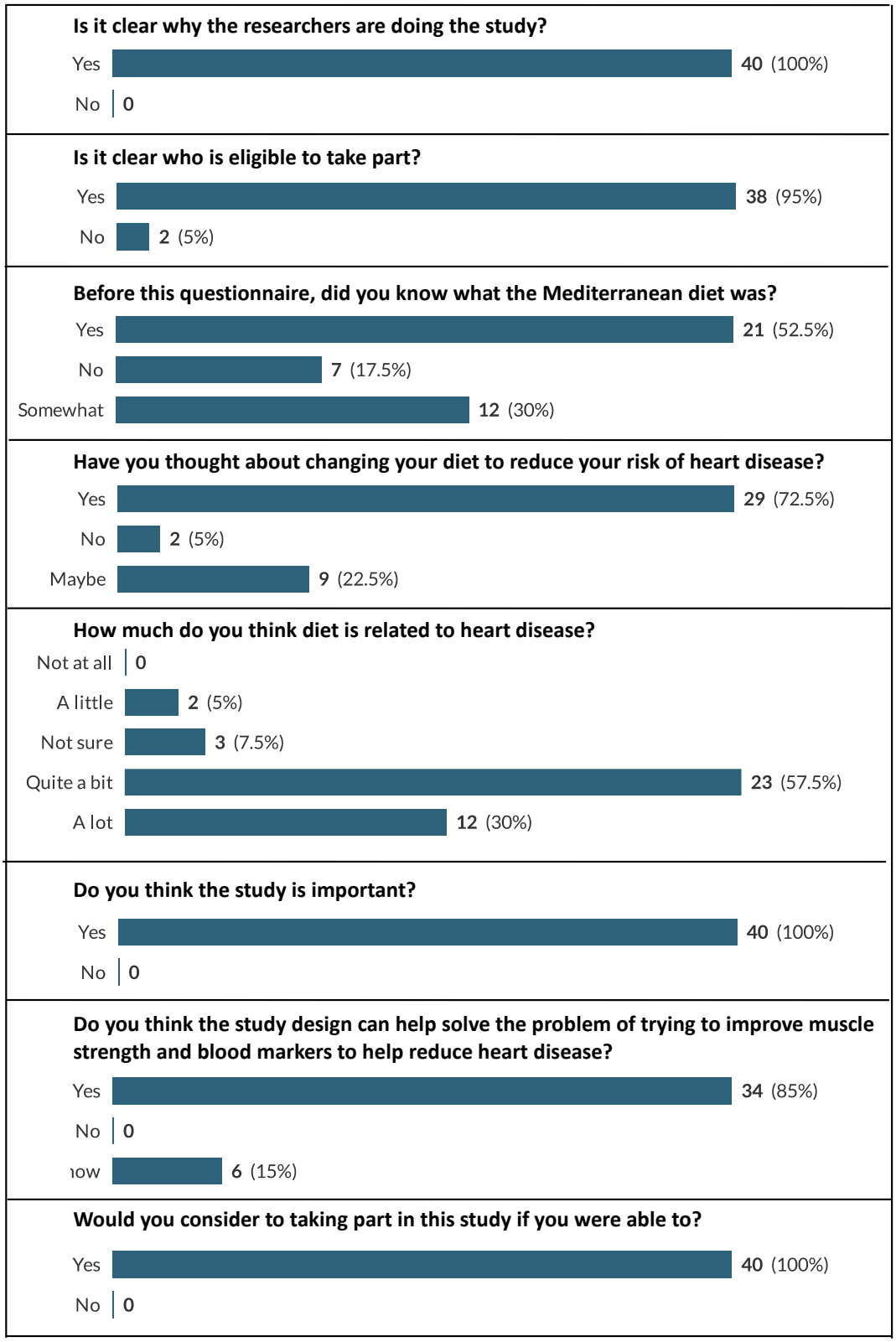


Figure 8.1. Participant responses to questions relating to intervention importance and willingness to participate. Displayed as number and (percentage) of respondents who selected each answer option (*e.g.*, 100% would represent that all this question's respondents chose that option)

Protocol requirements

Data related to participants' willingness to undertake the interventions dietary and exercise requirements, and their willingness to undertake the required laboratory procedures is displayed in Figure 8.2. The majority of participants (90% and above) were willing to participate in all aspects of the proposed intervention.

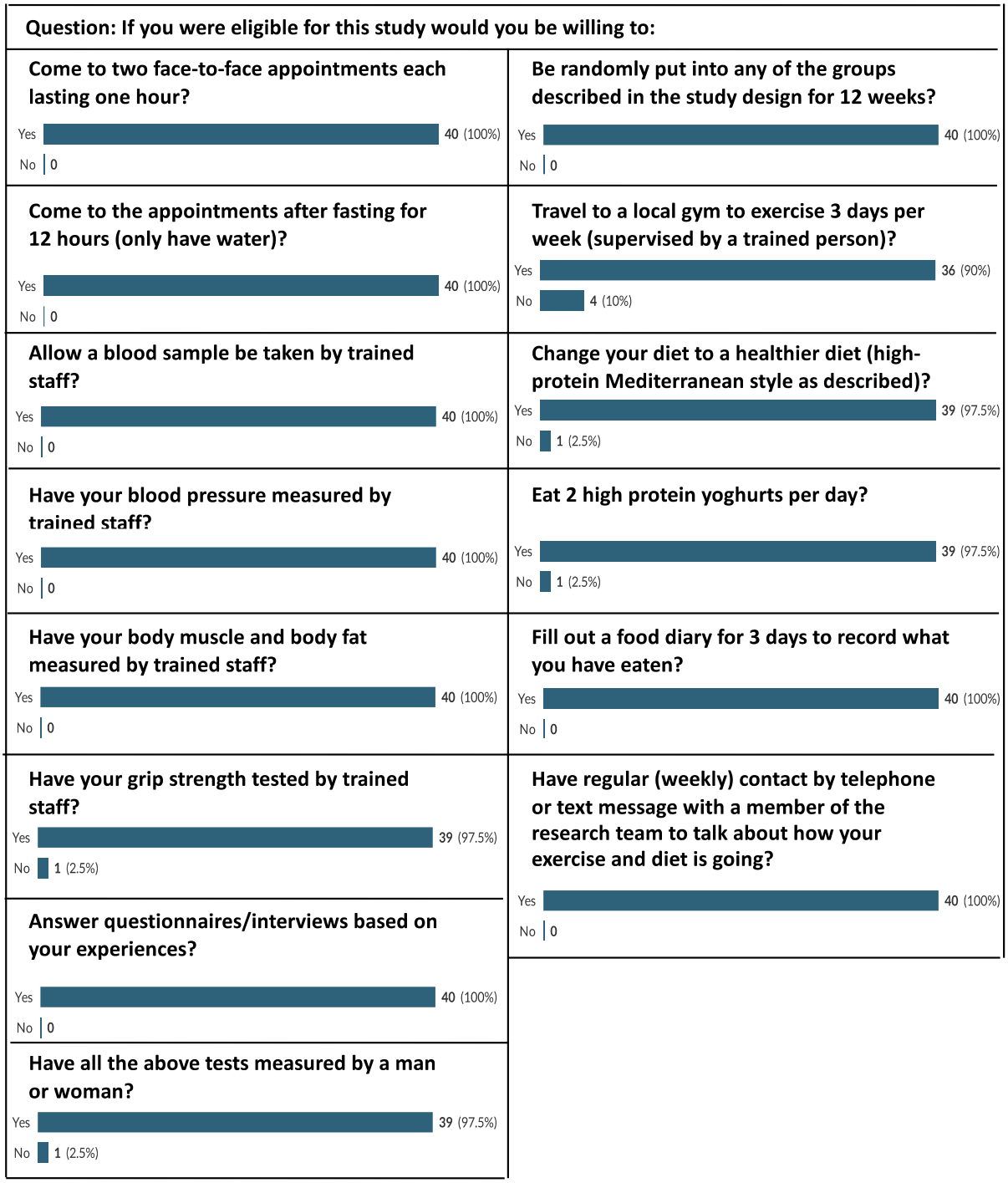


Figure 8.2. Participant responses to questions relating to intervention protocol requirements. Displayed as number and (percentage) of respondents who selected each answer option (*e.g.*, 100% would represent that all this question's respondents chose that option)

Proposed recipes

A total of 12 participants completed the recipe-related questionnaires. As each participant was instructed to try and provide feedback on as many recipes as they wished, a total of 38 responses were received. The recipes trialled, and the frequency of use of each recipe are presented in Table 8.2. Due to the large number of recipes trialled by the participants it was decided to pool the results from the recipe-related questionnaires to give an overview of participants' impressions of all the recipes trialled. A breakdown of participants' gustatory ratings of the recipes can be seen in Figure 8.3. In general, the recipes were well received by the participants with 79% stating that, overall, they "liked very much" or "extremely liked" the recipes they trialled (Figure 8.3). Participants' ratings of the ease/convenience of making the recipes are displayed in Figure 8.4. In general, 73.6% of respondents either agreed or strongly agreed that they would regularly make the recipe(s) they trialled.

Table 8.2. Recipes trialled by participants

Recipe	n
Beans and eggs on toast	6
Protein porridge	4
Herby roast potatoes	3
Chicken fried rice	3
Healthy fish and chips with mushy peas	2
Protein smoothie	2
Roasted vegetable and sausage bake	2
Fresh fruit and protein yoghurt	2
Simple peas and onions	2
Easy chicken pitta	2
Heart healthy burgers	1
Salmon and bean salad	1
Mushroom, spinach and cheese omelette	1
Tomato salsa	1
Quick frozen veg stir-fry	1
Chili con carne	1
Easy BBQ chicken and veg pizza	1
Chicken and veg omelette bites	1
Prawn & veggie spaghetti	1

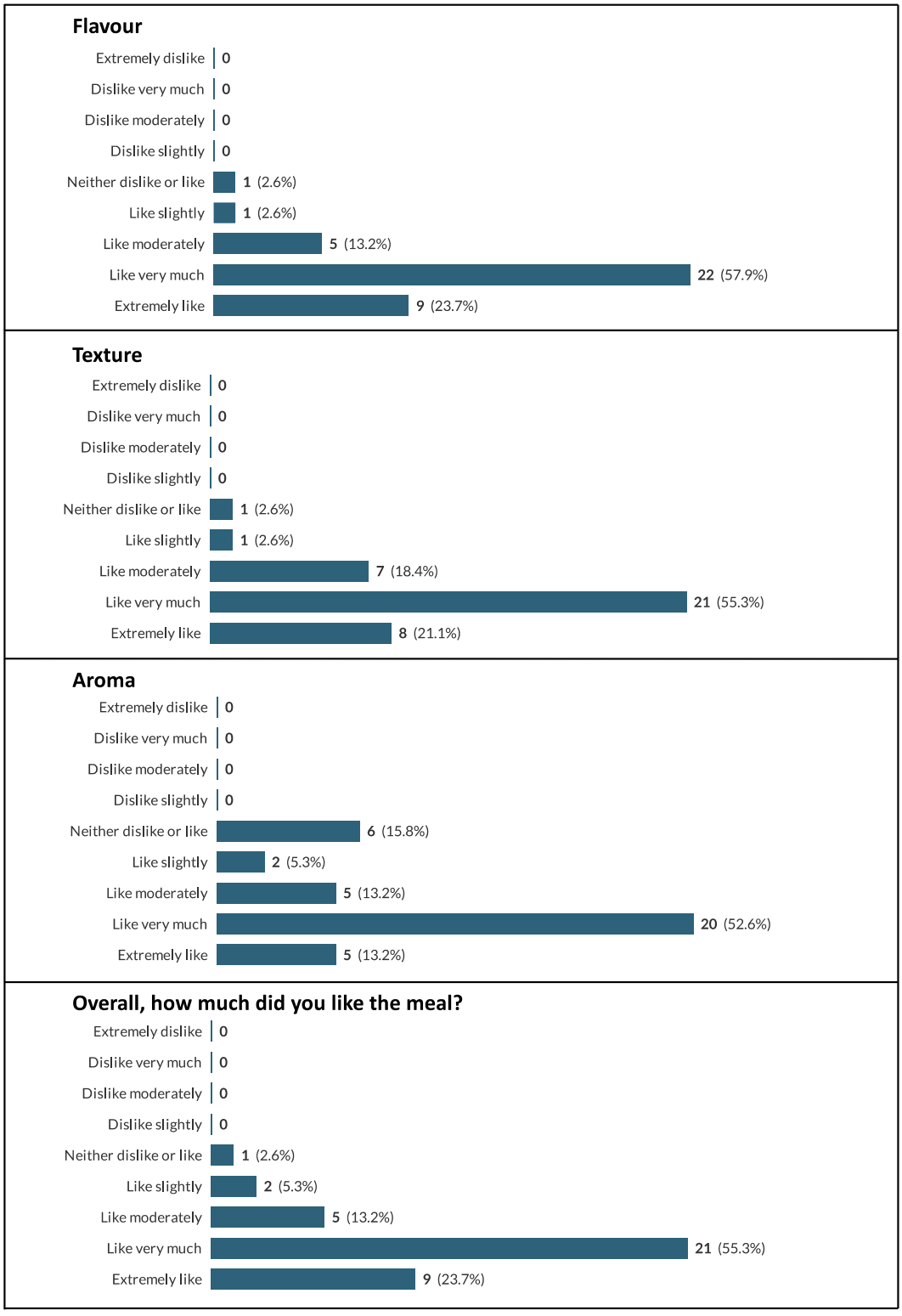


Figure 8.3. Pooled participant responses to questions relating to gustatory impressions of the proposed recipes. Displayed as number and (percentage) of responses to each answer option (*e.g.*, 100% would represent that all this question's responses chose that option)

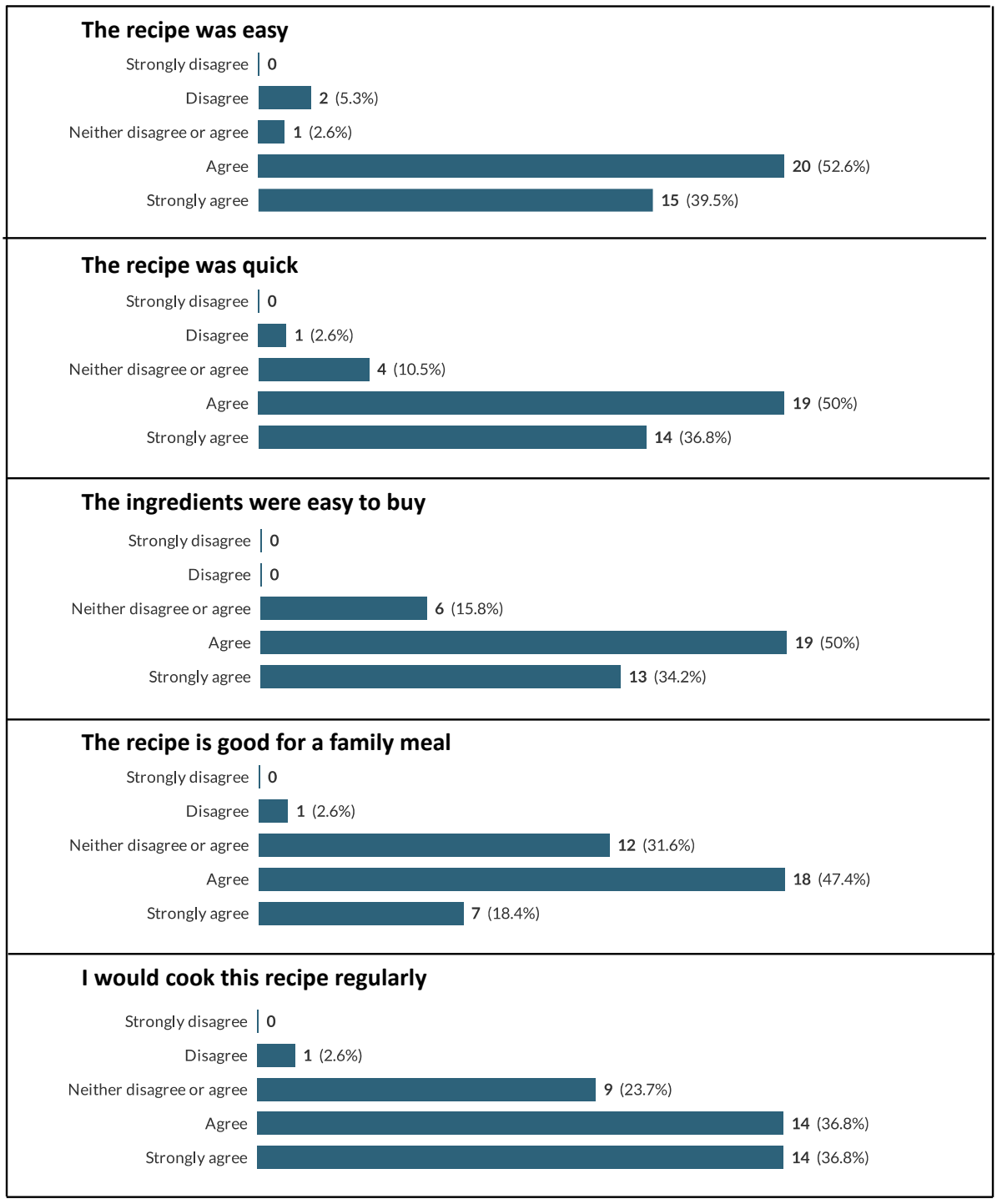


Figure 8.4. Pooled participant responses to questions relating to ease of preparation of the proposed recipes. Displayed as number and (percentage) of responses to each answer option (*e.g.*, 100% would represent that all this question's responses chose that option.)

Proposed exercises

A total of 18 participants completed the RE-related questionnaires. Results for participants' perceptions of RE, in general are displayed in Figure 8.5. The majority of participants (61.1% and above) disagreed or strongly disagreed with a number of common negative perceptions of RE such as "resistance exercise will make you look bulky or big" and "resistance exercise is bad for joints" (Figure 8.5). However, 94.5% of participants agreed or strongly agreed that "Resistance exercise is not good for older people" (Figure 8.5). Participants responded positively to statements about their willingness to participate in the various aspects of the proposed RE intervention with 88.9% of participants agreeing or strongly agreeing with the various participation questions (Figure 8.5).

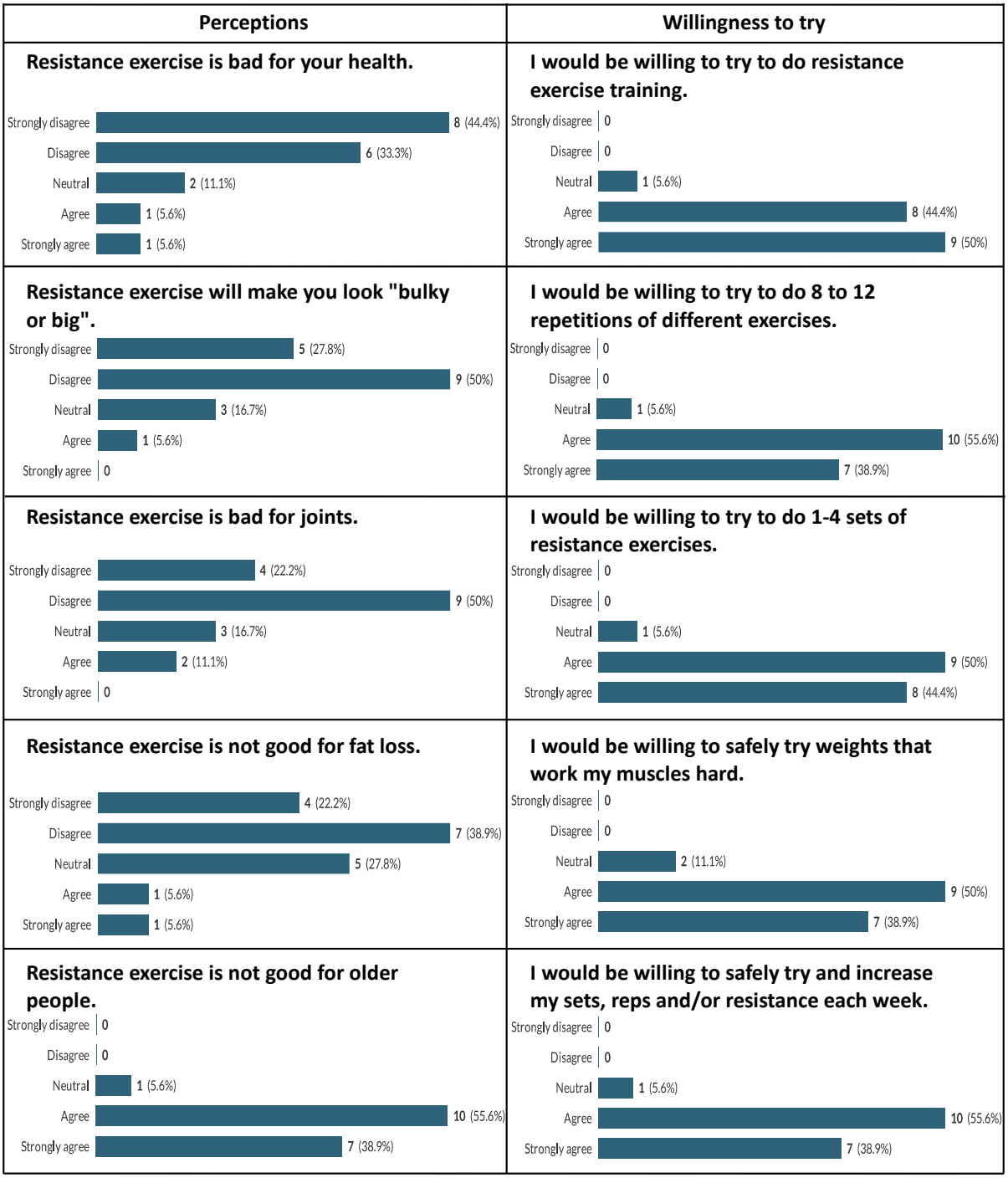


Figure 8.5. Pooled participant responses to questions relating to perceptions of and willingness to try resistance exercise. Displayed as number and (percentage) of responses to each answer option (*e.g.* 100% would represent that all this question's responses chose that option)

Participants also watched videos of the individual REs for the proposed intervention (leg press, Smith machine deadlift, machine chest press, machine row, machine shoulder press, lat pulldown, leg extension, hamstring curl, chest fly machine, horizontal cable row, and shoulder press machine). Due to the large number of exercise videos watched (n=11) by the participants it was decided to pool the results from the RE-related questionnaires to give an overview of participants' impressions of all the exercises viewed, and the results are displayed in Figure 8.6. In general, the exercises were well received by the participants with 96.5% of responses agreeing or strongly agreeing that they would be willing to perform the exercises under trained supervision, and 75.8% of responses agreeing or strongly agreeing that they would enjoy the exercises.

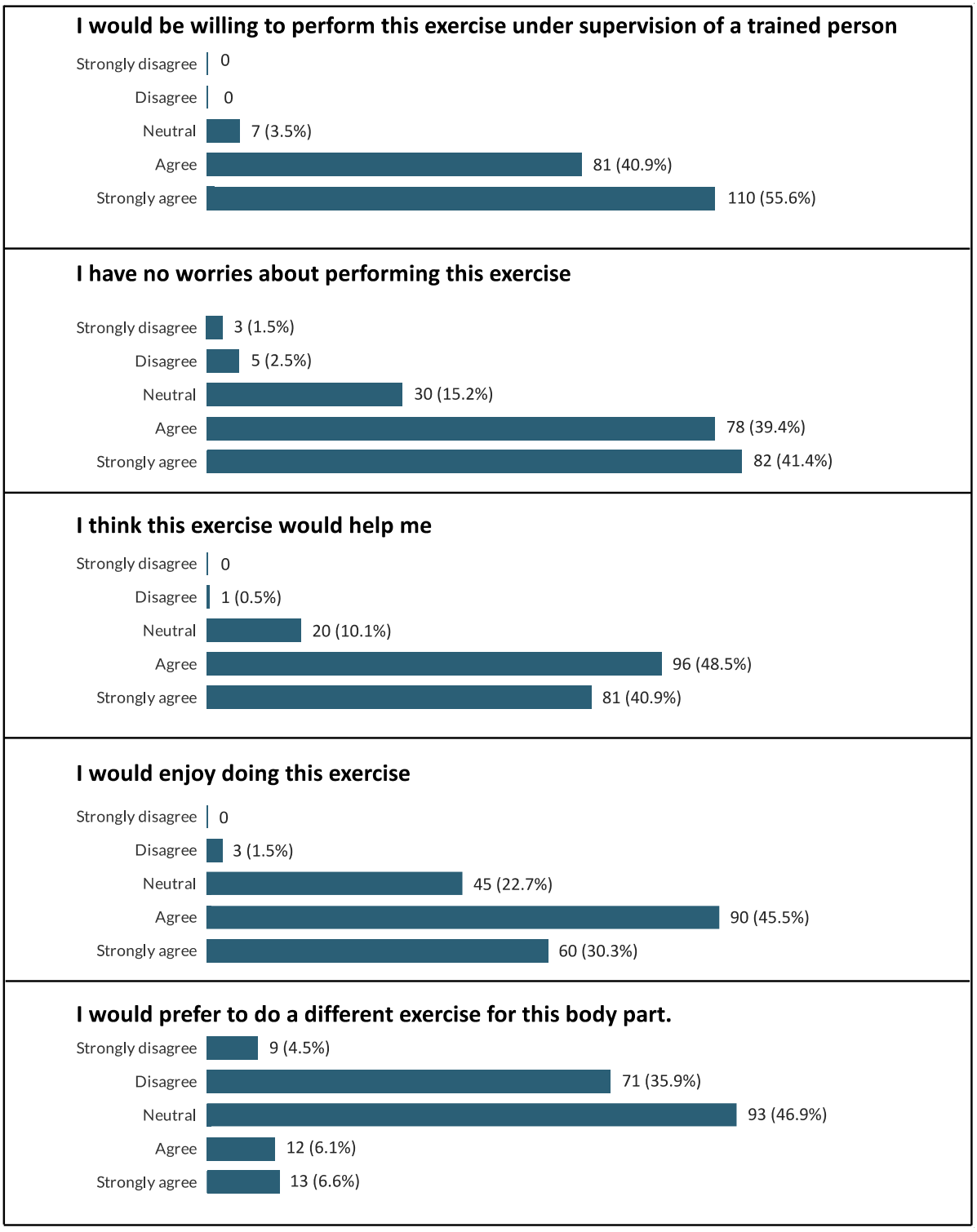


Figure 8.6. Pooled participant responses to questions relating to impressions of the proposed resistance exercises. Displayed as number and (percentage) of responses to each answer option (*e.g.*, 100% would represent that all this question's responses chose that option)

8.5.3 Qualitative Findings

Theme 1. “Pleasantly surprised”: Support for research and practical recommendations for improvements

Overall participants welcomed the concept of this research proposal. The research materials were considered appropriate and well explained. The resources were generally seen as a supportive reminder to prioritize their health.

A few practical recommendations for information formatting and providing hard copies of resources as opposed to digital versions, were suggested. Finally, some participants requested further information on the recommendations for heart health and how it relates to this to dietary requirements (Table 8.3).

Table 8.3 Quotes to support theme 1

Supportive of research	Practical recommendations
"anything that refreshes your memory and helps put you in the right direction doing the right stuff is always good" (P1)	*"improved somewhat by perhaps a little bit more of an index, perhaps by adding a bit more nutritional information. And if it's from my point of view as a standalone book, these sort of references to the rest of the cardiac rehab thing"* (P6)
"Like many overweight people I have a history of dieting and trying different things. And so, my ideal scenario based on past experience… it sounds like that combined approach sounds very attractive" (P7)	*"it was alright. It was simple. There was nothing difficult, well the things that I did. There was nothing difficult. I did change one or two things put in a little bit here and there to make it more personal."* (P3)
"I thought the instructions were easy to follow couldn't fault this at all" (P5)	*"on the front page, it says food and exercise for a healthier heart. But there's nothing about exercise in the recipe book. So it is part of the prime trial, that's fine. But as a standalone, it's, it's a bit confusing."* (P6)
"if you can come up with the ideal diet and exercise programme to help people recover. Yeah." (P1)	*"the introduction, it says the idea of this recipe book is to give you an idea of blah blah blah by following this particular way of eating, but it doesn't actually tell you what this particular way of eating is, gives you lots of recipes. So I think, I don't know how you're going to use it"* (P6)
"I was pleasantly surprised" (P7)	*"some inconsistency with some of the text in the recipe book"* (P4)
"my point of view, which is from the point of view of a non-expert cook. It was it was very good" (P6)	*"And sometimes I struggled to for me being an older person perhaps it might have been handy to have (a hard copy)"* (P4)
"People need to know and find out how exercise, how diet affects an unhealthy or a healthy heart, and to do that you need to do all the things that you're going to do (in the study)" (P3)	
"Mediterranean diet is admired for improving people's wellbeing and longevity, and I haven't selected that type of foods most of the time. Yeah." (P4)	

P = Participant

Theme 2: "I definitely would eat that" evaluation of the dietary approach

This theme is explained by an overall positive response to the proposed dietary intervention. Participants typically considered they were aware of, or already engaged in similar healthy eating practices, or had aspirations to do similar. Participants described how they made tweaks to personalize recipes, but overall recipes were 'not difficult', considered easy to follow, and often used 'everyday' readily available ingredients. The adapted Mediterranean dietary intervention proposed was therefore considered acceptable and appropriate to recommend to this patient population.

In addition, this theme outlines participants' queries and recognizes that participants offered practical recommendations particularly in relation to the dietary element of the intervention. For example, there were recommendations that the recipes should use European measurements as opposed to American cup measurements for the ingredients and the recipes could include more nutritional information (*e.g.*, the calorie and macronutrient information). It is noteworthy that participants made 'tweaks' to recipes to increase perceived 'healthiness', such as reducing the amount of fat or oil used within the ingredients list. These amendments related to their (mis)understanding of the dietary approach, and it might be helpful to offer participants further information on the development and background evidence-base upon which these dietary recipes are based (Table 8.4).

Table 8.4 Quotes to support theme 2

Supportive of dietary approach and Recipe book	Queries and amendments to dietary approach
"master some of these recipes. They were well written and simple to follow…. There was nothing in it that I was frightened of, or I wouldn't have tried" (P6)	*"it listed on there, sort of how many calories there were in each dish, because I'm diabetic as well. And I'd like to keep checking the calories. So that would you know if you pick up a packet in the supermarket, it tells you how many calories you're eating".* (P6)
"the Pictures were very appealing. So I like to see what I'm supposed to be making I like to see what it's supposed to look like. So having the picture and the one page instructions were an easy thing to do" (P7)	*"I find confusing it just seems to me the recipes maybe originated in the States. So that a lot of the measurements. And I find that confusing because everybody's got different cups"* (P4)
"was something that we would have normally done with a little bit of a twist on it." (P3)	*"I would prefer measurements of the tomatoes to be quantified. I don't like the American cup measurements. It would be beneficial for me to have carbohydrate value too"* (open response)
"super easy, super easy. I'm always on the lookout for new recipes but these were...most of the stuff we already had in, you know the basics, just normal stuff you have in the cupboard .... I liked the way everything was on one page so you just print out the page" (P7)	*"it seemed more than necessary and tasted a bit 'greasy' to me. Probably too much food and too much time for a breakfast meal, at least for me! Maybe better for dinner!"* (open response)
"most appealing to me, but also, probably, they seem like an easy thing to do. you could do it quickly" (P1)	*"We might have tried that but we cut down on one or two things because they're on the higher fat side. So most of most of the stuff we have is chicken- based. I like Fish but my wife doesn't so we don't have as much as we should do."* (P2)
"they weren't expensive meals. Which I think is important." (P5)	*"more oily fish dishes might be appropriate." (P4)*
"And I try and eat relatively healthy. So it was interesting for me to see some of the things that I thought Oh, yes, I definitely would eat that." (P4)	*"getting used to doing these things. I mean, they're not terribly complex. But I found with trying to eat the right food. Now I have a problem with the high cholesterol"* (P1)
"I tried a few of the recipes, I should go on using the recipes." (P6)	*"if it (recipe) says it takes 10- 15 minutes. You know it's gonna be double that."* (P2)
	"a lot of people just don't have the time these days for food prep. You know, especially on a day like this, it's gorgeous over here in Manchester so you don't want to spend like 3 hours in the kitchen." (P2)
	"I was a bit concerned about how many calories would be in the sausages. So I left that one out." (P6)

P = Participant; open responses are from the quantitative questionnaires and cannot be attributed to a specific participant

Theme 3. "Finding that right balance" exercise at home

Participants acknowledged that home-based exercise was feasible though most suggested that they were not motivated or did not regularly engage in physical activity at home. There was a sense that participants typically referred to other people doing or being able to exercise at home, but little acknowledgement that this was relevant to them as individuals. There was a call for more detailed information on the benefits and need of RE in this patient group.

Participants also queried the concept of home-based exercise and typically considered an external 'trip to the gym' as more motivational as it offers structure and social support. There were concerns raised regarding the safety of RE at home, with thoughts that having professionals who can monitor and offer instruction, being more appropriate. References to the exercise videos were positive overall, with reference to clear instructions, although again, respondents expressed a need for confirmation that a particular exercise was suitable to their individual health status. Further consideration on the practical 'how to do' and 'what to do' is warranted, alongside consideration for the role of social and motivational support for a future research trial in this patient group (Table 8.5).

Table 8.5 Quotes to support theme 3

Sample evidence quotations for "Finding that right balance" exercise at home
"more detail on the sort of exercise you're talking about?" (P1)
"That's a motivation thing. I mean you've got to be motivated to go to the gym anyway, its having the motivation after a hard day's work, you got to come in and then find the motivation to do exercise at home. So unless you can find a way of motivating people, and the NHS have things like the couch potato to five k, where you can join in on social media to find other groups support to support you." (P3)
"from a motivation perspective, actually going to the gym is better" (P3)
"I have a treadmill at home that I occasionally use when I'm too ... I prefer to go out"" (P6)
"I go a couple of times a week to exercise in the gym only cardiovascular stuff, only static cycling, a few bits of weights. But I like to do it away from.. I prefer doing it away from home. Rather than at home. I regard doing it at home as better than not doing it. but I'd rather to go out. it makes it a bit more special and gets me out of the door. But I do I do try to exercise." (P6)
"if you've not got an instructor or, or somebody else in the gym that knows what you're doing. So he can say, hey mate, you need to do this to improve this or that. If you're doing it at home and doing it wrong. You could end up doing yourself more damage." (P3)
"I think it's hard for me to do that at home. But as I'm not going to the gym at the moment, I should be doing something." (P4)
"I think one one commits better in a group than as an individual, you know, you think oh, I'll just do five minutes." (P5)
"Looks like a simple exercise and not overly difficult" (open response)
"Only concern is I could damage my back. It sounds as if this has been thought about!" (open response)
"Having had open heart surgery last August I would be a bit apprehensive of this exercise if the weight was too heavy as I wouldn't want to exert too much pressure on the internal wound" (open response)
"I have some slight shoulder pain on my left side when extending my arm in this way. I'm happy to do the exercise but would take advice on whether it's appropriate for me" (open response)

P = Participant; open responses are from the quantitative questionnaires and cannot be attributed to a specific participant

8.6 Discussion

To our knowledge, this is the first mixed-methods study to determine the acceptability of using a high-protein Mediterranean-style diet and RE protocol to improve lean mass, strength and cardiometabolic risk in a UK CR population. Both our quantitative and qualitative analyses highlight the recognition of the importance of, acceptability of and willingness to participate in the research protocol presented to the participants. However, a desire for clarification on certain aspects of the protocol's diet and exercise components and requests for more personalized guidance relating to these components were also highlighted.

The proposed research protocol presented to participants was developed in collaboration with CR practitioners and a hospital-based service-users group (LHCH SURE group) to ensure the research proposal was both understandable and applicable to the end users. This was intended not only to make the research more acceptable but to improve potential participants "health literacy" in relation to the aims and methods of the protocol. Health literacy has been described as 'the cognitive and social skills which determine the motivation and ability of individuals to gain access to, understand and use information in ways which promote and maintain good health' (Nutbeam, 2000). According to the American Medical Association, "health literacy entails more than a patient being able to read written instructions; it requires the ability to comprehend and apply the information ascertained" (Parker et al., 1999). As such, ensuring the materials provided to participants improve health literacy related to an intervention should be considered a vital aspect of intervention design. The qualitative research presented here has highlighted several areas where the proposed intervention can be improved. The formatting/presentation of study materials has been highlighted with participants requesting, for example, inclusion of an index, provision of more nutritional information for recipes (calories, carbohydrate content etc.) for recipes and inclusion of more

information related to the dietary pattern recommended in the intervention. Other suggestions included changes to/standardization of the measurements used for the recipes, particularly focused on avoiding the use of cup-measures (which are not commonly used in the UK).

Of particular note were comments from participants related to the quantity of oil used in some of the recipes with some participants reluctant to use so much oil when cooking. The Mediterranean dietary pattern is characterized by its use of olive oil as the primary culinary oil, which is believed to be partially responsible for some of the noted health benefits of this way of eating (Trichopoulou et al., 2014; D'Alessandro and De Pergola, 2018). The inclusion of educational material explaining the potential health benefits of olive oil (and other aspects of the Mediterranean dietary pattern) may be useful to assuage any concerns participants may have regarding the use of olive oil. It should also be noted that the participants in this study only received the recipe booklet and not the full dietary guide for the proposed research intervention, which does contain such information. Of further note is the quantitative result that almost half of the participants in this study did not know or only somewhat knew what a "Mediterranean" diet was. The provision of such information in the participant guides/materials should be considered for future iterations of the intervention.

While 88.9% of participants agreed or strongly agreed with the various participation questions related to the RE intervention, in contrast, 94.5% of participants agreed or strongly agreed that "Resistance exercise is not good for older people". This is another potential educational aspect that is worth elaborating on in future versions of the protocol. Resistance exercise has been shown to have multiple benefits for older adults including improving cardiometabolic risk markers, reducing measures of frailty and improving quality of life (Bray et al., 2016; Hart and Buck, 2019; McLeod et al., 2022). It should also be noted that appropriately instructed and monitored RE is safe in older adults and even those with CVD (Kirkman, Lee and Carbone,

2022). The provision of such information in an easy-to-understand format may be useful in encouraging participation in such interventions.

Participants also commented that the act of going to a gym to perform exercise may be more beneficial and is a concept worthy of further exploration. Performing in-person/gym-based exercise may increase the likelihood of vicarious experiences (observing others be successful) and verbal persuasion (verbal cues and/or feedback that may encourage success) (Bandura, 1977), which may lead to greater self-efficacy. Self-efficacy theory proposes that a favorable impression of one's results can help to encourage individuals to adhere to endeavors such as exercise (Desharnais, Bouillon and Godin, 1986). As such, the perception that results of in-person/gym-based exercise may be more beneficial may help individuals adhere to exercise programs such as CR (Kwasnicka et al., 2016), and accordingly, the benefits of such exercise should be elaborated on in any material/instruction provided to participants. Providing of such information along with contact with peers and CR exercise providers in in-person/gym-based settings might help encourage self-efficacy (McAuley et al., 2003; Jackson, 2010) and exercise maintenance.

8.6.1 Strengths and Limitations

This study presents a number of strengths and limitations. A particular strength of this study is the high proportion of female participants (50%), which is notably higher than the proportion of female CR attendance in England, which ranges from only 15% to 38% (Rehabilitation, 2019). Another strength of this study is the use of the mixed methodology approach to seeking feedback and engagement for this intervention. This offers a safe forum for participants to express their experiences and not be biased by researcher expectations. As such, feedback and analysis can be considered more reflective of participants own perceptions. The general

agreement of both quantitative and qualitative results in terms of the acceptability of the proposed intervention is also a strength of this research.

The majority of participants were of White British ethnicity (90%), and this is broadly considered a representative sample of CR participants in the UK, based on a recently published report of CR demographics that reported 83.8% of participants as white (Rehabilitation, 2019). However, it is noteworthy that the findings may not be representative of the diverse ethnic population of the UK as a whole or globally and as such further engagement and exploration of the intervention in a more diverse patient population group is warranted. It should be noted that this study does not have data on the socio-economic status or household income of the participants. Without such information these data cannot determine if the proposed recipes and exercises would be acceptable in different socio-economic groups and as such, further research is warranted in these population groups. Furthermore, as the majority of participants had class 1 obesity research with larger numbers of participants in more diverse BMI classifications may be beneficial for tailoring the diet and exercise guidelines.

A further limitation is that participants had the freedom to choose which recipes to make, which may have biased the results of their ratings of the recipes, as participants would naturally choose recipes they expect to agree with their palate and personal tastes. Finally, participants were recruited from RftF who are likely to be a subset of people/patients very willing to participate in research and may not be representative of the wider clinical population in the UK.

8.6.2 Conclusions

This mixed-methods study found that the proposed high-protein, Mediterranean-style diet and resistance exercise protocols for cardiac rehabilitation participants were generally found to be acceptable, with a high degree of willingness to participate from potentially eligible participants. Several potential areas of improvement were highlighted, particularly in regards to clarification around the benefits of the diet and exercise components and provision of more comprehensive information in participant-facing guides/documents. This information will be vital for improving future iterations of the proposed intervention protocol to help ensure acceptance and compliance in the target population, helping to increase the likelihood of positive health outcomes.

8.7 Supplementary material

Research Plan:

Heart disease is responsible for 1 in 4 deaths in the UK. The risk of heart disease can be increased by many causes. After a cardiac event such as a heart attack, people attend cardiac rehab to stop the disease getting worse. Normally for cardiac rehab, patients are helped to lose weight by eating fewer fatty foods and sugar and go to aerobic exercise sessions. The aerobic exercise sessions involve raising your heart rate for 30-45 minutes by doing exercises such as cycling to improve fitness.

Some people who are at a higher risk of death from heart disease have a normal body weight. Some people have less muscle and more fat than normal, and we believe that these patients don't look overweight – this is known as "sarcopenic obesity" (sarcopenia meaning loss of muscle, and obesity, excessive body fat). Loss of muscle is normal as we get older, but it might put patients at further risk of heart attacks.

Because of this it might be better to change to a diet and exercise programme that helps to improve muscle size and strength and blood markers of future risk of heart disease. If we are correct, our research could lead to improvements in current cardiac rehab (CR), meaning people will be less likely to have another cardiac event (like a heart attack).

To do this study, patients will need to meet the following requirements:

- must have been referred to cardiac rehab
- must be willing and able to do resistance exercise (exercise with weights)
- must be willing and able to eat dairy products (like yoghurt)
- must not have chronic kidney disease,
- must not have a congenital (from birth) or drug/alcohol-related heart condition.

The healthy diet we would ask cardiac rehab patients to follow is a a high-protein Mediterranean-style. This just means:

- eating **more** fruit and vegetables,
- **eating fewer** commercial pastries, and refined carbohydrate foods (white bread, white rice, white pasta)
- **eating more** wholegrains (wholegrain bread, rice and pasta),
- **replacing** butter and margarine with **olive oil** in some meals and dishes,
- **reducing** fatty meat and **eating more** lean meat (chicken), fish, and legumes (peas, beans, lentils),
- **eating more** high-protein, low fat foods, such as low-fat dairy (participants will be provided with 2 high-protein yoghurts to eat each day).

A trained person will give out recipes and guidance on how to make these affordable, healthy and tasty meals.

The exercise we would ask people to do is called resistance exercise. This means working your muscles hard to push or pull a light or heavy weight. Machines or weights can be used to do this. This type of exercise helps make muscles stronger and healthier. A trained person would safely guide the participant through the exercises 3 times per week.

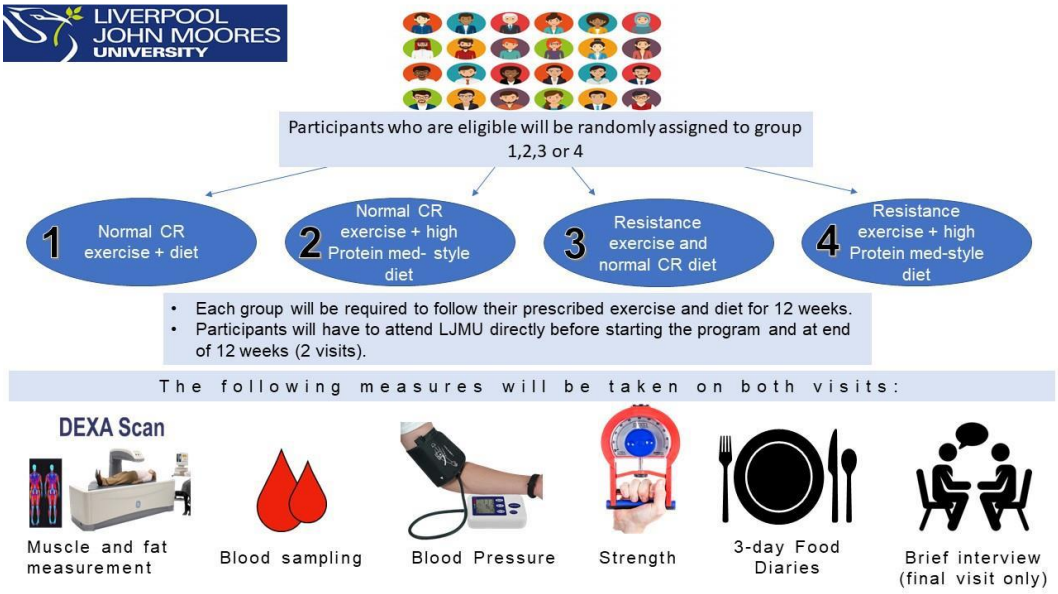
To find out of if this diet and resistance is exercise programme is better for health, we would need to measure eligible patients at the start and end of a 12-week period. At the start we will put participants into 1 of 4 groups.

1 – Normal Cardiac Rehab (CR) diet and aerobic exercise (treadmills, rowing machines, elliptical trainers)

2 – High-protein Mediterranean style diet and normal CR aerobic exercise.

3 – Normal CR diet and resistance exercise (machines and weights).

4 – High-protein Mediterranean style diet and resistance exercise.



Supplementary Figure 1 Study flow diagram

9 Chapter 9: Thesis Discussion

This final chapter briefly summarises and draws together an overview of the key findings of the studies presented in chapters 2-8. This discussion aims to identify the relevance of the studies' outcomes on future research and potential clinical practice, as well as critically evaluate the strengths and weaknesses of the research.

At the time of writing this discussion, a small-scale feasibility study, based on the protocol described in chapter 7, is being carried out. However, the results of this study will not be included in this PhD due to the COVID-19-imposed constraints explained in chapter 1. Instead, this PhD puts forward multiple lines of reasoning for the use of a high-protein, Mediterranean-style diet and resistance exercise in a cardiac rehabilitation (CR) population. The results of those studies and their unique contributions are summarised here, before further discussing their relevance.

9.1 Unique contributions to the scientific literature

Study 1 (chapter 2) (Kirwan et al., 2020) was a narrative review of the significance, causes, and potential countermeasures to age and inactivity-related sarcopenia, viewed through the lens of the COVID-19 pandemic. The relevance of inactivity, illness-induced bed rest, stress, disrupted sleep, and inadequate nutrition in the development of sarcopenia were discussed to highlight the risks to muscle mass and long-term health due to greater time spent at home as a result of pandemic-related control measures. Furthermore, the role of exercise in combatting this potential loss of muscle mass and function was highlighted as was the role of higher protein diets along with a number of other nutritional supplementation strategies including creatine, leucine and vitamin D.

Study 2 (chapter 3) (Kirwan et al., 2021b) was a systematic review and meta-analysis of the effect of protein interventions, with and without resistance exercise for the promotion of lean-mass and strength in older populations. This was the first such publication to only include studies using only supplemental protein or high protein diets, *i.e.*, without the use of isolated amino acids or other substances that might promote hypertrophy, in order to better isolate the effects of whole protein. The results of this study highlighted that any intervention using protein for the improvement of muscle mass and strength in older adults might only be successful by the concomitant use of a resistance exercise protocol. Sub-group analysis in this study also revealed that these effects were observed in adults with lower levels of muscle or physical function (sarcopenia, sarcopenic obesity [SO] and frailty). This is further evidence to suggest that such interventions may be of benefit to muscle mass and strength in those with the lowest levels of muscle and physical function, *i.e.*, those that may need it most, for example, CR participants with SO.

Study 3 (chapter 4) (Kirwan et al., 2021a) was the first Mendelian randomisation analysis of the relationship between serum vitamin D concentrations and lean mass using data from the UK Biobank study. Its results were the first to illustrate that Vitamin D is potentially causally associated with increased total, trunk, and upper body appendicular lean mass (ALM) but not with lower body ALM. Together with evidence from both population-based and intervention studies, this research strengthens the case for the use of vitamin D in reducing muscle loss/ augmenting muscle mass, and potentially function, throughout the life course. This may be of particular importance due to the prevalence of vitamin D insufficiency in both the general population and particularly, in older adults.

Study 4 (chapter 5) was the first Mendelian randomisation analysis of the relationship between both ALM and handgrip strength (HGS) with lipoprotein particle diameter, specifically LDL, VLDL and HDL. The results highlight the potentially causal role of greater levels of muscle mass and strength with improved lipoprotein particle size, which may indicate potential mechanisms by which muscle mass and strength may contribute to a lower risk of atherosclerosis and cardiovascular disease (CVD) in general. The relevance of lipoprotein particle diameter on cardiovascular health is a rapidly developing field and these results contribute to the rationale for aiming for increased muscle mass and strength as important outcomes in diet and exercise interventions, for the reduction of heart disease risk.

Study 5 (chapter 6) (Kirwan et al., 2022) was an investigation of the impact of COVID-19-related lockdown restrictions on participation in CR in the United Kingdom. It was also, to our knowledge, the first such publication to investigate the subjective opinions of CR participants in relation to their motivation and willingness to continue with CR in its peri-COVID-modified format. The results indicate that while there was some cessation of CR practice due to the pandemic, a majority of CR participants maintained their CR exercise habit, albeit with considerable alterations to factors such as location, goals, supervision, duration and enjoyment. Importantly, while most participants were willing to continue with CR in its COVID-modified form, almost 30% indicated they were less likely to do so than prior to the pandemic. The data gleaned from this research highlights that further research is warranted to develop and improve strategies to implement in times when individuals cannot attend CR in person, in order to maintain motivation and participation.

Study 6 (chapter 7) (McCullough et al., 2021) was the publication of the full PRiMER protocol for a high-protein Mediterranean-style diet and resistance exercise in CR. The protocol follows the original plan, conceived at the beginning of this PhD prior to the changes imposed by

COVID-19-related restrictions, for both a cross-sectional and a four-arm intervention study. When carrying out this study becomes feasible, the data collected and synthesised from study 7 (chapter 8) will be invaluable for better tailoring the diet and exercise intervention to the participants needs, thus increasing the likelihood of adherence to the protocol and attainment of the proposed outcomes.

Study 7 (chapter 8) is the first mixed-methods study to determine the feasibility of using the high-protein Mediterranean-style diet and resistance exercise protocol developed over the course of this PhD, in a UK CR population. The study used both quantitative surveys and qualitative interviews with thematic analysis to better understand the strengths/facilitators and weaknesses/barriers to implementing the protocol. The results showed both the diet and exercise protocols to be very acceptable to members of the target population while also providing valuable feedback on potential ways to improve and make the protocol more flexible for the varied needs of potential participants.

9.2 Synthesis of findings

Low muscle mass or sarcopenia is associated with a greater risk of chronic cardiometabolic (CM) conditions such as CVD and type 2 diabetes mellitus (T2DM) (Bahat and İlhan, 2016; Scott, de Courten and Ebeling, 2016), and a greater risk of mortality in cardiac populations (Atkins et al., 2014; Gusmao-Sena et al., 2016). Current CR practices focus predominantly on aerobic style exercise (Price et al., 2016; British Association for Cardiovascular Prevention and Rehabilitation, 2017) and as such, may not adequately address the issue of reduced muscle mass in this population. Additionally, there is a lack of evidence regarding the potential use of protocols aimed at augmenting muscle mass and strength in the secondary prevention of CVD. The research presented in this thesis aimed to investigate not only the potential

benefits of augmenting muscle mass, but also to methodically develop an acceptable protocol to effectively do so.

Chapters 1 and 2 (Kirwan et al., 2020) highlight the relevance of lean mass in the context of CM risk. From lower levels of inflammation (Hida et al., 2018) to improved glycaemic control (Hirasawa et al., 2019) and reduced risk of frailty and inactivity (Xu et al., 2020), an argument can be made for the maintenance of healthy levels of lean mass in individuals as they age. Chapter 5 further adds to this rationale by revealing the potentially causal relationship of greater levels of lean mass with favourable lipoprotein particle sizes, which can be speculated to be beneficial for reducing the risk of CVD.

Such evidence may therefore warrant the development of strategies to augment lean mass in populations at greater risk of CVD, particularly those with reduced muscle mass. To address this, chapter 3 (Kirwan et al., 2021b) highlights the importance of resistance exercise (RE), particularly when combined with elevated protein intakes, for increasing both appendicular lean mass (ALM) and strength in older adults with lower levels of muscle mass. Furthermore, chapter 4 (Kirwan et al., 2021a) reveals a potentially causal relationship between serum vitamin D levels and fat-free mass and as such, may highlight a possible role for vitamin D supplementation for improving lean mass.

Knowledge that RE, high-protein diets and other specific nutrients can help to increase lean mass is, however, not sufficient for implementing an effective intervention, and the development of tailored strategies for improving acceptability of, and adherence to, interventions is necessary to ensure their success. For example, chapter 6 (Kirwan et al., 2022) highlighted the potential loss of motivation or desire to continue with CR which occurred when CR participants were carrying out their exercise at home during the COVID-19

pandemic. To counteract this, centre-based CR may be more viable in the long-term than home-based CR. To apply the findings from the preceding chapters, chapter 7 (McCullough et al., 2021) outlines a diet and exercise protocol designed by consulting the recent literature regarding successful dietary and exercise interventions aimed at improving both CM health and inducing improvements in muscle size and strength. The design of this protocol was further improved by collaboration with community CR providers, British Association of Cardiac Prevention and Rehabilitation (BACPR) RE experts, and hospital service users and patients' groups. Finally, chapter 8 outlines the further refinement of the protocol using quantitative and qualitative input from members of the target population. As such, this thesis provides a comprehensive rationale for and methodology to achieve a high-protein Mediterranean-style diet and RE intervention in a CR population.

9.3 Recommendations for future research

The research presented in this thesis provides multiple streams of rationale and methodology for the development of a feasibility study to investigate the effects of the proposed intervention on lean mass, strength and cardiometabolic risk markers in a cardiac rehabilitation population. Ultimately, carrying out this intervention, collecting data on feasibility and outcomes and analyzing those data will provide more useful evidence for the clinical applicability of this intervention.

9.3.1 Isolation of beneficial protocol components and mechanisms

However, both within and outside the scope of this intervention, there are many more research directions that deserve further development and study. The originally planned, four-arm protocol was designed as a pragmatic intervention, *i.e.*, to practically deal with issues common to cardiac populations, including sarcopenia, frailty and dyslipidaemia (Pöss et al., 2011; Chin

et al., 2013; Bandeen-Roche et al., 2015) through different combinations of diet and exercise. However, while such an approach might help identify whether diet alone, exercise alone or the combination of both, is most effective for improving the proposed outcomes, some questions may remain unanswered. Primarily, issues may arise in attempting to isolate the effects of the high-protein aspect of the diet from the MedDiet aspect. This thesis has already discussed the numerous cardiovascular benefits of the MedDiets, many of which have diverse mechanisms related to the diversity of nutrients provided by this dietary pattern (Schwingshackl, Morze and Hoffmann, 2020). Alternatively, high-protein diets may exert cardiovascular benefits through their impact on weight loss and improvements in associated CVD risk factors such as blood pressure and glycaemic control (Dong et al., 2013). Furthermore, protein in combination with RE, may offer cardiovascular benefits through increases in LBM which may positively affect some aspects of blood lipids, such as those discussed in chapter 5. As such, further research comparing MedDiets, high-protein diets and combinations of both, with and without an RE component, may help to isolate the effects of each. Furthermore, measurement of additional CM markers, such as cytokines, other markers of inflammation, and lipoprotein particle sizes, along with metabolomic approaches may lead to better understanding of the mechanisms through which such interventions may work. This in turn can lead to further refinement of protocols aimed at reducing the risk of CVD and other chronic conditions.

9.3.2 Application for other cardiometabolic diseases

With respect to other chronic diseases (CDs) related to lifestyle, it may be prudent to not limit the scope of the protocol developed in this thesis to individuals with, or at risk of CVD alone. As previously mentioned, insulin resistance and dyslipidaemia are contributors to CVD risk (Ormazabal et al., 2018) and are also part of the diagnostic criteria for metabolic syndrome (MetS) (Alberti, Zimmet and Shaw, 2006). MetS is itself associated with wide variety of

lifestyle-related CDs including non-alcoholic fatty liver disease (NAFLD), chronic kidney disease (CKD), hypertension and dementia amongst others (Mendrick et al., 2017). Considering the mechanisms by which the diet and exercise intervention outlined in this thesis may exert its benefits in CVD, it may be speculated that such an intervention or modified versions thereof, might also be beneficial for those suffering from or at risk of CDs associated with MetS. This opens up the potential use of this intervention for a growing population (Hirode and Wong, 2020) with a diverse array of conditions including CVD, diabetes, NAFLD, CKD and neurological conditions (Mendrick et al., 2017). Further research investigating similar interventions in these clinical populations is warranted.

9.3.3 Cardiac prehab and role in early prevention

Research into the benefits of the diet and exercise protocol outlined in this thesis, shouldn't be limited to rehabilitation or secondary prevention of CVD. Increases in the prevalence of obesity in children have led to concerns, due to the increasingly early development of dyslipidaemia, hypertension, insulin resistance, and MetS in children, adolescents and young adults (Halpern et al., 2010; Reese et al., 2022). With the strong association of MetS with incident CVD (Mottillo et al., 2010) and the temporal component involved in the development of atherosclerosis (Kharbanda and MacAllister, 2005; Brown and Goldstein, 2006), a focus on primary prevention of CVD or cardiac prehab may be prudent. This might take the form of research, such as prospective cohort studies, investigating the long-term effects of protocols similar to that discussed in this thesis, in younger populations at elevated risk of CVD. Should such interventions be found effective for reducing the risk of developing CVD in the first place, this may lead to further investigation as to how to successfully promote such diet and exercise interventions in at-risk groups and the general population.

9.3.4 Long-term adherence

While the research carried out in this thesis has made considerable effort to develop an acceptable and effective diet and exercise protocol, further research will be necessary to understand what contributes to, and how to promote, long-term adherence to diet and exercise protocols. The effects of poor lifestyle habits may take years to manifest clinically as CM diseases and correspondingly, a 12-week lifestyle intervention as described in this thesis is not sufficient for maintaining life-long reduced risk of such conditions. There is a dearth of research involving long-term (two years or longer) follow-up of phase IV CR, *i.e.*, once patients have been discharged from phase III (immediately post-cardiac event), and as such, there are not sufficient data on the numbers of CR participants that maintain lifestyle changes in the long-term. However, existing data on benefits of long-term CR exercise maintenance are encouraging, highlighting that more than 3 years of CR was associated with a 33% lower mortality risk compared with lesser durations of CR (Taylor et al., 2017). However, in terms of dietary change, available research does indicate initial changes in dietary habits tend to return baseline food intakes within 2 years of beginning CR (Twardella et al., 2006). Outside of CR, while changes to diet and exercise habits may initially prove successful, regression to pre-intervention habits is common (Middleton, Anton and Perri, 2013). The long-term efficacy of the protocol outlined in this thesis will very much depend on long-term adherence and as such, long-term and follow-up studies should be considered a priority for improving participant outcomes.

9.3.5 Potential for home-based resistance exercise

Finally, while the exercise intervention in this thesis was designed for implementation in modern gym facilities, consideration should also be given to methods of encouraging

efficacious RE at home. Traditional (aerobically focused) CR at home has been shown to be as effective as centre-based CR regarding outcomes such as blood pressure, total cholesterol, psychological status and exercise capacity (Jolly et al., 2009; Buckingham et al., 2016). There may be concern that lack of access to resistance-machines or free-weights may present a barrier to sufficient stimulation of muscle hypertrophy and strength adaptations in older populations due to anabolic resistance (Phillips et al., 2017; Morton et al., 2018). However, research into the use of bodyweight exercise or exercise involving low-cost home equipment such as resistance bands is needed to assess this exercise modality's feasibility for improving lean mass, strength and CM outcomes in CR. Such research would be beneficial for those individuals who do not wish to attend centre/gym-based CR or those who are unable to attend due to distance or accessibility issues.

9.4 Personal reflections

In doing this PhD, I originally intended to develop, trial and analyse the results of a high-protein diet and RE intervention in a CR population. While I had heard many, many times that PhDs rarely go to plan, I properly learned that lesson thanks to the impact of the COVID-19 pandemic on research. While I finally have begun a feasibility study for my intervention, the data generated from that research do not appear in this thesis. However, even though my original plans were considerably affected and delayed, the delay gave me the opportunity to take part in research that I would otherwise not have had the opportunity to consider. Some of that research was unrelated to the research goals of this thesis and I feel it has given me the opportunity to consider other avenues of research that might interest me. On the other hand, much of that research was directly related to the PhD and I believe it has greatly contributed to my protocol design and the rationale for that design. Much of this research might not have happened had it not been for COVID. At the time of writing these final paragraphs, I have a strong rationale for using a high-protein Mediterranean-style diet and RE in CR and an even stronger desire to carry out the intervention and analyse the data-generated.

Reflecting on the past 4 years, I have learned a great deal about myself and what I want to achieve in academia. I've had the chance to share my PhD journey along the way via my social media accounts and I've learned that I genuinely love breaking down research and making it accessible to non-academics. Just like many disciplines, nutrition and exercise research is difficult to understand for the general public, which means it is far too easy for mis- and dis-information to be taken as fact. This is incredibly frustrating, but I hope that I can continue to make factual and non-sensational, easy-to-understand nutrition information available to the general public. I also think this ability to simplify more complex topics will greatly aid me when working with research participants in the future, and if I have the opportunity to work as an educator at university level.

In working with many collaborators over the last 4 years I've learned that I am incredibly impatient because "I want results, now, because I want to publish, now". Thankfully, I've gotten a lot better at managing that impatience because I've learned how vital collaboration is to science. There are so many different researchers in so many different fields that I want to work with in the years to come. The input of different ways of thinking and different research backgrounds is essential for developing innovative nutrition science for years to come.

A field that I knew very little about prior to this project is the application of qualitative research and patient and public involvement (PPI) and I am very grateful for the opportunities I have been presented with to learn more about these methods and apply them in my PhD. It is very easy in nutrition or exercise science to become overly focussed on effect sizes and p-values and that can sometimes distract us from the fact that the participants we are trying to help are people. Understanding how those people "feel" (a word that is almost considered "dirty" in science) is key to improving both how we carry out research, and health outcomes of those

we are trying to help. I look forward to learning more about and applying qualitative research methods in my future research and I hope others will do the same.

As much as this research has strengthened my conviction that promoting healthy eating and exercise are essential for reducing the risk of a multitude of chronic conditions and improving the health span and quality of life of a huge portion of the population, I cannot help but feel disheartened by the challenge that poses. I am now more than ever aware that educating people about healthy eating and better exercise are unlikely to make any significant change for most people, when we live in an environment where poor food choices and sedentarism are the more affordable or easy options. While individuals can and certainly do make these changes, I believe the only way we will see change on a societal level (where it is needed most) is when changes are made to government policy related to, amongst many other factors, the marketing, availability, and cost of both ultra-processed and whole foods. I have chosen a career in science because I hope I will have the opportunity to contribute to research in this field in the future. Research that will genuinely benefit and improve the lives of many.

Finally, I am very aware that every day, the more I learn, the more I realise how much I don't know. That is both humbling and exciting. I hope one day I'll get to a point where I feel I actually know something.

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11 Appendices

11.1 Appendix 1: Other publications

11.2 Appendix 2: Ethics documentation

11.3 Appendix 3: Participant-facing documents

Appendix 1

Other publications completed by the candidate during the PhD tenure

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Review article

Prevalence of mental health problems among children and adolescents during the COVID-19 pandemic: A systematic review and meta-analysis

Lu Ma [a,1], Mohsen Mazidi [b,1], Ke Li [a], Yixuan Li [a], Shiqi Chen [a], Richard Kirwan [d], Haixia Zhou [a], Na Yan [a], Atif Rahman [c], Weidong Wang [e,*], Youfa Wang [a,**]

[a] Global Health Institute, School of Public Health, Xi'an Jiaotong University Health Science Center, Xi'an, Shaanxi, China
[b] Department of Twin Research and Genetic Epidemiology, King's College London, London, United Kingdom
[c] Department of Primary Care and Mental Health, Institute of Population Health, University of Liverpool, Liverpool, United Kingdom
[d] School of Biological and Environmental Sciences, Faculty of Science, Liverpool John Moores University, Liverpool, United Kingdom
[e] Department of Sociology, School of Sociology and Population Studies, Renmin University of China, Beijing, China

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ABSTRACT

Background: This systematic review and meta-analysis examined the prevalence of depression, anxiety, sleep disorders, and posttraumatic stress symptoms among children and adolescents during global COVID-19 pandemic in 2019 to 2020, and the potential modifying effects of age and gender.

Methods: A literature search was conducted in PubMed, Web of Science, PsycINFO, and two Chinese academic databases (China National Knowledge Infrastructure and Wanfang) for studies published from December 2019 to September 2020 that reported the prevalence of above mental health problems among children and adolescents. Random-effects meta-analyses were used to estimate the pooled prevalence.

Results: Twenty-three studies (21 cross-sectional studies and 2 longitudinal studies) from two countries (i.e., China and Turkey) with 57,927 children and adolescents were identified. Depression, anxiety, sleep disorders, and posttraumatic stress symptoms were assessed in 12, 13, 2, and 2 studies, respectively. Meta-analysis of results from these studies showed that the pooled prevalence of depression, anxiety, sleep disorders, and posttraumatic stress symptoms were 29% (95%CI: 17%, 40%), 26% (95%CI: 16%, 35%), 44% (95%CI: 21%, 68%), and 48% (95%CI: -0.25, 1.21), respectively. The subgroup meta-analysis revealed that adolescents and females exhibited higher prevalence of depression and anxiety compared to children and males, respectively.

Limitations: All studies in meta-analysis were from China limited the generalizability of our findings.

Conclusions: Early evidence highlights the high prevalence of mental health problems among children and adolescents during the COVID-19 pandemic, especially among female and adolescents. Studies investigating the mental health of children and adolescents from countries other than China are urgently needed.

1. Introduction

The COVID-19 (Coronavirus Disease 2019) pandemic has affected the mental health (e.g., depression, anxiety, sleep disorders, and posttraumatic stress symptoms) of children and adolescents (Golberstein et al., 2020). As of April 8, 2020, schools have been suspended nationwide in 188 countries (Lee, 2020). Prolonged school closures, strict social isolation from peers, teachers, extended family, and community networks, economic shutdown, and the pandemic itself have contributed to the mental health problems of children and adolescents (Holmes et al., 2020; Tan et al., 2020). While some children may benefit from increased interaction with parents and siblings, many have experienced elevated levels of emotional distress (Sprang and Silman, 2013; Xie et al., 2020). Being confined to home leads to disturbances in sleep/wake cycles and physical exercise routines, and promotes excessive use of technology (Xie et al., 2020). The pandemic may increase family financial stressors and parental unemployment, which were associated with short- and long-term consequences on child mental

* Corresponding author at: Department of Sociology, School of Sociology and Population Studies, Renmin University of China, Beijing 100000, China.
** Corresponding author at: Global Health Institute, School of Public Health, Xi'an Jiaotong University Health Science Center, Xi'an, Shaanxi 710061, China.
E-mail addresses: wwd@ruc.edu.cn, i@wangweidong.com (W. Wang), youfawang@gmail.com (Y. Wang).
[1] Equal contributors

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health (Costello et al., 2003). There is also an increased risk of seeing or experiencing domestic violence and emotional, physical and/or sexual abuse (Costello et al., 2003). It is assumed that relaxing lockdown restrictions and returning to school might improve the mental health status of children as the economy and social practices begin to normalize globally (Tan et al., 2020). Understanding the psychological impact of the COVID-19 pandemic on children and adolescents would provide a theoretical basis for designing interventions, planning resources, and promulgating policies necessary to protect young people from such occurrences in future (Pappa et al., 2020).

Several original studies have found high levels of mental health problems among children and adolescents during the COVID-19 pandemic (Duan et al., 2020; Pınar Senkalfa et al., 2020; Türkoğlu et al., 2020). However, to the best of our knowledge to date, no systematic review to synthesize the impact of the pandemic on their mental health has been performed. While there are some systematic reviews on the psychological impacts of COVID-19 on patients and healthcare workers (Pappa et al., 2020; Luo et al., 2020; Rogers et al., 2020), evidence in children and adolescents is lacking.

The aim of this systematic review and meta-analysis was to examine the emerging evidence of the effects of the COVID-19 outbreak on the mental health of children and adolescents aged 18 years and under. In particular, we aimed to examine the prevalence of depression, anxiety, sleep disorders, and posttraumatic stress symptoms among uninfected/not known to be infected children and adolescents during the active phase of the pandemic during 2019 to 2020. The potential modifying effects of age and gender on the prevalence were also examined.

2. Methods and materials

This study was developed and reported according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) (Moher et al., 2009) and other standards (Johnson and Hennessy, 2019). The study protocol was registered with the International Prospective Register of Systematic Reviews, PROSPERO (registration no: CRD42020205166).

3. Literature search and study selection

A systematic search was performed in three English electronic bibliographic databases: PubMed, PsycINFO, and Web of Science, and two Chinese academic databases: China National Knowledge Infrastructure (CNKI) and Wanfang. The following search terms were used: ("Novel coronavirus" OR "SARS-COV-2" OR "COVID-19" OR "2019-nCov") AND ("depression" OR "anxiety" OR "sleep*" OR "posttraumatic stress symptoms", "mental health*" OR "psychological*" OR "psychiatry" OR "insomnia"). The specific search algorithm is provided in **Supplemental Table 1**. Studies reported the prevalence of self-reported mental health problems and symptoms were included. Two authors independently searched the same database with these search terms to ensure that none of the relevant studies was missed.

Titles and abstracts of the articles identified were screened against the study selection criteria by two independent reviewers. Potentially relevant articles were retrieved for an evaluation of the full text. Inter-rater agreement was assessed using the Cohen's kappa (k=0.64). Disagreements were reviewed and resolved through discussion with third author to resolve persistent inconsistencies.

This search strategy was further supplemented with hand searching of reference lists of included articles and through tracking the citations of eligible references in Google Scholar. Articles identified from the reference lists were further screened and evaluated by using the same criteria. Reference searches were repeated on all newly identified articles until no additional relevant articles were found.

3.1. Study selection criteria

Studies were included if they: (a) evaluated the prevalence of depression, anxiety, sleep disorders, and posttraumatic stress symptoms using validated assessment method among children and adolescents aged 18 years and under; (b) were written in English or Chinese; (c) were carried out between December 2019 to September 2020; and (d) were cross-sectional or longitudinal studies. When there were studies involving the same participants, only the most comprehensive or recent publication was included.

Studies were excluded if they: (a) were qualitative studies, case reports, editorials, protocols, meta-analysis, or reviews, (b) computer-based simulation studies with no human participants, c) included participants with COVID-19 infected, d) studies that did not provide data on the levels of the outcomes of interest, or e) studies focused on the prevalence of suicidal behaviours, suidal ideations and attempts among children and adolescents during COVID-19 pandemic.

3.2. Data extraction and preparation

A standardized data extraction form was developed to extract the following data from each article: author, study design, country, survey years, average age of participants, sample size (percentage of male participants), sampling strategy, mental health problems, diagnostic or screening instrument used, specific diagnostic criteria or screening instrument cutoff, and reported prevalence estimates of mental health problems. The data were extracted independently by two independent reviewers, and disagreements were reviewed and resolved through discussion with third reviewer to resolve persistent inconsistencies.

3.3. Study quality assessment

Two authors independently assessed the quality of the articles using the U.S. National Heart, Lung, and Blood Institute's Quality Assessment Tool for Observational Cohort and Cross-Sectional Studies. (Study Quality Assessment Tools, 2021) The assessment tool rates each study based on 14 criteria. For each criterion, a score of one was assigned if "yes" was the response, whereas a score of zero was assigned otherwise (i.e., an answer of "no," "not applicable," "not reported," or "cannot determine"). Overall quality was rated based on the total score of the scale: "7 $\leq$ total score" = good, "4<total score$\leq$6" = fair, "total score<4" = poor. The risk of bias of each study decreased with the increase in the total score.

3.4. Statistical analysis

Prevalence estimates of mental health problems were calculated by pooling the study-specific estimates using random-effects (using the DerSimonian-Laird method) meta-analyses that accounted for between-study heterogeneity (Borenstein et al., 2010). When studies reported point prevalence estimates made at different periods within the year, the overall period prevalence was used.

Study heterogeneity was assessed using the I^2 index and Tau-squared (T^2). The level of heterogeneity represented by I^2 was interpreted as modest ($I^2 \leq 25\%$), moderate ($25\% < I^2 \leq 50\%$), substantial ($50\% < I^2 \leq 75\%$), or considerable ($I^2 > 75\%$). Sensitivity analyses was performed by serially excluding each study to determine the influence of individual studies on the overall prevalence estimates.

Results from studies grouped according to prespecified study-level characteristics were compared using stratified meta-analysis (gender, diagnostic criteria or screening instrument, region, and country).

Publication bias was assessed by a visual inspection of contour-enhanced funnel plots and Egger's regression tests. All statistical analyses were conducted in STATA with specific commands (e.g., Metan and Metareg) (Version 14.0; Stata Corp., College Station, Texas, U.S.). All analyses used two-sided tests, and *p-value* < 0.05 was considered

Table 1
Characteristics of the 23 studies included in the review

Author/Survey time (Year, month)	Study design	Country	Age, years (mean±SD or range)	Sample size (Boys, %)	Participant type [a]	Assessment method & cutoff score	Mental health problems (n, %/M±SD) Depression	Anxiety	Sleep disorders	Posttraumatic stress symptoms
1.Türkoğlu S/ 2020, May (Türkoğlu et al., 2020)	Cross-sectional	Turkey	Mean:7.89/4-17	46(82.6%)	Autism Spectrum Disorder	AuBC, CSHQ (>41); Diagnosed by health providers	NR	NR	Total CSHQ scores increased from 47.82 ± 7.13 to 50.80 ± 8.15	NR
2.Senkalfa BP/ 2020, April (Pınar Senkalfa et al., 2020)	Cross-sectional	Turkey	Cystic Fibrosis group 0-18 Control group 0-18	Cystic Fibrosis group 45 (51.1%) Control group 90 (51.1%)	Cystic Fibrosis	STAI; Diagnosed by health providers	NR	Children aged 13–18 years in the control group:29.0 (27.8-32.3); Age-matched children with Cystic Fibrosis 41.5 (35.5-46.3)	NR	NR
3.Chen F/2020, April (Chen et al., 2020)	Cross-Sectional	China	6-15 Children:6-12 Adolescents:13-15	1036 (51.0%)	General	DSRS-C (≥15), SCARED (≥25); Self-reported by participants	122(11.8%)	196(18.9%)	NR	NR
4.Chen IH/2019, October-2020, March Chen et al., 2020	Longitudinal	China	10.88±0.72	543 (49.0%)	General	DASS-21; Self-reported by participants	Mean:1.22 95% CI: (1.19,1.25)	NR	NR	NR
5. Qi M/2020, March Qi et al., 2020	Cross-sectional	China	Adolescents:14-18	7202 (46.4%)	General	PHQ-9 (≥5), GAD-7 (≥5); Self-reported by participants	3207(44.5%)	2736(38.0%)	NR	NR
6.Zhou SJ/2020, March Zhou et al., 2020	Cross-sectional	China	Adolescents:12-18	8079 (46.5%)	General	PHQ-9 (≥5), GAD-7 (≥5); Self-reported by participants	3533(43.7%)	3020(37.4%)	NR	NR
7.Xie X/ 2020, February-2020, March Xie et al., 2020	Cross-sectional	China	NR	1784 (56.7%)	General	CDI-S (≥7), SCARED (≥23); Self-reported by participants	403(22.6%)	337(18.9%)	NR	NR
8.Zhu KH/2020, February-2020, March Zhu et al., 2020	Cross-sectional	China	NR	1264(55.9 %)	General	SCARED (≥23); Self-reported by participants	NR	234(18.5 %)	NR	NR
9.Lin L/2020, February Lin et al., 2020	Cross-sectional	China	NR	76(NR)	General	ISI(≥10), PHQ-9 (≥10), GAD-7 (≥10), ASDS (≥28); Self-reported by participants	NR	NR	24(31.6%)	NR
10.Liu Z/2020, February Vindegaard and Benros, 2020	Longitudinal	China	Children: 4-6	1619 (48.9%)	General	CSHQ (≥41); Reported by caregivers of participants	NR	NR	900(55.6%)	NR
11.Qi H/2020, February Qi et al., 2020	Cross-sectional	China	Adolescents:11-20	9554(NR)	General	GAD-7 (≥5); Self-reported by participants	NR	1814(19.0%)	NR	NR
12.Zhou J/2020, February Zhou et al., 2020	Cross-sectional	China	Adolescents:11-18	4805 (0.0%)	General	CES-D (≥16); Self-reported by participants	1899(39.5%)	NR	NR	NR
13.Li SW/2020, February Li et al., 2020	Cross-sectional	China	12.82±2.61/8-18	396 (50.3%)	General	SCARED(≥25); Self-reported by participants	NR	87(22.0%)	NR	NR
		China			General		NR	1045(19.4%)	NR	NR

(*continued on next page*)

Table 1 (*continued*)

Author/Survey time (Year, month)	Study design	Country	Age, years (mean±SD or range)	Sample size (Boys, %)	Participant type [a]	Assessment method & cutoff score	Mental health problems (n, %/M±SD) Depression	Anxiety	Sleep disorders	Posttraumatic stress symptoms
14.Mo DM/2020, February Mo et al., 2020	Cross-sectional		7-16 Children:7-12 Adolescents:13-16	5392 (54.5%)		SCARED(≥23); Self-reported by participants				
15.Tang S/2020, February Tang and Pang, 2020	Cross-sectional	China	640 primary school students and 233 junior high school students: NR	873 (52.3%)	General	SAS(standard score ≥50) CDI(>19); Self-reported by participants	Children: 41 (6.4%); Adolescents: 61 (26.2%)	Children: 19(3.0%); Adolescents:46(19.7%)	NR	NR
16.Wang Y/2020, February Wang et al., 2020	Cross-sectional	China	12.82±2.61/8-18	396 (50.3%)	General	DSRS(≥15); Self-reported by participants	41(10.4%)	NR	NR	NR
17.Yu QX/2020, February Yu et al., 2020	Cross-sectional	China	NR	2074 (52.4%)	General	Psychological Questionnaire for Sudden Public Health Events (each factor score≥2); Self-reported by participants	53(2.6%)	13(0.6%)	NR	NR
18.Zhang Y/ 2020, February Zhang et al., 2020	Cross-sectional	China	NR	4225 (47.4%)	General	PCL-C(≥39); Self-reported by participants	NR	NR	NR	448(10.6%)
19.Liu X/ 2020, January-2020, February Liu et al., 2020	Cross-sectional	China	NR	34(NR)	General	STAI, SDS (≥50); Self-reported by participants	13(38.2%)	NR	NR	NR
20.Hou TY/2020, NR Hou et al., 2020	Cross-sectional	China	NR	859 (61.4%)	General	PHQ-9 (≥10), GAD-7 (≥8), IES-R(≥26); Self-reported by participants	614(71.5%)	468(54.5%)	NR	735(85.5%)
21.Li D/ 2020, NR Duan et al., 2020	Cross-sectional	China	7-18 Children:7-12 Adolescents:13-18	3613 (50.2%)	General	SCAS, CDI(≥19); Self-reported by participants	805(22.3%)	Children: 23.87 ± 15.79 Adolescents:29.27 ± 19.79	NR	NR
22.Tang L/2020, NR Tang and Ying, 2020	Cross-sectional	China	14.01±1.56	3512 (49.1%)	General	MMHI-60(each factor score ≥2); Self-reported by participants	924(26.3%)	1047(29.8%)	NR	NR
23.Wang NX/ 2020, NR Wang and Xu, 2020	Cross-sectional	China	NR	410 (31.5%)	General	GAD-7(≥5); Self-reported by participants	NR	197(48.0%)	NR	NR

NR: Not reported.

DSRS-C: Depression Self-Rating Scale for Children; SCARED: Screen for Child Anxiety Related Emotional Disorders; DASS-21: Depression, Anxiety, Stress Scale 21; PHQ-9: 9-item Patient Health Questionnaire; GAD-7: 7-item Generalized Anxiety Disorder Scale; IES-R: Impact of Events Scale - Revised; SCAS: Spence Child Anxiety Scale; STAI: State and Trait Anxiety Inventory; SDS: Self-rating Depression Scale; CSHQ: Children's Sleep Habit Questionnaire; SCL-90: Symptom Checklist-90; AuBC: Autism, Behavior Checklist; CDI-S: Children's Depression Inventory–Short Form; ISI: Insomnia Severity Index; CES-D: Center for Epidemiologic Studies Depression Scale; SAS: Self-Rating Anxiety Scale; DSRS: Depression Self-rating Scale for Children; PCL-C: The PTSD Cheeklist-CivilianVersion; MMHI-60: Mental Health Inventory of Middle-school students.

Table 2
Survey and sampling method of the 23 studies included in the review.

Author/Survey time (Year, month)	Survey method	Sampling method Probability sampling	Nonprobability sampling
1. Türkoğlu S/ 2020, May	Teleconference survey		Purposive sampling
2. Senkalfa BP/ 2020, April	Teleconference survey		Control group: Purposive sampling Age-matched children with Cystic Fibrosis: Snowball sampling
3. Chen F/2020, April	Online survey		Purposive sampling
4. Chen I/2019, October-2020, March	Online survey		Purposive sampling
5. Qi M/2020, March	Online survey		Purposive sampling
6. Zhou S/2020, March	Online survey		Purposive sampling
7. Xie X/ 2020, February-2020, March	Online survey		Purposive sampling
8. Zhu KH/ 2020, February-2020, March	Online survey	Random cluster sampling	
9. Lin L/2020, February	Online survey		Snowball sampling
10. Liu Z/2020, February	Online survey		Convenient sampling
11. Qi H/2020, February	Online survey		Snowball sampling
12.Zhou J/ 2020, February	Online survey		Snowball sampling
13. Li SW/ 2020, February	Online survey		Snowball sampling
14. Mo DM/ 2020, February	Online survey		Purposive sampling
15.Tang S/ 2020, February	Online survey		Purposive sampling
16. Wang Y/ 2020, February	Online survey		Snowball sampling
17. Yu QX/ 2020, February	Online survey		Purposive sampling
18. Zhang Y/ 2020, February	Online survey		Purposive sampling
19. Liu X/ 2020, January-2020, February	Online survey		Snowball sampling
20. Hou T/ 2020, NR	NR	Random cluster sampling	
21. Li D/ 2020, NR	Online survey		Convenient sampling
22. Tang L/ 2020, NR	Online survey		Purposive sampling
23. Wang NX/ 2020, NR	Online survey		Purposive sampling

NR: Nor reported.

statistically significant.

4. Results

4.1. Characteristics of included studies

A total of 23 studies were included in the systematic review, the characteristics of which are summarized in Table 1. These studies were published predominantly from February to May 2020 with one longitudinal study including data from October 2019. The vast majority of studies were from China (21 studies), with the remaining studies from Turkey (2 studies). The sample size of these studies varied greatly, ranging from 46 to 9,554 participants.

Two of the studies used teleconference survey, the others used online survey. Two of the studies used random cluster sampling, the others used purposive sampling, snowball sampling, and convenient sampling (**Table 2**).

The study design and populations were diverse. There were 21 cross-sectional studies and 2 longitudinal studies. The majority of studies were carried out in healthy populations (21 studies), in a population with cystic fibrosis (1 study) and autism spectrum disorder (1 study). Some studies included adult participants in which case only data from child/adolescent participants was used in these analyses and participants' ages ranged from 0 to 18 years.

Of particular note is the diversity of mental health-related scales used among these studies which included. A brief description of each mental health scale follows (in order of descending frequency):

- 7-item Generalized Anxiety Disorder Scale (GAD-7) (6 studies): a self-report screening tool for generalized anxiety symptoms in the primary care setting consisting of 7 questions and validated in adolescents (Mossman et al., 2017);
- Screen for Child Anxiety Related Emotional Disorders (SCARED) (5 studies): a self-report instrument for children and their parents that screens for several types of anxiety disorders including generalized anxiety disorder, separation anxiety disorder, panic disorder, and social anxiety disorder (Monga et al., 2000);
- 9-item Patient Health Questionnaire (PHQ-9) (4 studies): a self-questionnaire consisting of nine items that assess the presence and severity of depressive symptoms based on the DSM-IV criteria for major depressive disorder (MDD) (Richardson et al., 2010);
- Depression Self-Rating Scale for Children (DSRS-C) (4 studies) is widely used to measure children's depressive symptoms and consists of 18 items (Ivarsson et al., 1994);
- Children's Depression Inventory (including short form) (CDI-S) (3 studies): a self-report scale consisting of 27-items which evaluates the severity of depression in children and adolescents (Allgaier et al., 2012).
- State and Trait Anxiety Inventory (STAI) (2 studies): assesses state and trait anxiety in children for the determination of anxiety disorder and contains two scales of 20 items each (Nunn, 1988);
- Self-rating Depression Scale (SDS) (2 studies): is used to assess depressive syndrome and is validated in Chinese urban children (Su et al., 2003);
- Children's Sleep Habit Questionnaire (CSHQ) (2 studies): a parent administered survey to assess children's sleep problems and consists of as 48 items divided into 5 scales focusing on different aspects of sleep behaviour (Tan et al., 2018);
- Autism, Behavior Checklist (AuBC) (1 study): designed for the identification of children suspected of having autism and consisting of a list of atypical behaviors characteristic of the pathology (Sevin et al., 1991);
- Center for Epidemiologic Studies Depression Scale (CES-D) (1 study): screens for depressive disorders in population-based samples and is based on a multidimensional approach to measuring depression in children and adolescents aged 6 and 17 years (Li et al., 2010);

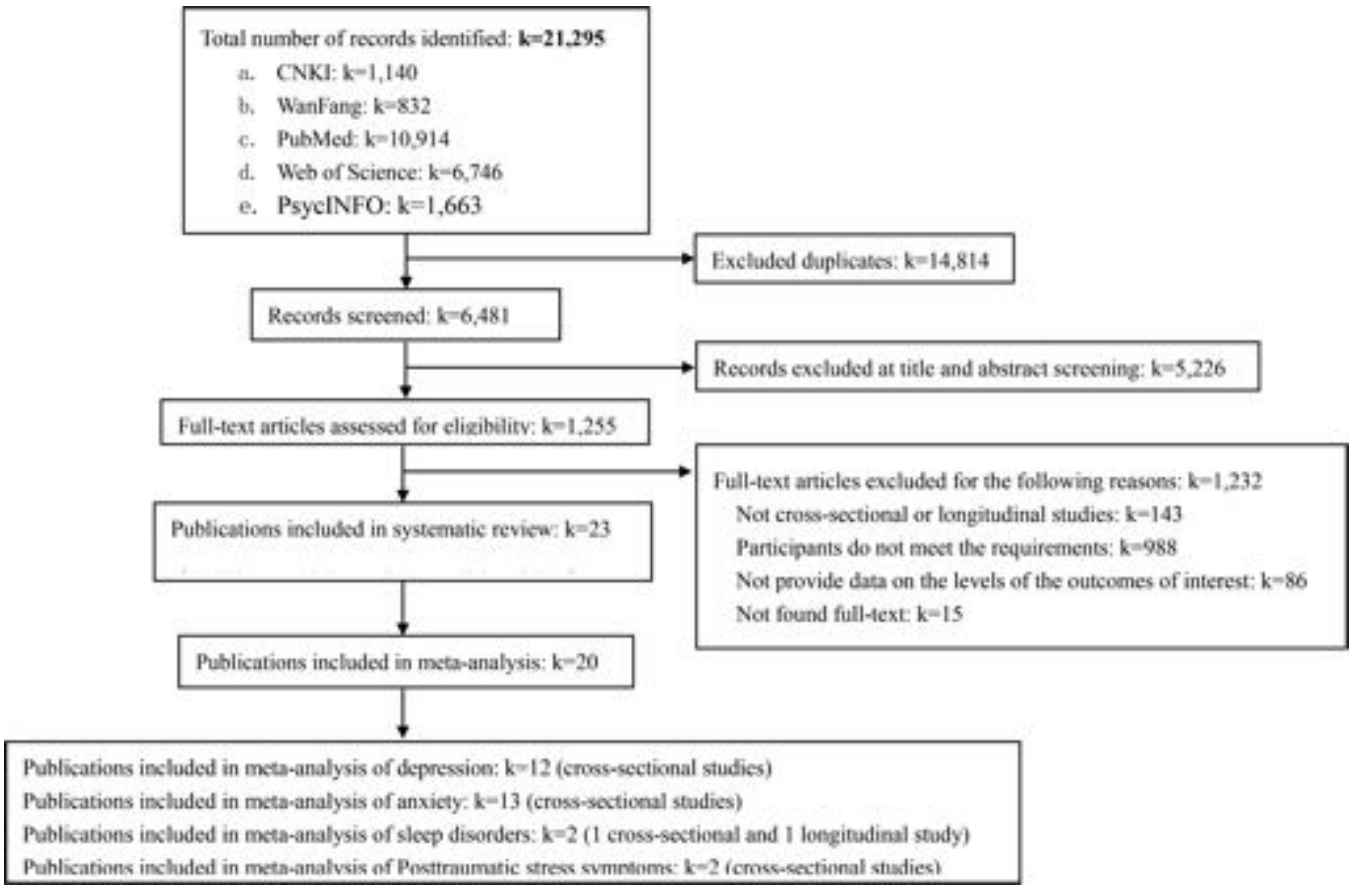

Fig. 1. Flowchart of the literature search and study selection according to the PRISMA standard

- Self-Rating Anxiety Scale (SAS) (1 study): a norm-referenced screener that, in conjunction with the Self-rating Depression Scale has been shown to discriminate anxiety from mood disorders (Dunstan and Scott, 2020);
- Impact of Events Scale-Revised (IES-R) (1 study):a widely used, 22 item questionnaire used to determine the degree of distress a patient feels in response to trauma and for identifying traumatic stress (Creamer et al., 2003);
- Spence Child Anxiety Scale (SCAS) (1 study): a 38-item parents-report measure of anxiety symptoms for children and adolescents developed using community samples (Wang et al., 2016);
- Insomnia Severity Index (ISI) (1 study): a brief self-report instrument measuring the patient's perception of both nocturnal and diurnal symptoms of insomnia and comprising seven items (Gagnon et al., 2013);
- Depression, Anxiety, Stress Scale 21 (DASS-21) (1 study): a set of three self-report scales designed to measure the emotional states of depression, anxiety and stress with each scale containing 7 items (Wang et al., 2016);
- The PTSD Checklist-Civilian Version (PCL-C) (1 study): a standardized self-report rating scale comprising 17 items that correspond to the key symptoms of PTSD (Blanchard et al., 1996);
- Mental Health Inventory of Middle-school students (MMHI-60) (1 study): a total of 60 items in the scale are used to measure the level of mental health of middle school students (Wang et al., 1997);
- Psychological Questionnaire for Sudden Public Health Events (PQSPHE) (1 study): a total of 25 items to measure depression, neurosism, fear, obsessive anxiety, and hypochondria among adolescents (Zhang, 2005).

4.2. Prevalence of mental health problems among children and adolescents

4.2.1. Depression

12 studies provided data on the prevalence of depression among children and adolescents during the COVID-19 pandemic. Meta-analysis of the results from these studies showed that the pooled prevalence of depression among children was 29% (95%CI: 17%, 40%) with a pooled heterogeneity of 99.9% ($p < 0.001$). The prevalence of depression reported in individual study ranges from 10% to 71% (**Fig. 2**).

Sub-group analysis by age indicated that the prevalence of depression in adolescents age 13-18 years (34.4%, 95%CI: 18.2%, 50.7%; $p<0.001$) was higher than that of children age ≤ 12 years (11.8%, 95% CI: 1.3%, 22.3%, $p=0.028$). Sub-group analysis by gender showed that the prevalence of depression in females (33.9%, 95%CI: 24.6%, 43.1%, $p<0.001$) was higher than that in males (28.9%, 95%CI: 14.1%, 43.7%, $p<0.001$) (**Table 3**).

4.2.2. Anxiety

A total of 13 studies provided data on the prevalence of anxiety among children and adolescents during the pandemic. Meta-analysis of the results from these studies showed that the pooled prevalence of anxiety among children and adolescents was 26% (95%CI: 16%, 35%) with a pooled heterogeneity of 99.9% ($p< 0.001$). The prevalence of anxiety reported in individual study ranges from 7% to 55% (**Fig. 3**).

Sub-group analysis by age indicated that prevalence of anxiety in adolescents age 13-18 years (29.1%, 95%CI: 17.1%, 41.1%, $p<0.001$) was higher than that in children age ≤ 12 years (15.7%, 95%CI: 9.0%, 22.3%, $p<0.001$). Sub-group analysis by gender showed that the prevalence of anxiety of females (27.4%, 95%CI: 20.3%, 34.6%, $p<0.001$) was higher than that of males (22.3%, 95%CI: 14.2%, 30.4%, $p<0.001$)

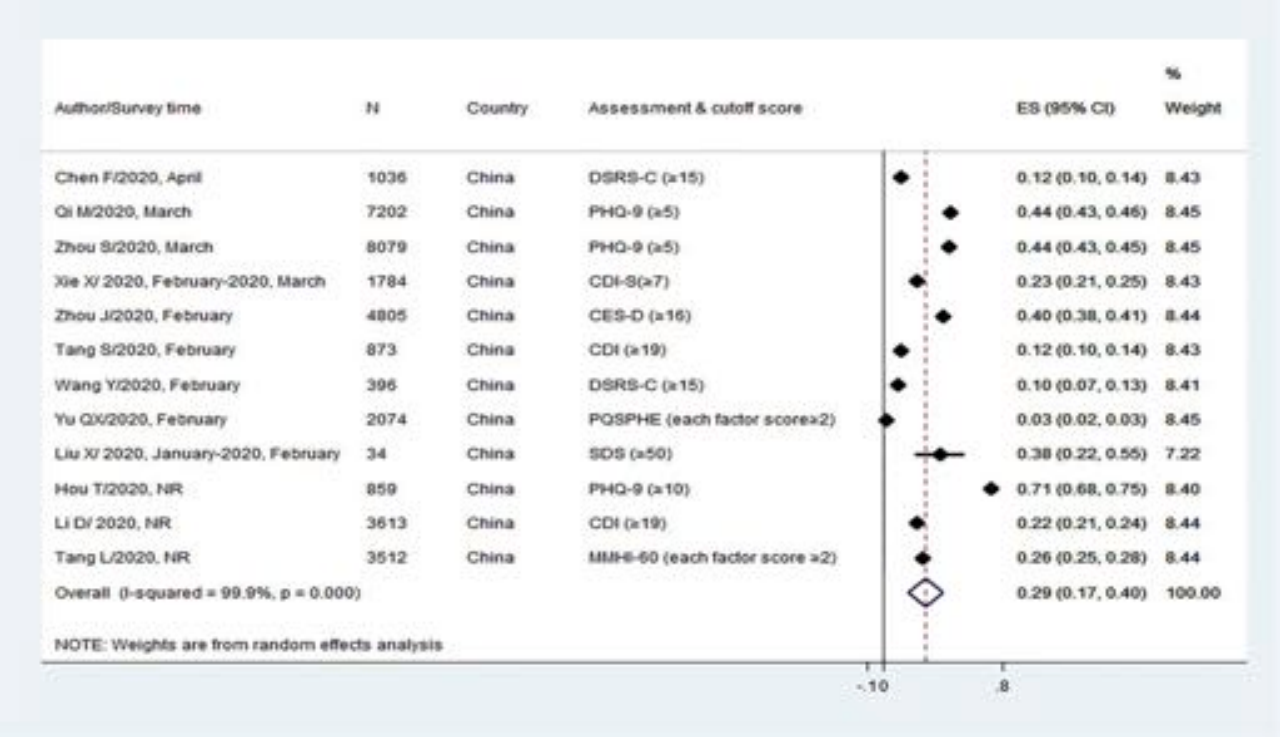

Fig. 2. Meta-analysis of the pooled prevalence of depression among children and adolescents (n=13)
Abbreviations: DSRS-C: Depression Self-Rating Scale for Children; PHQ-9: 9-item Patient Health Questionnaire; CDI-S: Children's Depression Inventory–Short Form; CES-D: Center for Epidemiologic Studies Depression Scale; CDI: Children's Depression Inventory; DSRS-C: Depression Self-Rating Scale for Children; PQSPHE: Psychological Questionnaire for Sudden Public Health Events; SDS: Self-rating Depression Scale; MMHI-60: Mental Health Inventory of Middle-school students. Prevalence was calculated based on the random-effect models.

Table 3
Total and subgroup meta-analysis of pooled prevalence [d] (%, 95%CI) of depression, anxiety, sleep disorders, and posttraumatic stress symptoms among children during the COVID-19 pandemic based on the included studies [a]

Type of analysis	Groups	N of studies	Prevalence (%, 95% CI)	P value	Heterogeneity			
					I^2 (%)	χ^2	P	Tau-squared
Depression	Total	12	28.6 (17.2, 40.1)	<0.001	99.9	8025.91	<0.001	0.0405
Anxiety	Total	13	25.5 (16.0, 35.1)	<0.001	99.9	10690.46	<0.001	0.0307
Sleep disorders [b]	Total	2	44.2 (20.7, 67.7)	<0.001	94.8	19.22	<0.001	0.0273
Posttraumatic stress symptoms [c]	Total	2	48.0 (-25.4, 121.4)	0.200	100	3364.24	<0.001	0.2804
Depression	Children (≤12 years)	3	11.8 (1.3, 22.3)	0.028	98.9	183.35	<0.001	0.0085
	Adolescents (13-18 years)	8	34.4 (18.2, 50.7)	<0.001	99.9	7695.86	<0.001	0.0548
Anxiety	Children	6	15.7 (9.0, 22.3)	<0.001	98.7	389.71	<0.001	0.0066
	Adolescents	11	29.1 (17.1, 41.1)	<0.001	99.9	10269.07	<0.001	0.0407
Depression	Male	4	28.9 (14.1, 43.7)	<0.001	99.6	670.02	<0.001	0.0228
	Female	5	33.9 (24.6, 43.1)	<0.001	99.2	506.21	<0.001	0.0110
Anxiety	Male	7	22.3 (14.2, 30.4)	<0.001	99.1	650.31	<0.001	0.0118
	Female	7	27.4 (20.3, 34.6)	<0.001	98.6	431.08	<0.001	0.0091

[a] All the studies included in meta-analysis were from China and among general children and adolescents, so no subgroup meta-analysis was conducted based on country and pre-existing conditions of children and adolescents.
[b] Only two articles were found on sleep disorders, one was conducted among children and both boys and girls, the age and gender of participants in the other study were not reported, so no subgroup analysis was conducted based on age and gender.
[c] Only two articles were found on posttraumatic stress symptoms, both studies did not report the age of participants and the gender-stratified prevalence, so no subgroup meta-analysis was conducted based on age and gender.
[d] Prevalence was calculated based on the random-effect models.

(Table 3).

4.2.3. Sleep disorders

Only 2 studies provided data on the prevalence of sleep disorders among children and adolescents. Meta-analysis of the results of the two studies showed that the pooled prevalence of sleep disorders was 44% (95%CI: 21%, 68%) with a pooled heterogeneity of 94.8% (p<0.001). The prevalence of sleep disorders of the two studies were 32% to 56%, respectively (Fig. 4). Sub-group analyses by age and gender were not performed due to lack of data.

4.2.4. Posttraumatic stress symptoms

Only 2 studies provided data on the prevalence of post-traumatic stress symptoms among children and adolescents. In the pooled analysis, the prevalence of post-traumatic stress symptoms were not be statistically significant in children and adolescents (pooled prevalence 48% (95%CI: -0.25, 1.21, p=0.200) with a pooled heterogeneity of 100% (p<0.001) (Fig. 5). Sub-group analyses by age and gender were

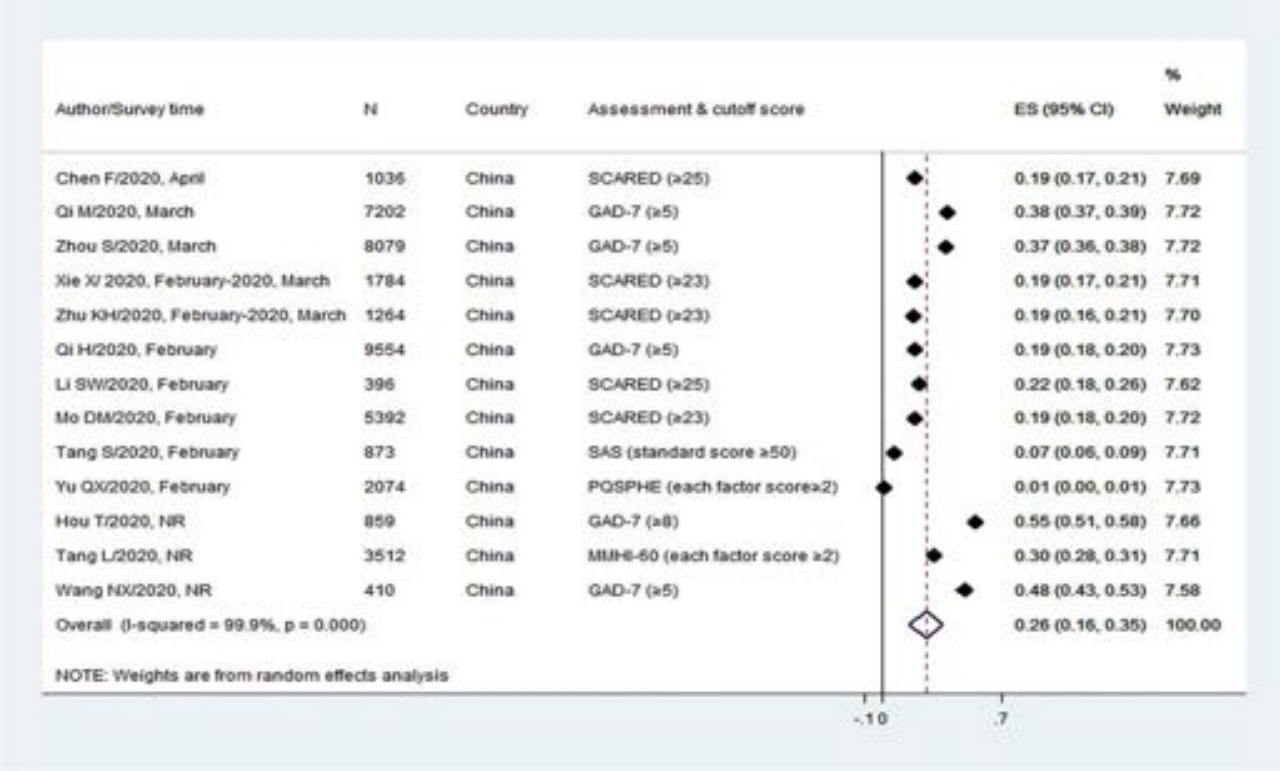

Fig. 3. Meta-analysis of the pooled prevalence of anxiety among all children and adolescents (n=12)
Abbreviations: SCARED: Screen for Child Anxiety Related Emotional Disorders; GAD-7: 7-item Generalized Anxiety Disorder Scale; SAS: Self-Rating Anxiety Scale; PQSPHE: Psychological Questionnaire for Sudden Public Health Events; MMHI-60: Mental Health Inventory of Middle-school students. Prevalence was calculated based on the random-effect models.

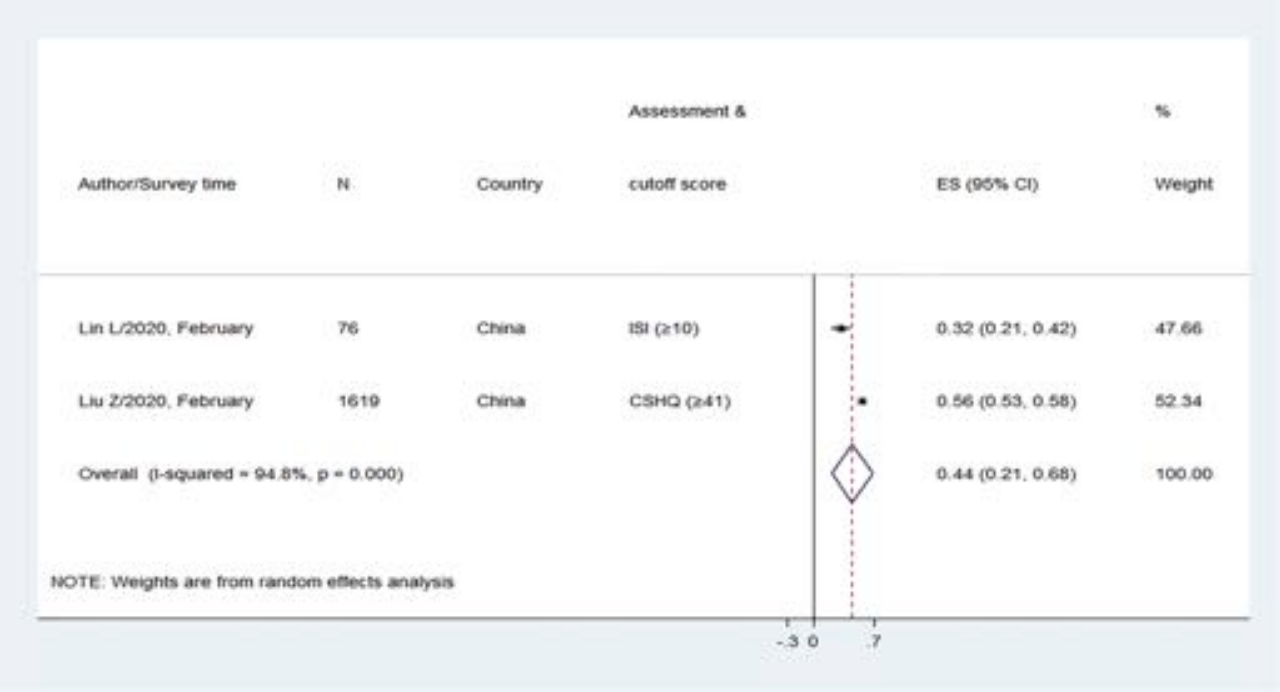

Fig. 4. Meta-analysis of the pooled prevalence of sleep disorders among all children and adolescents (n=2)
Abbreviations: ISI: Insomnia Severity Index; CSHQ: Children's Sleep Habit Questionnaire. Prevalence was calculated based on the random-effect models.

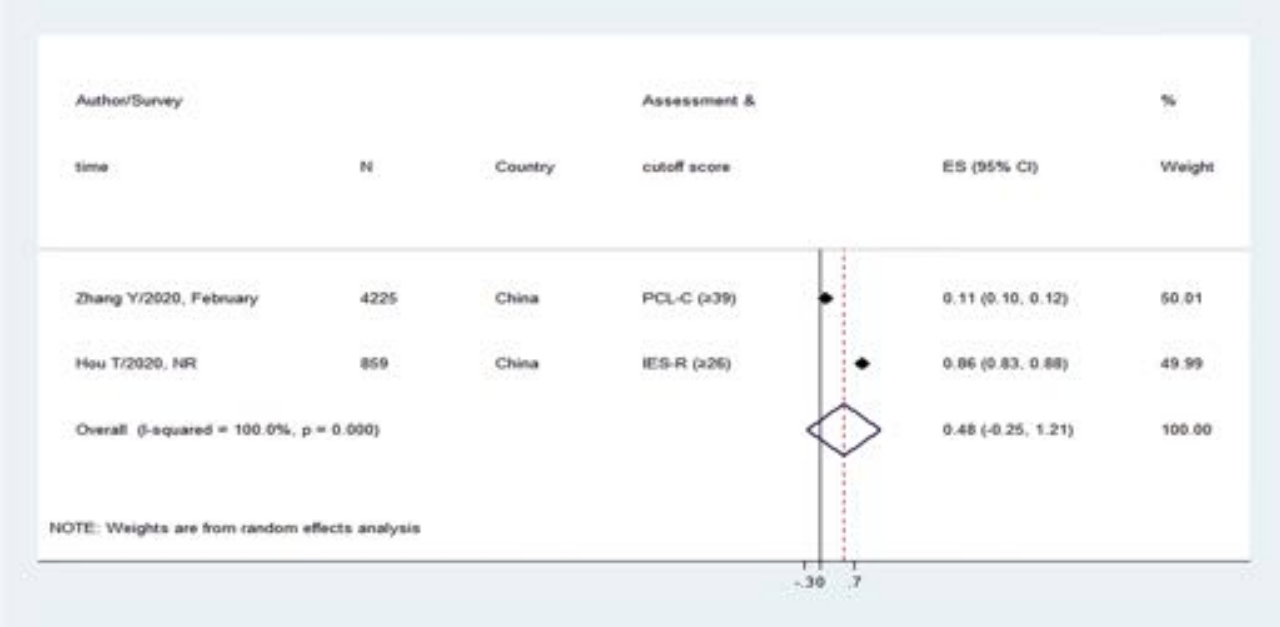

Fig. 5. Meta-analysis of the pooled prevalence of posttraumatic stress symptoms among all children and adolescents (n=2)
Abbreviations: PCL-C: The PTSD Cheeklist-CivilianVersion; IES-R: Impact of Events Scale-Revised. Prevalence was calculated based on the random-effect models.

not performed due to lack of data.

4.3. Results of sensitivity analysis and meta-regression analysis

Sensitivity analysis consistently showed that removing individual studies from the meta-analysis did not lead to any change in the prevalence of depression or anxiety. Because only two studies were included in meta-analyses of sleep disorders and posttraumatic stress symptoms, sensitivity analysis was not conducted (**Supplemental Table 3**).

Meta-regression analysis was performed on the prevalence of depression (12 studies) and anxiety (13 studies). Results indicated that neither age nor sample size were significant factors contributing to the heterogeneity of studies (Depression: β=-0.02, 95%: -0.07 to 0.03, p=0.378; Anxiety: β=-0.01, 95%: -0.01, 0.01, p=0.285). However, the questionnaire used for assessment of anxiety or depression did significantly contribute to heterogeneity of studies. In the analysis of depression prevalence, PHQ-9 and CES-D, and in the analysis of anxiety prevalence, SCARED, SAS and PQSPHE contributed to heterogeneity (**Table 4**).

4.4. Assessment of publication bias

The funnel plot and assessment of Egger's and Begg's tests did not reveal any significant publication bias in the prevalence of depression, anxiety, sleep disorders or post-traumatic stress symptoms (**Supplemental Table 2, Supplemental Figure 1**).

5. Discussion

A recent position paper in The Lancet Psychiatry identified the long-term consequences of COVID-19 for the younger generations are unknown and must be a priority (Holmes et al., 2020). This systematic review and meta-analyses of 23 studies and a total of 57,927 participants provides evidence that 28.6%, 25.5%, 44.2%, and 48.0% of children and adolescents experienced depression, anxiety, sleep disorders, and post-traumatic stress symptoms, respectively, during the COVID-19 pandemic. All the studies included in meta-analysis were from China and conducted among general children and adolescents. The prevalence of depression and anxiety was higher among adolescents and females compared with children and males, respectively.

The prevalence of depression and sleep disorders in children and adolescents during the COVID-19 were higher than the respective rates 19.9% for depression (Rao et al., 2019) and 21.6% for sleep disorders (Xiao et al., 2019), reported for the children and adolescents prior to the pandemic in China. However, no data on the prevalence of anxiety and posttraumatic stress symptoms were found among children and adolescents prior to the pandemic in China, thus, no comparisons could be made. Social isolation, school closures, and socioeconomic effects of the policies (increasing unemployment, financial insecurity, and poverty) during the COVID-19 pandemic have been reported to contribute to the mental health problems among children and adolescents (Holmes et al., 2020; Lee, 2020). While there was some research on the psychological impact of severe acute respiratory syndrome (SARS) and middle east respiratory syndrome coronavirus (MERS) on patients and health-care workers, such evidence in children and adolescents is scarce (Lee, 2020). Therefore, no direct comparison of the prevalence of mental health problems with previous pandemics could be made. However, COVID-19 is much more widespread than SARS, MERS, and other previous epidemics. As the pandemic continues, monitoring young people's mental health status over the long term and implementation of interventions and policies to support them are urgent and important.

Our study revealed that sleep disorders and posttraumatic stress symptoms were the most severe mental health problems among children and adolescents, and about half of them experienced these disorders during the COVID-19 pandemic. These findings indicate that the COVID-19 pandemic has a substantial impact on young people's sleep. Many children and adolescents may be exposed to unconstrained sleep schedules, prolonged screen exposure, and limited access to outdoor activities and peer interactions and these could have contributed to reported sleep disorders (Liu et al., 2020). Sleep disturbances are often a precursor to other more severe mental problems and it is necessary and urgent to disseminate sleep health education and sleep hygiene behavior interventions to children and adolescents (Lin et al., 2020). The COVID-19 pandemic is a traumatic event, and it is well known that surviving critical illness can induce posttraumatic stress symptoms (Vindegaard and Benros, 2020). The COVID-19 pandemic may be an independent factor that cause posttraumatic stress symptoms in children and adolescents. Children might also be exposed to greater interpersonal

Table 4
Results of meta-regression analyses on the prevalence of depression and anxiety based on 12 studies on depression and 13 studies on anxiety [a,b]

Type of analysis		β	95% CI	P
Depression (n=12)	Age	-0.02	-0.07, 0,03	0.378
	Sample size Assessment (Reference: CDI (≥19))	0.01	-0.01, 0.01	0.285
	DSRS-C (≥15)	-0.06	-0.20, 0.08	0.270
	PHQ-9 (≥5)	0.27	0.13, 0.41	**0.008**
	PHQ-9 (≥10)	0.54	0.37, 0.72	**0.002**
	CDI-S (≥7)	0.06	-0.12, 0.23	0.382
	CES-D (≥16)	0.22	0.05, 0.39	**0.025**
	PQSPHE (each factor score≥2)	-0.14	-0.31, 0.02	0.072
	SDS (≥50)	0.21	-0.10, 0.52	0.122
	MMHI-60 (each factor score≥2)	0.09	-0.08, 0.26	0.184
Anxiety (n=13)	Age	-0.01	-0.05, 0.03	0.722
	Sample size Assessment (Reference: GAD-7 (≥5))	0.01	-0.01, 0.01	0.813
	SCARED (≥25)	-0.15	-0.33, 0.03	0.092
	SCARED (≥23)	-0.16	-0.32, -0.01	**0.045**
	SAS (standard score≥50)	-0.28	-0.51, -0.05	**0.026**
	PQSPHE (each factor score≥2)	-0.35	-0.58, -0.12	**0.011**
	GAD-7 (≥8)	0.19	-0.04, 0.43	0.094
	MMHI-60 (each factor score≥2)	-0.06	-0.29, 0.18	0.581

DSRS-C: Depression Self-Rating Scale for Children; SCARED: Screen for Child Anxiety Related Emotional Disorders; PHQ-9: 9-item Patient Health Questionnaire; GAD-7: 7-item Generalized Anxiety Disorder Scale; SDS: Self-rating Depression Scale; CDI-S: Children's Depression Inventory–Short Form; CES-D: Center for Epidemiologic Studies Depression Scale; SAS: Self-Rating Anxiety Scale; MMHI-60: Mental Health Inventory of Middle-school students; PQSPHE: Psychological Questionnaire for Sudden Public Health Events.

[a] : Because only two articles were included for sleep disorders and posttraumatic stress symptoms, so no meta-regression analyses were conducted.

[b] : Meta-regression analysis was used to evaluate the heterogeneity of different studies, adjusting age, gender, and measurement scale of depression and anxiety.

Numbers in bold indicate significance.

violence and abuse, and this too might contribute to the high prevalence. However, the evidence is limited as only two studies reported on posttraumatic stress symptoms in this age group.

The subgroup meta-analysis revealed that the prevalence of depression and anxiety may be higher among adolescents and females. The higher prevalence among female reflects the already established gender gap for anxiety and depressive symptoms (Pappa et al., 2020). Again, adolescents exhibited much higher prevalence estimates both for depression and anxiety compared to younger children in our study. This may attribute to education is highly valued and regarded as the main path to success in traditional Chinese culture (Hou et al., 2020). As adolescents face the most important tests of their lives (e.g., the college or high school entrance examination), the uncertainty and potential negative effects on academic development of prolonged school closure had more adverse effects on adolescents than children (Zhou et al., 2020), thus, adolescents had more depressive and anxiety symptoms. However, no subgroup meta-analysis based on age and gender could be conducted for sleep disorders and posttraumatic stress symptoms, because there is no data available. Future such studies should take the potential modifying effects of age and gender into consideration.

No subgroup meta-analysis based on preexisting conditions and country could be conducted in this study. Only two studies included were conducted among children and adolescents with preexisting conditions (i.e., Autism Spectrum Disorder and Cystic Fibrosis). However, the two studies were not used for meta-analysis because they did not report the prevalence of mental health problems. All the studies included in our meta-analysis were from China. In our review, only two studies included were conducted in other countries (i.e., Turkey). However, the two studies from Turkey did not report the prevalence of mental health problems, thus they were not included in the meta-analysis. Though the fact that China was severely affected, our findings may provide a reliable indication of the effects of COVID-19 pandemic on the mental health of children and adolescents globally. However, considering the severity of COVID-19, economic status, and healthcare systems vary greatly between countries, more such studies from other countries are warranted.

We found that the most frequently used scale to measure depression and anxiety were PHQ-9 (3 of 13) and GAD-7 (5 of 12), respectively. However, each of the two studies used a different scale to measure sleep disorders and posttraumatic stress symptoms. The PHQ-9 is a simple, widely used, and highly effective self-assessment tool for depressive symptoms during the last 2 weeks (Kroenke et al., 2001). GAD-7 measured seven anxiety symptoms that bothered participants during the last 2 weeks (Zhou et al., 2020). Both PHQ-9 and GAD-7 are widely used among children and adolescents. Using the same scale and cutoff point for specific mental health problems could be better for comparison across studies.

This study has several limitations. First, all the studies included in meta-analysis were conducted in China, thus, the generalizability of findings to other countries is limited. Moreover, most of the studies used online survey method and nonprobability sampling, which further limit its generalizability. Second, a variety of assessment scales were utilized to measure mental health problems and different cut-offs were used even though several studies used the same tests. Third, due to the limited number of studies, we could not explore the potential modifying effects of preexisting conditions and country on the prevalence of mental health problems. Fourth, only two studies focused on children age <6 years were included; thus, the mental health status of these children warrants further research.

To advance research in this area, future studies target children and adolescents are warranted to improve the following aspects. First, longitudinal studies to examine the long-term implications of COVID-19 pandemic on mental health are needed. Second, further studies to examine the prevalence of sleep disorders and posttraumatic stress symptoms are needed. Third, besides the general children and adolescents, more studies are needed to focus on children and adolescents with preexisting conditions, such as chronic diseases and psychiatric conditions. Fourth, studies from countries other than China are needed to provide insight on the global impacts of COVID-19 pandemic on mental health.

Despite its limitations, this study is the first to examine the pooled prevalence of depression, anxiety, sleep disorders, and posttraumatic stress symptoms among children and adolescents during the COVID-19 pandemic. We conducted comprehensive literature search based on both English and Chinese databases, the findings have important clinical and public health implications. Furthermore, our subgroup analysis of depression and anxiety based on age and gender provided additional valuable insights of potential particular vulnerabilities.

In conclusion, our study highlighted the high prevalence of depression, anxiety, sleep disorders, and posttraumatic stress symptoms among children and adolescents during the COVID-19 pandemic, in particular, among the females and adolescents. Further research is needed to identify strategies for preventing and treating these disorders in this

population.

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Authors' contribution

The authors's responsibilities were as follows: YFW and WDW designed the research; LM and MM wrote the protocol; KL, SQC and HXZ managed the literature searches and selection; RK and KL performed data extraction; NY performed verification of data extraction, YXL performed meta-analysis; SQC assessed the quality of the included articles; LM wrote the first draft of the manuscript; ML, AR, and MM revised the manuscript; and all authors read and approved the final manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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None.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jad.2021.06.021.

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ORIGINAL ARTICLE

Genetically determined blood lead is associated with reduced renal function amongst individuals with type 2 diabetes mellitus: insight from Mendelian Randomisation

Mohsen Mazidi[1] · **Richard Kirwan**[2] · **Ian G. Davies**[3]

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Abstract
Some observational studies indicate a link between blood lead and kidney function although results remain controversial. In this study, Mendelian randomisation (MR) analysis was applied to obtain unconfounded estimates of the casual association of genetically determined blood lead with estimated glomerular filtration rate (eGFR) and the risk of chronic kidney disease (CKD). Data from the largest genome-wide association studies (GWAS) on blood lead, eGFR and CKD, from predominantly ethnically European populations, were analysed in total, as well as separately in individuals with or without type 2 diabetes mellitus. Inverse variance weighted (IVW) method, weighted median (WM)-based method, MR-Egger, MR-Pleiotropy RESidual Sum and Outlier (PRESSO) as well as the leave-one-out method were applied. In a general population, lifetime blood lead levels had no significant effect on risk of CKD (IVW: $p = 0.652$) and eGFR (IVW: $p = 0.668$). After grouping by type 2 diabetes status (no diabetes vs. diabetes), genetically higher levels of blood lead had a significant negative impact among subjects with type 2 diabetes (IVW = Beta: −0.03416, $p = 0.0132$) but not in subjects without (IVW: $p = 0.823$), with low likelihood of heterogeneity for any estimates (IVW $p > 0.158$). MR-PRESSO did not highlight any outliers. Pleiotropy test, with very negligible intercept and insignificant p-value, indicated a low likelihood of pleiotropy for all estimations. The leave-one-out method demonstrated that links were not driven by a single SNP. Our results show, for the first time, that among subjects with type 2 diabetes, higher blood lead levels are potentially related to less favourable renal function. Further studies are needed to confirm our results.

Key messages

What is already known about this subject?

- Chronic kidney disease is associated with unfavourable lifestyle behaviours and conditions such as type 2 diabetes.
- Observational studies have reported an association between blood lead and reduced estimated glomerular filtration rate, but the relationship between lead exposure and renal function remains controversial.

What is the key question?

- Using Mendelian randomisation with data from 5433 individuals from the UK and Australian populations, does genetically determined blood lead have a potentially causal effect on estimated glomerular filtration rate and the risk of chronic kidney disease?

What are the new findings?

- Blood lead levels have a potentially causal effect on reduced renal function in individuals with type 2 diabetes.
- In subjects without diabetes, no such causal relationship was identified.

Extended author information available on the last page of the article

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How might this impact on clinical practice in the foreseeable future?

- This highlights the risk of elevated blood lead, for example, due to environmental exposure, amongst those with type 2 diabetes, which may predispose them to impaired renal function.

Keywords Mendelian randomisation · Blood lead · Chronic kidney disease · Estimated glomerular filtration rate · Diabetes · Nephrology

Introduction

Chronic kidney disease (CKD) is an age-associated decline in renal function, diagnosed by impaired glomerular filtration rate (GFR) or increased urinary albumin excretion (albuminuria) [1]. Up to 13% of the global population is estimated to suffer some degree of CKD with increasing age associated positively with reduced renal function such that over one-third of those 70 years or older are affected [2, 3]. Chronic kidney disease is a frequently observed comorbidity in multiple cardiometabolic conditions such as type 2 diabetes, hypertension (HT), obesity and cardiovascular disease (CVD) [4–14], considerably adding to the burden of these conditions. As the aforementioned conditions are also components of metabolic syndrome (MetS) [15], it is not surprising that CKD is also frequently associated with this diagnosis [16, 17] which is estimated to affect 20–25% of western populations [18, 19]. Of particular interest in patients with diabetes is the development of diabetic nephropathy, with diabetes being a primary cause of end-stage renal disease in 40–60% of cases, globally [20]. Furthermore, recent research has illustrated that environmental lead exposure may accelerate progressive diabetic nephropathy, and that reductions in body lead levels by chelation therapy can reduce this rate of progression [21].

Similar to MetS and its constituent conditions, the incidence of CKD is associated with unfavourable dietary patterns and lifestyle behaviours such as low levels of physical activity [22–25]. Interestingly, a number of observational studies have found an association between blood lead levels and reduced estimated glomerular filtration rate (eGFR) [26–29], although not to a clinically significant degree, and this finding is not consistently observed [30–32]. Lead exposure may also be associated with a slight hyperfiltration state, which has been found to attenuate the age-related decline in baseline creatinine clearance, a measure of GFR and even increased eGFR [32]. Thus, the relationship between lead exposure and renal function remains controversial, and further investigation is required. While randomised controlled trials (RCTs) are reliable determinants of causal inferences in nutrition science, not all exposure-outcome interactions can be tested. This is due to both a cost and time perspective and also because of ethical considerations brought about by exposing participants to presumed risk factors, in this case, lead.

Alternatively, Mendelian randomisation (MR) analysis uses functional polymorphisms (single nucleotide polymorphisms (SNPs)) associated with specific changes in exposures (e.g. lead) as genetic instruments and can provide unbiased and robust evidence on mechanisms of disease pathogenesis. Thus, MR studies can overcome this shortcoming of RCTs [33]. Unlike conventional observational studies and risk factor–based epidemiology, MR studies are considerably less prone to confounding, residual bias and reverse causation [34]. Therefore, we used MR analysis to obtain unconfounded estimates of the casual association of genetically determined blood levels of lead with renal function.

Methods

Study design

A two-sample MR study design was used, in which summary statistics from different genome wide association studies (GWAS) were analysed for the exposures (blood lead) and outcomes (renal function), to estimate the effects of exposure on outcome [35]. Essentially, we applied genetic predictors of blood lead to extensively genotyped case–control studies of renal function (eGFR and the risk of CKD) to obtain estimates of the association of exposure to our clinical outcomes.

Genetic predictors of exposures

We retrieved summary data for the association between SNPs and circulating lead from the GWAS carried out by the Queensland Institute of Medical Research (QIMR), Australia ($n = 2603$, mean age 47.2 years, 59% women), and from the Avon Longitudinal Study of Parents and Children (ALSPAC) (2830 unrelated mothers, mean age 28.4 years) [36]. Genotyping, quality control and imputation procedures are described elsewhere [36]. If a SNP was unavailable for the outcome GWAS summary statistics, we identified proxy SNPs with a minimum linkage disequilibrium (LD) $r^2 = 0.8$.

We used 13 independent SNPs with a p-value $< 5 \times 10^{-6}$. To minimize bias in effect estimates induced by correlation between SNPs, we restricted our genetic instrument to independent SNPs not in linkage disequilibrium ($p = 0.0001$). We refer to a set of SNPs that proxy blood lead as "genetic instruments."

Genetic predictors of outcomes

Genetic associations with renal function were obtained from the largest available extensively genotyped study based on a meta-analysis ($n = 133{,}413$ individuals with replication in up to 42,166 individuals) (full details of all studies included are available in the original article) [37]. eGFR was estimated using the four-variable modification of diet in renal disease (MDRD) equation [37]. CKD was defined as eGFR < 60 ml/min/1.73 m^2. Type 2 diabetes was defined as fasting glucose ≥ 126 mg/dl, antidiabetic drug treatment or by self-reported history. Kidney function and type 2 diabetes were assessed simultaneously.

For GWAS analysis, a centralized analysis plan was applied with each study regressing sex- and age-adjusted residuals of the logarithm of eGFR on SNP dosage levels. Furthermore, logistic regression of CKD was performed on SNP dosage levels adjusting for sex and age. For all traits, adjustment for appropriate study-specific features, such as study site and genetic principal components, was included in the regression and family-based studies appropriately accounted for relatedness. There was no overlap between the exposure sample size and outcome sample size.

Statistics

We combined the effect of instruments using the inverse variance weighted (IVW) method as implemented in the TwoSampleMR package running under *R*. Heterogeneity was assessed using *Q* value for IVW. To address the potential effect of pleiotropic variants on the final effect estimate, we performed sensitivity analysis including weighted median (WM) and MR-Egger. Sensitivity analysis was conducted using the leave-one-out method to identify instruments that might drive the MR results. The WM estimate provides correct estimates as long as SNPs accounting for ≥ 50% of the weight are valid instruments. Inverse variance is used to weight the variants, and bootstrapping is applied to estimate the CIs [35]. MR-Egger is able to make estimates even under the assumption that all SNPs are invalid instruments, as long as the assumption of instrument strength independent of direct effect (InSIDE) is satisfied [35]. However, the InSIDE assumption cannot be easily verified. Average directional pleiotropy across genetic variants was assessed from the p value of the intercept term from MR-Egger [35]. Causal estimates in MR-Egger are less precise than those obtained by using IVW MR [38]. Analysis using MR-Egger has a lower false-positive rate, but a higher false-negative rate, than IVW, i.e. it has a lower statistical power [39].

Heterogeneity between individual genetic variant estimates was assessed by the use of the Q′ heterogeneity statistic [40]. The Q′ statistic uses modified 2nd-order weights that are a derivation of a Taylor series expansion, taking into account the uncertainty in both numerator and denominator of the instrumental variable ratio [40].

To assess the instrumental variable analysis "exclusion-restriction" assumption, we used Ensembl release (http://useast.ensembl.org/index.html) that contains a base of SNP phenotypes and PhenoScanner (Ensembl gives SNP phenotypes, PhenoScanner also gives phenotypes of correlated SNPs.).

Sensitivity analysis

As sensitivity analysis, we used MR-Egger and MR pleiotropy residual sum and outlier (MR-PRESSO) test [40]. MR-Egger and MR-PRESSO may provide correct estimates as long as the instrument strength independent of direct effect assumption is satisfied [40]. MR-Egger can be imprecise, particularly if the associations for SNPs on exposure are similar, or the number of genetic instruments is low [40]. A non-null MR-Egger intercept suggests that the IVW estimate is invalid. MR-Egger does not explicitly identify outliers. MR-PRESSO detects, and if necessary, corrects for potentially pleiotropic outliers [40]. The MR-PRESSO framework detects effect estimates that are outliers and removes them from the analysis by regressing the variant-outcome associations on variant-exposure associations. A global heterogeneity test is then implemented to compare the observed distance between residual sums of squares of all variants to the regression line with the distance expected under the null hypothesis of no pleiotropy [41]. Furthermore, MR-Robust Adjusted Profile Score (RAPS) was applied. This method can correct for pleiotropy using robust-adjusted profile scores. We consider as results causal estimates that agreed in direction and magnitude across MR methods, passed nominal significance in IVW MR, and did not show evidence of bias from horizontal pleiotropy using heterogeneity tests. All analyses were done using the R software (version 3.4.2 R Core Team, 2017).

Ethics

This investigation uses published or publicly available summary data. No original data were collected for this manuscript. Ethical approval for each of the studies included in the present analysis can be found in the original publications (including informed consent from each participant).

The study conforms to the ethical guidelines of the 1975 Declaration of Helsinki.

Results

Demographic characteristics of the study participants are shown in Online Resource 1. The genetic instruments and observed phenotypes are shown in Online Resource 2, and the instrument associations for blood lead levels are shown in Online Resource 3. The instruments have F-statistics higher than threshold, making significant bias from use of weak instruments unlikely [42]. The results, expressed as beta-coefficient for blood lead per 1 standard deviation (SD) increase in outcomes, are presented in Table 1.

Genetically higher blood lead levels had no significant effect on risk of CKD (IVW = Beta: 0.02677, $p = 0.652$: Table 1; Fig. 1) or level of eGFR (IVW = Beta: −0.001514, $p = 0.668$, Table 1) in this sample. After grouping subjects based on type 2 diabetes status (no type 2 diabetes vs. type 2 diabetes), genetically determined levels of blood lead had no significant impact on subjects without type 2 diabetes (IVW = Beta: 0.0008706, $p = 0.823$: Table 1; Fig. 2). However, in subjects with type 2 diabetes, a significant effect on eGFR was observed (IVW = Beta: − 0.03416, $p = 0.0132$: Table 1; Fig. 3).

Heterogeneity results and pleiotropy bias are also shown in Table 1. Estimation based on both MR Egger and IVW was higher than 0.05, which indicated no chance of heterogeneity (all IVW $p > 0.158$, all MR Egger $p > 0.175$). Further, the results of the MR-PRESSO did not indicate any outliers for all the estimates. The horizontal pleiotropy test, with very negligible Egger regression intercept, also indicated a low likelihood of pleiotropy for all of our estimations (all $p > 0.139$). The results of the MR-RAPS were identical with the IVW estimates, highlighting again a low likelihood of pleiotropy. The results of the leave-one-out method demonstrated that the links were not driven by single SNPs.

Discussion

In this study, we have analysed a set of genetic variants that were demonstrated to be associated with blood lead levels in order to determine their relationship with renal function. Mendelian randomisation analyses showed that higher blood lead might be linked with less favourable renal function but only amongst individuals with type 2 diabetes.

Lead is commonly used for industrial purposes, and chronic exposure to lead, either through industrial or environmental means, has been responsible for numerous cases of lead toxicity or plumbism [43–45]. Concerns over the toxicity of lead have led to the phasing out of some of its use in industry and consumer goods [46–48]. In particular, lead in petrol and paint is believed to have been one of the principle contributors to increased blood lead levels in humans and was phased out of use in the USA from the late 1970s [49].

Table 1 Results of the Mendelian randomisation (MR) analysis for effects of blood lead on CKD and eGFR

Exposures		MR				Heterogeneity			Pleiotropy		
		Method	Beta	SE	*p*	Method	*Q*	*p*-value	Intercept	SE	*p*
Blood lead	**CKD**	**MR Egger**	0.2227	0.2405	0.397	**MR-Egger**	6.389	0.272	**−0.025**	**0.029**	**0.430**
		WM	−0.02288	0.07127	0.7482						
		IVW	0.02677	0.05943	0.6524	**IVW**	7.036	0.293			
		RAPS	0.02344	0.06442	0.716						
	eGFR (overall)	**MR Egger**	−0.01488	0.01406	0.3381	**MR-Egger**	6.642	0.248	**0.0017**	**0.0017**	**0.377**
		WM	−0.00294	0.004129	0.4766						
		IVW	−0.00151	0.003539	0.6688	**IVW**	7.793	0.245			
		RAPS	−0.00197	0.003753	0.5989						
	eGFR (No T2DM)	**MR Egger**	−0.01441	0.01536	0.3913	**MR-Egger**	7.854	0.164	**0.0019**	**0.0019**	**0.355**
		WM	−0.00098	0.004262	0.8183						
		IVW	0.000871	0.003896	0.8232	**IVW**	9.485	0.148			
		RAPS	0.000262	0.00407	0.9486						
	eGFR (T2DM)	**MR Egger**	0.07222	0.04948	0.2043	**MR-Egger**	2.113	0.832	**−0.013**	**0.006**	**0.096**
		WM	−0.03251	0.01742	0.06207						
		IVW	−0.03416	0.0138	0.01328	**IVW**	7.068	0.314			
		RAPS	−0.03816	0.01433	0.007746						

WM weighted median, *IVW* inverse variance weighted, *SE* standard error, beta beta-coefficients, *MR* Mendelian randomisation, *CKD* chronic kidney disease, *eGFR* estimated glomerular filtration rate, RAPS robust adjusted profile score, *T2DM* type 2 diabetes mellitus

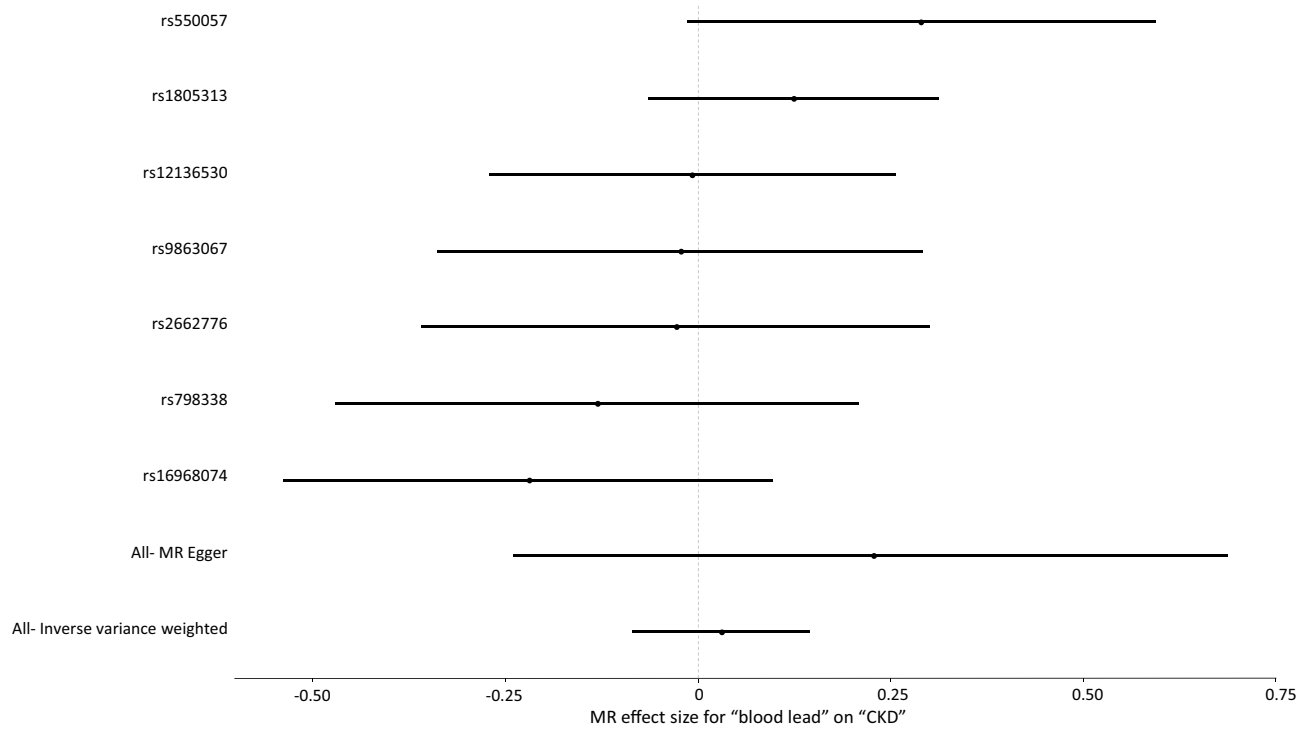

Fig. 1 Forest plot of overall and individual SNP effects on CKD. SNP single nucleotide polymorphism; CKD chronic kidney disease

While the relation between lead exposure and CVD is well established [50, 51], the role of blood lead levels in the development of CKD and reduced renal function remains controversial. Indeed, cross-sectional studies of lead-exposed workers often report changes in markers of kidney function, such as increases in creatinine clearance, without clinically significant reductions in eGFR or diagnosis of renal failure [28, 29, 52, 53]. In a sample of 803 Korean lead workers, blood lead levels were significantly associated with increased uric acid (UA) levels (which is known to be nephrotoxic) in the oldest tertile of workers with serum creatinine greater than the median [28]. Similarly, in a sample of 229 Chinese lead battery factory workers, there was an increasing trend in the dose–response relationship between blood lead levels and indicators of renal function of blood-urea nitrogen (BUN) and UA [29]. However, only those with longer periods of occupational lead exposure had a higher possibility of reduced renal function. Cardenas et al. [52] compared data from 50 Belgian, lead-exposed workers with age-matched controls and reported no indication of significantly increased proteinuria in those exposed to lead. However, blood lead was associated with altered urinary excretion of 6-keto-PGF and thromboxane, eicosanoids which may contribute to the pathologies involved in renal failure and hypertension [54]. Pollock and Ibels [53] presented a case study of 6 men exposed to lead from paint in Australia and suffering from lead intoxication. While some measures related to renal function, such as serum uric acid, urinary protein and creatinine clearance, were abnormal in some cases, these were not consistently observed in the majority of the cases presented. Thus, it can be seen that while lead exposure may have effects on renal-related parameters, a conclusive relationship between lead and CKD in otherwise healthy populations cannot be drawn. Furthermore, such cross-sectional data is not sufficient to determine a causal relationship between lead exposure and CKD, and thus, sufficiently controlled, longitudinal studies as well as mechanistic evidence for a causal effect would be needed. However, the use of MR analysis can overcome the limitations of observational studies as MR is a powerful tool for the detection of causation [34]. As such, the results of this study provide evidence that small, life-long changes in genetically determined blood lead do not impact the development of CKD in individuals without type 2 diabetes.

As such, our study did find an association between genetically determined blood lead and decreased eGFR in those presenting with type 2 diabetes. Renal tubule damage is a common feature of type 2 diabetes and is considered to be a pathway to glomerular dysfunction associated with proteinuria and the development of CKD in those with type 2 diabetes [55]. It could be speculated that the nephrotoxic effects of substances such as UA, which are elevated in lead-exposed individuals, might contribute to the development of diminished kidney function in those already experiencing

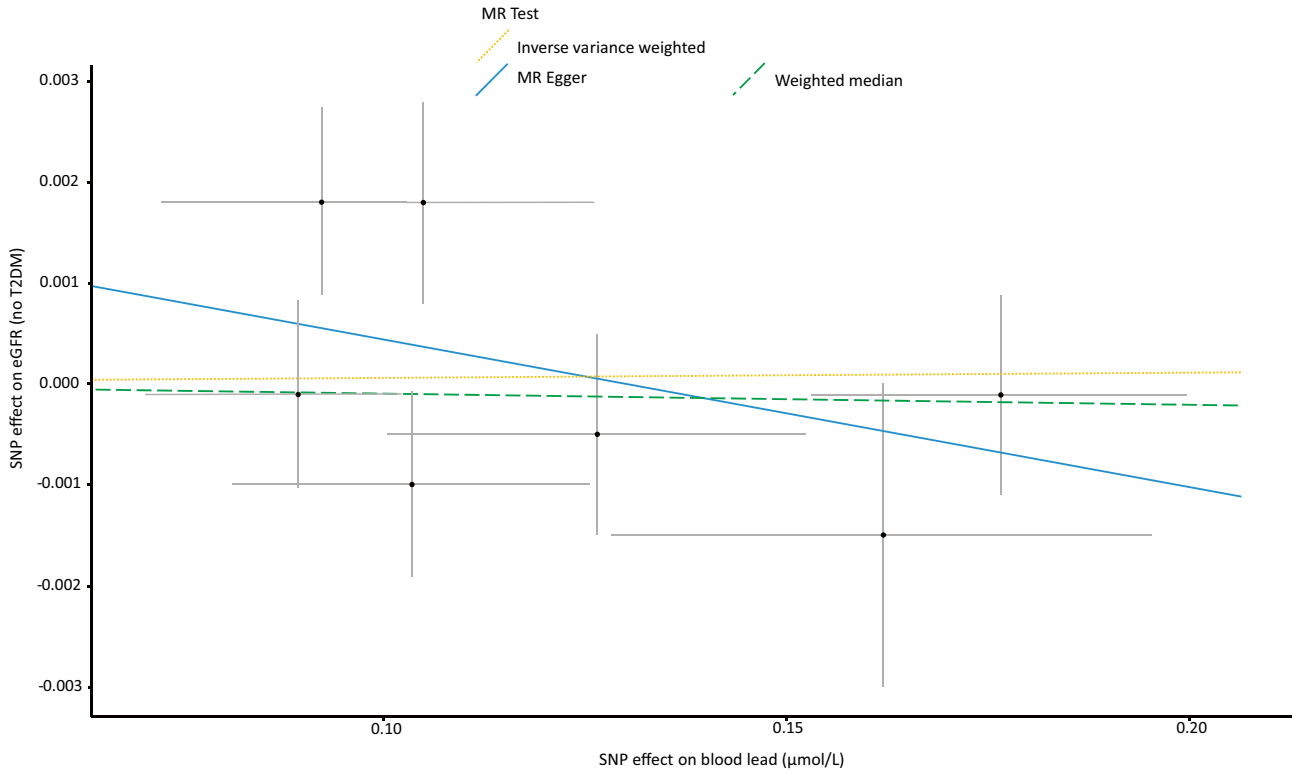

Fig. 2 Scatter plot of the association of the effect of SNP-determined blood lead on eGFR in individuals without T2DM. Each black point represents an SNP, plotted by the estimate of SNP on blood lead level (x-axis, nmol/L) and the estimate of SNP on eGFR (y-axis, mL/min). The slopes of each line represent the potential causal associations for each method. SNP single nucleotide polymorphism; T2DM type 2 diabetes mellitus; eGFR estimated glomerular filtration rate

renal tubule damage due to type 2 diabetes [28, 29]. This might explain why high blood lead is only seen to contribute to CKD in those with type 2 diabetes, i.e. those with pre-existing damage to renal tubules.

We believe this to be the first study to report that there is a relationship between genetically determined blood lead levels and reduced eGFR in individuals with diabetes. Indeed, diabetes is a frequent comorbidity in CKD and is believed to contribute to the development of impaired renal function [7, 20]. It has been observed that individuals with earlier onset type 2 diabetes, and consequently longer duration of diabetes, have a 2.6-fold higher risk of CKD, compared to those with later-onset diabetes [56]. Lead is known to contribute to oxidative stress in those exposed to high levels [57, 58], and more specifically, lead has been reported to lead to oxidative stress and apoptosis in in vitro human mesangial cells which may be a possible mechanism for lead-induced nephrotoxicity [59]. Similarly, lead exposure is known to affect the immune system resulting in altered cytokine metabolism and a proinflammatory response [60]. We propose that as the diabetic state is associated with metabolic derangement such as elevated oxidative stress [61] as well as elevated levels of proinflammatory cytokines [62, 63] and renal tubule damage [55], lead exposure may accelerate and augment these detrimental processes (which may not be present in those without diabetes) and lead to renal dysfunction more readily in subjects with type 2 diabetes. Further research is needed to investigate the mechanisms of the blood lead–related renal dysfunction amongst those with diabetes.

A major strength of our study is the large sample population with access to individual participant data of high validity for eGFR and CKD status, and with the relevant SNPs available for blood lead concentration. Additionally, the use of MR methods allows us to examine the potential causal effects of blood lead, largely without the disadvantages of confounding or reverse causation. We checked for known pleiotropy using Ensembl and found few known phenotypes of the genetic predictors of blood lead apart from multiple associations for rs550057 (ABO) (Online Resource 2). A potential limitation of this study is the use of a predominantly white, ethnically European population which limits the generalizability of the results. As such, ethnically diverse GWAS and MR studies are necessary to generalize MR results to people of different ancestries. Furthermore, while this MR analysis provides evidence on the effect of smaller life-long, genetically determined

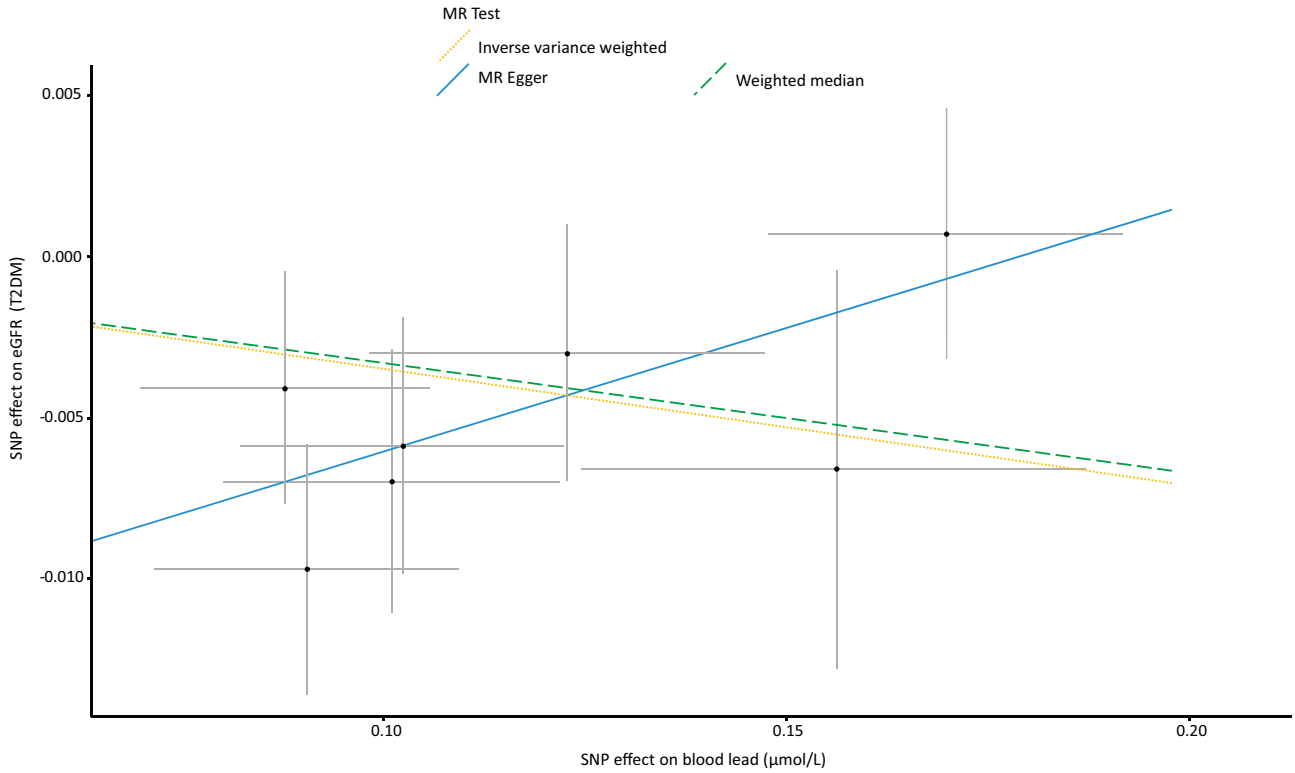

Fig. 3 Scatter plot of the association of the effect of SNP-determined blood lead on eGFR in individuals with T2DM. Each black point represents an SNP, plotted by the estimate of SNP on blood lead level (*x*-axis, nmol/L) and the estimate of SNP on eGFR (y-axis, mL/min). The slopes of each line represent the potential causal associations for each method. SNP single nucleotide polymorphism; T2DM type 2 diabetes mellitus; eGFR estimated glomerular filtration rate

blood lead levels, it may not necessarily apply to short-term larger changes in blood lead, due to environmental factors. Another potential concern with MR analysis is the risk of stratification bias, which would only be an issue if type 2 diabetes resulted from both elevated blood lead levels and the presence of CKD. Finally, due to the limited number of shared SNPs identified by both the QIMR and ALSPAC studies ($n = 3$) (Online Resources 4 and 5), it is not possible to perform a sensitivity analysis to determine differences between the results of both datasets. As such, future research should endeavour to perform such sensitivity analyses, as sufficient data on relevant SNPs becomes available.

In conclusion, this investigation found evidence to support a potential causal association between genetically determined blood lead levels on renal function in individuals with type 2 diabetes. However, in subjects without diabetes, no such causal relationship was identified. While further investigation is required to investigate the link between lead exposure and indices of renal function in those with diabetes, this novel data also contributes to the current understanding that the relationship between lead exposure and CKD in non-diabetic individuals may simply be associative.

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Author contribution MM designed the study, acquired the data and performed the analyses. MM, RK and IGD interpreted the findings and drafted the manuscript, and all the authors contributed to critical reading and revision of the draft report. All the authors approved the final version to be published.

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Availability of data and material The datasets analysed in this study are publicly available summary statistics.

Declarations

Ethics approval Not applicable.

Consent to participate Not applicable.

Consent for publication Not applicable.

Conflict of interest RK has received speaker's honoraria from the British Association for Parenteral and Enteral Nutrition and fees for media content creation from Myprotein. MM and IGD declare that they have no conflict of interest.

Disclaimer The study funder was not involved in the design of the study; the collection, analysis, and interpretation of data; writing the report; and did not impose any restrictions regarding the publication of the report.

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Authors and Affiliations

Mohsen Mazidi[1] · Richard Kirwan[2] · Ian G. Davies[3]

✉ Richard Kirwan
r.p.kirwan@2018.ljmu.ac.uk

[1] Department of Twin Research and Genetic Epidemiology, King's College London, London, UK

[2] School of Biological and Environmental Sciences, Liverpool John Moores University, Liverpool, UK

[3] Research Institute of Sport and Exercise Science, Liverpool John Moores University, Liverpool, UK

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Review

A Mitocentric View of the Main Bacterial and Parasitic Infectious Diseases in the Pediatric Population

Sonia Romero-Cordero [1], Richard Kirwan [2,*], Antoni Noguera-Julian [3,4,5,6], Francesc Cardellach [4,7,8,9], Clàudia Fortuny [3,4,5,6] and Constanza Morén [4,7,8,9,*]

1 Faculty of Medicine, Pompeu Fabra University and Universitat Autònoma de Barcelona, 08002 Barcelona, Spain; sonia.romero.cordero@gmail.com
2 School of Biological and Environmental Sciences, Liverpool John Moores University, Liverpool L2 2QP, UK
3 Malalties Infeccioses i Resposta Inflamatòria Sistèmica en Pediatria, Unitat d'Infeccions, Servei de Pediatria, Institut de Recerca Pediàtrica Hospital Sant Joan de Déu, 08950 Barcelona, Spain; ton@hsjdbcn.org (A.N.-J.); cfortuny@hsjdbcn.org (C.F.)
4 Faculty of Medicine and Health Sciences, University of Barcelona, 08036 Barcelona, Spain; fcardell@clinic.cat
5 Centro de Investigación Biomédica en Red de Epidemiología y Salud Pública (CIBERESP), 28029 Madrid, Spain
6 Red de Investigación Translacional en Infectología Pediátrica (RITIP), 28029 Madrid, Spain
7 Muscle Research and Mitochondrial Function Laboratory, Cellex-IDIBAPS, Faculty of Medicine and Health Sciences, University of Barcelona, 08036 Barcelona, Spain
8 Centro de Investigación Biomédica en Red de Enfermedades Raras (CIBERER) (ISCIII), 28029 Madrid, Spain
9 Internal Medicine Department-Hospital Clínic of Barcelona (HCB), 08036 Barcelona, Spain
* Correspondence: r.p.kirwan@2018.ljmu.ac.uk (R.K.); cmoren1@clinic.cat (C.M.)

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Abstract: Infectious diseases occur worldwide with great frequency in both adults and children. Both infections and their treatments trigger mitochondrial interactions at multiple levels: (i) incorporation of damaged or mutated proteins to the complexes of the electron transport chain, (ii) mitochondrial genome (depletion, deletions, and point mutations) and mitochondrial dynamics (fusion and fission), (iii) membrane potential, (iv) apoptotic regulation, (v) generation of reactive oxygen species, among others. Such alterations may result in serious adverse clinical events with great impact on children's quality of life, even resulting in death. As such, bacterial agents are frequently associated with loss of mitochondrial membrane potential and cytochrome c release, ultimately leading to mitochondrial apoptosis by activation of caspases-3 and -9. Using Rayyan QCRI software for systematic reviews, we explore the association between mitochondrial alterations and pediatric infections including (i) bacterial: *M. tuberculosis*, *E. cloacae*, *P. mirabilis*, *E. coli*, *S. enterica*, *S. aureus*, *S. pneumoniae*, *N. meningitidis* and (ii) parasitic: *P. falciparum*. We analyze how these pediatric infections and their treatments may lead to mitochondrial deterioration in this especially vulnerable population, with the intention of improving both the understanding of these diseases and their management in clinical practice.

Keywords: antibiotics; infections; mitochondria; pediatrics

1. Introduction

The burden of infectious diseases worldwide has considerable impact resulting in 350,000 deaths in 2017 according to the WHO [1]. As such, fully understanding the main molecular, subclinical events associated with such infections is of vital importance to clinical research. This systematic review focuses on the mitochondrial changes associated with the principal bacterial and parasitic infections, as well as their treatments, in the pediatric population, an especially vulnerable group. Here, we review how both infections and their treatments promote molecular mitochondrial damage at multiple levels, including depletion, deletions, and point mutations in the mitochondrial genome, mutated mitochondrial proteins, and alterations in mitochondrial dynamics, clearance, membrane potential,

apoptosis, and oxidative damage. All these molecular events may compromise cell viability, which ultimately may result in serious adverse clinical events, considerably impacting quality of life or even resulting in death. To provide a complete overview of these molecular alterations resulting from the main pediatric bacterial and parasitic infections (including *M. tuberculosis*, *E. cloacae*, *P. mirabilis*, *E. coli*, *S. enterica*, *S. aureus*, *S. pneumoniae*, *N. meningitidis*, and *P. falciparum*), we will first guide the readers through some of the main considerations regarding mitochondrial physiology and pathology, which retain multiple features of their bacterial ancestry, a key issue when considering the mitochondrial effects precisely related to bacterial infections. We will then review, for the first time to our knowledge, the specific mechanisms by which mitochondria are affected by the aforementioned bacterial and parasitic infections, as well as their corresponding treatments in children.

2. Mitochondria

Mitochondria are semi-autonomous, maternally inherited organelles present in the cytoplasm of virtually all eukaryotic cells [2,3]. They are essential for cell viability due to their involvement in cellular respiration, apoptosis, catabolism and anabolism of metabolites, calcium homeostasis, heat production, and energy, through the formation of adenosine triphosphate (ATP) molecules [4] (Figure 1). Mitochondria are present in variable numbers within cells depending on the energy requirements of the tissue. The greater the energy demand, the greater the number of mitochondria, with the greatest numbers reported in nervous and muscular tissue [5]. With respect to this review, the endosymbiotic theory of Lynn Margulis, claiming a common bacterial and mitochondrial origin [6], deserves special attention and is supported by the shared characteristics of both the microorganisms and the organelle, many of which are detailed below.

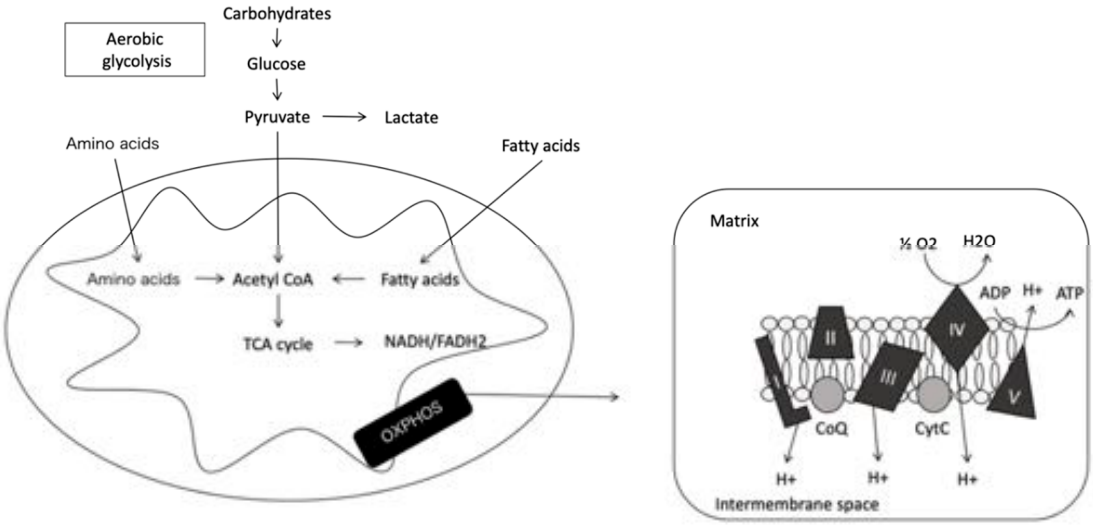

Figure 1. Simplified general summary of the main mitochondrial metabolic pathways. ADP, adenosine diphosphate; ATP, adenosine triphosphate; I, complex I; II, complex II; III, complex III; IV, complex IV; CoQ, coenzyme Q; CytC, cytochrome C; FADH, flavin and adenine dinucleotide; H+, proton; NADH, nicotinamide adenine dinucleotide hydrogen; OXPHOS, oxidative phosphorylation system; TCA, tricarboxylic acid and V, V complex.

2.1. Mitochondrial Structure

Mitochondria are not static structures within cells; but dynamic, capable of fusion and fission. They consist of:

2.1.1. Outer Mitochondrial Membrane (OMM)

Permeable to ions, metabolites, and polypeptides, due to porins and/or voltage-dependent channels [3].

2.1.2. Inner Mitochondrial Membrane (IMM)

Impervious to almost all molecules and ions, highly selective and rich in cardiolipin. Cardiolipin is a phospholipid found exclusively in the bacterial membrane or in the IMM of eukaryotic cells and represents 10–20% of the total phospholipid content. Transmembrane transport is carried out using proteins specific to certain molecules such as: pyruvic acid, adenosine diphosphate (ADP), ATP, oxygen, water, and fatty acids. Of particular note are adenine nucleotide translocase (ANT), which transports cytosolic ADP to the mitochondrial matrix and ATP (once synthesized) to the cytosol and phosphate translocase, which transfers cytosolic phosphate plus a proton (H+) to the matrix. This phosphate is essential for the phosphorylation of ADP in the oxidative phosphorylation (OXPHOS) process. The IMM presents many folds or invaginations, called mitochondrial cristae, that greatly extend the surface where the enzymatic complexes of the OXPHOS process are embedded [5] (Figure 2).

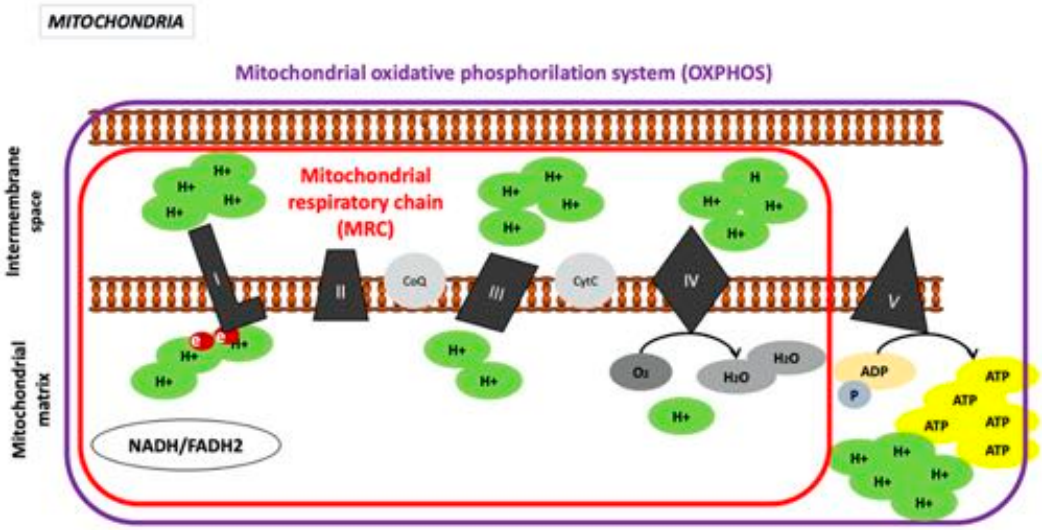

Figure 2. Mitochondrial respiratory chain and oxidative phosphorylation system in the mitochondria. ADP, adenosine diphosphate; ATP, adenosine triphosphate; I, complex I; II, complex II; III, complex III; IV, complex IV; V, complex V; CoQ, coenzyme Q; CytC, cytochrome C; e, electrons; FADH, flavin and adenine dinucleotide; H+, proton; NADH, nicotinamide adenine dinucleotide hydrogen and OXPHOS, oxidative phosphorylation system.

Oxidative Phosphorylation System

Oxidative phosphorylation is the process of ATP synthesis coupled with oxygen consumption, whereby electrons are transferred in stages, through 4 enzymatic complexes (complex I or CI, CII, CIII, and CIV), 2 carriers of mobile electrons (coenzyme Q (CoQ) and cytochrome C (CytC)), which make up the mitochondrial respiratory chain (MRC), and a fifth complex, called ATP synthase or complex V (CV) [3].

In this process, oxygen is consumed and an electrochemical gradient is established, driving ATP synthesis [7]. The electrons flow through the MRC through oxidation–reduction (or redox) reactions ending in complex IV, where oxygen is the final receptor for the electrons and is reduced to H_2O:

$$\text{ADP (matrix)} + \text{inorganic phosphate (Pi) (matrix)} + 3H^+ \text{ (intermembrane)} \rightarrow 2\text{ATP (matrix)} + H_2O + 3H^+ \text{ (matrix)}$$

Complex I, Nicotinamide Adenine Dinucleotide Hydrogen (NADH) Dehydrogenase or NADH$^-$CoQ Reductase (CI)

It contains flavin mononucleotide (FMN) and transfers electrons to CoQ or ubiquinone:

$$NADH + H^+ + CoQ + 4H^+ \text{ (matrix)} \rightarrow NAD^+ + CoQH_2 + 4H^+ \text{ (intermembrane space)}$$

The goal is to oxidize the NADH, obtained through the Krebs cycle in the mitochondrial matrix, and transfer the electrons to reduce CoQ. The plant-based insecticide rotenone and the antibiotic piericidin A are specific inhibitors of CI [5].

Complex II, Succinate Dehydrogenase or Succinate-CoQ Reductase (CII)

The smallest complex, composed of 4 peptides. The 2 largest peptides make up the peripheral portion of the complex and function as the enzyme "succinate dehydrogenase" in the Krebs cycle. The electrons from the oxidation of succinate to fumarate are channeled through this complex to ubiquinone. Therefore, complex II links the Krebs cycle directly to the MRC. Cofactors, cytochrome b558, and metal ions also constitute CII. A flavin is covalently linked to the largest peptide, producing the flavoprotein subunit (Fp). The overall reaction catalyzed by CII is as follows

$$\text{Succinate} + \text{CoQ} \rightarrow \text{fumarate} + \text{CoQH}_2.$$

It should be noted that in this complex, there is a lack of proton pumping, which takes place at the CI, CIII, and CIV levels. CoQ acts as a mobile carrier of electrons between complexes and receives electrons from CI and CII and transfer them to CIII. The analogue substrate malonate is a specific CII inhibitor, which binds competitively and specifically at the active site in the Fp subunit of the complex [3,5].

Complex III or $CoQH_2$, Known as Cytochrome c Reductase

Composed of some cytochromes (Cyt b562, Cyt b566, and Cyt c1), as well as an iron–sulfur (Fe-S) group, and transfers electrons from $CoQH_2$ to CytC:

$$CoQH_2 + 2CytC^{3+} + 2H^+ \text{ (matrix)} \rightarrow CoQ + 2CytC^{2+} + 4H^+ \text{ (intermembrane space)}.$$

CytC is a mobile electron carrier protein located on the outer face of the IMM. Its function is to transfer the electrons from the CIII to the CIV [3,5]. Antibiotic antimycin A is a CIII specific inhibitor [5].

Complex IV or Cytochrome C Oxidase (COX)

Transfers electrons to an oxygen molecule reducing it to two water molecules

$$4CytC_2{}^+ + 8H^+ + O_2 \rightarrow 4CytC_3{+} + 4H^+ + 2H_2O.$$

Molecular oxygen is the terminal electron receptor, the CytC mobile carrier is re-oxidized, and two protons are transferred to the intermembrane space. Potassium cyanide (KCN) is the specific inhibitor of CIV [5].

Complex V or ATP Synthase Complex

Responsible for ATP synthesis through phosphorylation of the ADP molecule. This endergonic reaction is coupled to redox reactions, and electron transport is driven by a proton gradient and constitutes the OXPHOS system. The CV presents two functional subunits: F0 and F1. F0 is a hydrophobic structure and contains a transmembrane channel where the protons are found, enabling the passage from the intermembrane space to the mitochondrial matrix. F1 contains catalytic synthase activity and is composed of five polypeptide chains (α, β, ɣ, δ, and ε).

The general reaction that takes place in this complex is:

$$\text{ADP (matrix)} + \text{Pi (matrix)} + 3H^+ \text{ (intermembrane)} \rightarrow 2\text{ATP (matrix)} + H_2O + 3H^+ \text{ (matrix)}.$$

The ATP produced is the energy required to power all energy-consuming cellular processes [2]. The antibiotic oligomycin is a highly specific inhibitor of the CV F0 subunit.

2.1.3. Intermembrane Space

The space between the OMM and IMM with a similar composition to that of the cytosol and a high concentration of protons as a result of the pumping carried out by the MRC [5].

2.1.4. Mitochondrial Matrix

Contains ions, oxidizable metabolites, and the genetic material of the mitochondria. Along with chloroplasts in the plant cell, mitochondria are the only organelles that contain their own deoxyribonucleic acid (DNA) or mitochondrial DNA (mtDNA) and an autonomous transcriptional and translational capacity, which encodes for some proteins of the OXPHOS [5]. mtDNA lacks introns and does not follow the universal genetic code. Most of the mitochondrial proteins are encoded by nuclear genes and imported to the mitochondria [8] and follow the usual Mendelian inheritance patterns [7]. The mitochondrial genome is found in the matrix as a double-stranded circular DNA, similar to a bacterium's genome, and synthesizes 2 ribosomal ribonucleic acids (rRNAs), 22 transfer ribonucleic acids (tRNAs), and 13 mRNA that will constitute the MRC subunits. mtDNA can present with heteroplasmy, which is the coexistence of mutated and wild-type mitochondrial genome molecules. This is due to the random distribution that occurs when these organelles divide [5], as mtDNA is randomly distributed into daughter cells by mitotic segregation during cell division [3,5,7].

2.2. Mitochondrial Physiology

Mitochondrial functions respond to a series of genetic, metabolic, and neuroendocrine signals with functional and morphological changes and, in turn, generate signals that influence a large number of cellular functions that contribute to the complexity of physiology and pathology. This places the mitochondria in a privileged position, as a "portal" at the intersection of the cell and its environment [8]. Thus, mitochondria have been implicated in aging, regulation of cell metabolism, control of the cell cycle, cell development, defense responses to infections and signal transduction, among other processes [9].

The tricarboxylic acid (TCA) cycle, also called the Krebs cycle or citric acid cycle, which takes place within the matrix of the mitochondria, is a series of eight enzymatic steps that consumes and then regenerates, citrate. It links the metabolism of carbohydrates, fats, and proteins, since the catabolism of these compounds generates acetyl CoA. This key molecule enters the TCA cycle and oxidizes and produces flavin and adenine dinucleotide hydrogen (FADH) and NADH, reducing molecules that feed MRC and OXPHOS [7].

2.3. Mitochondrial Pathology

The first patient with mitochondrial disease was described in 1962 [10]. Human mitochondrial diseases are actually a very large collection of hundreds of very heterogeneous and rare diseases, since changes in literally thousands of genes can affect mitochondrial function [7]. Hence, mitochondrial research is on the rise in the medical sciences. As evidence, the number of medical publications related to mitochondriopathies has surpassed those related to other alterations in other organelles, including the endoplasmic reticulum, the Golgi apparatus, and the nucleus [8]. Mitochondrial disorders represent a major challenge in medicine [10]. In the same way, the origin of pleiotropic and multisystemic symptoms in mitochondrial disorders is still poorly understood and often makes it difficult to diagnose this type of disease [8]. Thus, oxidative tissues, with high energy demand (brain, muscle, retina, cochlea, liver, and kidney) are the most vulnerable to OXPHOS defects [10].

Clinical presentations in childhood include allergy, hypotonia, development of mental retardation, conduction failure, seizures, cardiomyopathy, hearing or visual impairment, movement disorders, and lactic acidosis [11].

2.3.1. Anaerobiosis

In abnormal conditions, such as hypoxia or alterations in mitochondrial function, the metabolic pathways are readjusted to continue obtaining the reducing power necessary for energy production via an anaerobic process (Figure 3). Under these conditions, the pyruvate resulting from catabolism of the metabolites is not imported into the mitochondria, but is converted to lactate through the enzyme lactate dehydrogenase [5].

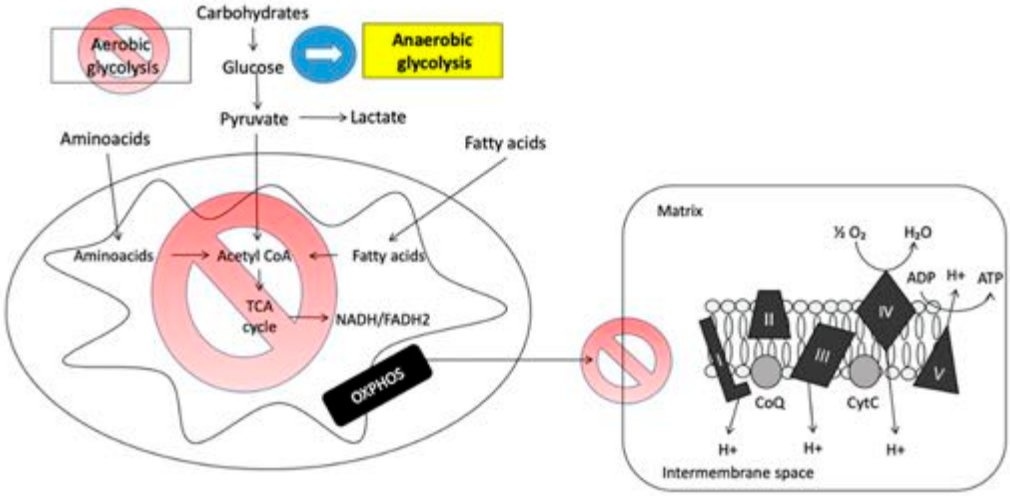

Figure 3. Mitochondrial anaerobiosis state during mitochondrial dysfunction. ADP, adenosine diphosphate; ATP, adenosine triphosphate; I, complex I; II, complex II; III, complex III; IV, complex IV; V, complex V; CoQ, coenzyme Q; CytC, cytochrome C; FADH, flavin and adenine dinucleotide hydrogen; H+, proton; NADH, nicotinamide adenine dinucleotide hydrogen; OXPHOS, oxidative phosphorylation system; TCA, tricarboxylic acid.

In this pathological context, lactate concentration increases in the blood stream, which comes from its synthesis in skeletal, liver, nervous, and lymphoid tissues. Lactate concentrations under normal conditions range from 0.5 to 2.4 mmol/L [5]. Under conditions of hyperlactatemia, the blood pH falls and acidification occurs [5]. Interestingly, a recent study reported increased lactate in the cerebrospinal fluid of children suffering from meningitis and this may serve as a biomarker to distinguish the bacterial or viral origin of this pediatric infection [12].

2.3.2. Reactive Oxygen Species

Reactive oxygen species (ROS) are intermediate metabolites derived from oxygen generated in the mitochondria during OXPHOS dysfunction. ROS are highly oxidizing free radicals with decoupled electrons that can damage cellular structures, such as proteins, lipids, carbohydrates, genetic material, and also mitochondria, which are particularly vulnerable. ROS are derived from O_2^- (in the MRC at CI and CIII) and include superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl anion (OH^-), and peroxynitrite ($ONOO^-$) [5]. There are many antioxidants that counteract the deleterious effects of ROS, such as superoxide dismutase (SOD), catalase or peroxidase, and glutathione peroxidase. In addition, there are many non-enzymatic antioxidants, molecules such as vitamins E and C, carotenes, quinones, glutathione, and other metallic elements, such as selenium, zinc, iron, or copper, among others [13]. All of them are capable of reducing ROS levels. Under normal conditions, all antioxidant mechanisms minimize ROS production and therefore, act as a protective system against oxidative stress. However, in the presence of mitochondrial dysfunction, ROS increase beyond the detoxification threshold. Mitochondria and ROS are thus a nexus of multiple pathways that determine the response of cells to disruptions in cellular homeostasis such as infection [14]. Specifically, ROS release may be associated with the presence of exogenous toxic compounds affecting the mitochondria. Nitric oxide

(NO) and reactive oxygen species (ROS) produced during bacterial infections are involved critically in host defense mechanisms [15].

2.3.3. Apoptosis

Apoptosis is a naturally occurring programmed cell death mechanism, which occurs through extrinsic (external trigger) and the intrinsic (internal trigger) pathways and is mainly driven by caspases (serine proteases). Mitochondria play a central role, especially in the intrinsic pathway [16]. Depending on the site of action, apoptotic caspases can be classified into different groups (Figure 4):

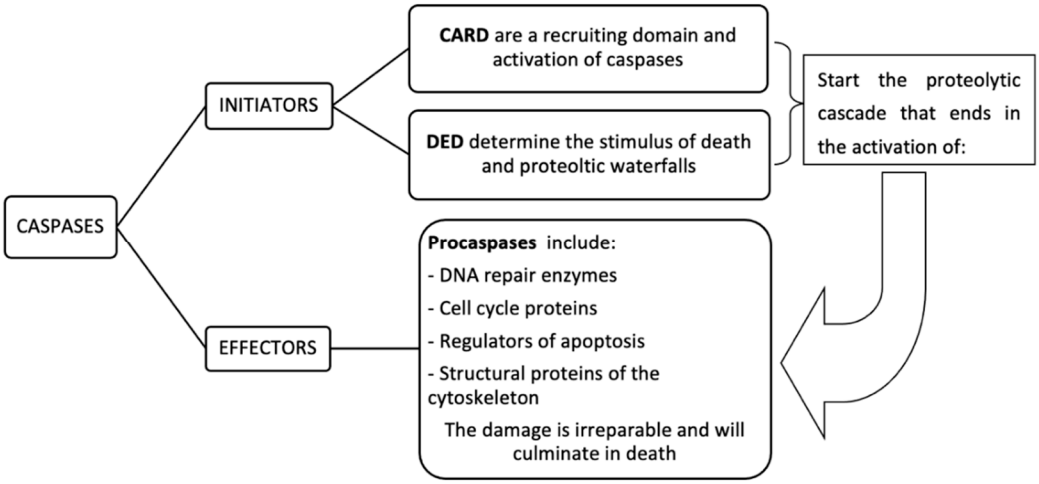

Figure 4. Types of caspases: classification and main functions. Initiator caspases, including CARD [17] and DED [18], and effector caspases, including procaspases, as well as their functions are represented. CARD, caspase activation and recruitment domain; DED, death effector domain.

In the extrinsic pathway, the stimulus can be external, received by a cell surface receptor. The binding of the apoptosis-inducing ligand to the corresponding receptor leads to the activation of caspase-8 in the cytosol, which, in turn, activates the proapoptotic protein Bid by proteolysis. In this case, mitochondrial involvement in extrinsic apoptosis enhances activation. In the intrinsic pathway, regulated by the mitochondria, the stimulus is internal, as it is the result of the action of a drug, toxin, radiation damage, food shortage or, in general, a situation of stress.

Of course, drugs, toxins, radiation, etc. are also ultimately external influences, but the difference between these two mechanisms, extrinsic and intrinsic, becomes apparent during the next phase, i.e., signal transduction [16]. Active and healthy mitochondria exhibit a mitochondrial transmembrane potential ($\Delta\psi m$) through the IMM where an electrochemical and pH gradient is established as a result of electron transport through the electron transport chain (ETC), with an excess of positive charge in the intermembrane space. In cells undergoing apoptosis, a drop in $\Delta\psi m$ is observed as one of the relatively early events, in many different cell types, prior to DNA fragmentation. When induction of the apoptotic process occurs, there are changes in the ratio of proapoptotic (e.g., Bax, Bak, t-Bid, Bim, Bad, and Bik) and antiapoptotic (e.g., bcl-2) proteins. This relationship regulates the permeability of the OMM and its imbalance. Subsequently, the opening of a permeability transition pore (PTP) occurs. If a massive opening of such pores occurs, it can collapse the $\Delta\psi m$, stop OXPHOS, stop the importation of proteins into the mitochondria, and induce leaks of CytC and other apoptogenic mitochondrial proteins, such as SMAC-DEVIL, which exit into the cytosol. On the other hand, mitochondria release an apoptogenic protein, apoptosis-inducing factor (AIF), an intermembrane protein with protease activity but without nuclease activity. CytC binds the cytosolic protein apoptosis protease-activating factor-1 (APAF-1) which, in turn, interacts with procaspase-9 to form a multiprotein complex called the apoptosome. The apoptosome induces the activation

of caspase-9 by proteolysis, and caspase-9 activates the caspase-3 effector. This induces irreversible activation of a caspase-catalyzed cascade of reactions and ultimately, deoxyribonuclease (DNAse) is activated by a caspase and cleaves DNA leading to cell death [5]. Mitochondrial alterations can be classified into primary or genetic, when the origin is a genetic alteration, which affects a mitochondrial protein, or secondary or acquired, when the cause is external or environmental, e.g., the bacterial infections [19] and their corresponding antibiotic treatments [20].

Here, we will review those bacterial and parasitic infections and treatments that are associated with mitochondrial involvement and that can be considered of relevance in the pediatric population, with a focus on improving both the understanding of these diseases and their management in clinical practice.

3. Bacterial Infectious Processes and Mitochondrial Involvement

3.1. Tuberculosis (TB)

TB is a contagious infectious disease caused mainly by *Mycobacterium tuberculosis* (*Mtb*) in humans, which represents one of the top 10 causes of death worldwide and the leading cause of death from a single infectious agent (ranking above HIV/AIDS) [21]. Almost 9 million people present active TB and 2 million die from this disease every year [22,23]. Nonetheless, the probability of developing TB is much higher among people presenting risk factors such as HIV, under-nutrition, diabetes, smoking, and alcohol consumption and also in young children [21]. These groups are also at higher risk of severe and disseminated forms of the disease.

Mtb is spread essentially through the air and enters the human organism mainly through the respiratory route after inhalation. These volatile particles are small enough to reach the lower airways. Infection and the development of the pulmonary form of TB (lungs are the main target of this bacterium) proceed as follows: (i) phagocytosis of the bacilli, (ii) intracellular multiplication, (iii) a latent contained phase of infection, and (iv) active lung infection. These steps can progress towards spontaneous cure, development of disease, latent TB infection and later re-activation, or re-infection [24]. Although the pathogen typically affects the lungs (pulmonary TB), it can also affect other sites (extrapulmonary TB) [21].

Symptoms of TB in other parts of the body depend on the area affected. TB in the lungs may cause symptoms such as a chronic cough, chest pain, and hemoptysis. Other unspecific symptoms of TB are weakness or fatigue, weight loss, loss of appetite, chills, fever, and night sweats [24].

3.1.1. Structure and Replication Cycle

Mtb infects the alveolar macrophage, through the deposition of infected aerosols in the pulmonary alveoli [25,26]. Each alveolus presents its own alveolar macrophages that are dedicated to constantly cleaning this space [27–30]. The function of alveolar macrophages is to keep the alveolus clean to allow gas exchange and to avoid any inflammatory development at all costs. The alveolar space is drained with a surfactant generated by pneumocytes, which entails a negative counterpart, and prevents the entry of antibodies [31].

The viable bacillus is phagocytosed by an alveolar macrophage, and then it displays its pathogenic capacity by secreting a 6 kDa early secretory antigenic target (ESAT-6). This peptide is essential to (i) prevent phagosome-lysosome binding, (ii) prevent apoptosis, and (iii) finally, to allow the entry of the bacillus into the cytoplasm. Therefore, the bacillus takes full advantage of its multiplication capacity in a single alveolar macrophage, approximately between 5 and 6 division cycles, to achieve a concentration of between 32 and 64 bacilli per cell. This process takes place for approximately 5–6 days, considering that each division cycle in *Mtb* requires approximately 24 h, causing alveolar macrophage necrosis. To control *Mtb* replication, alveolar macrophages attempt to initiate apoptosis [31]. However, virulent *Mtb* strains are capable of triggering a necrosis-like cell death that is associated with higher mycobacterial replication, strong inflammation, dissemination, and disease progression. Intracellular replication of *Mtb* plays a critical role in determining the fate of the infected

cell. If >25 bacilli per cell are achieved, the host cell undergoes an atypical, necrotic-like cell death characterized by lysosomal permeabilization, mitochondrial damage, and membrane destruction. In contrast, macrophages with a low bacillary burden undergo apoptosis, leading to a more advantageous outcome for the host [31].

As the infective process evolves, thousands of bacilli are generated, promoting the synthesis of enough chemokines by infected alveolar macrophages to trigger an inflammatory response [25,32]. With inflammation, there is an imbalance due to an exudate that destroys the alveolar tension and allows the entry of polymorphonuclear cells, normally neutrophils and monocytes, in differential proportions depending on the type of chemokines and cytokines secreted. At the same time, there is more vigorous lavage of the affected alveoli, draining into the lymph nodes through the afferent lymphatic capillaries. In this way, *Mtb* infects the macrophages of the nodules, generating lymphadenitis and dendritic cells [25,33]. As hypersensitivity develops, the inflammatory response becomes more intense, and regional lymph nodes often enlarge. The parenchymal portion of the primary infectious complex is often completely cured by fibrosis or calcification after caseous necrosis and encapsulation.

The tubercle bacilli of the primary infectious complex can spread through the bloodstream and lymphatic vessels to many parts of the body leading to the development of parenchymal injury and accelerated cassation caused by the development of hypersensitivity [34]. *Mtb* can potentially colonize any organ, and recolonize previously generated lesions that, being in an inflammatory process, have a greater vascularity and permeability, and can also pass into the venous capillaries, reach the left atrium and ventricle, and spread systemically [25,33]. The organs in which endothelial cells allow greater permeability, such as bone tissue, especially in developing children, or the kidney, are common target organs. This spread can involve large numbers of bacilli, leading to disseminated (miliary) TB, or small numbers of bacilli that leave microscopic tuberculous foci scattered in various tissues. These metastatic foci are not clinically apparent initially but are the origin of extrapulmonary TB and pulmonary TB reactivation in some children and in many adults. Extrapulmonary TB represents approximately 30% of cases of TB and usually denotes a delay in the immune response, mainly affecting children under 5 years of age or people suffering from immunosuppression, although there is significant geographic variability, a fact that can be interpreted as the possible existence of some genetic factor that favors it [25,35].

3.1.2. TB in the Pediatric Population

One million children develop TB each year, and 210,000 die from complications of the disease. Increased case rates of childhood TB have been associated with a simultaneous increase in TB rates among HIV-infected adults in the community. Childhood TB is very different from adult TB in epidemiology and clinical and radiographic presentation [36] (Table 1).

Table 1. Clinical differences in children vs. adults.

TB	Children	Adults
Cause	A complication of the pathophysiologic events surrounding the initial infection	A reactivation of organisms that were lodged in the apices of the lungs during hematogenous dissemination at the time of primary infection
Incubation period	Often only weeks to months	Often long (years to decades)
Pulmonary and extrapulmonary TB	Children are more prone to extrapulmonary disease but rarely develop contagious pulmonary disease	Adults are more prone to developing contagious pulmonary disease
Pulmonary location	Anywhere (25% multilobar)	Apical
Adenopathy	Usual	Rare (except HIV related)
Cavitation	Rare (except adolescents)	Common
Signs and symptoms	Unspecific	Typical

A fairly predictable timetable for primary TB and its complications in infants and children is apparent (34). The incubation period in children between the time the Mtb enters the body and the development of cutaneous hypersensitivity is usually 2–12 weeks and most often 4–8 weeks. The onset of hypersensitivity may be accompanied by a febrile reaction that lasts from 1 to 3 weeks. During this phase of intensified tissue reaction, the primary infectious complex may become visible on a chest radiograph. Massive lymphohematogenous dissemination leading to meningitis, miliary, or disseminated disease occurs in 0.5–2% of infected children, usually no later than 2–6 months after infection. Clinically significant lymph node or endobronchial TB usually appears within 3–9 months. Lesions of the bones and joints usually take at least a year to develop; renal lesions may be evident 5–25 years after infection. In general, intrathoracic complications of the primary infection occur within the first year. Reactivation of TB is rare in infants and young children and, among adolescents, it affects females twice as often as males for unknown factors. Finally, the age of the child at acquisition of TB infection has a significant effect on the occurrence of both primary and reactivated TB.

However, if young children do not suffer early complications, their risk of developing reactivated TB later in life is low. Conversely, older children and adolescents rarely experience complications of the primary infection but have a much higher risk of developing reactivated pulmonary TB as an adolescent or adult. Although protective immunity to TB in children is incompletely understood, several key attributes have been identified. As evidenced by children with underlying immunodeficiency, immune control of mycobacteria is dependent upon cell-mediated immunity (*Mtb*-specific T lymphocytes, dendritic cells, Toll-like receptors, γ-interferon (IFNγ), Tumor Necrosis Factor-α (TNFα), and interleukin), as well as macrophages and neutrophils [37]. Additionally, there is a distinctive risk profile of TB among children as younger children, and adolescents are at higher risk of progressing from infection to disease than children between 5 and 10 years old. As immune maturation proceeds, the risk for progressing to disease decreases.

HIV is a significant risk factor for the development of TB disease. There are limited data on incidence rates of TB in HIV-infected children, and rates vary significantly depending on the prevalence of HIV and TB in the community [38]. A study conducted in South Africa demonstrated that the incidence of TB in HIV-infected children was 42 times higher than in HIV-uninfected children [39].

3.1.3. Mitochondrial changes in TB infection

Necrosis (involving cell swelling and lysis of plasma membranes) often coexists with apoptosis and both types of cell death are simultaneously observed in many infections. Although apoptosis of *Mtb*-infected macrophages is associated with diminution of the infection, preponderance of necrosis has been associated with increased bacterial growth [23,24,32]. There has been some indication that the condition of the mitochondria is the branch point leading either to necrosis or to apoptosis and Ca^{2+} acts as an intracellular messenger involved in cell death [23,24,32].

There are two main strains of *Mtb: Mtb* H37Ra (avirulent) or *Mtb* H37Rv (virulent), and infection of macrophages with H37Ra or H37Rv causes OMM permeabilization, characterized by CytC release [40]. Experiments with *Mtb* H37Rv suggest that these pathogens are able to override the detrimental effects of apoptosis by inducing necrosis, which results in uncontrolled mycobacterial growth. Mycobacteria have apoptotic as well as anti-apoptotic properties, and these seemingly contradictory findings have been attributed to the different virulence of *Mtb* strains [26]. The balance between proapototic and antiapoptotic proteins determines the flow rate of ions and water and, in consequence, the integrity of mitochondrial membranes [26,40].

An increase in intracellular Ca^{2+} protects mitochondria from irreversible *Mtb*-derived damage, promotes apoptosis, and inhibits alveolar macrophage necrosis and mycobacterial survival [41]. The effect of Ca^{2+} depletion on the mitochondria themselves is of critical importance leading to disruption of the $\Delta\psi m$ and therefore the necrosis of the alveolar

macrophage [41]. Transient dissipation of Δψm 6 h after infection is essential for the induction of macrophage necrosis by *Mtb*, a mechanism that allows further dissemination of the pathogen and development of the disease [42,43].

It is also important to note that the observed mitochondrial damage is a consequence of the NAD^+ glycohydrolase activity of the TB necrotizing toxin (TNT) and does not require a direct interaction of TNT with the mitochondria, in contrast to other *Mtb* proteins associated with mitochondria [31]. TNT accounts for more than half of the loss of NAD^+ in *Mtb*-infected macrophages. Low levels of cytosolic NAD^+ directly decrease the concentration of NAD^+ in the mitochondria, ultimately compromising ETC/MRC function and reducing the H^+ gradient across the IMM. In this context, perturbations in the mitochondrial functioning of CII, CIII, and CIV among extrapulmonary gastrointestinal TB have been documented [44].

Of note, it is well known that mycobacterial infection of macrophages increases inducible nitric oxide synthase (iNOS) gene expression and, consequently, nitric oxide (NO) production. Remarkably, NO is also a potent inhibitor of cell respiration, by CIV inhibition. Blockade of respiratory chain CIV by NO, although related to mitochondrial dysfunction, may initiate a protective mitochondrial action by maintaining its Δψm, which results in prevention of apoptosis [26]. Some mycobacterial molecules involved in macrophage apoptosis have been identified [45] and are displayed, together with their main mitochondrial interactions (Table 2).

Table 2. *Mtb* molecules of direct or indirect mitochondrial interactions.

Name	Characteristics	Mechanism of Action
Cpn60.2	*Mtb* chaperone protein	• Essential for bacterial growth • Interacts with host mortalin (a mitochondrial protein that protects cells from apoptosis and is over-expressed in cancer cells). It has a strong anti-apoptotic activity dependent on its interaction with mitochondrial mortalin, for promoting *Mtb* survival [46]
LpqH	*Mtb* protein	• Triggers an intrinsic or mitochondrial apoptosis pathway, with the participation of CytC and the AIF, a previously unrecognized mechanism [45]
ESAT 6	*Mtb* protein	• A potent immunomodulator, which can induce cytolysis by disrupting mitochondrial membrane bilayers [47]
Cyclophilin D (CypD)	Mitochondrial matrix protein	• Plays a key role in necrosis by regulating the PTP • Its pharmacological inhibition in human macrophages leads to the inhibition of necrosis and reduction in *Mtb* growth • A critical checkpoint of T cell metabolism for regulating disease tolerance in TB [40]

Importantly, *Mtb* infection may induce a quiescent energy phenotype in human monocyte-derived macrophages and decelerated flux through glycolysis and the TCA cycle. *Mtb* reduces mitochondrial dependency on glucose and increases mitochondrial dependency on fatty acids, shifting this dependency from endogenous fatty acids in uninfected cells to exogenous fatty acids in infected macrophages for survival under stress conditions. *Mtb* uniquely decelerates both glycolysis and OXPHOS to enter a state of metabolic quiescence and consequently decreases the rate of ATP production of the macrophage [48].

3.1.4. TB Treatment in the Pediatric Population and Mitochondrial Involvement

Due to the common origin of mitochondria and bacteria, antibiotics are known to affect mitochondrial protein synthesis as an off-target consequence of their anti-bacterial function. In treating TB, it is of utmost importance to ensure adherence to medication to avoid potential re-infections and resistance in children. TB disease can be treated by the intake of several drugs for 6–9 months. There are 10 TB drugs currently approved by the US Food and Drug Administration (FDA). The first-line anti-TB agents isoniazid (INH), rifampin (RIF), ethambutol (EMB), and pyrazinamide (PZA) constitute the core of the treatment [49], and their associated mitochondrial dysfunction is shown (Table 3).

Table 3. Anti-TB first-line treatments, their mechanism of action, related mitochondrial dysfunction, and derived clinical adverse events.

Anti-TB Family	Characteristics	Mechanism of Action	Mitochondrial Dysfunction	Clinical Secondary Effects	Pediatric Studies
Isoniazid (INH)	• Bacteriostatic • It enters the mycobacterial cell by passive diffusion. Once inside, it acts as a prodrug and is activated by a mycobacterial enzyme, namely, KatG. • Subsequent to its activation, the generated metabolite produces ROS [50]	• It blocks the biosynthesis of mycolic acids [51]	• Mitochondrial free radical generation • Loss of mitochondrial Δψm • Affects mitochondrial PTP • Altered mitochondrial dynamics • Impairs mitochondrial biogenesis [52]	• It promotes liver injury mediated by a toxic reactive intermediate, acetylhydrazine, 2 weeks to 6 months after the initial treatment [53] • Sometimes related to psychiatric manifestations [54]	• Improving adherence to INH among children is vital in the fight to reduce world-wide TB mortality, especially in endemic areas with diagnostic difficulties [55] • Quantification of the magnitude of INH-resistant TB and variation in frequency of INH resistance associated mutations is important [56]
Rifampin (RIF)	• Bactericide from the rifamycin group • Metabolism is mainly hepatic with 13–24% of the drug excreted unchanged in the urine [57]	• Inhibits the essential *rpoB* gene product b-subunit of DNA-dependent RNA polymerase activity, acting early in transcription. Binding of the drug suppresses chain formation in RNA synthesis [57]	• Mitochondrial free radical generation • Loss of mitochondrial Δψm • Affects mitochondrial PTP • Altered mitochondrial dynamics [52]	• Induces many drug metabolizing enzymes such as CYP1A2, 2C9, 2C19, and 3A4 and therefore increases chances of liver injury caused by additional anti-TB drugs [53] • Hepatoxicity is initially characterized by an increase in plasma AST and ALT activities as well as bilirubin levels within 1–6 weeks of initiation [53]	• Among children under the age of 18 years, treatment for 4 months had similar rates of safety and efficacy but a better rate of adherence than 9 months of treatment with INH [58] • Pediatric patients were significantly more likely to complete latent TB infection treatment using RIF than with a 9-month INH regimen [59]
Ethambutol (EMB)	• Bacteriostatic, although it also shows bactericidal effects if the concentrations are high enough • Is active only in bacteria in the active multiplication phase [60,61]	• Inhibits arabinosyl transferases involved in cell-wall biosynthesis [61,62]	• Triggers disruption of mitochondrial CIV activity through a copper-chelating action [63–66] • Loss of mitochondrial Δψm • Fragmentation, disturbed calcium homeostasis, and the accumulation of abnormal intracellular vacuoles [66,67]	• Optic neuropathy • Hepatotoxicity • Pruritus • Joint pain • Gastrointestinal upset and abdominal pain • Malaise • Headache • Dizziness • Psychiatric alterations such as mental confusion, disorientation, and hallucinations [62]	• For children aged 5 years or more, EMB can be recommended at a dosage of 20 mg/kg/day for routine treatment, without taking more precautions than for adults; this should be included in official recommendations. For younger children, EMB can also be used without undue fear of side effects [68,69]

Table 3. *Cont.*

Anti-TB Family	Characteristics	Mechanism of Action	Mitochondrial Dysfunction	Clinical Secondary Effects	Pediatric Studies
Pyrazynamide (PZA)	• Fundamentally bacteriostatic, although it can also act as a bactericide [70]	• A prodrug that needs to be activated to its active form by PZA (whose mutation generates bacterial resistance) • It inhibits the FAS I system in the synthesis of mycolic bacteria mycolic acid • Activated in an acidic medium, at the tubercular necrotic edges in which the inflammatory cells produce lactic acid [70]	• Translocation of CytC from mitochondria to the cytosol, and consequent induction of the apoptotic pathway [71]	• One of the most hepatotoxic tuberculostatics	• The absorption and the clearance of PZA is slower, the elimination half-life longer and the volume of distribution higher in children compared with the reported values in the adult population [72] • PZA is distributed uniformly in the body and serum levels are related to body weight, and a dose of 30 mg/kg bodyweight is appropriate in children [73] • Lowering of PZA dosage is suggested in children for better patient compliance along with reduction in cost, side effects and toxicity without compromising its efficacy [74]

ALT, alanine aminotransferase; Anti-TB, antituberculosis; ARV, antiretrovirals; AST, aspartate aminotransferase; CI, complex I; CIII, complex III; CIV, complex IV; CytC, cytochrome C; DNA, deoxyribonucleic acid; EMB, ethambutol; ETC, electron transport chain; FAS, fatty acid synthetase; HIV/AIDS, human immunodeficiency virus infection and acquired immune deficiency syndrome; INH, isoniazid; KatG, catalase-peroxidase; NADH, nicotinamide adenine dinucleotide hydrogen; PI, protease inhibitors; PTP, permeability transition pore; PZA, pyrazynamide; RIF, rifampin; RNA, ribonucleic acid; ROS, reactive oxygen species; rpoB, beta subunit of RNA polymerase; TB, tuberculosis; $\Delta\psi m$, mitochondrial transmembrane potential.

Rifapentine (RPT) is a bactericidal first-line treatment of TB but it has not been included due to the lack of data on its association with mitochondrial dysfunction [75–79]. Furthermore, it is not currently approved in Europe. In the United States, it is only used as treatment of latent infection. There are several clinical trials ongoing.

On the other hand, among second-line anti-TB drugs, not included in Table 3, both levofloxacin and moxifloxacin have been found to impair mitochondrial function. The former inhibits activities of mitochondrial ETC CI and CIII, leading to inhibition of mitochondrial respiration and reduction in ATP production [80,81]. Moxifloxacin promotes airway smooth muscle cell apoptosis by altering mitochondrial $\Delta\Psi m$ [82]. Other second-line anti-TB drugs are aminoglycosides, cycloserine, clofazimine, ethionamide, or the more recent bedaquiline and delamanid/pretomanid and the commonly used, linezolid, for which the off-target mitochondrial effects will be briefly discussed later in the text.

Children who have been exposed to smear-positive adults with pulmonary TB but are free of symptoms, have negative immunodiagnostic TB tests (i.e., tuberculin skin test or interferon-gamma release assays -IGRA-) and a normal chest X ray are often put on INH primary chemoprophylaxis, if younger than 5 years of age or if they have other risk factors for the rapid development of the disease, such as being immunocompromised [36,83]. Failure to do so may result in rapid development of severe TB, during the incubation period. The child is treated for 8–12 weeks and the TST or IGRA is repeated; if the second test is positive, infection is documented and INH should be continued for 9 months, but if the second test is negative, TB infection is ruled out and the treatment can be stopped. All children with latent TB should also receive treatment to prevent the development of disease in the near or distant future [36]; the most common regimens are INH + RIF for 3 months, RIF for 4 months, or INH for 6–9 months. As a summary, children must receive anti-TB medication for several months.

3.1.5. Pediatric Studies of Mitochondrial Interaction in TB Infection

Difficulty in treating adults and children who have multidrug-resistant TB has led to increased interest in linezolid. Prolonged linezolid use is associated with high rates of hematologic and neurologic side effects based on inhibition of mitochondrial enzymes. In particular, the inhibition of mitochondrial protein synthesis diminishes respiratory chain enzyme content and thus limits aerobic energy production [84] without ultrastructural mitochondrial abnormalities and without mutations or depletion of mtDNA [85].

3.2. Enterobacteria

The *Enterobacteriaceae* family comprises aerobic Gram-negative rods and facultative anaerobes. They are microbiologically characterized by not forming spores, fermenting glucose, not producing oxidase, and presenting variable mobility. They present a cytoplasmic membrane, a peptidoglycan coating and a complex cell wall that includes the capsule, which contains lipopolysaccharides and channels for the penetration of antibiotics and nutrients [86,87]. Enteric pathogens are a major source of morbidity and mortality throughout the world, including in children. It has been estimated that there are more than 3 million deaths associated with Gram-negative enteric pathogens worldwide due to diarrhea and enteric fever each year. The differences in disease manifestations are related to the different virulence factors present in the bacteria and the altered phenotypes that these virulence factors allow the organisms to employ during disease pathogenesis [88].

In this review, we are focusing on *Enterobacter cloacae*, *Proteus mirabilis*, *Escherichia coli*, and *Salmonella enterica* as their mitochondrial interactions in the pediatric population are widely studied and reported in the literature.

First, *Enterobacter cloacae* can cause urinary tract and surgical wound infections, enteritis (especially in children), and even bacteremia. However, the most frequent presentations are nosocomial infections in immunocompromised patients [89], including children [90]. This pathogen presents a polysaccharide that potently inhibits cell proliferation of human osteosarcoma cells by inducing apoptosis through a loss of $\Delta\Psi m$, release of CytC from the

mitochondria into the cytosol, activation of caspase-9 and-3, cleavage of poly (ADP-ribose) polymerases (PARP), elevated ratio of Bax/Bcl-2 protein and overexpression of p53. As is known, the release of CytC from mitochondria into the cytosol is tightly regulated by Bcl-2 family proteins, which include pro-apoptotic Bax protein and anti-apoptotic Bcl-2 protein. Bax, known as a pro-apoptotic protein, is believed to have the ability to generate pores in the OMM, thus allowing the release of CytC into the cytoplasm to activate the pro-apoptotic caspase cascade. In contrast, Bcl-2, as one type of anti-apoptotic protein, inhibits CytC release, resulting in prevention of apoptosis initiation. These two apoptosis-modulating proteins are also regulated by the gene p53, the tumor suppressor gene, which functions as a cellular emergency response system to induce cell growth arrest or apoptosis via the equilibrium between Bax and Bcl-2. Polysaccharide from *E. cloacae* induces apoptosis in osteosarcoma cells via alteration of Bax, Bcl-2, and p53 protein expression [91].

Second, *Proteus mirabilis* is a common cause of urinary tract infection in childhood, after *E. Coli* [92]. This pathogen has been related to pathological changes in the mitochondria, in particular, intra-mitochondrial crystals. This is due to the large stores of calcium in the mitochondria and the endoplasmic reticulum. When the pH level increases greater than 7.3 and the solubility of calcium phosphate is exceeded, calcium precipitation occurs. Activity and mechanical damage by crystals eventually leads to nuclear destruction and disruption of calcium levels. In infected cells, mitochondrial swelling, inflammation, and destruction is visible, suggesting apoptosis involving ROS, CytC, caspase-9, and caspase-3 [93].

Third, *Escherichia coli* is a main causative agent of urinary tract infections worldwide and of childhood diarrhea in developing countries. The EspF effector protein is the product of the espF gene found at the enterocyte effacement site, the key pathogenicity island carried by enteropathogenic *E. coli* and enterohemorrhagic *E. coli.* Importantly, EspF, whose N-terminus is a mitochondrial targeting signal, plays a role in the permeabilization of the mitochondrial membrane induced by enteropathogenic *E. coli* infection. Furthermore, EspF is associated with the release of CytC from the mitochondria to cytoplasm and cleavage of caspase-9 and caspase-3. These findings indicate a role for EspF in initiating the mitochondrial death pathway, thus suggesting that intracellular EspF is targeted to mitochondria. Mitochondrial proteins often reside in an N-terminal mitochondrial targeting signal that can be cleaved by a specific peptidase after import into the mitochondrial matrix [94].

Fourth, *Salmonella enterica* is one of the top four causes of diarrheal disease. Most cases of salmonellosis are mild, although they can sometimes be fatal. Every year 550 million people get sick, of whom 220 million are children under the age of 5 [95]. There are a few studies mentioning mitochondrial changes in children with salmonellosis, although interestingly and related to therapeutic approaches, mitochondrial components of monocytes from young children with salmonellosis (6 months to 2 years), have been assessed in order to test the effectiveness of low intensity lasers on functional cell status. These have reported normalization of mitochondrial components and improvement of clinical symptoms [96].

Pediatric Studies of Mitochondrial Interaction in Enterobacteria

From the Enterobacteriaceae family, *E. cloacae*, *P. mirabilis*, *E. Coli*, or *S. enterica* have the same mitochondrial influence, as the pathogens are associated with loss of $\Delta\Psi m$, release of CytC, and activation of caspase-3 and -9 [92,94,95]. Most of the aforementioned alterations are due to the precipitation of intra-mitochondrial calcium and the increase in ROS, as reported in children [93].

3.3. Staphylococcus aureus

Staphylococcus is a group of bacteria that includes 30 types, from which *Staphylococcus aureus* (*S. aureus*) is the principal cause of staph infections. *S. aureus* is a Gram-positive member of the *Firmicutes* phylum of the *Micrococcaceae* family within the Bacillus class [97]. Humans are a natural reservoir of *S. aureus*. Between 30% and 50% of healthy adults are colonized, and between 10% and 20% permanently colonized [97,98]. *S. aureus* is a commensal organism and also an important opportunistic human pathogen, causing a

variety of community and hospital-associated pathologies, including bacteremia-sepsis, endocarditis, pneumonia, osteomyelitis, arthritis, and skin diseases [97,99]. Under normal conditions, *S. aureus* does not cause infections, this only occurs in immunocompromised patients in whom the persistence of the bacteria in the host leads to disease risks [100]. Pathogenic infections are generally initiated by penetration of skin or mucosal barriers, allowing bacteria to access adjacent tissues or the bloodstream [101]. The release of toxins derived from *S. aureus* into the skin and other organs can cause various types of skin rashes and general symptoms, as in the case of toxic shock syndrome or acute diarrheal disease.

The urgency of *S. aureus* over the past decade in many settings has been facilitated not only by mechanisms of resistance to bacterial antibiotics but also by the emergence of new clonal types of *S. aureus* with increased expression of virulence factors and their ability to neutralize the host's immune response [97,100]. Prevention of the spread of *S. aureus* infection is based on the use of adequate contact precautions and infection control procedures that have, so far, not been fully effective [101].

3.3.1. Structure and Replication Cycle

The genus Staphylococcus is formed by Gram-positive cocci, with a diameter of 0.5–1.5 μm, grouped as single cells, in pairs, tetrads, short chains, or forming bunches of grapes. They are non-mobile bacteria, not sporulated, mostly lacking capsule, although there are some strains that develop a slime capsule, and are facultative anaerobes. Most staphylococci produce catalase, the enzyme capable of splitting hydrogen peroxide into water and free oxygen; a main feature used to differentiate the *Staphylococcus* genus from the *Streptococcus* and *Enterococcus* genera, which are catalase negative [100]. The cell wall of staphylococci has teichoic acids attached, which do not exist in the micrococci and, finally, another difference is the composition of the cytochrome and menaquinone of the respiratory chain present in staphylococci [100].

S. aureus is widely distributed among primates but is not restricted to them exclusively, e.g., it produces mastitis in cattle and sheep. In humans, the nasal location of *S. aureus* allows its spread and, as a consequence, the spread of its multi-resistance to antibiotics such as methicillin (MRSA) [100].

The complex physiology of *S. aureus* makes the organism highly versatile, adaptable, and capable of resisting many host defense mechanisms [99]. The pathogenicity of infections by *S. aureus* is related to various components of the bacterial surface; in general, peptidoglycans and teichoic acids, in addition to protein A. Thus, the pathogenesis caused by this microorganism arises when there exists a combination of these virulence factors with decreased defenses of the host [102]. These conditions favor *S. aureus*, allowing it to present important virulence characteristics resulting in particular damage.

Patients with *S. aureus* infections often become infected with the same strain that colonizes their nostrils; colonization also allows transmission between individuals in the hospital and in the community. For an adequate survival and invasion of the host, this whole system of virulence factors must form part of a cell–cell communication system, mediated by proteins produced by the bacteria and depending on environmental factors. This system can activate a large number of genes coding for virulence factors. *S. aureus* has proteins on its surface known for inhibiting phagocytosis and opsonization by the human complement system. The recognition by complement and immunoglobulins of receptors are blocked by protein A, on the cell wall, which binds to a certain portion of the IgG immunoglobulin. *S. aureus* also produces molecules that can inhibit neutrophil recruitment, phagocytosis, and bacterial recognition [100]. Perhaps one of the most salient features of *S. aureus* is its ability to produce a variety of toxins that are targeted at human blood cells. These toxins include hemolysin-α, hemolysin-β and hemolysin-γ, Panton–Valentine leukocidin, and phenol-soluble β-modulin, which have been recently discovered [100,103].

3.3.2. *Staphylococcus aureus* in the Pediatric Population

Even though *S. aureus* is known to be a common cause of bacteremia in children leading to significant morbidity and mortality, there are quite few studies of *S. aureus* in pediatrics. Little is known about the frequency of nasal colonization by MRSA in young children, but in some cohorts 17.5–18.1% of the children were colonized with *S. aureus* and 1.3% with MRSA, and the bacteremia case-fatality ratio has been described as high as 14.1%, in some developing populations [104,105]. MRSA infection has recently become a difficult problem worldwide, which requires special attention in pediatrics. Strain characteristics of pediatric MRSA-infected children have been assessed through whole genome sequencing, describing a particular tropism of a specific clone in pediatric patients and while susceptible to fluoroquinolones and tetracyclines, these should be prescribed in children with caution [106].

3.3.3. Mitochondrial Changes in *S. aureus* Infection

S. aureus promotes mitochondrial alterations by a panoply of interactions with the organelle, as described below. *S. aureus* infection activates mitochondrial changes when, subsequently to phagocytosis of the bacterium, caspase-8 is activated followed by the progressive interruption of $\Delta\psi m$ [107], ultimately leading to the production of ROS. Mitochondrial ROS generation has been shown to drive bactericidal macrophage activity against MRSA [108]. Of note, the reversible caspase-8 inhibitor, IETD-FMK, prevents disruption of $\Delta\psi m$ and prevents CytC release from monocytes exposed to *S. aureus* [109].

Another example of mitochondrial interactions occurs during acute infection of lung lesions, in which *S. aureus* activates a ubiquitin E3 ligase component, the so-called Fbxo15, which mediates proteasomal degradation in epithelia, resulting in decreased availability of cardiolipin, the main lipid in the IMM, and altered mitochondrial function [110]. Importantly, *S. aureus* secretes a pore-forming toxin, Panton–Valentine leucocidin, that has recently been associated with necrotizing pneumonia. Panton–Valentine leucocidin-induced apoptosis has been associated with rapid disruption of mitochondrial homeostasis and activation of caspase-9 and caspase-3, suggesting that this induced apoptosis is preferentially mediated by the mitochondrial pathway [111–113]. Finally, MRSA actively prevents the recruitment of mitochondria to the area around the vacuoles in which the bacteria reside to prevent intracellular death, prompted by caspase-11, allowing for the survival of MRSA within macrophages [114].

3.3.4. *S. aureus* Treatment in the Pediatric Population and Mitochondrial Involvement

Invasive *S. aureus* infections are a leading cause of morbidity and mortality in both hospital and community settings, especially with the widespread emergence of virulent and resistant *S. aureus* strains [115,116]. The clinical use of methicillin has led to the appearance of MRSA [117]. However, there are other antibiotics available to treat this infection [118]. Again, taking into account the common origin of mitochondria and bacteria, antibiotics are known to affect mitochondrial protein synthesis. Anti-*S. Aureus* drugs along with the mitochondrial damage related to their administration are depicted below (Table 4).

Table 4. Antibiotic therapy of *S. aureus* infection, mechanism of action, associated mitochondrial damage, and relevant pediatric studies.

Drug	Antibiotic Type	Mechanism of Action	Mitochondrial Damage	Mitochondrial Interactions and Pediatric Studies
Gentamicin	Aminoglycoside	• Bactericide • Penetrates the bacteria and binds to the 30S and 50S ribosomal subunits, inhibiting protein synthesis [108]	• Increases lactate production and inhibits Δψm [119] • Induces mitochondrial ROS causing DNA damage [120,121]	• Aminoglycoside antibiotics, in particular gentamicin and tobramycin, are still used in pediatric clinical practice. Acute kidney injury may occur in between 20% and 33% of children exposed to aminoglycosides. Cytoplasmic aminoglycoside then acts both directly and indirectly on the mitochondria, activating the intrinsic pathway of apoptosis via CytC, which, in turn, leads to the disruption of electron transport and ATP production and the formation of ROS [122]
Linezolid	2-oxazolidone	• Bacteriostatic • Inhibits bacterial protein synthesis by binding to 23S rRNA in the large ribosomal subunit and preventing the fusion of 30S and 50S ribosomal subunits and the formation of the translation initiation complex [123,124]	• Inhibition of protein synthesis in mitochondria [123–125] • Decrease in mitochondria-derived CIV impairs cellular OXPHOS and increases glycolysis [126] • Deficient CV activity, which may be additive or synergistic in contributing to mitochondrial dysfunction and production of lactic acidosis [123,124,126,127] • Mitochondrial dysfunction may lead to dysregulation of insulin secretion from pancreatic beta-cells, causing systemic hypoglycemia [125]	• Linezolid-associated lactic acidosis by means of depressed mitochondrial CIV activity. Linezolid administration has been associated with lactic acidosis in adults; however, the same phenomenon has not been reported in children [125]
Doxycycline	Tetracycline	• Bacteriostatic • Inhibits bacterial protein synthesis by binding to the 30S ribosomal subunit [127]	• Disrupts mitochondrial functions by decreasing Δψm and mitochondrial respiration. • Decreases levels of ATP and the elevated levels of mitochondrial superoxide, intracellular ROS, protein carbonylation, and lipid peroxidation [128] • Inhibits mitochondrial protein synthesis by reducing oxygen and increasing glucose consumption [129]	• Doxycycline is a tetracycline-class antimicrobial for children >8 years of age for many common childhood infections. Doxycycline is not labeled for children $\leq$8 years of age, due to the association between tetracycline-class antibiotics and tooth staining, although doxycycline may be used off-label in severe conditions [130]

Table 4. *Cont.*

Drug	Antibiotic Type	Mechanism of Action	Mitochondrial Damage	Mitochondrial Interactions and Pediatric Studies
Tigecycline	Tetracycline	• Bacteriostatic • Inhibits protein translation in bacteria by binding to the 30S ribosomal subunit [131]	• Inhibits mitochondrial OXPHOS • Inhibits mitochondrial translation possibly by interacting with mitochondrial ribosome • Deceases in mitochondrial respiration is the primary effect, while increased glycolysis flux is the secondary effect • Induces mitochondrial ROS [132,133]	• N/A
Vancomycin	Glycopeptide	• Bactericide • Inhibits biosynthesis of the bacterial cell wall, interferes with RNA synthesis, and damages the bacterial cell membrane [134]	• Inhibits CI activity • Causes apoptotic cell death by enhancing mitochondrial superoxide production leading to mitochondrial membrane depolarization followed by the caspase activities [135]	• Higher vancomycin use does not improve outcomes in pediatric healthcare-associated *S. aureus* bacteremia but is associated with increased nephrotoxicity [136]. Vancomycin dosing strategies in pediatric patients should consider age and weight as well as renal function and indication [137].

ATP, adenosine triphosphate; CI, complex I; CIV, complex IV; CV, V complex; CytC, cytochrome C; DNA, deoxyribonucleic acid; OXPHOS, oxidative phosphorylation system; PTP, permeability transition pore; RNA, ribonucleic acid; ROS, reactive oxygen species; rRNA, ribosomal ribonucleic acid; *S. aureus, Staphylococcus aureus* and Δψm, mitochondrial transmembrane potential. Note: rifampin is also a therapeutic option that has not been included in Table 4, since it has been considered in Table 3. Quinolones, cotrimoxazole, and teicoplanin are also common treatments of pediatric TB but have not been included in this table due to limited information on their mitochondrial effects.

Of note, the bactericides oxacillin, cloxacillin, dicloxacillin, and nafcillin, belonging to the beta-lactam family, which prevent the formation of the bacterial cell wall [109,110] as well as the bacteriostatic clindamycin, belonging to the lincosamide family, have not been considered in the table due to the lack of pediatric studies (neither has clindamycin been associated with mitochondria in the literature). However, it should be mentioned that the former promote mitochondrial oxidative impairment through ROS overproduction [138] via the disruption of the TCA cycle and ETC [139], as well as decrease $\Delta\psi m$ [139]. Daptomycin is another antibiotic from the lipopeptic family, which has been demonstrated to present high clinical success rates against a wide variety of infections and is well tolerated in children and adolescents [140]. It has been excluded from Table 4 due to the lack of information on its mitochondrial impact.

In general, antibiotics in the beta-lactam family are the first choice for the treatment of methicillin-sensitive infections. Vancomycin has been used for decades for the antibiotic treatment of methicillin resistance [101].

3.3.5. Pediatric Studies of Mitochondrial Interactions in *S. aureus* Infection

High risk groups for *S. aureus* infection include not only patients undergoing surgical procedures and individuals undergoing immunosuppressive or cancer therapy but also infants with low birth weight and young children. Occasionally, it has been observed that people who have *S. aureus* infection have Panton-Valentine leukocidin localized to the mitochondrial membrane, inducing mitochondrial apoptosis [116]. Bacterial sepsis induces mitochondrial injury resulting in depressed metabolism, while biogenesis restores mitochondrial content and function [141].

3.4. Meningitis

Meningitis is an infection characterized by inflammation of the meninges that in 80% of cases is caused by viruses, in 15–20% of cases, it is caused by bacteria and in the rest of cases, it is due to poisoning, fungi, medications, and other diseases. Here, we classify meningitis according to the causative agent [142] and relevance in the pediatric population [143] (Table 5).

Table 5. Different causal pathogens of meningitis.

Meningitis Type		Microorganisms Responsible
Bacterial meningitis	In newborns and young infants (<3 months)	Group B *Streptococcus* *E. coli* *Listeria monocytogenes*
	In infants (>3 months) and children	*Hemophilus influenzae* type b * *Neisseria meningitidis* *Streptococcus pneumoniae*
	Others	Syphilis TB
Viral meningitis		Poliovirus * Enterovirus (e.g., coxsackie virus and echovirus) Mumps (paramyxovirus) * Herpes Simplex Virus (HSV)
Other organisms that can also cause meningitis		*Borrelia burgdorferi* (Lyme disease) Fungi such as candida, *Aspergillus*, or *Cryptococcus neoformans* in immunosuppressed patients

* uncommon due to vaccination campaigns.

The microorganisms that cause 95% of the cases of bacterial meningitis, i.e., *Neisseria meningitidis (N. meningitidis*, meningococcus) and *Streptococcus pneumoniae (S. pneumoniae, pneumococcus*), are habitual residents of the nasopharynx and oropharynx sites, where they

do not normally cause damage. However, for unknown reasons, they can eventually pass into the blood reaching and colonizing the meninges [144]. Herein, we review the potential mitochondrial interactions of the two most relevant pathogens for meningitis in pediatrics, *N. meningitidis* and *S. pneumoniae*.

N. meningitidis is a Gram-negative anaerobic pathogen belonging to the β subgroup of proteobacteria that colonizes the nasopharynx in up to 35% of healthy people. They only colonize human hosts with no other known reservoirs [145]. It infects 500,000 to 1.2 million people and kills between 50,000 and 135,000 per year [146–148] causing significant morbidity and mortality in children and young adults worldwide through epidemic or sporadic meningitis and/or sepsis [146]. On the contrary, *S. pneumoniae* is a spherical, facultative anaerobic member of the genus Streptococcus, which is part of the normal upper respiratory tract flora and the second most common cause of meningitis in children older than 2 years [149]. Both pathogens are usually found in pairs (diplococci) [150,151].

Direct transmission of both meningococci and pneumococci occurs by sharing respiratory and throat secretions (saliva or spit) [145,152] and, in exceptional cases, can be a cause of neonatal infections. The most frequent symptoms associated with meningitis by both pathogens are headache, neck stiffness, fever, photophobia or phonophobia, and altered consciousness [153]. Often, particularly in young children, only nonspecific symptoms such as irritability and drowsiness occur. The existence of a skin rash is common in meningococcemia [154]. Bulging of the anterior fontanel can also occur in infants [155].

Infections due to *N. meningitidis* can cause sepsis and meningitis once it reaches the bloodstream and nervous system, respectively, [156] and present as a spectrum of clinical disease, with meningitis and septicemia being the most common, but it also includes pneumonia, septic arthritis, pericarditis, conjunctivitis, and urethritis [148]. Infections due to *S. pneumoniae* mainly cause pneumonia and otitis media, but also other diseases and symptoms including pneumococcal meningitis, fever and chills, cough, rapid breathing, difficulty breathing, chest pain, and sepsis [157].

There are 13 serogroups of *N. meningitidis* based on different capsular polysaccharide structures, but only six of them (A, B, C, W-135, X, and Y) cause the majority of life-threatening disease. During some epidemics, the incidence increases in older children and adults [147]. In endemic situations, serogroup B is more common in infants, serogroup C in adolescents, and serogroups B or Y in older adults, although this depends on the geographical zone. Serogroup A carriage has been observed as the most prevalent in older children and young adults in African cohorts [147]. All age groups are at risk of invasive meningococcal disease, but infants and adolescents are particularly vulnerable due to the disappearance of maternal antibodies early in life and the high rate of nasopharyngeal colonization [155].

There are more than 90 known serotypes of *S. pneumoniae*, but only 12 (1, 3, 4, 5, 6, 7, 8, 9,14, 18, 19, and 23) present the highest clinical impact, responsible for >80% of invasive pneumococcal infections [158]. Specifically, serotypes: 6, 9, 14, 18, and 23 [159] are the ones that most often cause meningitis. Resistance to antibiotics has been mainly associated with serotypes 6, 14, 19, and 23, and these are the most frequently isolated serotypes in children under 2 years of age are associated with prolonged carrier states, and are easily reacquired [160].

Meningitis progresses very rapidly, so early diagnosis and early treatment are important to prevent serious sequelae and death [161]. Death occurs in 6–10% of cases and sequelae in 4.3–11.2% of cases.

3.4.1. Structure and Replication Cycle

The infection may be acquired when these micro-organisms residing in the normal nasopharynx and oropharynx habitat end up colonizing the meninges through the blood, through nearby injury (after fractures, fissures, and lumbar punctures, among others) or through contiguous spread from a nearby suppurative focus [144].

The expression of the capsule polysaccharide plays a key role in meningococcal pathogenesis [148]. The virulence of *N. meningitidis* is influenced by multiple factors, such as capsule polysaccharide expression, surface adhesive protein expression (outer membrane proteins including pili, PorA and B porins, Opa and Opc adhesion molecules), sequestration mechanisms and endotoxin production (lipooligosaccharide, LOS) [146]. Once meningococci penetrate the mucosal barrier of the upper respiratory tract and adhere to human epithelial cells, a series of interactions take place, resulting in an effect on the epithelial surface and the formation of microcolonies. Viable meningococci can be phagocytosed by respiratory or non-ciliated epithelial cells or escape, thus reaching the submucosa or directly invading damaged epithelial surfaces [147].

The pathogenicity of *S. pneumoniae* is characterized by its capsule, which is essential due to its ability to block opsonization through the complement system, and phagocytosis by cells of the immune system; along with the wall, made up of a network of peptidoglycan chains, lipids, and teichoic acids. They play an important role in the processes of colonization, adherence, inflammation, and bacterial invasion [162,163].

Once in the bloodstream, bacteria enter the subarachnoid space at places where the blood–brain barrier is vulnerable, such as the choroid plexus. The large-scale inflammation that occurs in the subarachnoid space during meningitis is not a direct result of bacterial infection but can largely be attributed to the response of the immune system to the entry of bacteria into the central nervous system. When the components of the bacterial cell membrane are identified by the cells of the brain's immune system (astrocytes and microglia), they respond with the release of large amounts of cytokines. The blood–brain barrier presents changes, leading to "vasogenic" cerebral edema (swelling of the brain due to leakage of fluid from the blood vessels). Large numbers of leukocytes entering the cerebrospinal fluid cause inflammation of the meninges and lead to interstitial edema (edema due to intercellular fluid). In addition, the walls of the blood vessels themselves become swollen (cerebral vasculitis), leading to decreased blood flow and a third type of edema, the so-called cytotoxic edema [159].

3.4.2. Meningitis in the Pediatric Population

In regards of meningococcal disease, the highest incidence of the disease occurs in childhood and the most susceptible children are from 6 to 24 months, in whom passively transferred maternal antibodies have already disappeared [164]. Despite the advances in the treatment of this disease, mortality in the United States is as high as 8% with neurological sequelae observed in up to 31% of cases [165].

3.4.3. Mitochondrial Changes Derived from Meningitis Pathogens

In regards of *N. meningitidis*, the virulence factor NHBA binds heparin through a conserved region rich in arginine that is the target of two proteases, the meningococcal NalP and human lactoferrin, responsible for the binding of heparin and heparan sulfate. Binding to heparin improves the survival of the bacterium in human serum, while binding to heparan sulfate has often been linked to the ability to bind and invade host cells; heparan sulfate is produced as a proteoglycan on the surface of many types of cells [155,156].

The NHBA can be cleaved by NalP, generating a fragment called C2 that maintains an arginine-rich domain or on the other hand, it can be cleaved by human lactoferrin, generating the fragment called C1, in which the arginine-rich domain is absent. Both fragments are released from the bacteria into the surrounding environment. Since the C2 fragment retains the domain responsible for binding to heparin, it is plausible that it exercises its own biological role. The C2 glue accumulates rapidly in the mitochondria where it induces the production of ROS. This is necessary for phosphorylation of the binding protein and for its internalization, which, in turn, is responsible for endothelial leakage/permeability [156]. Therefore, the integrity of the endothelial barrier can be disturbed by ROS, which worsens the pathological conditions in the presence of the bacteria.

On the other hand, *S. pneumoniae* is the most common and aggressive cause of bacterial meningitis and induces a novel AIF–dependent form of brain cell apoptosis. Specifically, two pneumococcal toxins, pneumolysin and H_2O_2, produce mitochondrial damage and apoptosis. Both toxins induce an increase in intracellular Ca^{2+} and trigger the release of AIF from mitochondria [166,167].

3.4.4. Meningitis Treatment in the Pediatric Population and Mitochondrial Involvement

The treatment of meningitis in the pediatric population depends on the causative agent. For instance, the inclusion of conjugated vaccines against *H. influenzae* type b; meningococcal serogroup B, C, ACWY; and pneumococcus in systematic vaccination schedules has caused a significant decrease in the incidence of these diseases. For bacterial meningitis, different antibiotics, with subsequent mitochondrial associated changes, are available for treatment (Table 6). As previously depicted, due to the common origin of mitochondria and bacteria, antibiotics are known to affect mitochondrial protein synthesis as an off-target consequence of their anti-bacterial function. Specific mitochondrial interactions derived from these antibiotics are provided (Table 6).

Table 6. Antibiotic therapy against bacterial meningitis infection and associated mitochondrial effects.

Drug	Antibiotic Type	Mechanism of Action	Mitochondrial Damage	Pediatric Studies
Ampicillin	Betalactamic	• Bactericide • Inhibits the synthesis and repair of the bacterial wall [168]	• Promotes ROS overproduction [138]	Ampicillin is a "broad-spectrum penicillin" used as therapy for suspected bacterial meningitis. Penetrates the blood–brain barrier sufficiently with an adequate dose and inflamed meninges. This drug revolutionized the treatment of bacterial meningitis in children [169,170]
Cefotaxime	Cephalosporin (also Betalactamic)	• Bactericide • Inhibits bacterial cell wall synthesis [171]	• Apoptosis [172].	High-dose cefotaxime, while safe, is not reliably sufficient therapy for cephalosporin-non-susceptible pneumococcal meningitis, and combination therapy is recommended in children [173]
Ceftriaxone		• Bactericide • Broad spectrum and long acting • Inhibition of cell wall synthesis [174]	• Decreases $\Delta\psi m$ [175] • Reduces Ca^{2+} influx [176]	Ceftriaxone, widely used in children in the treatment of sepsis, is not reliably sufficient therapy for cephalosporin-non-susceptible pneumococcal meningitis [177]

Ca^{2+}, calcium ion; CytC, cytochrome C; DNA, deoxyribonucleic acid; ROS, reactive oxygen species and $\Delta\psi m$, mitochondrial transmembrane potential.

In epidemic conditions in areas of Africa with limited resources or poor health infrastructure, the drug of choice is ceftriaxone [150,178,179]. In children with bacterial meningitis caused by pneumococci or *H. influenzae* type B, adjuvant treatment with dexamethasone significantly reduces the risk of sensorineural hearing loss and ataxia [178].

In regards of *N. meningitidis*, there are three types of vaccine, some of which have been available for more than 30 years. Vaccines can be monovalent (group C), bivalent (groups A and C), trivalent (groups A, C, and W), or tetravalent (groups A, C, Y, and W135). The first vaccine against group B has been recently developed and combines 4 protein components [148,179]. This vaccine, named "Bexsero" and "Trumemba," specifically

targets a surface-exposed lipoprotein ubiquitously expressed by *N. meningitidis* strains, the so-called Neisseria heparin-binding antigen (NHBA).

In the case of *S. pneumoniae*, several vaccines have been developed. The WHO recommends routine childhood pneumococcal vaccination. Thus, it is incorporated into the childhood immunization schedule, along with the meningococcal conjugate vaccine, in a number of countries [180].

3.4.5. Pediatric Studies of Mitochondrial Interaction in Meningitis Infection

In meningitis, brain cells produce cytokines, chemokines, and other pro-inflammatory molecules in response to bacterial stimuli, and polymorphonuclear leukocytes are attracted and activated and release large amounts of O^{2-} and NO, leading to $ONOO^{-}$ formation generating oxidative stress. This cascade leads to lipid peroxidation, mitochondrial damage, and breakdown of the blood–brain barrier, thus contributing to cell injury during neonatal meningitis [181]. Nitric oxide, which is a specific inhibitor of CIV MRC, is very likely to play a role in the physiopathological mechanisms of bacterial meningitis in children. As shown in in vitro studies, NO is toxic to endothelial cells, as well as to neurons and, thus, may be responsible for neurological sequelae in bacterial meningitis. Increased levels of NO can also inhibit mitochondrial respiration, enhancing anaerobic glycolysis [182].

4. Parasitic Infectious Processes and Mitochondrial Involvement

4.1. Malaria

Malaria is one of the three major global infectious health threats [183] causing approximately 2.7 million deaths per year, mainly among young children under the age of 5 [184]. The disease is caused by *Plasmodium* parasites, and mosquitoes are essential for the spread of the disease [185]. There are different malarial parasites, including *Plasmodium falciparum, Plasmodium malariae, Plasmodium brasilianum, Plasmodium inui, Plasmodium vivax, Plasmodium ovale, Plasmodium cynomolgi, and Plasmodium bergei* [186]. Of these species, *P vivax* and *P falciparum* cause 95% of infections [187,188], although the latter is the main cause of malaria in humans [188].

Symptoms include fever, headaches, and vomiting, and they appear 10–15 days after the bite from a mosquito carrying the parasite. In general, malaria causes hemolysis and alters the contribution of blood to vital organs, putting the patient's life in danger [189].

Severe or complicated malaria occurs in most cases by *P. falciparum* and *P. knowlesi*. It presents with an alteration of consciousness, delirium, seizures or coma (cerebral malaria), extreme prostration, inability to feed, respiratory distress, circulatory collapse, jaundice, muco-cutaneous hemorrhages (petechiae), and acute kidney failure (rare in children). Regarding hematological alterations, there is severe anemia (Hb < 5 gr%) due to acute intravascular hemolysis (sometimes triggering hemoglobinuria) and hypersplenism (common in children and predominantly those under 2 years), secondary hyperbilirubinemia, thrombopenia, and disseminated intravascular coagulation. Lactic acidosis and severe hypoglycemia (blood glucose < 40 mg/dL) also occur due to increased consumption of glucose by the parasite and liver disease. Children with severe malaria should be hospitalized, since it is fatal in most cases without specific treatment and in spite of treatment still reaches mortality rates of 10–20% [190].

4.1.1. Structure and Replication Cycle

Every patient infected with malaria (regardless of whether or not they show symptoms) has the parasite go through the exact same life-cycle, morphological changes, and human–parasite interactions [186]. From the time of the mosquito bite to approximately 1 week later, the patient remains asymptomatic [188]. The replication cycle is highly complex and has been represented to facilitate understanding (Figure 5). Specifically, the vector is the female mosquito, of the *Anopheles* genus and the *Culicidae* family, which carries *Plasmodium* in its salivary glands so that after the mosquito's bite, the sporozoites enter the circulation. In approximately 60 min, the sporozoites are transported through the blood

to hepatocytes in the liver, thereby initiating the exo-red cell cycle. There, they rapidly multiply within hepatocytes by multiple asexual division and transform into merozoites that enter the bloodstream. Once these merozoites leave the liver, they invade the red blood cells, thus initiating the erythrocytic cycle. As the nucleus begins to divide, the trophozoite is now called a developing schizont. The mature schizont contains merozoites that are released into the bloodstream. Although many merozoites are destroyed by the immune system, others immediately invade red blood cells, in which a new cycle of erythrocytic schizogony begins [187].

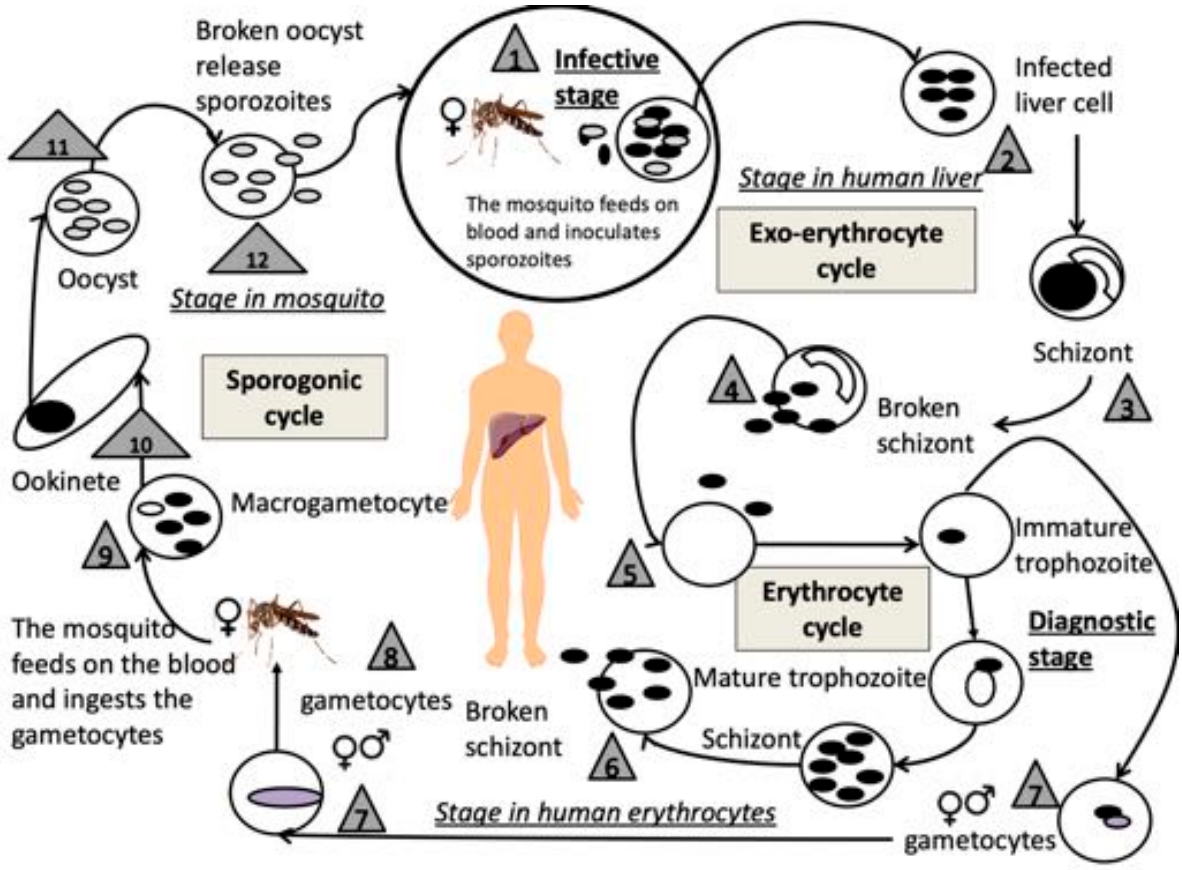

Figure 5. Replication stages in malaria infection. The sporozoites enter the circulation after the mosquito's bite. They are then transported through the blood to hepatocytes in the liver, initiating the exo-red cell cycle. There, they rapidly multiply within hepatocytes by multiple cycles of asexual division and transform into merozoites that enter the bloodstream and leave the liver. Merozoites invade red blood cells, initiating the erythrocytic cycle. As the nucleus begins to divide, the trophozoite is now called a developing schizont. The mature schizont contains merozoites that are released into the bloodstream. Although many merozoites are destroyed by the immune system, others immediately invade red blood cells, in which a new cycle of erythrocytic schizogony begins. After several generations of erythrocytes, male and female gametocytes develop from some merozoites (sexual cycle). With the union of the gametes, the egg is generated in the mosquito's intestine. The egg is mobile and will give rise to an oocyst that will divide again and give sporozoites.

After several generations of erythrocytes, male and female gametocytes are developed from some merozoites. When an uninfected female Anopheles bites a patient and acquires gametocytes, the Plasmodium sexual cycle begins. With the union of the gametes, the egg is generated in the mosquito's intestine. The egg is mobile and will give rise to an oocyst that will divide again and give sporozoites ready to infect again, upon reaching the mosquito's salivary glands [187].

Plasmodium parasites are eukaryotes and therefore contain mitochondria, since they live in aerobic hosts [185]. Most parasites do not use the oxygen available within the host to generate ATP, but instead use anaerobic metabolic pathway systems [191]. Furthermore, all

parasites have their own life cycle and, in many cases, use aerobic metabolism during "their free life stage" outside the host. Plasmodium depends mainly on monocytosolic glycolysis but not on OXPHOS, although this is essential for its survival. For this reason, there is very little complete oxidation of glucose nor a subsequent increase in by-products such as lactate and pyruvate [192]. The mitochondria of the malaria parasite *Plasmodium falciparum* are morphologically different between the asexual and sexual blood stages (gametocytes). However, Plasmodium has all the genes necessary for TCA cycle and the existence of CI, CII, CII, and CIV suggests that the biochemically active ETC operates in this parasite. There is also an alternative branching pathway for electron transport, which includes an anaerobic function of CII [188,193]. Some of the functions of the mitochondria in the parasite include the coordination of pyrimidine biosynthesis, the ETC, and the utilization of oxygen through dihydroorotate dehydrogenase and CoQ [188].

4.1.2. Malaria in the Pediatric Population

Every 2 min, a child dies from malaria and every year more than 200 million new cases are estimated to occur in children [194]. Although some countries have drastically reduced the number of cases and deaths since 2000, in recent years, there has been a stagnation in reduction and, worryingly, malaria is progressing in some countries. Children under the age of 5 are the most vulnerable group; in 2018, they accounted for 67% (272,000) of all malaria deaths worldwide [194]. Furthermore, malaria is one of the most severe and life-threatening infections affecting international pediatric travelers, and it is more frequent in child travelers who return to their countries of origin (often less developed than their country of residence) for the purpose of visiting friends and relatives. Children can develop severe parasitemias rapidly and are at increased risk for associated complications, such as seizures, shock, coma, and death, with the added problem that they may initially present with nonspecific symptoms that often lead to delayed diagnosis [195].

4.1.3. Mitochondrial Changes in Malaria Infection

Despite the fact that evolutionary pressure from *Plasmodium falciparum* malaria has favored a large number of human gene adaptations, there are surprisingly a few investigations on the effects of malaria on human mitochondrial sequence variation. *Plasmodium falciparum* infection can cause severe malaria anemia with insufficient tissue oxygenation, lactic acidosis, and death [196].

Asymptomatic children can temporarily maintain their status by orchestrating active gene regulation through chromatin remodeling [197]. This likely affects the production of immunoglobulin chain transcripts found repressed in asymptomatic children, but specifically activated in uncomplicated pediatric malaria. On the contrary, in symptomatic malaria, basophil and eosinophil transcripts remain suppressed. More specifically, interferon-alpha inducible protein 27 (IFI27) levels have been found to correlate with clinical parameters and thus could represent a potential indicator of parasitemia, along with hemoglobin and lactate levels. The latter occurs because persistent oxygen deficiency leads to a replacement of intracellular aerobic respiration with anaerobic glycolysis and excessive production of lactic acid [197]. IFI27 is associated with, or inserted into, the mitochondrial membrane and its transient expression leads to a decrease in the number of viable cells and an increased sensitivity to apoptosis induced by DNA damage. This suggests the involvement of IFI27 in the mechanisms of apoptosis that generate lymphopenia during severe anemia due to malaria.

Approximately 50% of Ugandan children with severe malarial anemia present elevated blood lactate levels [196]. In this study, mitochondrial gene sequences among a cohort of children with or without lactic acidosis in the context of severe malaria anemia were investigated [196]. It has been suggested that the determinants of high blood lactate levels in severe malarial anemia patients may be related to genetic polymorphisms outside the mitochondrial genome, or to other factors. Alternatively, variation in other nuclear genes such as those in the glycolytic pathway, Krebs cycle, or those that affect NAD^+/NADH

levels may influence blood lactate levels during severe anemia. In addition, severe malarial anemia lactic acidosis may also be related to factors unrelated to genetics, such as microvascular physiology, nutritional status, or duration of disease. Increased blood lactate, an important marker for decreased survival in severe malarial anemia, despite being a consequence of mitochondrial impairment, does not appear to be strongly associated with mitochondrial DNA sequence variation, but could be related to variations in those nuclear genes influencing mitochondrial function [196].

4.1.4. Malaria Treatment in the Pediatric Population and Mitochondrial Involvement

Chemoprophylaxis depends on the species of the parasite, area of origin (and Plasmodium resistance rates in that country), and clinical situation of the child (severity criteria) [190]. The parasite's mitochondria has been detected as a main potential target for antimalarial drugs and, in particular, the mitochondrial ETC, as it has a relatively limited function while the parasite is developing [188] (Table 7). Of note, it is conceivable that if the treatment targets the mitochondria of the parasites, it may cause off-target effects in the mitochondrial function of the patients.

Depending on the parasite involved and the clinical situation at diagnosis, the treatment can be administered on an outpatient basis or intravenously.

If malaria is diagnosed early, it can usually be cured within about 2 weeks. However, many people living in areas where malaria is endemic are repeatedly infected and never completely recover between consecutive bouts of this disease. Without treatment, the disease can be fatal, especially in malnourished children [207].

Finally, there is a vaccine, known as Mosquirix, which is an injectable option that provides partial protection against malaria in young children and that is being evaluated in sub-Saharan Africa as an instrument of complementary control that could be added to other preventive, diagnostic, and therapeutic measures recommended by the WHO [190].

Table 7. Antimalarial treatment in children and associated mitochondrial effects.

Type	Parasite and Resistance	Drug	Parasite's Mitochondria Involvement
Uncomplicated malaria (no signs of severity)	*P. falciparum* or chloroquine-resistant strains	Atovaquone/proguanil	• Inhibits the cyt bc1 complex, a key mitochondrial enzyme that catalyzes the transfer of electrons that maintain the membrane potential of the parasite's mitochondria [188,190]
		Quinine sulfate + clindamycin or doxycycline (>8 years old)	• Quinine sulfate affects parasitized erythrocytes and has a schizonticidal action [198,199]
		derivatives of artemisin, in Spain dihydroartemisin + piperaquine	• Acts by depolarizing the mitochondrial membrane • Increases production of ROS to alter mitochondrial functions • ETC has some interactions with artemisinin, and probably plays an important role in its activation [200]
		Mefloquine	• Blood schizonticide • Breaks down hemoglobin in a food vacuole, producing a free heme pool and increasing ROS [201]
	P. vivax, *ovale*, *malariae*, or *falciparum* from chloroquine-sensitive area	Chloroquine	• Blocks detoxification of heme, a by-product of hemoglobin degradation. During the asexual intraerythrocytic-stages, the parasite imports host cell hemoglobin into its food vacuole. Proteases in the food vacuole degrade hemoglobin into free amino acids, which are utilized in various growth processes. Heme is released during hemoglobin digestion and is essential for parasite growth as a cofactor for cytochromes in the parasite's ETC [200]
		Primaquine	• Eradicates hypnozoites that remain quiescent in the liver and prevents relapse [202]
Severe malaria	Normally caused by *P. falciparum*	Quinine gluconate IV diluted in glucose 5% + clindamycin IV (>8 years old)	• Quinine sulfate affects parasitized erythrocytes and has a schizonticidal action [198,199]
		Artesunate	• Is a semisynthetic analogue of artemisinin [203] • Disrupts redox homeostasis in parasites [204,205] • Increases ROS [206]

ETC, electron transport chain; ROS, reactive oxygen species.

5. Discussion

To our knowledge, this is the first time that such mitochondrial molecular events, both at the genetic and at the functional level, associated with the main bacterial infections and their treatments, are reviewed in the pediatric population. In fact, a major observation of the present review, is the lack of information relating to the role of mitochondria in most bacterial infective processes in the pediatric population.

Both intracellular and extracellular bacteria have been shown to modify host metabolism by disturbing mitochondrial homeostasis and function. Recently, several intracellular bacterial pathogens have been shown to modulate mitochondrial functions to maintain their replicative niche [208]. Thus, infection induces mitochondrial changes in infected macrophages, triggering modifications of the host metabolism that lead to important immunological reprogramming.

Importantly, mitochondrial interactions and toxicity are ultimately determined by bacterial load as well as by the drug selected to treat such infections. These interactions eventually and frequently turn out to be reversible once the pathogen is eradicated or the therapeutic agent is interrupted.

A main mitochondrial hallmark related to most infective processes is the increase in mitochondrial ROS that occurs in many bacterial (e.g., enterobacteria) and parasitic (e.g., malaria) infections. For example, during meningitis, the integrity of the endothelial barrier can be disturbed by ROS, which worsens pathological conditions in the presence of the bacteria [156].

Interestingly, ROS not only are a by-product of oxidative respiration but also regulate signaling pathways such as signal transducers and activators of transcription, and phosphoinositide 3-kinase pathways. Hence, an increase in ROS as a cellular stress signal has to be avoided or counteracted during the establishment of a persistent infection [209]. ROS generation and subsequent oxidative stress often lead to apoptosis. Mitochondrially driven apoptosis caused by bacterial infections has also been documented. Such is the case of the Enterobacteriaceae family, and others such as *S. aureus*, which also cause mitochondrial impairment. Interestingly, all these infections are characterized by promoting loss of $\Delta\Psi m$, release of CytC, activation of caspase-3 and -9 [94], ultimately leading to mitochondrial driven cell death [31,190]. In addition, worth noting is the increase in Bax/Bcl-2 proteins in *E. clocae* and the overexpression of p53 [91] in *P. mirabilis*, also associated with intramitochondrial calcium precipitation and ROS increase [93], the latter also observed in *S. aureus* [26,210].

However, mitochondrial damage derived from bacterial infections goes beyond ROS generation and apoptosis. First, many infectious processes caused by different pathological agents not only are related to ROS overproduction but also share some other similar molecular events, such as inflammatory mechanisms [211]. Second, it is known that disruption of mitochondrial integrity has been identified as a key virulence strategy of bacterial pathogens [212]. Third, most products derived from bacterial infection, such as nitric oxide, are widely known to be specific inhibitors of complex IV of the mitochondrial respiratory chain [213]. Lastly, metabolic switching from an aerobic to anaerobic state and vice versa, has been documented during bacterial infections [208]. Mitochondrial performance is highly adaptive during an infectious process of either bacterial or parasitic origin. In malaria, for example, oxygen deficiency leads to a replacement of intracellular aerobic respiration with anaerobic glycolysis and excessive production of lactic acid [197]. Interestingly, sometimes the mitochondrial and cellular changes triggered by the infective process are destined to protect the cell. In TB, the increase in intracellular Ca^{2+} protects the mitochondria from irreversible damage caused by the pathogen, *Mtb*, and inhibits macrophage necrosis. Of course, deleterious effects leading to mitochondrial damage are also present in TB, mainly related to the H37Rv*Mtb* strain, by exerting changes in the $\Delta\psi m$, release of CytC, and modification of mitochondrial dynamics [26,31].

As observed in this review, not only the pathogens but also their treatments are frequently associated with mitochondrial changes. This is explained, as already mentioned, by

the shared bacterial-mitochondrial origin [6]. Accordingly, antibiotics have been reported to inhibit mitochondrial protein synthesis, due to the common origin of mitochondria and bacteria, described by Margulis in the endosymbiont hypothesis [214]. This is the case of linezolid-derived inhibition of cytochrome-c-subunit protein of complex IV, as reported by our group [123].

In clinical practice, it is occasionally difficult to differentiate whether mitochondrial abnormalities are exclusively related to the infection itself or to its antimicrobial treatment [3] but, importantly, molecular events have been correlated with the onset of clinical symptoms in the pediatric population, meaning mitochondrial alterations are more evident in children presenting with clinical manifestations than in those without [215]. The vulnerability of pediatric patients highlights the importance of longitudinal studies assessing mitochondrial changes and their derived clinical consequences over time.

In other cases, mitochondria of the pathogen itself turns out to be the main therapeutic target to treat the infection and pharmacological inhibition of a given mitochondrial function may represent a key step to avoid pathogen replication. Such is the case with pharmacological inhibition of complex III, a well-defined drug target for the treatment of malaria [188]. Surprisingly and against all odds, in the case of malaria, despite the fact that the parasites mitochondria are a therapeutic target, there is little investigation of the potential secondary effects of antibiotic therapies on the human mitochondrial genome and function [196]. Mitochondria-targeted pathogen products and the mitochondrial pathways affected by them provide potential novel targets for the rational design of drugs. Pathogen products may alter oxidative balance, mitochondrial transition pore permeability, mitochondrial membrane potential, electron transport chain, and ATP production [216].

Mitochondrial changes associated with bacterial infections and their antibiotic treatments most likely occur in the same manner in children and adults. However, some infections prevail during childhood and mitochondrial features may differ in children vs. adults. We have focused our review on those bacterial and parasitic infections presenting higher incidence rates in children, considering the most relevant characteristics of such infections in this population group. For each infection described in this review, a summary of the main mitochondrial assessments conducted in children presenting with the specific bacterial or parasitic infection and/or the antibiotic treatment, has been provided. It should be noted that there is a lack of studies on mitochondrial changes related to other pediatric infectious processes, herein not included, for which scarce data are available in children (e.g., regarding infection with *Neisseria gonorrhoeae*, *Clostridium botulinum*, *Streptococcus pneumoniae*, and *Clostridium tetani*).

In many cases, the number of children infected and receiving drug therapy against a given infection is increasing. In addition, it is likely that if treatment is given as indicated in pregnant women with acute infections, the number of treatment-exposed newborns will also increase. Since studies and information are limited, especially in pediatric populations, it is essential to accurately assess the potential mitochondrial toxicity of such pharmacologic therapeutic options in a population as susceptible as newborns and infants. Remarkably, this has been studied in bacteriostatic antibiotics, which do not stimulate ROS production, suggesting that only bactericidal antibiotics result in major production of ROS [139]. Thus, mitochondrial dysfunction and oxidative damage induced by bactericidal antibiotics in mammalian cells may be alleviated by antioxidants or prevented by preferential use of bacteriostatic antibiotics [139].

In the near future, the identification of pathways or metabolites that are common to multiple pathogens remains an important challenge. Additionally, metabolic alterations that are directly involved in pathogen replication and not just a consequence of the infection need to be identified. More data and, in particular, longitudinal follow-up studies will be needed to contribute to the rather complex interaction of pathogens and treatments with mitochondrial metabolism [209], especially in children.

6. Conclusions

Infants are an especially susceptible population group, particularly vulnerable during specific infective processes. Mitochondria play a major role in specific infections, due to: (i) molecular and/or ultrastructural alterations directly caused by the pathogen, (ii) molecular and/or ultrastructural changes caused by the treatments, (iii) their role as a therapeutic target in the disease, (iv) their implication in further clinical manifestations, (v) their identification as key targets of the infection process, (vi) their high adaptability during the infection process, and (vii) their protective role during the infection process. There is an urgent need to carry out longitudinal studies monitoring the long-term effects of bacterial and parasitic infections, which target mitochondria in developing children to further our understanding of these diseases.

7. Selection Criteria and Outcomes

We searched for scientific publications in three main database sources including Pubmed (MEDLINE), Web of Science, and SCOPUS. We included the common search terms: "mitochondria AND pediatric OR childhood OR infant OR children" for all the infectious diseases. For each infectious disease, we added the following terms: tuberculosis (TB), *Mycobacterium tuberculosis*; enterobacteria and enteroviruses, *Enterobacter cloacae*, *Proteus mirabilis*, *Escherichia coli* and *Salmonella enterica*; *Staphylococcus aureus*; meningitis, *Neisseria meningitidis and Streptococcus pneumoniae*; AND malaria, *Plasmodium falciparum*. We searched papers published in English between 1984 and 2020. We used the Rayyan QCRI software for systematic reviews (http://rayyan.qcri.org (accessed on 20 May 2020)), a free web and mobile app, that helps expedite the initial screening of abstracts and titles using a process of semi-automation while incorporating a high level of usability [217]. The studies were assessed for relevance to the topics and selected by two authors independently. With respect to the inclusion criteria, all randomized controlled studies in human models were included, as well as case reports and review articles. Animal models were excluded for this review.

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Abbreviations

AIF	Apoptosis-Inducing Factor
ANT	Adenine Nucleotide Translocase
APAF-1	Apoptosis Protease-Activating Factor-1
ATP	Adenosine Triphosphate
ADP	Adenosine Diphosphate

EMB	Ethambutol
ER	Endoplasmic Reticulum
ETC	Electron Transport Chain
CoQ	Coenzyme Q
CytC	Cytochrome C
DNAse	Deoxyribonuclease
FADH	Flavin and Adenine Dinucleotide Hydrogen
FMN	Flavin Mononucleotide
Fp	Flavoprotein
CI	Complex I
CII	Complex II
CIII	Complex III
CIV	Complex IV
CV	Complex V
COX	Cytochrome C Oxidase
CypD	Cyclophilin D
ESAT-6	Early Secretory Antigenic Target
ETC	Electron Transport Chain
HIV	Human Immunodeficiency Virus
HSV	Herpes Simplex Virus
IGRA	Interferon-Gamma Release Assays
IFNγ	Interferon-γ
IMM	Inner Mitochondrial Membrane
INH	Isoniazid
iNOS	Inducible Nitric Oxide Synthase
KCN	Potassium Cyanide
LOS	Lipooligosaccharide
MRC	Mitochondrial Respiratory Chain
MRSA	Methicillin Resistant *Staphylococcus Aureus*
Mtb	*Mycobacterium tuberculosis*
mtDNA	Mitochondrial Deoxyribonucleic Acid
mtRNA	Mitochondrial Ribonucleic Acid
NADH	Nicotinamide Adenine Dinucleotide Hydrogen
NHBA	Neisseria Heparin-Binding Antigen
NO	Nitric Oxide
OMM	Outer Mitochondrial Membrane
OXPHOS	Oxidative Phosphorylation System
PARP	Poly ADP-Ribose Polymerase
Pi	Inorganic Phosphate
PTP	Permeability Transition Pore
PZA	Pyrazinamide
RIF	Rifampin
ROS	Reactive Oxygen Species
rRNA	Ribosomal Ribonucleic Acid
SOD	Superoxide Dismutase
TB	Tuberculosis
TCA	Tricarboxylic Acid
TNFα	Tumor Necrosis Factor-α
TNT	TB Necrotizing Toxin
tRNA	Transfer Ribonucleic Acid
TST	Tuberculin Skin Test
Δψm	Mitochondrial Membrane Potential

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Association of Dietary Intakes and Genetically Determined Serum Concentrations of Mono and Poly Unsaturated Fatty Acids on Chronic Kidney Disease: Insights from Dietary Analysis and Mendelian Randomization

Mohsen Mazidi [1,2,3,*], Andre P. Kengne [4], Mario Siervo [5] and Richard Kirwan [6]

1 Medical Research Council Population Health Research Unit, University of Oxford, Oxford OX3 7LF, UK
2 Clinical Trial Service Unit and Epidemiological Studies Unit (CTSU), Nuffield Department of Population Health, University of Oxford, Oxford OX3 7LF, UK
3 Department of Twin Research & Genetic Epidemiology, South Wing St Thomas', King's College London, London SE1 7EH, UK
4 Non-Communicable Disease Research Unit, South African Medical Research Council, University of Cape Town, Cape Town 7505, South Africa; andre.kengne@mrc.ac.za
5 School of Life Sciences, Queen's Medical Centre, The University of Nottingham Medical School, Nottingham NG7 2RD, UK; mario.siervo@nottingham.ac.uk
6 School of Biological and Environmental Sciences, Liverpool John Moores University, Liverpool L3 3AF, UK; r.p.kirwan@2018.ljmu.ac.uk
* Correspondence: mohsen.mazidi@ndph.ox.ac.uk; Tel.: +44-7714-16-6923

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Abstract: Polyunsaturated fatty acid (PUFA) intake is generally associated with better renal function, while the association of monounsaturated fatty acids (MUFAs) remains unconfirmed. Mendelian randomization (MR) analysis was used to obtain unconfounded estimates of the causal association of dietary intake and genetically determined serum PUFA and MUFA levels with measures of renal function. Data from participants of the National Health and Nutrition Examination Surveys (NHANES) from 2005 to 2010 were used. Data from the largest genome-wide association studies (GWAS) on MUFAs, PUFAs, eGFR, and chronic kidney disease (CKD) were analysed for the entire sample. A total of 16,025 participants were included. eGFR improved across increasing quartiles of total PUFA intake from 86.3 ± 0.5 (Q1) to 96.2 ± 0.5 mL/min/1.73 m^2 (Q4), ($p < 0.001$). Conversely, there was no association between MUFA intake and measures of renal function (all $p > 0.21$). In multivariable models, the top quartile of PUFA intake had a 21% lower risk for CKD, but there was no significant association between CKD risk and MUFA intake. Genetically determined serum MUFA (heptadecenoate (17:1), myristoleic acid (14:1), and palmitoleic acid (16:1)) and PUFA (α-linolenic acid and eicosapentaenoic acid) concentrations had no significant association with eGFR and CKD risk. Additionally, no association was found in the analyses stratified by diabetes status. Higher dietary PUFA intake is associated with lower risk of CKD, while there was no association with serum levels of MUFAs or PUFAs. Additional studies including clinical trials are warranted.

Keywords: mendelian randomization; serum fatty acids; monounsaturated fatty acids; chronic kidney disease; polyunsaturated fatty acids; renal function

1. Introduction

Chronic kidney disease (CKD) is defined as significantly impaired kidney function, identified by a reduced glomerular filtration rate (GFR) or increased urinary albumin excretion (albuminuria) that are confirmed on two or more occasions at least 3 months apart [1]. The global prevalence of CKD has been estimated between 11 to 13%, with prevalence increasing with age, and up to 34% of subjects older than 70 years old have a low eGFR indicating the presence of CKD [2,3]. CKD is known to be associated with

and contribute to the disease burden of multiple comorbidities including diabetes [4–6], hypertension [7,8], obesity [9,10], and especially cardiovascular diseases [11–14], and as such, it is associated with a higher all-cause mortality [13].

Both low plasma concentrations and dietary intakes of n-3 and n-6 polyunsaturated fatty acids (PUFA) have previously been associated with impaired renal function [15–17], while a lower saturated fatty acid (SFA) intake has been associated with improved renal function [18]. The association between monounsaturated fatty acids (MUFA) and risk of CKD, however, remains poorly understood. A potential mechanism through which PUFAs may play a protective role in kidney function is by downregulating certain aspects of the inflammatory response, for example, a reduction in proinflammatory cytokines [19]. Indeed, lower levels of plasma markers of chronic inflammation such as CRP and tumor necrosis factor alpha have been observed in older adults with higher serum levels of PUFAs [20]. However, such observational data cannot be used to determine the causality of serum PUFAs in the etiology of CKD.

Mendelian randomization (MR) analysis using functional single nucleotide polymorphisms (SNPs) associated with specific changes in physiological exposures (such as serum MUFAs and PUFAs) as genetic instruments of analysis are capable of providing unbiased and robust evidence on the mechanisms of the pathogenesis of disease and the efficacy of treatments. Compared with conventional risk-factor epidemiology, these studies are considerably less prone to confounding, residual bias, and reverse causation [21]. While randomized controlled trials (RCTs) are considered useful for the determination of causality, they are often limited by cost, time, and ethical constraints, depending on the characteristics of the exposure and disease state being studied. MR studies can be considered a way to avoid these inherent issues with RCTs and, in addition to this, the data from such studies can be used to improve the development of pilot RCTs and strategies for clinical trials by elucidating the potential efficacy of an intervention or even the magnitude of effect in selected individuals and groups [22].

Therefore, national survey data (Nutrition and Health Examination Surveys (NHANES)) and Mendelian randomization (MR) analysis were used to determine unbiased estimates of the casual association of genetically determined serum levels and dietary intake of MUFAs and PUFAs with renal function.

2. Materials and Methods

2.1. National Survey

2.1.1. Population

We used data from the NHANES, which were previously published in detail [23]. In brief, repeated cross-sectional surveys are conducted by the US National Center for Health Statistics (NCHS). These consist of home visits where questionnaires are used to collect data on demographics and health habits such as diet. Complex multistage probability sampling procedures are employed by NHANES to ensure adequate racial/ethnic representation, as well as recruitment from diverse locations [23]. The NCHS Research Ethics Review Board approved the protocol and all participants provided informed consent.

The methods for the specific analyses can be found in the NHANES Laboratory/Medical Technologists Procedures Manual [24–27]. Blood was drawn from an antecubital vein following a standard protocol. The Jaffe rate method (kinetic alkaline picrate) was used in the D×C800 modular chemistry side in order to determine the concentration of creatinine in the serum. The creatinine calibration was traceable to an isotope dilution mass spectrometry reference method [28]. Urinary creatinine and urinary albumin (which was assessed using a solid-phase fluorescent immunoassay of a random urine sample) [29] were used to calculate the urine albumin to creatinine ratio (ACR). eGFR was calculated using the CKD Epidemiology Collaboration (CKD-EPI) equation (in mL/min/1.73 m^2). Prevalent CKD was identified as an eGFR <60 mL/min/1.73 m^2 [29].

2.1.2. Dietary Intake

Dietary intake was assessed though 24 h recall, obtained with the assistance of a trained interviewer, through the use of the United States Department of Agriculture Automated Multiple-Pass Method (AMPM), which is an interview system with standardized probes that is aided by a computerized system [30,31]. Briefly, the type and quantity of all foods and drinks ingested in the 24 h period prior to the dietary interview (from 12 am to 12 am) were collected using the AMPM. The AMPM is designed to ensure more complete and accurate data collection while reducing the burden on participants [31,32]. In the current study, we used the data on the total MUFA intake as well as the intake of specific MUFAs: 16:1 (Hexadecenoic, commonly known as palmitoleic acid), 18:1 (Octadecenoic, commonly known as oleic acid (OA)), 20:1 (Eicosenoic), and 22:1 (Tetracosenoic). We also analyzed data on the intake of total PUFAs, as well as the individual PUFAs: PUFA 18:2 (octadecadienoic, commonly known as linoleic acid (LA)), PUFA 18:3 (octadecatrienoic, commonly known as α-linolenic acid (ALA)), PUFA 18:4 (octadecatetraenoic), PUFA 20:4 (eicosatetraenoic (ETA)), PUFA 20:5 (eicosapentaenoic (EPA)), PUFA 22:5 (docosapentaenoic (DPA)), and PUFA 22:6 (docosahexaenoic (DHA)).

2.1.3. Statistical Analysis

CDC guidelines for the analysis of complex NHANES data, using the appropriate weighting methods and accounting for the masked variance, were employed [33]. Mean and standard error of mean (SEM) were used for continuous (analysis of variance) measures and percentage for categorical variables (chi-square). In order to evaluate the normality of data, the Kolmogorov−Smirnov test was applied. The adjusted mean of specific kidney function markers (serum creatinine, ACR, and eGFR) across MUFA and PUFA quartiles were estimated using analysis of covariance (ANCOVA). These models were adjusted for age, sex, race, poverty to income ratio, fasting blood glucose, systolic and diastolic blood pressure, energy intake, red meat intake, body mass index (BMI, kg/m^2), diabetes (DM) (self-reported history of DM or fasting plasma glucose $\geq$126 mg/dL), and hypertension (HTN), diagnosed in individuals with systolic blood pressure $\geq$140 mmHg, diastolic blood pressure $\geq$90 mmHg, or in those on antihypertensive drugs [34]. Log transformations were performed for variables with departure from normal distribution. Logistic regressions models with three different levels of adjustments (model 1: age, sex, race and poverty to income ratio; model 2: age, sex, race, poverty to income ratio, fasting blood glucose, systolic and diastolic blood pressure, and hypertension (HTN); and model 3: age, sex, race, poverty to income ratio, fasting blood glucose, systolic and diastolic blood pressure, HTN, triglycerides (TG) and high density lipoprotein cholesterol (HDL), diabetes mellitus (DM), body mass index (BMI), and C-reactive protein (CRP)), were then used to derive the odds ratio (OR) and 95% confidence interval (CI) for the association with prevalent CKD across MUFA and PUFA quartiles. The lowest quartile was always used as the reference value. Variance inflation factors (VIF) were applied at each step to assess multi-collinearity for the multiple linear regressions [35]. Multi-collinearity was considered high when the VIF was greater than 10 [35]. All of the tests were two sided, and a p-value of less than 0.05 characterized significant results.

2.2. Mendelian Randomization

2.2.1. Study Design

This study employed a two-sample MR study design. Briefly, summary statistics from different genome wide association studies (GWAS) were analyzed for the exposures (serum MUFAs and PUFAs) and outcomes (renal function) of interest, to estimate the effects of the former on the latter [36]. That is to say, genetic predictors of serum MUFAs and PUFAs were applied to extensively genotyped case-control studies of renal function (eGFR and the risk of CKD) to obtain estimates of their association.

2.2.2. Genetic Predictors of Exposures

We retrieved summary data for the association between SNPs and four circulating MUFAs (myristoleic acid (14:1 (tetradecenoic)), palmitoleic acid (16:1 (hexadecenoic)), 10-heptadecenoate (17:1), oleic acid (18:1 (octadecenoic))) from the GWAS (7824 adults of European ancestry) (Supplementary Table S1). Genotyping, quality control, and imputation procedures have been previously elaborated [37]. For analysis of the serum PUFAs, we retrieved summary data for the association between SNPs and circulating ALA and EPA concentration from the CHARGE meta-GWAS (n = 8866 adults, European descent) [38]. In instances where an SNP was not available for the outcome GWAS summary statistics, proxy SNPs were identified. A minimum linkage disequilibrium (LD) $r^2 = 0.8$ was required for such proxy SNPs. Bias in effect estimates can induced by correlation between SNPs, and in order to minimize this bias, our genetic instruments were limited to independent SNPs not in linkage disequilibrium ($p = 0.0001$). We refer to a set of SNPs that proxy serum MUFAs and PUFAs as "genetic instruments".

2.2.3. Genetic Predictors of Outcomes

A meta-analysis consisting of the largest genotyped study sample (n = 133,413 with replication in up to 42,166 participants) was used to obtain genetic associations with eGFR [39], which was determined using the four-variable Modification of Diet in Renal Disease (MDRD) Study Equation [39]. The determination of CKD was an eGFR <60 mL/min/1.73 m^2 and that of type 2 diabetes (T2 D) was fasting glucose $\geq$126 mg/dL, antidiabetic drug treatment, or self-reported. Kidney function and T2 D were assessed simultaneously. For the GWAS analysis, a centralized analysis plan was applied with each study regressing sex- and age-adjusted residuals of the logarithm of eGFR on the SNP dosage levels. Furthermore, logistic regression of CKD was performed on SNP dosage levels, adjusting for sex and age. For all traits, adjustment for appropriate study-specific features, such as study site and genetic principal components, was included in the regression, and family-based studies appropriately accounted for relatedness.

2.2.4. Statistics

We combined the effect of instruments using the inverse variance weighted (IVW) method. The Q value for IVW was used to determine heterogeneity. As the final effect estimate may be potentially affected by pleiotropic variants, a sensitivity analysis including weighted median (WM) and MR-Egger and using the leave-one-out method, was performed [36]. Causal estimates in MR-Egger are less precise than those obtained using IVW MR [40] due to a lower false-positive rate and an associated higher false-negative rate, leading to a lower statistical power [41].

The Q′ heterogeneity statistic was used to determine the heterogeneity between individual genetic variant estimates [42]. Furthermore, the instrumental variable analysis "exclusion-restriction" assumption was assessed by using Ensembl release (http://useast.ensembl.org/index.html, accessed on 2 April 2020) and PhenoScanner (SNP phenotypes are provided by Ensembl and the phenotypes of correlated SNPs are provided by PhenoScanner).

2.2.5. Sensitivity Analysis

Sensitivity analysis was performed using MR-Egger and the MR pleiotropy residual sum and outlier (MR-PRESSO) test [42]. Outlier effect estimates were detected by the MR-PRESSO framework, which subsequently removed them from the analysis. This was done by regression of the variant−outcome associations on variant−exposure associations. Furthermore, the MR-Robust Adjusted Profile Score (RAPS) was applied in order to correct for pleiotropy. To qualify as a result, the causal estimates must agree in both direction and magnitude across MR methods, must have nominal significance in IVW MR, and must not show any evidence of bias from horizontal pleiotropy. All analyses were done using R software (version 3.4.2 R Core Team, 2017).

3. Results

3.1. Dietary Intake

A total of 16,025 of the NHANES participants fulfilled the criteria for inclusion in the analysis; 6.8% had prevalent CKD. The characteristics of participants for the whole sample and by CKD status are summarized in Table 1. Overall, 51.2% of the participants were women, with no significant gender difference observed between those presenting with or without CKD ($p = 0.412$). Compared with those without CKD, participants with CKD were more likely to be non-Hispanic Whites (82.9 vs. 68.4%), and less likely Mexican-Americans (2.5 vs. 9.0%), non-Hispanic Black (8.1 vs. 11.2%), other Hispanic (3.0 vs. 5.4%); $p < 0.001$ for differences in the distribution of ethnicity by CKD status. The mean age was 45.8 years; participants with CKD were older than those without (69.1 vs. 44.6 years, $p < 0.001$). Those presenting with CKD had a higher BMI ($p < 0.001$) and higher serum CRP, TG, and fasting glucose ($p < 0.001$ for all comparisons), as well as being more likely to have DM and HTN ($p < 0.001$ for all comparisons, Table 1).

Table 1. Demographic characteristics of subjects for the whole sample and stratified by chronic kidney disease (CKD) status.

Characteristics		Overall	With CKD	Without CKD	*p*-Value
Sex	Men (%)	48.8	37.7	49.2	<0.001
	Women (%)	51.2	62.2	50.9	
Age (years), mean (± SEM)		45.8 ± 0.1	69.1 ± 0.2	44.6 ± 0.2	<0.001
Race/Ethnicity	White (non-Hispanic) (%)	68.4	82.9	68.6	<0.001
	Non-Hispanic Black (%)	11.5	8.1	11.2	
	Mexican-American (%)	8.1	2.5	9.0	
	Other Hispanic (%)	5.2	3.0	5.4	
Body mass index (kg/m^2)		28.5 ± 0.1	29.1 ± 0.1	28.7 ± 0.1	<0.001
Serum Triglycerides (mg/dL)		155.8 ± 3.0	179.3 ± 3.9	152.3 ± 2.3	<0.001
Serum Total cholesterol(mg/dL)		196.6 ± 0.7	192.9 ± 1.0	196.5 ± 0.8	0.096
Serum High density lipoprotein (mg/dL)		53.0 ± 0.5	53.2 ± 0.4	53.1 ± 0.2	0.483
Serum CRP (mg/dL)		0.33 ± 0.03	0.55 ± 0.02	0.29 ± 0.01	<0.001
Fasting blood glucose (mg/dL)		99.3 ± 0.2	113.1 ± 0.8	97.6 ± 0.3	<0.001
Hypertension (%)		15.4	34.7	13.7	<0.001
Diabetes (%)		8.9	21.5	7.8	<0.001
MUFA intake(gm/d)		27.3 ± 0.6	25.6 ± 0.9	27.9 ± 0.6	<0.001
PUFA intake(gm/d)		15.6 ± 0.8	14.9 ± 1.1	16.1 ± 0.4	<0.001

CKD: chronic kidney diseases; CRP: C-reactive protein; Continuous values are expressed as a mean ± SEM.

Adjusted (age, sex, race, fasting blood glucose, systolic and diastolic blood pressure, energy intake, red meat intake, BMI, DM, and HTN) mean levels of kidney function by quartiles of total MUFAs and PUFAs are shown in Table 2. Across increasing the total PUFA quartiles, renal function improved, with, for example, the adjusted mean of eGFR changing from 86.3 ± 0.5 in Q1 to 96.2 ± 0.5 mL/min in Q4 ($p < 0.001$). Across increasing the total MUFA quartiles, the mean urine albumin, log ACR, and eGFR did not change significantly (all $p > 0.213$, Table 2). Across quartiles of different types of MUFAs (hexadecenoic (16:1), octadecenoic (18:1), eicosenoic (20:1), and docosenoic (22:1)) and PUFAs (PUFA 18:2 (octadecadienoic), PUFA 18:3 (octadecatrienoic), PUFA 18:4 (octadecatetraenoic), PUFA 20:4 (eicosatetraenoic), PUFA 20:5 (eicosapentaenoic), PUFA 22:5 (docosapentaenoic), and PUFA 22:6 (docosahexaenoic)) urine albumin, log ACR, and eGFR did not change significantly (all $p > 0.412$, data not shown). Multiple potential confounders, arranged into three separate

models, were used to evaluate the odds of CKD across the quartiles of the total MUFAs and PUFAs (Table 3). In the model adjusted for age, sex, race, and poverty to income ratio, compared with the lowest quartile of the total PUFA, the OR (95%CI) for CKD was 0.60 (0.40–0.81) for the top quartile (p = 0.012 for trend, Table 3).

Table 2. Age, sex, race, fasting blood glucose, systolic and diastolic blood pressure, energy intake, red meat intake, body mass index, diabetes, and hypertension—adjusted mean of markers of kidney function across quartiles of monounsaturated fatty acids and polyunsaturated fatty acids consumption.

Variables	Quartiles of Monounsaturated Fatty Acid (MUFA) Consumption (gm)					Quartiles of Polyunsaturated Fatty Acid (PUFA) Consumption (gm)				
	1	2	3	4	*p*-Value [a]	1	2	3	4	*p*-Value [a]
Median (25th–75th)	11.4 (8.4–14.0)	20.9 (18.7–23.0)	30.5 (27.6–33.5)	47.1 (41.5–56.2)		6.2 (4.4–7.7)	11.7 (10.4–13.0)	17.6 (16.0–19.6)	28.8 (24.8–35.9)	
Serum Creatinine (mg/dL)	0.79 ± 0.001	0.81 ± 0.003	0.78 ± 0.001	0.81 ± 0.001	0.186	0.91 ± 0.03	0.83 ± 0.04	0.81 ± 0.05	0.76 ± 0.06	<0.001
Log Urea Albumin (ug/mL)	2.16 ± 0.01	2.20 ± 0.02	2.19 ± 0.01	2.10 ± 0.01	0.415	2.23 ± 0.01	2.17 ± 0.03	2.08 ± 0.01	2.04 ± 0.02	<0.001
Glomerular filtration rate (mL/min/1.73 m^2)	84.5 ± 0.36	89.6 ± 0.30	85.4 ± 0.41	86.1 ± 0.37	0.359	86.3 ± 0.5	90.2 ± 0.5	91.4 ± 0.3	96.2 ± 0.5	<0.001
Log Albumin-Creatinine Ratio (mg/dL)	2.10 ± 0.01	2.11 ± 0.01	2.10 ± 0.02	2.09 ± 0.01	0.635	2.17 ± 0.01	2.12 ± 0.03	2.11 ± 0.01	2.04 ± 0.02	<0.001

Values expressed as estimated mean and standard error. [a] p-values for linear trend across quartiles of MUFA and PUFA consumption. Variables were compared across quartiles of MUFA and PUFA consumption using an analysis of co-variance (ANCOVA) test.

Table 3. Adjusted logistic regression to examine the association between quartiles for mono and polyunsaturated fatty acids and the risk of chronic kidney disease (CKD).

Variables	Likelihood of CKD with Different Models					
	Age, sex, race, poverty to income ratio		Age, sex, race, poverty to income ratio, alcohol intake, energy intake, smoking, physical activity, fasting blood glucose, systolic and diastolic blood pressure, HTN, and DM		Age, sex, race, poverty to income ratio, alcohol intake, energy intake, smoking, physical activity, fasting blood glucose, systolic and diastolic blood pressure, HTN, DM, TG and HDL, and CRP	
	Odds Ratio	Lower Bound-Upper Bound	Odds Ratio	Lower Bound-Upper Bound	Odds Ratio	Lower Bound-Upper Bound
MUFA (Q2)	1.06	(0.62–1.49	0.85	(0.61–1.17)	0.76	(0.55–1.09)
MUFA (Q3)	1.10	(0.58–2.13)	0.96	(0.40–2.13)	0.88	(0.35–2.61)
MUFA (Q4)	0.98	(0.50–1.90)	1.02	(0.29–3.96)	0.96	(0.40–2.13)
PUFA (Q2)	1.01	(0.69–1.43)	1.02	(0.76–1.28)	0.97	(0.78–1.20)
PUFA (Q3)	0.76	(0.69–0.83)	0.81	(0.78–0.86)	0.85	(0.61–1.17)
PUFA (Q4)	0.60	(0.40–0.81)	0.73	(0.65–0.84)	0.79	(0.68–0.88)

The first quartile was always used as a reference. Q2: second quartile; Q3: third quartile; Q4: fourth quartile; CKD: chronic kidney disease; HTN: hypertension; TG: triglyceride; HDL: high density lipo-protein; DM: diabetes; CRP: C-reactive protein; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids.

Employing the expanded models and following adjustment for age, sex, race, poverty to income ratio, fasting blood glucose, systolic and diastolic blood pressure, HTN, DM, TG, HDL, and CRP the top quartile of total PUFA intake had a 21% lower likelihood of CKD (OR = 0.79 (0.68–0.88)). With regard to MUFA intake, in three different models with range-varied confounders (age, sex, race, poverty to income ratio, fasting blood glucose, systolic and diastolic blood pressure, HTN, DM, TG, HDL, and CRP), we found no association between intake of MUFA and prevalent CKD (Table 3).

3.2. Mendelian Randomization

The instrument associations for serum MUFA and PUFA levels are shown in Supplementary Table S1. The instruments had F-statistics ranging from 142 to 236, making significant bias from use of weak instruments unlikely (42). The results, expressed as beta-coefficient for serum MUFA/PUFA per 1 standard deviation (SD) increase in outcomes, are presented in Supplementary Tables S2–S7.

3.2.1. MUFAs

Genetically higher serum heptadecenoate (17:1), myristoleic acid (14:1), oleic acid (18:1), and palmitoleic acid (16:1) levels had no significant effect on risk of CKD (IVW = Beta: >0.2052, $p > 0.5888$, Supplementary Table S2) or level of eGFR (IVW = Beta: >-0.0098, $p > 0.6569$, Supplementary Table S2). Genetically determined levels of serum heptadecenoate (17:1), myristoleic acid (14:1), oleic acid (18:1), and palmitoleic acid (16:1) had no significant impact on CKD in either diabetic subjects (IVW = Beta: >-0.00512, $p > 0.9542$, Supplementary Table S2) or non-diabetic subjects (IVW = Beta: >-0.00174, $p > 0.937$, Supplementary Table S2).

3.2.2. PUFAs

With regards to the impact of PUFAs on renal function, we found that genetically higher levels of serum ALA and EPA had no significant impact on the risk of CKD (IVW = Beta: 0.3791, $p > 0.8125$), nor the level of eGFR (IVW = Beta: -0.04827, $p > 0.486$). Genetically determined levels of serum ALA and EPA had no significant impact on either diabetic subjects (IVW = Beta: -0.3987, $p > 0.1593$, Supplementary Table S6) or normal subjects (IVW = Beta: -0.03081, $p > 0.6565$, Supplementary Table S6).

Heterogeneity analyses and pleiotropy bias are also shown in Supplementary Tables S2–S7. The estimation based on both MR Egger and IVW was higher than 0.05, which indicted a low chance of heterogeneity (all IVW $p > 0.075$, all MR Egger $p > 0.063$). Furthermore, the results of the MR-PRESSO did not indicate outliers for all of the estimates. The horizontal pleiotropy test, with very negligible Egger regression intercept, also indicated a low likelihood of pleiotropy for all of our estimations (all $p > 0.212$). The results of the MR-RAPS were identical with the IVW estimates, again highlighting a low likelihood of pleiotropy (Supplementary Tables S2–S7). The results of the leave-one-out method demonstrated that the links were not driven by single SNPs. A graphical summary of methodology and results is displayed in Figure 1.

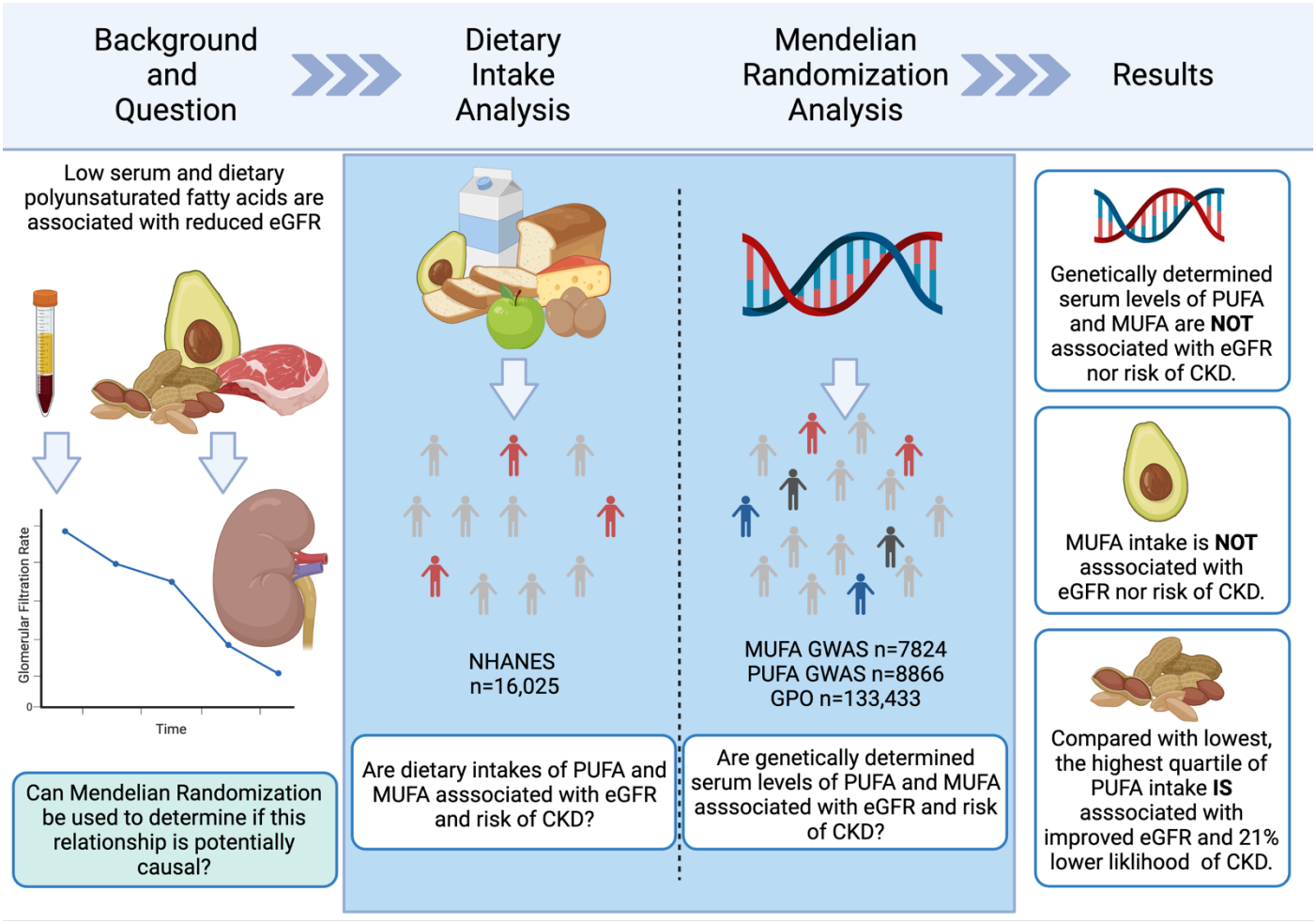

Figure 1. Graphical summary of the study methodology and results. eGFR: estimated glomerular filtration rate; NHANES: Nutrition and Health Examination Survey; PUFA: polyunsaturated fatty acid; MUFA: monounsaturated fatty acid; CKD: chronic kidney disease; GWAS: genome-wide association study; GPO: genetic predictors of outcomes.

4. Discussion

In this article, we analyzed dietary data on MUFAs and PUFAs intake, along with a set of genetic variants that have been demonstrated to be associated with four circulating serum MUFAs and PUFAs in order to determine their association with renal function. No significant association was observed between different dietary intakes of MUFAs. Conversely, the dietary intake of PUFAs was inversely associated with measures of kidney function and prevalent CKD. However, MR analyses did not support any causal effect of various MUFA or PUFA concentrations on CKD.

CKD is commonly observed as a comorbidity in chronic lifestyle diseases, including diabetes, hypertension, obesity, and cardiovascular disease [4–14], all of which are components of metabolic syndrome (MetS), which itself has been associated with CKD in multiple meta-analyses [43,44]. Lifestyle change involving dietary intervention is seen as a potentially cost-effective treatment for MetS [45,46]. In particular, modulation of dietary fatty acids, comprising replacement of saturated fatty acids (SFA) with MUFAs and PUFAs, has been shown to be beneficial for the prevention and improvement of MetS components [47–51]. Due to the prevalence and clinical significance of CKD [1,2], determining the role of specific macronutrients in its etiology should be considered important.

Although the cause−effect association between CKD and certain components of the MetS (diabetes and hypertension) is believed to be bidirectional [11] and higher ratios

of MUFA:SFA intake, along with increased intake of PUFAs, can lead to improvement of these conditions [50–52], there is little evidence to show a direct association between MUFA intake specifically and CKD. For example, while PUFA intake was associated with a lower risk of CKD in a non-diabetic population, such an association was not observed for MUFA [17]. Similarly, in an observational study in a diabetic population, MUFA intake was not associated with improvements in renal function, while an association was observed for the MUFA:SFA ratio and intakes of PUFAs [18]. There are a number of putative mechanisms through which an increased intake of PUFAs could potentially protect renal function, ranging from effects on regulatory molecules involved in renal inflammatory processes [53], reduction in proteinuria [54], improvement of blood pressure levels [55], reduction of serum triglycerides [56], and even improvements in blood vessel function [57].

We found no significant association between different dietary intakes of total and individual MUFAs and CKD. The addition of MR analysis makes our study superior to simple observational studies, as MR is a powerful tool for the detection of causation [21], and our results do not support a causal association between serum MUFAs and CKD. The MR analysis also showed no such association between genetically determined markers of serum ALA and EPA and renal function. This could potentially indicate that the results of the observational study of dietary intake could be affected by confounding or even reverse causation. Briefly, healthy dietary choices (such as increased PUFA intake) often occur together with other healthy dietary or lifestyle factors [58], leading to confounding of the interpretation of results. For example, healthy dietary patterns such as Mediterranean, DASH, and Prudent, which tend to be rich in fruits, vegetables, and whole grains, and lower in red and processed meat, refined grains, and added sugar, are associated with a reduced risk of chronic diseases such as obesity, diabetes, and cardiovascular disease [59], and subsequently with a reduced risk of CKD [60]. The overall composition of these healthier dietary patterns, as well as other healthy lifestyle behaviors that often occur together [58,61] and that may promote better kidney function, may confound a dietary analysis focused on specific nutrients, in this case PUFAs. Indeed, this may explain the difference in results for the dietary analysis and mendelian randomization in relation to PUFAs and risk of CKD. Similarly, reverse causality, whereby the knowledge of a disease status or disease-marker influences dietary choices, can be particularly problematic in retrospective studies [62]. However, another possible reason for the discrepancy in the dietary analysis and MR results should be considered. It should not be overlooked that serum fatty acids are known to be determined largely due to dietary intake [63]. Therefore, this study may highlight the fact that MR may not be a suitable analysis method for determining the role of such serum markers that are more dependent on diet as opposed to genetics.

Our study has some limitations. Firstly, the inability of the NHANES database to distinguish between MUFAs of an animal and plant origin, which could potentially contribute to the lack of association in this and other observational studies of CKD. In the "Western diet" pattern, MUFAs are predominantly supplied by foods of an animal origin, while in countries that typically follow a Mediterranean diet for example, extra virgin olive oil is the major source of these fatty acids [64]. Accordingly, dietary interventions high in MUFAs of a plant origin (from olive oil or nuts) have been shown to have benefits on cardiometabolic health, including hypertension and glucose control [46,65], whereas studies not differentiating between sources of MUFAs have found no benefit [16]. There is debate as to whether the observed benefits of MUFAs may be due to the fatty acids themselves or to other bioactive components in the fat sources or the dietary patterns [66–69]. Thus, elucidating the role of plant or animal MUFA intake in this particular cohort is not possible. A further limitation is that MR analysis is known to have limited statistical power, such that a lack of finding in our analysis might be due to small causal effects that were not detectable in our study. Finally, while we used the largest GWAS that is currently available in the literature for this analysis, the availability of future GWAS with a greater sample size, and thus providing more statistical power, may warrant further analysis of this topic at that time.

5. Conclusions

In conclusion, we found no evidence to support an association between the intake of total or individual MUFAs, nor a causal effect of serum MUFAs on CKD. While no causal effect of genetically determined serum PUFAs on prevalent CKD could be determined, a clear inverse association was observed between the dietary intake of PUFAs and CKD. While further investigation is required into the role of PUFAs in the development of CKD, these findings do not contradict the current evidence-base for the benefit of replacing dietary SFAs with MUFAs and, preferentially, PUFAs [70].

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/nu14061231/s1. Table S1: Summary results of the genetic loci of monounsaturated fatty acids (MUFAs) and polyunsaturated fatty acids (PUFAs). Table S2: Results of the Mendelian Randomization (MR) analysis for 10-heptadecenoate (17:1) and kidney function. Table S3: Results of the Mendelian Randomization (MR) analysis for Myristoleic acid (14:1) and kidney function. Table S4: Results of the Mendelian Randomization (MR) analysis for Oleic acid (18:1) and kidney function. Table S5: Results of the Mendelian Randomization (MR) analysis for Palmitoleic acid (16:1) and kidney function. Table S6: Results of the Mendelian Randomization (MR) analysis for alpha-linolenic acid (18:3) and kidney function. Table S7: Results of the Mendelian Randomization (MR) analysis for Eicosapentaenoic acid (20:5) and kidney function.

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Limitations of Self-reported Health Status and Metabolic Markers among Adults Consuming a "Carnivore Diet"

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Dear Editor:

We read with interest the publication by Lennerz et al. (1), which aimed to provide "descriptive data on the nutritional practices and health status of a large group of carnivore diet consumers." Indeed, high-quality research into the effects of this dietary pattern should be welcomed as, in agreement with the authors, little is known about the health status of people habitually following a carnivore eating pattern.

We understand the significant challenges the coronavirus disease 2019 (COVID-19) pandemic has placed on research and how this may have necessitated the reliance on survey-based methodologies. Online surveys may be adequate for evaluating satisfaction/acceptance of a given diet and potentially highlighting adverse effects. However, such methodology opens the door for considerable bias when trying to characterize dietary habits, describe health status and changes in said status, and assess nutritional deficiencies (2).

The authors highlighted that there are no validated instruments to assess food frequency on such a carnivore diet. However, the stated characteristics of the "carnivore diet" are an exclusively meat- and animal-based diet that eliminates most, or all, plant-based foods. Existing validated food-frequency questionnaires (FFQs) have shown good correlation coefficients between red meat consumption from FFQs and reference validation instruments across diverse populations (3–5). Any number of existing FFQs can be found at the Dietary Assessment Validation/Calibration Register maintained by the National Cancer Institute (6). We contend that the lack of "carnivore diet"–specific dietary assessment instruments did not preclude the use of validated food-frequency instruments that exhibit good correlations with animal meat intakes (3–5). The speculative methods used in the present study, such as the unvalidated modified Likert scales, should be treated with caution.

The data provided in relation to change in health status should also be interpreted with caution, primarily due to their unverified nature since beginning this dietary pattern. In more extensive epidemiological studies, such health status is normally verified through an interview by a trained interviewer or access to participant medical records (7). This is to confirm the specificity and accuracy of the participant's medical history details and ensure that they are true. No such verification was performed in this study, meaning that participants could, in theory, provide any information they wished, regardless of accuracy or integrity. It may also be considered dubious that participants would be able to accurately assess the presence or absence of nutritional deficiencies considering the possibility of subclinical manifestation, regardless of how likely they would or would not be expected to occur.

Furthermore, and of most concern, is the inclusion of self-reported data related to metabolic markers such as blood lipids. It is irregular for a study to include such data, which is, as previously mentioned, unverified and also subject to reporting bias, as will be discussed in the following paragraphs. In addition, we consider it highly unusual that such data would pass rigorous peer review due to their unverified nature, particularly the inconsistency in the use of current and pre-diet values. Given the selection bias inherent in the inclusion of adherents to a very specific dietary pattern, the lack of verified biomarker data should also be treated with caution and are of dubious scientific validity.

A further consideration is that those recruited (i.e., those who identify as followers of the carnivore diet) may have multiple other health behaviors that differentiate them from the general population, making generalization of any inferences from this study more difficult. Behavioral research posits that the more one self-identifies, for example, as a healthy person or a follower of a specific diet, the more likely one is to participate in other health-related behaviors (8). This may lead followers of specific diets to engage in other behaviors they deem to be healthy, such as regular exercise and stable sleep patterns, among many others, which may also have considerable effects on health markers and overall health status.

In recent years, research has noted the development of information and ideological echo chambers, segregated communities of online social media platforms, where individuals share a common interest or viewpoint and have little exposure to opposing views (9). While efficient, recruiting participants from such groups may increase the likelihood of information gerrymandering, whereby a small number of zealots may influence others' biased survey response outcomes (10). Indeed, as noted by the authors in the study limitations, selection bias for adults adhering to the carnivore diet may have led to selection of a particular subpopulation with high levels of affinity for the diet. It cannot be ruled out that such information gerrymandering did not occur in this study (intentionally or otherwise), leading to an increased likelihood of responses intended to paint this eating pattern in a positive light.

Of further concern is the number ($n = 28$) of duplicate survey responses identified by e-mail addresses, highlighting the risk of unverified respondents completing a survey multiple times (11). Considering

the aforementioned ideological echo chambers and desire to promote certain viewpoints, it may be speculated that specific individuals could complete the survey multiple times using alternate e-mail addresses. While this "stakeholder bias" is a potential concern for all such online survey-based research (11), it may be of particular concern among individuals aligned with specific dietary ideologies.

The authors are aware of many of the limitations of their study design and the generalizability of the results. It is also abundantly clear that higher-quality research is required to determine the carnivore diet's long-term positive and adverse health effects. However, considering the propensity of media outlets and the lay public to misinterpret, exaggerate, and disseminate findings from scientific research, we believe caution should be exercised when discussing the study's conclusions. In particular, discussion relating to the changes in health status and metabolic markers recorded in this study requires considerable reference to the unverifiable nature of the data.

We congratulate the authors on taking the first steps towards scientifically quantifying the health effects of the carnivore diet and welcome any future, high-quality studies that may provide valuable data to fill the sparse literature on this specific eating pattern.

Richard Kirwan
Gregg S Mallett
Lucy Ellis
Alan Flanagan

From the School of Biological and Environmental Sciences, Liverpool John Moores University, Liverpool, United Kingdom (RK, r.p.kirwan@2018.ljmu.ac.uk); School of Nursing and Health Sciences, Department of Exercise Science, Saint Xavier University, Chicago, IL, USA (GSM); School of Food Science and Nutrition, University of Leeds, Leeds, United Kingdom (LE); Department of Nutritional Sciences, Faculty of Health and Medical Science, University of Surrey, Surrey, United Kingdom (AF).

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Konstantinos Prokopidis ORCID iD: 0000-0002-6264-9388

Panagiotis Giannos ORCID iD: 0000-0003-1037-1983

Konstantinos S. Kechagias ORCID iD: 0000-0001-6681-8479

The impact of branched-chain amino acid supplementation on measures of glucose homeostasis in individuals with hepatic disorders: A systematic review of clinical studies

Konstantinos Prokopidis*[1]; Richard P. Kirwan[2]; Panagiotis Giannos[3]; Konstantinos K. Triantafyllidis[4]; Konstantinos S. Kechagias[5,6], Scott C. Forbes[7], Darren G. Candow[8]

[1]Department of Musculoskeletal Biology, Institute of Life Course and Medical Sciences, University of Liverpool, Liverpool, UK

[2]School of Biological and Environmental Sciences, Liverpool John Moores University, Liverpool, UK

[3]Department of Life Sciences, Faculty of Natural Sciences, Imperial College London, London, UK

[4]Department of Nutrition & Dietetics, Musgrove Park Hospital, Taunton & Somerset NHS Foundation Trust, Taunton, UK

[5]Department of Metabolism, Digestion and Reproduction, Faculty of Medicine, Imperial College London, UK

[6]Department of Nutrition & Dietetics, Chelsea and Westminster Hospital NHS Foundation Trust, London, UK

[7]Department of Physical Education Studies, Faculty of Education, Brandon University, Brandon, MB, Canada

[8] Faculty of Kinesiology and Health Studies, University of Regina, Regina, SK, Canada

*Corresponding author:

Konstantinos Prokopidis, University of Liverpool, Tel: +44 07464202561, email: k.prokopidis@liverpool.ac.uk

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Author Contributions Study concept and design: Mr Prokopidis, Mr Giannos. Acquisition of data: Mr Prokopidis, Mr Kirwan, Mr K. Triantafyllidis. Analysis and interpretation of data: Mr Prokopidis, Mr Kirwan, Dr S. Kechagias. Drafting of the manuscript: Mr Prokopidis, Mr Kirwan, Mr Giannos. Critical revision of the manuscript for important intellectual content: Mr Prokopidis, Mr Kirwan, Mr Giannos, Dr C. Forbes, Dr G. Candow.

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Biography:

Mr. Konstantinos Prokopidis: PhD(c) in musculoskeletal biology and clinical nutrition at University of Liverpool interested in sarcopenia, frailty, and inflammation.

Mr. Richard P. Kirwan: PhD(c) in clinical nutrition and post-doctoral researcher at LJMU with an interest in the role of protein, dietary patterns, and exercise on muscle mass and cardiometabolic disease risk.

Mr. Panagiotis Giannos: Biomedical scientist pursuing a PhD degree focused on mechanisms of sleep and sleep regulation at Imperial College London.

Mr. Konstantinos K. Triantafyllidis: Bariatric dietitian with an interest in nutritional assessment methods, bariatric surgery and gastroenterology.

Dr. Konstantinos S. Kechagias: Medical doctor working as a specialty trainee in obstetrics and gynaecology and pursuing a PhD degree at Imperial College London.

Dr. Scott C. Forbes: Associate professor at Brandon University, Manitoba, Canada. His main research area examines the interaction of nutrition and exercise

Dr. Darren G. Candow: Professor and Director of the Aging Muscle and Bone Health Laboratory at the University of Regina, Canada. Dr. Candow's research focuses on creatine monohydrate and resistance training on musculoskeletal aging.

KeyPoints

- Hepatic disorders such as liver cirrhosis, hepatic encephalopathy, and hepatocellular carcinoma, are characterized by an impaired circulating branched-chain amino acid (BCAA) profile.

- The aim of this systematic review was to explore the effects of isolated BCAA supplementation on markers of glucose metabolism in adults with hepatic disorders.
- Qualitative analysis revealed limited benefits of isolated BCAA supplementation on overall glucose homeostasis among individuals with hepatic disorders.
- BCAA supplementation as an independent strategy is not an effective tool in improving glucose homeostasis in this population group.

ABSTRACT

Background

Branched chain amino acid (BCAA) supplementation may influence glucose metabolism in individuals with impaired glycemic profile. This systematic review investigated the effects of isolated BCAA supplementation on measures of glucose homeostasis in individuals with hepatic disorders.

Methods

We searched PubMed, Web of Science, Cochrane Library, and Scopus for published clinical trials that investigated the effects of isolated BCAA supplementation on measures of glucose homeostasis, including serum glucose and insulin, glycated hemoglobin (HbA1c) levels, and Homeostatic Model Assessment for Insulin Resistance (HOMA-IR) scores.

Results

Eleven trials met the inclusion criteria. Only one study revealed a decrease in serum glucose from BCAA supplementation compared to three studies that showed increases. Five studies demonstrated no significant changes in serum glucose, and two studies displayed no changes in HbA1c following BCAA supplementation. Serum levels of insulin were decreased in three studies, remained unchanged in one, whilst increased in the remaining three studies. BCAA supplementation reduced HOMA-IR scores in two studies, increased HOMA-IR scores in another two or resulted in no changes in two other studies.

Conclusions

BCAA supplementation in isolation had no effect on overall glucose homeostasis in individuals with hepatic disorders, although some improvements on serum insulin levels and HOMA-IR scores were observed. Overall, there is little evidence to support the utilization of BCAA supplementation as a potential nutritional strategy for improving measures of glucose homeostasis in individuals with hepatic disorders.

Keywords: hepatic disorders, BCAA, branched chain amino acids, liver disease, nutritional supplementation

INTRODUCTION

Branched-chain amino acids (BCAAs: leucine, isoleucine, valine) are essential amino acids metabolized primarily in skeletal muscle (White, 2021). Despite their prominent role in skeletal muscle protein metabolism, BCAAs are fractionally catabolized in other organs, including the liver and adipose tissue (Brosnan and Brosnan, 2006), contributing to the upregulation of glucose transport and insulin secretion (Zhou et al., 2019). However, excessive BCAA consumption interferes with lipid oxidation in skeletal muscle (White et al., 2016), leading to impaired insulin signaling (Crossland et al., 2020, Tremblay et al., 2007, Jang et al., 2016, Zhou et al., 2019). Conversely, impaired insulin signaling may cause exacerbated skeletal muscle, adipose tissue, and liver proteolysis (Lake et al., 2015, Cheng et al., 2015, Lerin et al., 2016), which could potentially lead to high circulating levels of BCAAs (White et al., 2021). Epidemiological evidence has proposed that insulin resistance (IR) may drive increased circulating fasting BCAA levels, as opposed to BCAA consumption being the primary driver of IR (Mahendran et al., 2017). Indeed, a recent systematic review of observational studies has reported conflicting results on the association between intake of BCAAs and IR development, with two of the three reported studies suggesting a proportional relationship (Vieira et al., 2020).

BCAA supplementation has been reported to increase insulin secretion but with minimal influence on glycemic responses (Smith et al., 2015, Zhang et al., 2011), as opposed to protein supplements such as whey protein which may modulate glucose disposal in an insulin-dependent manner (Pal et al., 2010, Smith et al., 2015, Smith et al., 2020, Stevenson and Allerton, 2018). Particularly, improved oral glucose sensitivity index and postprandial insulin secretion have been observed in humans following short (1 week) (Ramzan et al., 2021) and longer (4 and 8 weeks) (Fontana et al., 2016, Karusheva et al., 2019) dietary BCAA intake restriction, however, longer trials may be warranted to elicit more clinically meaningful findings.

Hepatic disorders such as liver cirrhosis, hepatic encephalopathy, and hepatocellular carcinoma, are all characterized by decreased circulating BCAA levels (Tajiri and Shimizu, 2013). Hepatic disorders have long been linked with impaired glucose tolerance and IR, which has more recently been observed to improve upon BCAA supplementation (Sakaida et al., 2004, Kato et al., 1998, Sato et al., 2005, Park et al.,

2017). Indeed, BCAAs may increase peroxisome proliferator-activated receptor (PPAR)-γ and uncoupling protein 2 (UCP2) in the liver and UCP3 in skeletal muscle, stimulating free fatty acid oxidation and improving insulin sensitivity (Tajiri and Shimizu, 2013). The effects of BCAA consumption on glycemic profile may depend on dose, duration, and individual health status. These observations of improved IR and glucose tolerance with BCAA supplementation contrast considerably with the association of elevated serum BCAAs with IR in some chronic diseases. The aim of this systematic review was to investigate the effects of isolated BCAA supplementation on markers of glucose metabolism in adults with various hepatic disorders.

METHODS

This systematic review was performed based on the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) (Page et al., 2021) guidelines and the protocol was registered in the International Prospective Register of Systematic Reviews (PROSPERO) (Registration number: CRD42022304636).

Search strategy

Two independent reviewers (KP and RK) searched PubMed, Scopus, Web of Science, and Cochrane Library, using the following search terms: “BCAA” OR “branched chain amino acids” OR “leucine” AND “insulin” OR “blood glucose” OR “glycaemic” OR “blood sugar” OR “HbA1c” OR “HOMA-IR” AND “liver disease” OR “hepatic disorder” OR “cirrhosis” OR “hepatitis” OR “hepatocellular carcinoma” OR “portal vein embolization” OR “hepatic encephalopathy”. The full search strategy and search terms used are described in Table S1. Discrepancies in the literature search process were resolved by a third and fourth investigator (PG and KKT).

Study eligibility

Studies were included based on the following inclusion criteria: (1) human studies in populations with hepatic disorders; (2) clinical trials; (3) BCAAs as an intervention group; and (4) oral route of administration. Studies were excluded based on the following exclusion criteria: (1) non-clinical trials; (2) BCAA co-ingestion with a mixed meal; (3) acute studies lasting < 7 days; and (4) full text not published.

Data extraction and risk of bias

Two authors (KP and RPK) extracted data based on name of first author, publication date, country of origin, study design, participant health status, age, sex, sample size, outcome measures, supplemental form, dose, and duration. Disagreements between

authors were resolved by a third and fourth reviewer (PG and KSK). The quality of included studies was assessed using the Cochrane Risk-of-bias 2 (RoB2) for randomised trials tool and evaluated by three independent reviewers (KP, PG, and KKT). Appraisal of risk of bias using the RoB2 tool included assessment of the domains of bias in RCTs: (1) randomization process, (2) deviations from intended interventions, (3) missing outcome data, (4) measurement of the outcome, and (5) selection of the reported result (Higgins et al., 2011). According to the RoB2 tool scoring system, study quality was defined as low risk of bias, some concerns or high risk of bias. In addition, risk of bias assessment for the non-randomized (single arm) trials was performed using the Risk Of Bias In Non-Randomized Studies - of Interventions (ROBINS-I) tool that classifies studies based on bias due to: (1) confounding factors; (2) selection of participants into the study; (3) the classification of interventions; (4) deviations from intended interventions; (5) missing data; (6) outcome measurements; and (7) selection of the reported result.(Sterne et al., 2016) According to ROBINS-I tool, the quality of studies was categorized as low, moderate, or serious risk.

RESULTS

Search results

The literature search yielded 3403 publications. In total, 1318 duplicates were excluded, and 2085 publications were sought for retrieval. Following screening of tittles, abstracts, and full-texts, 20 studies were retrieved examining the effects of BCAA supplementation on markers of glucose metabolism. Of these, two studies had ineligible interventions, three had incompatible study population, and four had missing data. Overall, 11 studies were deemed eligible for inclusion in the review (Figure 1).

Characteristics of the included studies

All relevant information pertained to participant characteristics are summarized in Table 1. Of the 11 studies, seven studies were conducted in Japan (Kitajima et al., 2018, Beppu et al., 2015, Yoshiji et al., 2011, Ichikawa et al., 2010, Kawaguchi et al., 2008, Nakaya et al., 2007, Takeshita et al., 2012), two in Mexico (Ruiz-Margáin et al., 2018, Ocaña-Mondragón et al., 2018), one in Italy (Marchesini et al., 1990), and one in Spain (Hernández-Conde et al., 2021). Two studies were conducted in individuals aged between 50-60 years (Ruiz-Margáin et al., 2018, Takeshita et al., 2012) and nine in individuals ≥ 60 years (Kitajima et al., 2018, Beppu et al., 2015,

Yoshiji et al., 2011, Ichikawa et al., 2010, Kawaguchi et al., 2008, Nakaya et al., 2007, Ocaña-Mondragón et al., 2018, Marchesini et al., 1990, Hernández-Conde et al., 2021). All studies were cohorts of both males and females. Two studies did not provide relevant information pertained to the total number of males and females (Takeshita et al., 2012, Marchesini et al., 1990).

Further, four studies were RCTs (Beppu et al., 2015, Yoshiji et al., 2011, Ichikawa et al., 2010, Nakaya et al., 2007), two were double-blinded RCTs (Hernández-Conde et al., 2021, Marchesini et al., 1990), one was a crossover, open-label RCT (Takeshita et al., 2012), one was an open label RCT (Ruiz-Margáin et al., 2018), and three were clinical trials (Kitajima et al., 2018, Ocaña-Mondragón et al., 2018, Kawaguchi et al., 2008). Moreover, seven used BCAA supplementation alone (Kitajima et al., 2018, Ocaña-Mondragón et al., 2018, Beppu et al., 2015, Yoshiji et al., 2011, Takeshita et al., 2012, Ichikawa et al., 2010, Marchesini et al., 1990), three co-supplemented vitamins and minerals (Hernández-Conde et al., 2021, Kawaguchi et al., 2008, Nakaya et al., 2007) of which one followed a physical activity protocol (Hernández-Conde et al., 2021), and one followed a high-protein/high-fiber diet (Ruiz-Margáin et al., 2018). BCCA supplementation ranged from 4 weeks to 48 months in terms of duration and from 2.4 to 30 g/day in terms of dosage.

Amongst the comparator groups, two studies used placebo controls (Marchesini et al., 1990, Hernández-Conde et al., 2021), of which one included physical activity (Hernández-Conde et al., 2021), one used an isocaloric control snack (Nakaya et al., 2007), four used a usual diet regime (Beppu et al., 2015, Yoshiji et al., 2011, Takeshita et al., 2012, Ichikawa et al., 2010), one used a high-protein/high fiber diet (Ruiz-Margáin et al., 2018), while three were single arm trials (Kitajima et al., 2018, Ocaña-Mondragón et al., 2018, Kawaguchi et al., 2008).

Six studies included individuals with liver cirrhosis (Hernández-Conde et al., 2021, Ruiz-Margáin et al., 2018, Kitajima et al., 2018, Ichikawa et al., 2010, Nakaya et al., 2007, Marchesini et al., 1990), of which one experimented with sleep disturbance (Ichikawa et al., 2010), one with hepatocellular carcinoma (HCC) (Yoshiji et al., 2011), two with hepatitis (Ocaña-Mondragón et al., 2018, Takeshita et al., 2012), of which one included participants with insulin resistance (Takeshita et al., 2012), one with portal vein embolization (PVE) and sequential hepatectomy (Beppu et al., 2015).

Serum insulin

BCAA supplementation led to conflicting results regarding serum insulin levels. Specifically, 8 g/d of BCAA for 6 months, decreased serum insulin from 13.85 (6.6 – 18.6) U/mL to 7.9 (5.0 – 96.9) U/mL in patients undergoing PVE, however, similar changes were shown in the control group, which followed their usual diet (13.50 (4.4 – 18.8) U/mL to 9.2 (2.7 – 38.8) U/mL) (Beppu et al., 2015). Furthermore, another study in patients with liver cirrhosis showed that 3 months of 2.4 g/d BCAA slightly improved serum insulin (25 ± 17 μU/L to 23 ± 17 μU/l) compared to placebo (casein) group (19 ± 10 μU/l to 22 ± 17 μU/l), although no significant changes were observed (Marchesini et al., 1990). On the contrary, in patients with hepatitis C and insulin resistance, BCAA supplementation (12.5 g/d) increased serum insulin levels after 12 weeks (13.8 ± 1.6 μU/l to 17.8 ± 3.6 μU/l) as opposed to participants following their usual dietary patterns (23.3 ± 8.0 μU/l to 21.2 ± 4.6 μU/l) (Takeshita et al., 2012). Furthermore, another study showed a substantial increase of serum insulin (16.2 ± 6.8 μU/mL to 32.9 ± 34.5 μU/mL) compared to an isocaloric control snack (21.3 ± 19.5 μU/mL to 20.9 ± 14.4 μU/mL) in patients with liver cirrhosis (Nakaya et al., 2007). However, in this case the supplementary product consisted of BCAAs alongside vitamins and minerals. In the single arm studies, a high BCAA dose (30 g/d) was slightly effective in reducing serum insulin levels (16 (11 – 31) μU/l to 14 (9 – 22) μU/l) in patients with chronic hepatitis C when administered for 30 months (Ocaña-Mondragón et al., 2018), while another study displayed a significant decrease of serum insulin (22.8 ± 9.7 μU/mL to 13.3 ± 1.9 μU/mL) after BCAA supplementation (6.4 g/d) with vitamins and minerals after 90 days in patients with chronic liver disease (Kawaguchi et al., 2008). Finally, one study demonstrated a small increase in serum insulin (14.2 ± 11.8 μU/mL to 15.7 ± 16.5 μU/mL) following a low BCAA dose (4 g/d) in patients with liver cirrhosis for 48 weeks (Kitajima et al., 2018).

Serum glucose

Conflicting results were also observed on serum glucose after BCAA supplementation. In one study using 8.6 g/d BCAA (Ruiz-Margáin et al., 2018), a small increase in serum glucose levels in the intervention (110.8 ± 52.9 mg/dl to 112 ± 52 mg/dl) group was observed as opposed to the control group (104.3 ± 45.4 mg/dl to 94.1 ± 17.4 mg/dl) in patients with liver cirrhosis when administered 6 months. Likewise, another study displayed a similar trend following 12.3 g/d BCAA co-supplemented vitamins and minerals (107 ± 23 mg/dl to 118 ± 39 mg/dl) compared to

an isocaloric snack group (99 ± 26 mg/dl to 95 ± 10 mg/dl) (Nakaya et al., 2007). Furthermore, another study also showed a small increase in the intervention (92.1 ± 2.1 mg/dl to 96.6 ± 2.1 mg/dl) compared to the usual diet group (100.6 ± 2.9 mg/dl to 96.2 ± 2.0 mg/dl) (Takeshita et al., 2012). On the other hand, a significant decrease in serum glucose levels (126.0 (75 – 184) mg/dl to 98.0 (84 – 242) mg/dl) was reported after 6 months with 8 g/d BCAA supplementation compared to usual diet (101.0 (87 – 123 mg/dl to 104.0 (90- 125) mg/dl) in patients with PVE (Beppu et al., 2015). No changes were seen in serum glucose levels of patients with HCC between the intervention (102.7 ± 30.6 mg/dl to 95.4 ± 31.1 mg/dl) and the control group (113.4 ± 28.8 mg/dl to 107.8 ± 31.2 mg/dl) following 12 g/d for 48 months (Yoshiji et al., 2011). In addition, an identical trend was depicted in patients with liver cirrhosis and sleep disturbance after 13.5 g/d BCAA for 8 weeks (107.5 ± 27.2 mg/dl to 105.7 ± 73.2 mg/dl) against usual diet (115.4 ± 27.2 mg/dl to 111.6 ± 24.2 mg/dl) (Ichikawa et al., 2010). In the single arm studies, serum glucose was reduced in each trial, however, no significant decrease was displayed (113.6 ± 31.7 mg/dl to 108.5 ± 27.7) (Kitajima et al., 2018); (124.2 ± 9 mg/dl to 120.6 (109.9 – 133.3) mg/dl) (Ocaña-Mondragón et al., 2018); (104.5 ± 6.4 mg/dl to 102.8 ± 5.4 mg/dl) (Kawaguchi et al., 2008).

Glycated haemoglobin

No changes in HbA1c were observed following 12.45 g/d BCAA supplementation for 12 weeks compared to usual diet in IR patients with hepatitis C (5.0 ± 0.1% to 4.9 ± 0.1% vs. 4.9 ± 0.1% to 5.0 ± 0.1%) (Takeshita et al., 2012). Additionally, no changes on HbA1c were revealed after consumption of 6.4 g/d BCAA for 90 days (5.5 ± 0.2% to 5.4 ± 0.3%) (Kawaguchi et al., 2008).

Homeostatic Model Assessment for Insulin Resistance

The overall score of HOMA-IR was reduced following 5.2 g/d BCAA co-supplemented with vitamins, minerals, and physical activity after 12 weeks (4.9 ± 6.7 to 3.2 ± 1.8), however, no differences were observed compared to the physical activity and placebo group (6.3 ± 8.6 to 4.7 ± 3.2) (Hernández-Conde et al., 2021). Similarly, identical findings were identified following 12 g/d of BCAA supplementation for 12 weeks (3.55 ± 3.01 to 2.75 ± 2.08) against placebo (3.79 ± 2.92 to 3.61 ± 2.88) (Yoshiji et al., 2011). Interestingly, an increase in HOMA-IR score was demonstrated after 12.45 g/d for 12 weeks of BCAA (3.2 ± 0.4 to 4.5 ± 1.1) compared to usual diet that reduced HOMA-IR (6.1 ± 2.2 to 5.3 ± 1.3) (Takeshita et

al., 2012). In the single arm studies, BCAA supplementation led to a decrease in HOMA-IR after 90 days as observed in (5.5 ± 2.1 to 3.5 ± 0.6) (Kawaguchi et al., 2008) and (3.5 (2.6 – 7.9) to 3.2 (1.9 – 5.0) (Ocaña-Mondragón et al., 2018). Finally, a study revealed higher HOMA-IR scores following a 4 g/d BCAA dose for 48 weeks (3.9 ± 3.0 to 4.5 ± 5.4) (Kitajima et al., 2018).

Risk of bias

According to RoB2, risk of bias was high in one study (Beppu et al., 2015) due to lack of information relevant to treatment allocation concealment and participants and trial personnel knowing about the type of intervention. Finally, some concerns were raised in three studies due to participants possibly knowing about the type of intervention (Takeshita et al., 2012, Ichikawa et al., 2010, Nakaya et al., 2007). A detailed traffic light plot is presented in Figure 2.

According to ROBINS-I, moderate risk of bias was displayed in one study due to insufficient control for confounders (i.e., physical activity) (Kitajima et al., 2018). Serious risk of bias was observed in two studies due to no control for major confounding factors (i.e., diet and physical activity) (Ocaña-Mondragón et al., 2018). A detailed traffic light plot is presented in Figure 3.

DISCUSSION

In this systematic review, we identified 11 studies examining the effects of BCAA supplementation on markers of glucose metabolism in participants with hepatic disorders. Overall, BCAA supplementation resulted in small decreases in serum insulin and HOMA-IR scores with no effect on serum glucose levels or changes in HbA1c.

The maintenance of physiological serum glucose is an essential component of glucose homeostasis, with impaired glycaemic control linked to a greater risk of chronic diseases such as T2D and cardiovascular disease (Skyler et al., 2009, Nichols et al., 2013). A contributing factor to poor glycemic control is IR. Epidemiological data has shown that IR and clinical diagnoses of T2D and pre-diabetes are associated with elevated serum BCAAs (Long et al., 2020). In contrast to the observation of higher serum BCAA levels in those with IR or T2D, BCAA supplementation has been reported in some cases to improve measures of glucose homeostasis (Yoshizawa, 2012). Recent research using Mendelian randomisation analysis has further clarified that elevated serum BCAAs are likely driven by the presence of IR and not the other way around (i.e., elevated serum BCAA do not drive IR) (Mahendran et al., 2017).

Animal models have revealed that a mechanism for the potentially beneficial effects of BCAA supplementation on glycemic control is the activation of phosphoinositide-3 kinase (PI3K). This increase in insulin sensitivity and upregulation of glucose transporter protein 4 (GLUT4) may facilitate non-insulin mediated entry of glucose into cells (Zhu et al., 2021). Additional research in rat models has duplicated the observation of increased GLUT4 translocation to the skeletal muscle cell membrane as well as increased translocation of the GLUT1 glucose transporter protein (Nishitani et al., 2005). The same research group observed an upregulation of glycogen synthase activity in leucine treated rats, which resulted in increased glycogen content in soleus muscle compared to controls (Nishitani et al., 2005). Such increased synthesis of glycogen by taking excess serum glucose out of circulation and storing it in skeletal muscle, could assist with overall glycemic regulation.

Insulin sensitivity may be further affected by increased utilization of glucose as fuel through glycolysis, via upregulation of GLUT2 and glucokinase in the liver, leading to improved bioactivity of the glucose-sensing apparatus (Higuchi et al., 2011). Specifically, glucokinase is involved in the regulation of hepatic glycolysis and glucose oxidation, glycogen synthase, glycogenolysis and gluconeogenesis amongst others (Matschinsky, 2009). Therefore, BCAA supplementation may act as a partial substitute for insulin in glucose transport regulation by increasing glycogen synthesis in both skeletal muscle and liver. However, it should be noted that some research has reported conflicting results. Specifically, infusion of amino acids including leucine and isoleucine in human subjects has been reported to compete with glucose as an oxidative fuel, reducing glucose uptake (Schwenk and Haymond, 1987). Nevertheless, the aforementioned study involved venous infusion and not dietary supplementation of BCAAs, indicating that elevated serum levels of BCAAs may interfere with glycemic control and not necessarily dietary intake.

Moreover, increased adiposity and in particular, skeletal muscle and liver tissue triglyceride (TG) accumulation are known to interfere with GLUT4 translocation and glucose uptake, mediated via the activation of insulin-stimulated PI3K, which may lead to IR (Shulman, 2000). In mouse models, supplementation with the BCAA isoleucine has been reported to reduce accumulation of TG in both skeletal muscle and liver tissue (Nishimura et al., 2010, Arakawa et al., 2011). This is speculated to occur via upregulation of peroxisome proliferator-activated receptor (PPAR)-α and uncoupling protein (UCP) 2 in liver tissue and UCP3 in the skeletal muscle tissue.

Thus, leading to increased free fatty acid oxidation, which results in improvements of insulin sensitivity induced by lipotoxicity (Arakawa et al., 2011, Guerre-Millo et al., 2000).

Limitations

This systematic review is the first to examine the effects of isolated BCAA supplementation on markers of glucose metabolism in patients with hepatic disorders. The prevailing limitation of this review was the inability to produce a meta-analysis due to the heterogeneity in study designs. The large heterogeneity in protocols that can be observed in the populations included, the varied dosage of BCAA supplementation (2.4 – 30 g/day), and study duration (4 weeks to 48 months). Furthermore, of the 11 studies included, seven involved Japanese populations with the remaining four studies from the USA, Spain, Mexico, and Italy, which may raise concerns regarding the generalizability of the results to other geographical regions or ethnicities. Finally, inconsistencies among dietary intakes among studies, in which there was no control is a critical confounding factor in extrapolating more accurate conclusions regarding the effects of BCAA supplements in isolation.

CONCLUSIONS

This systematic review revealed limited effects of isolated BCAA supplementation on overall glucose homeostasis among individuals with hepatic disorders, however, some improvements on serum insulin and HOMA-IR scores were observed. Studies should be aware of controlling strictly for dietary intake to omit the potential impact of other nutrients on glucose homeostasis and incorporate a placebo group as a comparator that would reduce bias risk. BCAA supplementation as an independent strategy appears to may not be an effective tool in improving glucose homeostasis in patients with hepatic disorders.

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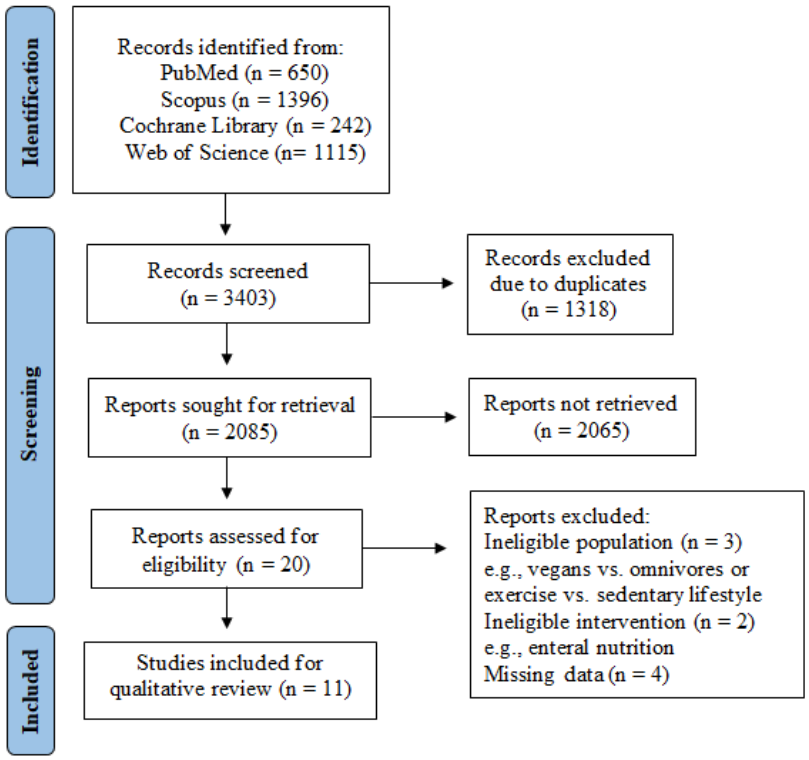

Figure 1. Flowchart of the employed literature search

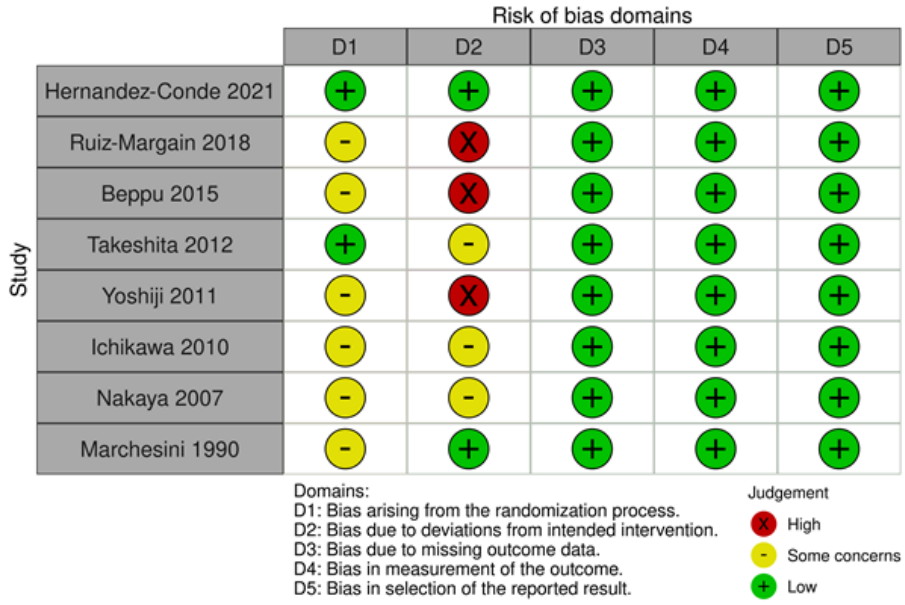

Figure 2. Quality assessment of the included studies according to the Cochrane risk-of-bias tool for randomised trials (RoB2).

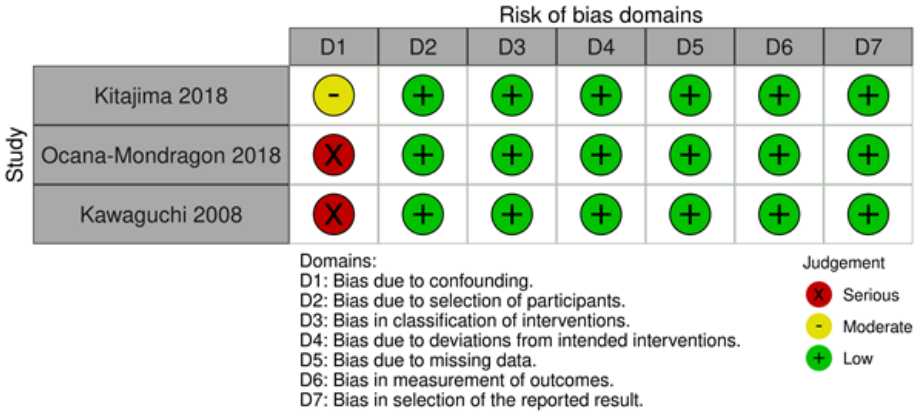

Figure 3. Quality assessment of the included non-randomized (single arm) studies according to the Risk Of Bias In Non-randomised Studies-of Interventions tool (ROBINS-I).

Table 1. Study and participant characteristics of the included studies.

Study year	Country	Study design	Total	BCAA		Comparator		Treatment dose (g/d)	Treatment duration	Health status	Reported outcomes
			n (M/F)	*n* (M/F)	Age (SD)	*n* (M/F)	Age (SD)				
Hernandez-Conde 2021	Spain	Double-blind RCT	32 (28/4)	17 (15/2)	69 (9.7)	15 (13/2)	61 (9.4)	5.2	12 weeks	Cirrhosis	HOMA-IR
Ruiz-Margain 2018	Mexico	Open-label RCT	72 (14/58)	37 (6/31)	54.9 (10.3)	35 (8/27)	47.8 (14.6)	8.6	6 months	Cirrhosis	Glucose
Kitajima	Japan	Clinical	21 (9/	21 (9/	71.3	-	-	4	48 week	Cirrhosis	Insulin

2018*		Trial	12)	12)	(7.9)				s		Glucose HOMA-IR Insulin Glucose HOMA-IR Insulin Glucose HbA1c Insulin Glucose HOMA-IR HbA1c Glucose HOMA-IR Gluc
Ocana-Mondragon 2018*	Mexico	Clinical Trial	20 (10/10)	20 (10/10)	53 (45.63)	-	-	30	3 months	Chronic Hepatitis C	
Beppu 2015	Japan	RCT	28 (19/9)	13 (9/4)	64 (47-83)	15 (10/5)	72 (56-78)	8	6 months	PVE	
Takeshita 2012	Japan	Crossover, open label RCT	24	13	58.6 (2.9)	11	64.2 (3.0)	12.5	12 weeks	Hepatitis C IR	
Yoshiji 2011	Japan	RCT	42 (26/16)	16 (10/6)	63.7 (10.8)	26 (16/10)	62.5 (11.5)	12	48 months	HCC	
Ichik	Japa	RCT	21	12	66.	9	67.	13.5	8	Cirrh	Gluc

awa 2010	n		(10 /11)	(5/ 7)	2 (8. 2)	(5/ 4)	4 (9. 9)		week s	osis Sleep Distur bance	ose
Kawa guchi 2008*	Japa n	Clini cal Trial	12 (5/ 7)	12 (5/ 7)	64. 3 (2. 4)	-	-	6.4	90 days	Chron ic Liver Disea se	Insul in Gluc ose HO MA-IR HbA 1c
Naka ya 2007	Japa n	RCT	38 (20 /18)	19 (13 /6)	67 (9)	19 (7/ 12)	67 (8)	12.3	3 mont hs	Cirrh osis	Insul in Gluc ose
Marc hesini 1990	Italy	Doub le-blind RCT	61	29	60 (44 - 70)	32	60 (4 3- 70)	2.4	3 mont hs	Cirrh osis	Insul in Gluc ose

Abbreviations: HbA1c, glycated haemoglobin; HCC, hepatocellular carcinoma; HOMA-IR, homeostatic model assessment for insulin resistance; IR, insulin resistance; PVE, portal vein embolization; RCT, randomized controlled trial.

*Studies with single-arm clinical trial design.

Increased COVID-19 Infection Susceptibility and Adverse Outcomes Due to Obesity: A Systematic Review and Meta-analysis

Xiaomin Sun
Xi'an Jiaotong University
Mohsen Mazidi
King's College London
Richard Kirwan
Liverpool John Moores University
Yixuan Li
Xi'an Jiaotong University
Bingtong Zhao
Xi'an Jiaotong University
Jayanthi Kandiah
Ball State University
Youfa Wang (youfawang@gmail.com)
Xi'an Jiaotong University

Research Article

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Abstract

Objectives: Study bidirectional relationships between weight gain, obesity and COVID-19 infection risk during the pandemic.

Methods: MEDLINE, Embase, MedRxiv, and WHO COVID-19 Database were searched till June 2, 2020. Newspaper and internet article sources were identified using a media database. Meta-analysis was conducted using random- and fixed effect models.

Results: Ten studies published from 5 countries met inclusion criteria; five studies (provided 17 analyses/types of results) were included in meta-analysis. The studies suggested bidirectional relationships and some dose-response relationships. Meta-analysis showed obesity was associated with increased COVID-19 infection risk (Odds ratio, OR=1.69, 95% CI: 1.41, 2.02). COVID-19 risk increased with obesity (OR=1.43 (1.18, 1.73)) and severe obesity (OR=1.96 (1.49, 2.59)). Obesity was positively associated with COVID-19 mortality (OR=1.64, 95% CI: 1.20, 2.25) and its severity: admission to intensive care unit (ICU) (OR=2.01 (1.25, 3.23)), and invasive mechanical ventilation (IMV) use (OR=8.20 (2.10, 31.91)). We also observed a stronger association in younger age groups (β=-0.29 (-0.47 to -0.10)).

Conclusions: Obesity was positively associated with higher COVID-19 infection risk, severity, and mortality. Appropriate treatment of COVID-19 patients with obesity and weight management are warranted.

Introduction

As of early June 2020, the SARS-CoV-2 (COVID-19) pandemic has become a global emergency of unprecedented proportions with more than 6.9 million confirmed cases and over 400,000 deaths globally (1). In response to this pandemic, governments are implementing widespread measures to reduce the spread of the disease including quarantine, isolation, social distancing and bans on travel, both domestically and internationally (2, 3). Such measures have affected people's daily life including their eating and physical activity, and thus may affect their body weight as well.

Some initial reports from multiple countries have highlighted obesity amongst COVID-19 infected patients and particularly in more severe cases that were admitted to intensive care units (ICU) (4, 5). Some research suggests that in populations with obesity, more severe degree of obesity classification as defined by body mass index (BMI) ≥35 kg/m^2) was associated with even more serious prevalence of the disease (6), and obesity was associated with greater mortality (7). The US Centers for Disease Control and Prevention (CDC) has suggested that individuals with obesity and conditions which are associated with obesity, are amongst those at greatest risk from COVID-19 (8).

However, the link between obesity and COVID-19 infection are poorly understood, not systematically studied yet. Therefore, a comprehensive review will help shed light on this link by systematically reviewing all the accessible studies in order to provide more clarity to the currently available body of scientific evidence.

Obesity has become a global public health crisis, and the prevalence was nearly tripled between 1975 and 2016 (9, 10, 11). As of 2016, the World Health Organization estimated that over 650 million people globally suffered with obesity indicating that a significant proportion of the population are at elevated risk of COVID-19 complications and mortality (12). This obesity problem is potentially exacerbated by the many measures being used to fight COVID-19, which increase risk for development of obesity. Increased time spent at home, highlighted by mobile phone tracking reports (13), may contribute to reductions in physical activity while increased levels of stress and anxiety brought on by quarantine (14, 15, 16) may result in unhealthy food choices leading to the overconsumption of hyperpalatable and energy dense convenience foods (17).

This study reviewed research on the role obesity plays in susceptibility to COVID-19 and the risk of developing more severe, life-threatening symptoms. We also examined the evidence for the increased risk of weight-gain and subsequent development of obesity during the COVID-19 pandemic. This body of knowledge will aid health care practitioners in deciding whether the use of greater vigilance, and tailoring their testing and treatment practices to individuals with obesity is warranted for better outcomes and reductions in mortality. Information on the role of obesity in COVID-19 progression may also help the public and government agencies in determining whether engaging in activities that can result in lower adiposity or prevent the progression of obesity are likely to result in reduced risk of COVID-19 infection and/or more severe disease symptoms.

Methods And Materials

1. **Study design and data collection**

This systematic review was conducted in accordance with the Preferred Reporting in Systematic Reviews and Meta-Analyses guidelines. MEDLINE (via PubMed), Embase, MedRxiv, and WHO COVID-19 Databases were searched from 1st April, 2020 through 2nd June, 2020. Newspaper and internet article sources were identified using a media database called Nexis (https://www.lexisnexis.com/en-us/products/nexis.page). The key search terms we used included 'coronavirus OR Severe acute respiratory syndrome OR covid-19 OR nCoV OR COVID OR SARS OR MERS OR middle east respiratory syndrome) AND (Obesity OR BMI OR body mass index OR weight OR overweight.'

To account for papers not yet indexed in databases, we also hand searched COVID-19 resource centers from the following journals and publishers, by manually trawling the collections for relevant titles: BMJ; Cambridge University Press; Elsevier; JAMA Network; The Lancet; New England Journal of Medicine; Oxford University Press; PLOS; Springer Nature; SSRN (reprints); Wiley. There was no restriction on publication type. This search was complemented by an exhaustive review of the bibliography of key articles. Results were restricted to English language articles. All retrieved studies were exported into EndNote to remove duplicates.

2. **Study/report inclusion and exclusion criteria**

All reported studies that reported results on the association between obesity and COVID-19 were included. In addition, the inclusion criteria incorporated entire comprehensive methodological study designs worldwide. We excluded articles that exclusively reported data on other respiratory viruses that did not relate to the COVID-19 clinical or epidemiological aspects, and those not written in English. We excluded editorials, correspondence letters, reviews, qualitative studies, theses, and non-full text articles.

Based on PRISMA guidelines, a flow chart was produced to facilitate transparency of the process (**Figure 1**). In total, 10 studies met our inclusion criteria and were included in this report. Of them, five studies provided adequate data and were included in meta-analysis.

3. **Data extraction**

Two authors independently assessed (screened titles and abstracts, reviewed full reports) the articles for inclusion and exclusion criteria and extracted data, with a third author resolving any differences. The reference lists from all identified studies and reviews were scrutinized for eligible articles. The data extraction was independently checked by the senior author.

The following data were retrieved from each study: country/setting, study sample size, age (mean or range), % of male participants, main findings relating to the association of obesity with outcomes (e.g. Odds ratio, OR), and other

information of importance, such as BMI cut-offs used for classifying body weight status including obesity. In addition, in studies related to people's weight gain and obesity related behaviors the following baseline data were gathered from each study: main findings on weight gain and eating habits and physical activity.

4. **Statistical analysis**

Meta-analysis was performed using findings from five studies, which provided needed information to estimate the pooled effects on the associations between COVID-19 and obesity. The OR and 95% confidence interval (CI) were used to determine the associations between COVID-19 and obesity. Study heterogeneity was assessed using the I^2 index. The level of heterogeneity represented by I^2 was interpreted as small ($I^2 \leq 25\%$), moderate ($25\% < I^2 \leq 50\%$), substantial ($50\% < I^2 \leq 75\%$), or considerable ($I^2 > 75\%$). In our meta-analysis, a fixed-effect model was estimated when modest to moderate heterogeneity was present, and a random-effects model was estimated when substantial to considerable heterogeneity was present. Additionally, meta-regression analyses were performed in order to determine whether subjects' age (in years), and gender (male) could be related with the associations between COVID-19 and obesity.

To investigate potential sources of heterogeneity, subgroup analyses were performed based on categorical variables including country, study design, obesity classification and the severity of COVID-19. In this review, obesity classification based on BMI was defined as: 1) obesity; 2) severe obesity. The severity of COVID-19 was classified as: 1) COVID-19 admission; 2) ICU admission; 3) IMV use; 4) acute care admission; and 5) death by COVID-19.

A pre-specified sensitivity analysis was conducted to investigate the influence of a single study on the overall pool estimate by omitting one study in each turn. Publication bias was assessed by visual inspection of the funnel plot and Begg's and Egger's tests.

All statistical analyses were conducted using the Stata software version 14 (Stata Corp., College Station, Texas, USA). All analyses used two-sided tests, and p-values□0.05 were considered statistically significant.

Results

1. **Characteristics of the included studies**

The characteristics of the 10 included studies are summarized in **Table 1**. These studies were published in the months of April and May 2020 from 5 countries, including China (2 studies), France (2 studies), Italy (1 study), the United Kingdom (3 studies), and the United States (2 studies). The sample size of these studies varied greatly, ranging from 41 to 17,425,445. Nine studies focused specifically on COVID-19 and obesity, and one study investigated obesity related behaviors (e.g., eating and physical activity).

The study design and populations were diverse. There were 8 cohort studies, 1 cross-sectional study and 1 case report. These studies included retrospective analysis of primary care electronic health records in the general population (1 study), retrospective analysis of cohorts of COVID-19 patients (5 studies), and prospective observational cohort studies (3 studies). The duration of data collected from these studies ranged from 1 week (2 studies), to 3 weeks (3 studies), 4 weeks (1 study), 6 weeks (1 study), 10 weeks (1 study) and 12 weeks (1 study). Some studies only included adults while others incorporated children. Subjects' ages ranged from 0 to 104 years.

Only one study examined the influence of COVID-19 on obesity-related behaviors. This was a longitudinal observational study in Italy with 41 children. It reported changes in eating, physical activity and inactivity behaviors.

2. **Effect of obesity on COVID-19 infection risks**

Nine studies provided results regarding the association between obesity and COVID-19 infection and severity risks. Three studies reported that obesity was more prevalent amongst COVID-19 infected patients. In a retrospective cohort study of SARS-CoV-2 confirmed patients, compared with a non-SARS-CoV-2-infected patient control group, Simonnet et al (4) observed that obesity (BMI 30-34.9kg/m^2) and severe obesity (BMI ≥35 kg/m^2) were significantly more frequent among COVID-19 infected patients than controls (47.6% vs 25.2% and 28.2% vs 10.8%, respectively). Williamson et al (18) used data from over 17 million UK National Health Service primary care records and observed that the incidence of COVID-19 advanced with increasing severity of obesity. They reported a dose-response relationship between degree of obesity and COVID-19 infection risk. Assuming non-obese individuals had a hazard ratio (HR) of 1.00, the fully adjusted HRs for obesity class I (BMI 30-34.9kg/m^2), obesity class II (BMI 35-39.9kg/m^2) and obesity class III (BMI ≥40 kg/m^2) were 1.27, 1.56 and 2.27, respectively. An intensive care admissions report of confirmed COVID-19 cases from the Intensive Care National Audit and Research Centre (19) showed the majority of ICU admissions (71.7%) also suffered from overweight or obesity. Specifically, 31.6% of ICU admissions had overweight (BMI 25-29.9kg/m^2), 32.8% had moderate obesity (BMI 30-39.9kg/m^2) and 7.3% had severe obesity (BMI ≥40 kg/m^2).

Six studies from different countries reported on the association between obesity and severity of COVID-19 infection based on varying criteria such as admission to acute or critical care such as ICU or requirement for IMV. Overall, they suggested a positive assocatoin. In a study of 291 French patients admitted to ICU for SARS-CoV-2, Caussy et al (20) observed a doubled risk of requirement for IMV in severe obese- (BMI ≥35 kg/m^2) compared to lean patients (81.8% versus 41.9%). In a retrospective analysis of COVID-19 hospitalized patients in Wuhan, China, Hu et al (21) observed that while obesity (BMI ≥30 kg/m^2) was not associated with diagnosis of disease severity (p=0.522), it was significantly associated with unfavorable clinical outcomes (OR=3.6, p=0.009). In a retrospective cohort study of 103 US patients admitted for COVID-19, Kalligeros et al (6) observed that obesity was associated with the severity of COVID-19 presentation. Severe obesity was associated with admission to ICU (adjusted OR=5.39) while obesity and severe obesity were associated with the need for IMV (adjusted OR=6.85 and 9.99, respectively). Similarly, Lighter et al (5) performed a retrospective analysis of COVID-19 patients in a New York hospital and found that in those under 60 years old, patients with obesity (BMI 30-34.9kg/m^2) and severe obesity (BMI 35-39.9kg/m^2) had a greater risk of admission to acute care (OR 2.0 and 2.2, respectively) and admission to ICU (OR 1.8 and 3.6, respectively). A similar trend was also observed in a French cohort (4) as its multivariate regression analysis showed OR for requirement for IMV was 7.36 in patients with BMI ≥35 kg/m^2 compared with those with BMI <25 kg/m^2 (p=0.021). Another study from China of patients with metabolic associated fatty liver disease (22) reported a significant association between overweight/obesity (BMI ≥25 kg/m^2) and COVID-19 severity even after adjusting for age, sex, smoking, diabetes, hypertension, and dyslipidemia (adjusted OR=6.32, p=0.033).

One study reported the association between obesity and COVID-19 mortality. Data from about 17,000 COVID-19 patients in the UK showed that any form of obesity (as defined by hospital staff) was associated with a 37% greater risk of COVID-19 mortality (HR=1.37). (7) Note that in the study from China, Hu et al (21) included death, along with progression to more severe disease presentation, in their criteria they found unfavorable outcomes, indicating mortality data alone was insufficient from this study.

2. **Effect of COVID-19 on obesity-related behaviors**

Only one published study investigated the effect of COVID-19 on obesity-related behaviors such as eating and physical activity. A longitudinal study of lifestyle factors involving 41 children and adolescents with obesity was based in Verona, Italy. Compared with previous data, lockdown was associated with an increase in number of meals a day (+1.15), an increase in number of servings of potato chips, red meat and sugary drinks (+0.54, 1.66 and 0.5,

respectively), an increase in screen time and sleep time (+4.85 hours and +0.65 hours, respectively) and a decrease in time dedicated to sports (-2.3 hours/week). Therefore, more research on this topic is needed.

3. **Meta-analysis of the associations between COVID-19 and obesity**

Figure 2 and **Table 2** show that five studies (which provided 17 analyses and types of results) were included in the meta-analysis. The overall pooled analysis showed that obesity was associated with increased risk of COVID-19 infection (OR=1.69, 95% CI: 1.41, 2.02), and with large heterogeneity (I^2= 87.3%, χ^2=126.48, p< 0.001). The ORs reported by the individual studies ranged from 0.90 to 9.99.

Our subgroup analyses showed between-country differences in the associations between obesity prevalence and COVID-19 (**Table 2**): in the US (OR=1.80, 95% CI: 1.32, 2.46), the UK (OR=1.57, 95% CI: 1.23, 2.02), and China (where national obesity/overweight prevalence was much lower than that in the US and the UK) (OR=6.32, 95% CI: 1.16, 34.48). When stratified by study design, obese/overweight people had 1.66 times higher risk of getting COVID-19 infection than those non-overweight (OR=1.66, 95% CI: 1.39, 1.99) in cohort studies. In contrast, in a cross-sectional study, the reported OR for obese patients was 6.32 (95% CI: 1.16, 34.48). When stratified by obesity classifications, obesity (OR=1.43, 95% CI: 1.18, 1.73) and severe obesity (OR=1.96, 95% CI: 1.49, 2.59) were positively associated with COVID-19 risk, and obesity was positively associated with COVID-19 mortality (OR=1.64, 95% CI: 1.20, 2.25) and its severity: ICU (OR=2.01, 95% CI: 1.25, 3.23), and IMV use (OR=8.20, 95% CI: 2.10, 31.91).

Meta-regression analysis indicated that age affected the association between obesity and COVID-19 infection (β=-0.29; 95%: -0.47 to -0.10), but not gender (**Table 3**).

4. **Sensitivity analysis and assessment of publication bias**

The sensitivity analysis consistently showed that removing individual studies from the meta-analysis did not change the association estimate (**Supplemental Table 1**). The funnel plot and assessment of Egger's and Begg's tests did not reveal any significant publication bias in the association (Egger P=0.148; Begg P=0.343) (**Figure 3**).

Discussion

We systematically investigated the relationship between obesity and COVID-19 infection risk and severity. This included an examination of the risk of weight-gain and subsequent development of obesity due to government mandated containment measures. The results indicated that obesity and in particular, severe obesity are consistently and significantly associated with elevated risk of COVID-19 (our meta-analysis showed that OR=1.43 and 1.96, respectively). Obesity was also associated with an increased risk of developing more severe COVID-19 symptomology, such as admission to acute care, admission to ICU (OR=2.01), use of IMV (OR=8.20), and mortality (OR=1.64). Meta-regression also revealed that age was an important mediating factor and negatively associated with the association between COVID-19 and obesity. The association was stronger in younger people. Given the high prevalence of overweight and obesity globally, these results indicate that a large proportion of the world population may be at an increased risk of COVID-19 complications and mortality (12).

To prevent the spread of COVID-19, the unprecedented response of global governments has included measures such as quarantine, isolation, social distancing and travel bans (2, 3) which may result in an extended period of time spent in one's home. Such time in quarantine is known to result in negative emotions such as fear, nervousness and sadness (23) and eating is a known coping mechanism for dealing with stress and negative emotions (24, 25). Thus, this increased likelihood of stress eating, combined with potentially increased use of hyperpalatable, calorie-dense ultra-processed foods (26, 27, 28) which may be preferentially purchased in times of anticipated food emergency (29), may

lead to overeating, body fat gain and the further development of obesity in the population. Indeed some of these obesity-promoting behaviors (increased consumption of UPFs, increased screen time and reduced physical activity) were observed in only one study to document such behaviors during a COVID-19 lockdown scenario (30). However, very limited research has reported about the impact of such measures on obesity-related behaviors such as eating and physical activity.

It is worth noting that the meta-regression revealed that age is a moderator in the association between obesity and COVID-19, which means the association of obesity and COVID-19 was greater in younger age groups. Compared with the elderly, younger people were unlikely to have diabetes, stroke and other chronic diseases, and are general considered a lower-risk group for COVID-19. Nevertheless, some studies and reports have pointed to obesity being a notable risk for COVID-19, especially among younger patients (5, 31).

The proposed mechanism by which obesity may augment the risks of COVID-19 infection and severity is yet to be elucidated, but may be multifaceted. Physically, those with obesity carry excess adipose tissue and in particular, central/abdominal distribution of this adipose tissue can make breathing more difficult, resulting in less oxygen from respiration entering the blood (32). It is known that SARS-CoV-2 gains cellular entry via the angiotensin converting enzyme 2 (ACE2) receptor (33). Highman et al observed increased ACE2 expression in the bronchial epithelium of chronic obstructive pulmonary disease (COPD) patients who are overweight compared to those not overweight, which may be a route for increased SARS-CoV-2 infection of the respiratory tract (34). Obesity patients are known to have chronically higher leptin (and lower adiponectin) concentrations resulting in an unfavorable, pro-inflammatory hormonal milieu which may lead to a dysregulation of the immune response and lead to obesity-linked complications in COVID-19 (35). Similarly, those with obesity typically have chronically elevated levels of pro-inflammatory cytokines, such as Tumor Necrosis Factor- alpha, Monocyte Chemoattractant Protein-1 and Interleukin-6, which can contribute to defective innate immunity (36). Lower levels of physical activity, which may be more likely observed in individuals with obesity, may also contribute to increased risk as regular physical activity improves immune function (37).

The implications of these results are significant for the general population. One also needs recognize that those with obesity may suffer from the additional burden of weight bias and stigma (38). During this COVID-19 pandemic, the acknowledgment of obesity as a risk factor for COVID-19 might led to fat-shaming amongst the general public and, in particular, on social media. Therefore, efforts need be made to prevent weight stigma. The ultimate risk of such weight stigma is that those with obesity may avoid healthcare services even when suffering symptoms of COVID-19, which may lead to a worsening of their condition and predisposing them to other chronic diseases, and post risks to others.

Obesity is considered a top public health concern, and the prevalence was nearly tripled from 1975 to 2016 (9, 10, 11). In 2016, 39% of adults worldwide were overweight, and 13% were obese (12). In addition to obesity-related many other non-communicable chronic diseases, our study indicated that having obesity will increase the risk of contracting COVID-19, its severity and mortality. Thus, countries like the US where the prevalence of overweight and obesity has reached nearly 70% and the reported COVID-19 cases are greater than 1.1 million, government agencies and health care professionals need to take extra effort to encourage their citizens with overweight/obesity issues to take extra precautions against COVID-19.

Our findings also support the importance of the ongoing efforts worldwide in fighting the global obesity epidemic. There are major concerns that the obesity epidemic will become worse with the many dramatic changes in people's daily life, including reduced physical activity and over-consumption of unhealthy food and reduced consumption of fresh vegetables and fruits. Many sectors in the society need pay attention on such issues while they are taking efforts to fight COVID-19 and save lives.

Regarding the limitations of the individual studies used in this review, many of the studies included in this article were single-center studies which increases the likelihood of admission bias and selection bias between studies. Furthermore, due to the urgent nature of the pandemic many of the included studies did not clarify their inclusion criteria, nor did they provide specific details on the severity of disease presentation. In addition, some of the included studies were retrospective studies, therefore the influence of other confounding factors cannot be ruled out. Finally, the heterogeneous nature of the reported studies must be noted. Further, investigation is required to determine the impact of body composition (adipose tissue/fat-free mass/muscle) on clinically relevant outcomes such as hospitalization, severity of disease and mortality associated with COVID-19, as well as the mechanisms involved, which may help improve COVID-19 treatment strategies.

This study has several important strengths. It is the first study to comprehensively investigate the link between COVID-19 and obesity. Second, we analyzed data from multiple nations and different ethnicities, which may improve the generalizability of the findings to other populations. Third, we conducted meta-analysis and meta-regression analysis. Combining those results with those of the larger data sets, makes our conclusions more robust. Finally, our findings are timely and will help guide ongoing public health efforts, to contain COVID-19 and save lives.

In conclusion, obesity is positively associated with a greater risk of COVID-19 infection and there is a dose-response relationship, while age is mediating factor. Obesity is also associated with increased severity of COVID-19 infection outcomes including like admission to ICU, requirement for IMV and death. These findings highlight the need for early detection and appropriate treatment of COVID-19 in patients with obesity. They also demonstrate the importance of obesity prevention. Government agencies and policy makers should consider the potential negative health repercussions induced by altered lifestyle behaviors during COVID-19 lockdowns and how best to reduce the risk of obesity development.

Abbreviations

ACE2, angiotensin converting enzyme 2; BMI, body mass index; CDC, The Center for Disease Control and Prevention; CI, confidence interval; HR, hazard ratio; COPD, chronic obstructive pulmonary disease; ICU, intensive care unit; IMV, invasive mechanical ventilation; OR, Odds ratio, aOR, adjusted OR.

Declarations

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Author contributions

YW and MM contributed to the study design, data collection, and drafting the manuscript. XS, YL, BZ and RK contributed to the meta-analysis and drafting of the manuscript. All authors contributed to interpretation of the data, commented on and revised the report, and approved the final version for publication.

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Author contributions

YW and MM contributed to the study design, data collection, and drafting the manuscript. XS, YL, BZ and RK contributed to the meta-analysis and drafting of the manuscript. All authors contributed to interpretation of the data, commented on and revised the report, and approved the final version for publication.

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Tables

Table 1. Main characteristics of studies and related findings on the associations between COVID-19 and obesity

Study ID	First author Publication Year	Country/ setting	Study design	Study sample size	Age (mean, median or range) (years)	Males (%)	Main findings on the associations, e.g. ORs 95%CI	Note: How obesity was measured
A. Associations between COVID-19 and obesity								
1	Hu et al, 2020	China	Cohort study	323	Mean 61 (range: 23-91)	51.4	Obesity prevalence: All patients: 4%, Non-severe cases: 3.8%, Severe cases: 4.3%, Critical cases 8% Outcome unfavorable: 10.7%, Favorable: 2.9%.	Obesity (BMI >30 kg/m^2).
2^	Zheng et al, 2020	China	Cross-sectional study	66	Mean 47 (range:18-75)	25.8	Compared to those with non-severe COVID-19, patients with severe disease were more obese (89.5% vs. 59.6%, p = 0.021). Association with obesity and COVID-19 severity (adjusted-OR 6.32, 95% CI 1.16–34.54).	Obesity: BMI >25 kg/m^2. All had metabolic associated fatty liver disease.
3	Caussy et al, 2020	France	Cohort study	291	NA	NA	Higher requirement for IMV in severe obesity ≥35 kg/m^2 compared to lean patients: 81.8% versus 41.9%, p=0.001.	Severe obesity: BMI ≥35 kg/m^2.
4	Simmonet et al, 2020	France	Cohort study	124	Median 60 (range: 51- 70)	73.0	Obesity and severe obesity were significantly more frequent	Obesity (BMI >30 kg/m^2) ; Class II obesity

							among SARS-CoV-2 participants than in non SARS-CoV-2 controls: 47.6% vs 25.2% and 28.2% vs 10.8%, respectively. Admission to ICU: 47.5% presented with obesity, including class II obesity in 13.7% and with class III obesity in 14.5%.	(BMI 35-39.9 kg/m^2); Class III obesity (BMI ≥40 kg/m^2).
5^	Docherty et al, 2020	UK	Cohort study	16,749	Median 72 (range: 0-104)	60.2	Mortality All obesity 1.37 (1.16-1.63, p<0.001).	as recognized by clinical staff
6	ICNARC	UK	Case report	196	Median 64 (range: 52-73)	70.9	28.6% of patients had a BMI of 25-30, 29.6% had a BMI of 30-40 and 6.6% had a BMI of 40 or higher.	Obesity (BMI >30 kg/m^2).
7^	Kalligeros et at, 2020	USA	Cohort study	103	Median 60 (range: 52-70)	61.1	1) Admission to ICU: obesity (aOR:2.65; 95% CI 0.64-10.95); and severe obesity (aOR: 5.39; 95% CI1.13-25.64). 2) Use of IMV: obesity (aOR: 6.85; 95% CI 1.05-44.82); and severe obesity (aOR: 9.99; 95% CI 1.39-71.69).	Obesity (BMI 30-34.9 kg/m^2); Severe obesity (BMI≥35 kg/m^2).
8^	Lighter et al, 2020	USA	Cohort study	3,615	<60 and ≥60	NA	1) <60 years: Admission to acute care:	Obesity BMI 30-34 kg/m^2;

							obesity OR: 2.0 (95% CI 1.6-2.6), and severe obesity OR: 2.2 (95% CI 1.7-2.9). Admission to ICU: obesity OR: 1.8 (95% CI 1.2-2.7), and severe obesity OR: 3.6 (95% CI 2.5-5.3). 2) ≥60 years Admission to acute care: obesity OR: 0.9 (95% CI 0.6-1.2), and severe obesity OR: 0.9 (95% CI 0.6-1.3). Admission to ICU: obesity OR: 1.1 (95% CI 0.8-1.7), and severe obesity OR: 1.5 (95% CI 0.9-2.3).	Severe obesity BMI >35 kg/m^2.
9^	Williamson et al, 2020	UK (England)	Cohort study	17,425,445 (5,683 COVID-19 deaths)	>18	49.9	Incidence Not obese 1.00 (ref) Obese class I: 1.27 (1.18-1.36); Obese class II: 1.56 (1.41-1.73); Obese class III: 2.27 (1.99-2.58).	Obese class I (30-34.9kg/m^2) Obese class II (35-39.9kg/m^2) Obese class III (≥40 kg/m^2)
B. Obesity related behaviors (e.g. eating and physical activity)								
10	Pietrobelli et al, 2020	Italy	Cohort study	41	Mean 13.0±3.1 (range: 6-18)	53.7	1) Meals (#/day) change: 1.15±1.56; 95% CI: 0.65/1.64	Baseline BMI was about 30.5 kg/m^2 in males and 29.7 kg/m^2 in females

							2) Potato chips change: 0.54±0.86; 95% CI: 0.26/0.81 3) Red meat change: 1.66±2.10; 95% CI: 1.00/2.32 4) Sugary drinks (#/day) change: 0.50±1.08; 95% CI: 0.16/0.84 5) Screen time (hrs/day) change: 4.85±2.40; 95% CI: 4.10/5.61 6) Sleep time (hrs/day) change: 0.65±1.29; 95% CI: 0.24/1.05 7) Sports (hrs/week) change: -2.30±4.60; 95% CI: -3.76/-0.85

Abbreviation: CI: confidence intervals; ICU: Intensive Care Unit; IMV: Invasive Mechanical Ventilation; COVID-19: Coronavirus Disease 2019; aOR, adjusted OR; WHO: World Health Organization.

^ These studies were included in our meta-analyses, because the effect sizes (OR and 95% CI) were reported.

Table 2. Overall results of meta-analysis and sub-group analysis of the associations between COVID-19 and obesity

	Number of studies/ Analyses*	Effect size (95%CI)	P value	Heterogeneity			
				I^2 (%)	χ^2	P value	Tau-squared
Total	17	1.69 (1.41, 2.02)	<0.001	87.3	126.48	<0.001	0.0838
Country							
USA	12	1.80 (1.32, 2.46)	<0.001	80.7	57.12	<0.001	0.1945
UK	4	1.57 (1.23, 2.02)	<0.001	95.1	61.29	<0.001	0.0605
China	1	6.32 (1.16, 34.48)	0.033	…	…	…	…
Study design							
cohort study	16	1.66 (1.39, 1.99)	<0.001	87.9	123.73	<0.001	0.0826
cross-sectional study	1	6.32 (1.16, 34.48)	0.033	…	…	…	…
Obesity classifications							
obesity	9	1.43 (1.18, 1.73)	<0.001	71.0	27.56	0.001	0.0417
severe obesity	8	1.96 (1.49, 2.59)	<0.001	86.7	52.55	<0.001	0.0997
COVID-19 severity							
COVID-19 admission	2	2.31 (0.56, 9.56)	0.248	67.6	3.09	0.079	0.7903
ICU admission	6	2.01 (1.25, 3,23)	0.004	77.4	22.16	<0.001	0.2290
IMV use admission	2	8.20 (2.10, 31.91)	0.002	0.0	0.07	0.786	0.0000
Acute care admission	4	1.40 (0.88, 2.22)	0.155	89.2	27.79	<0.001	0.1956
The death of COVID-19	3	1.64 (1.20, 2.25)	0.002	96.7	60.57	<0.001	0.0740

Abbreviation: CI: confidence intervals; ICU: Intensive Care Unit; IMV: Invasive Mechanical Ventilation; COVID-19: Coronavirus Disease 2019.

*Results from five studies (reported 17 analyses, including for different related outcomes and subgroups) were included in the meta-analysis. A fixed-effect model was fit when modest to moderate heterogeneity was present, and a random-effects model was fit when substantial to considerable heterogeneity was present.

Table 3. Results of meta-regression analyses of age and gender differences on the association between COVID-19 and obesity*

	β	95% CI	P-value	R^2
Age	-0.29	-0.47, -0.10	0.005	26.01
Gender	0.01	-0.15, 0.17	0.859	

*Meta-regression analyses based on results from five studies were performed to determine whether subjects' age (in years) and gender (% of males) affected the association between COVID-19 and obesity.

Figures

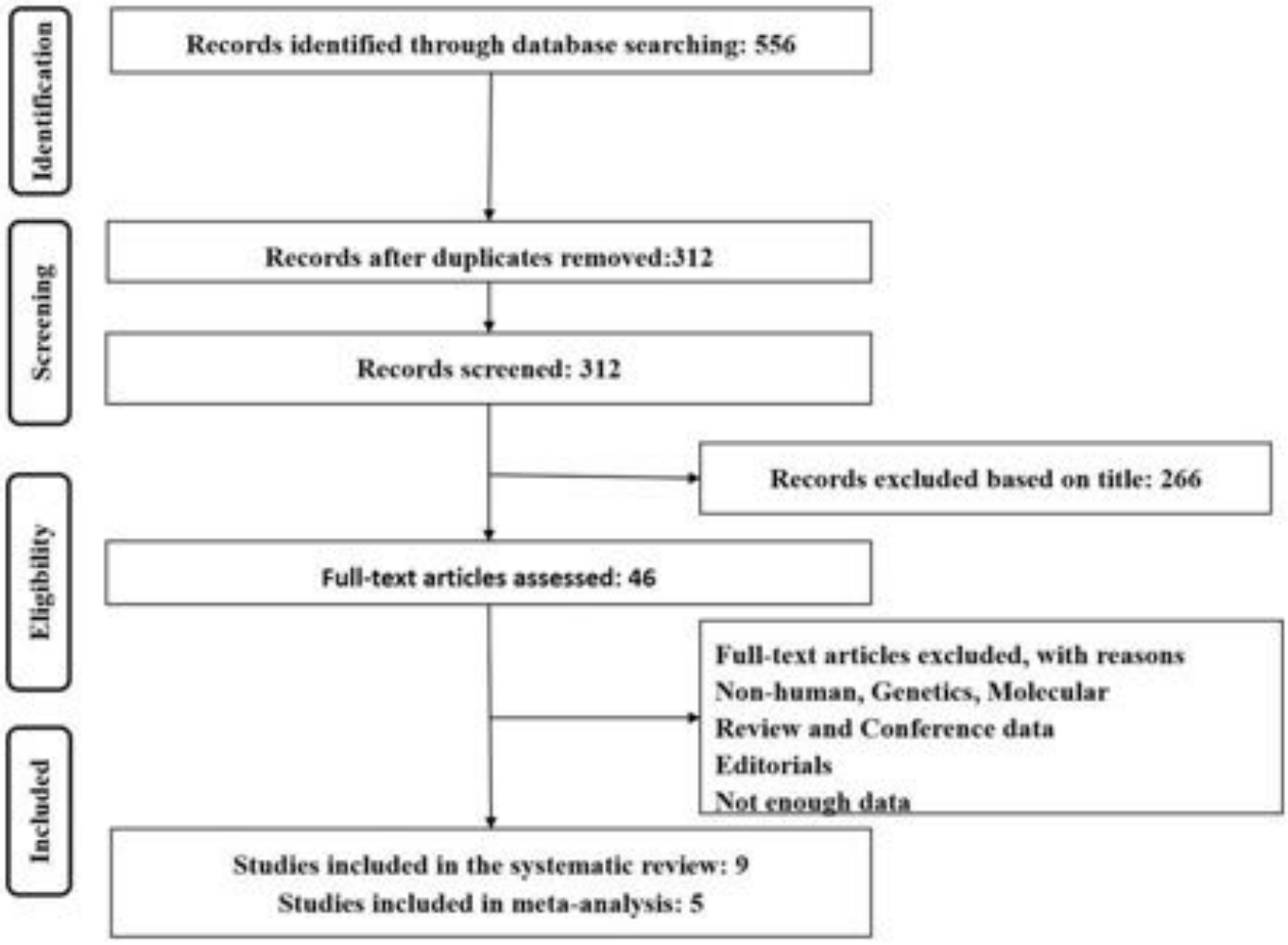
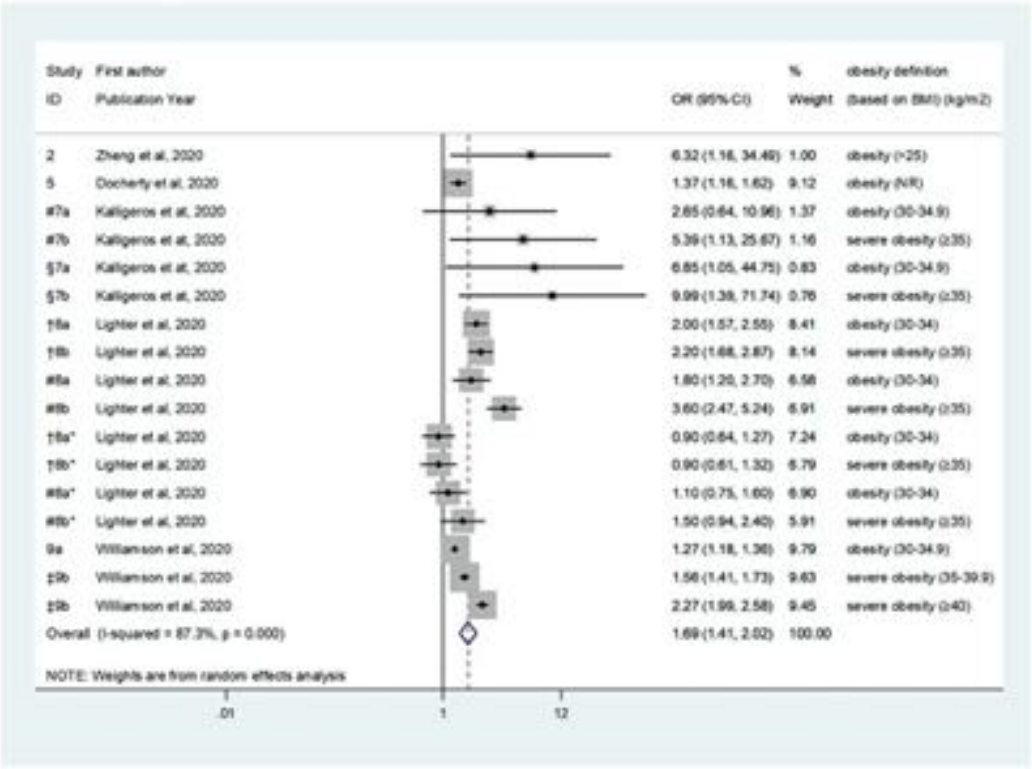


Figure 1

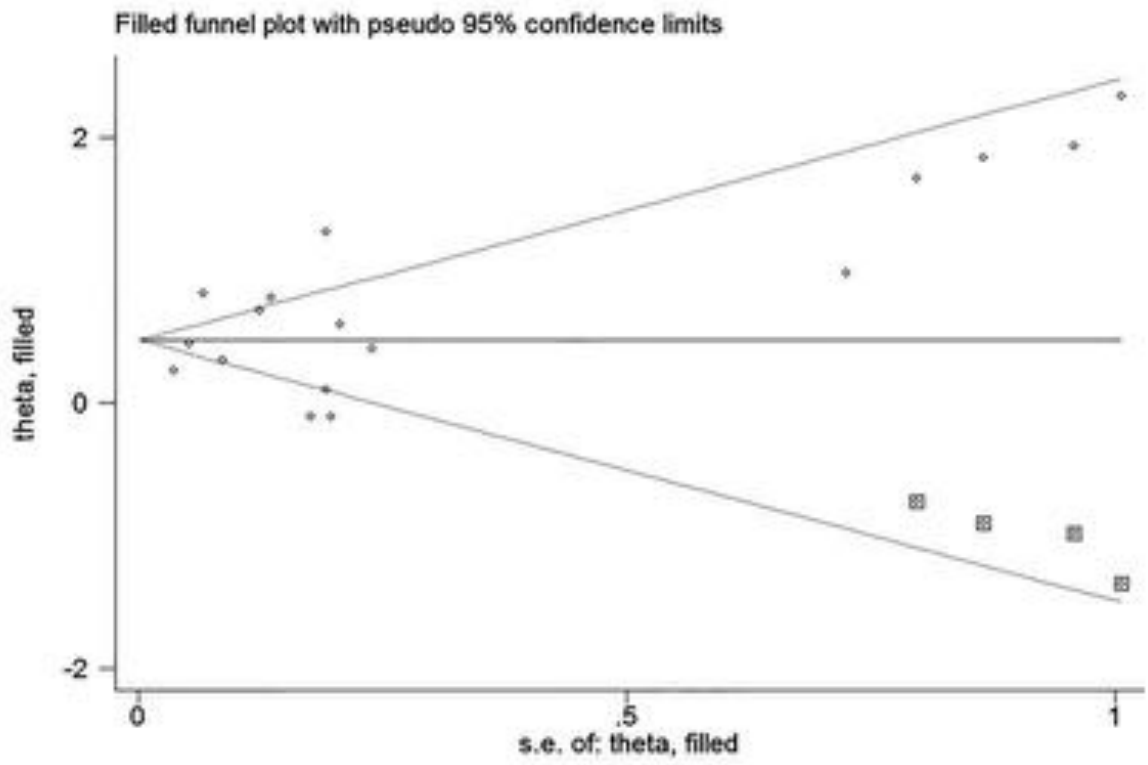
Flow chart diagram of studies selected for investigating the relationship between obesity and Covid-19



Five studies (17 analyses) were included. A fixed-effect model was estimated when modest to moderate heterogeneity was present, and a random-effects model was estimated when substantial to considerable heterogeneity was present.
a. obesity; b. severe obesity.
‡ Severe obesity was divided into two categories in this article: severe obesity I (35-39.9 kg/m^2) and II (≥40 kg/m^2).
# The COVID-19 patients in these articles needed Intensive Care Unit (ICU) admission;
§ The COVID-19 patients in these articles needed Invasive Mechanical Ventilation (IMV) use;
† The COVID-19 patients in these articles needed acute care admission.
* indicates age ≥60; age groups were classified as under and over 60 years in the study reported by Lighter et al, 2020.

Figure 2

Meta-analysis of associations between COVID-19 and obesity: Odds ratios and 95% confidence intervals based on mixed models Five studies (17 analyses and types of results) were included. A fixed-effect model was estimated when modest to moderate heterogeneity was present, and a random-effects model was estimated when substantial to considerable heterogeneity was present. a. obesity; b. severe obesity. ‡ Severe obesity was divided into two categories: severe obesity I (35-39.9 kg/m2) and II (≥40 kg/m2). # The COVID-19 patients in these articles needed Intensive Care Unit (ICU) admission; § The COVID-19 patients in these articles needed Invasive Mechanical Ventilation (IMV) use; † The COVID-19 patients in these articles needed acute care admission. * indicates age ≥60; age groups were classified as under and over 60 years in the study reported by Lighter et al, 2020.



Five studies (17 analyses) were included in the meta-analysis of the associations between COVID-19 and obesity). We used the trim method to make the funnel plot.

Figure 3

Funnel plot for publication bias Five studies (17 analyses) were included in the meta-analysis of the associations between COVID-19 and obesity). We used the trim method to make the funnel plot.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- XiaominCovidobesityTable.docx
- SupplementalTable1.pdf

Appendix 2

Documentation required for NHS ethics application

To Whom it may concern,

Please find attached our application to the LJMU REC for approval for NHS Ethics submission. The study is entitled “A high-protein Mediterranean diet and resistance exercise for cardiac rehabilitation: a pilot randomised controlled trial”.

We look forward to hearing your feedback in the near future.

Kindest Regards

Chief Investigator: Fatima Perez de Heredia

Date: 18/09/2019

Signature: 

To whom it may concern,

I am satisfied that the exposure to ionising radiation planned in this research study (as defined in A1 and/or B1) (IRAS ID 256927) is reasonable and that the risks are adequately described in the participant information sheet for the study.

I hereby authorize this IRAS form.

Signed: Date: 04/10/2019

Dr Marousa Ntouskou, Consultant Radiologist

Registration number: 7485185

Liverpool Heart and Chest Hospital NHS
NHS Trust

THOMAS DRIVE,
LIVERPOOL
L14 3PE
Tel: 0151 228 1616
Homepage: http://www.lhch.nhs.uk;
Email:webmaster2@ctc.nhs.uk

I, Fatima Perez de Heredia, as Chief Investigator for

Project Title: A high-PRotein Mediterranean diet and resistance Exercise for cardiac rehabilitation: a pilot randomised controlled trial

Confirm that I authroize the IRAS application No: 256927 as part of the post-graduate research of Richard Kirwan.

Chief Investigator: Fatima Perez de Heredia

Date: 18/09/2019

Signature: 

To whom it may concern,

I am satisfied that the information in sub-sections A and/or B and the assessment in sub-section C of the IRAS application (ID 256927) provide a reasonable estimate of the ionising radiation exposure planned in this research and the associated risks.

I hereby authorize this IRAS form.

Signed: Date: 23/09/19

Pete Cole, Radiation Protection Officer and MPE

Welcome to the Integrated Research Application System

IRAS Project Filter

The integrated dataset required for your project will be created from the answers you give to the following questions. The system will generate only those questions and sections which (a) apply to your study type and (b) are required by the bodies reviewing your study. Please ensure you answer all the questions before proceeding with your applications.

Please complete the questions in order. If you change the response to a question, please select 'Save' and review all the questions as your change may have affected subsequent questions.

Please enter a short title for this project (maximum 70 characters)
Pilot:High-Protein Mediterranean Diet, Exercise and Sarcopenia V.1

1. Is your project research?

(●) Yes ( ) No

2. Select one category from the list below:

( ) Clinical trial of an investigational medicinal product

( ) Clinical investigation or other study of a medical device

( ) Combined trial of an investigational medicinal product and an investigational medical device

(●) Other clinical trial to study a novel intervention or randomised clinical trial to compare interventions in clinical practice

( ) Basic science study involving procedures with human participants

( ) Study administering questionnaires/interviews for quantitative analysis, or using mixed quantitative/qualitative methodology

( ) Study involving qualitative methods only

( ) Study limited to working with human tissue samples (or other human biological samples) and data (specific project only)

( ) Study limited to working with data (specific project only)

( ) Research tissue bank

( ) Research database

If your work does not fit any of these categories, select the option below:

( ) Other study

2a. Will the study involve the use of any medical device without a CE Mark, or a CE marked device which has been modified or will be used outside its intended purposes?

( ) Yes (●) No

2b. Please answer the following question(s):

a) Does the study involve the use of any ionising radiation? (●) Yes ( ) No

• Does the study involve exposure to radioactive materials? ( ) Yes (●) No

b) Will you be taking new human tissue samples (or other human biological samples)? (●) Yes ( ) No

c) Will you be using existing human tissue samples (or other human biological samples)? ◯ Yes ◉ No

3. In which countries of the UK will the research sites be located? *(Tick all that apply)*

- [x] England
- [ ] Scotland
- [ ] Wales
- [ ] Northern Ireland

3a. In which country of the UK will the lead NHS R&D office be located:

- ◉ England
- ◯ Scotland
- ◯ Wales
- ◯ Northern Ireland
- ◯ This study does not involve the NHS

4. Which applications do you require?

- [x] IRAS Form
- [ ] Confidentiality Advisory Group (CAG)
- [ ] Her Majesty's Prison and Probation Service (HMPPS)

5. Will any research sites in this study be NHS organisations?

◉ Yes ◯ No

5a. Are all the research costs and infrastructure costs (funding for the support and facilities needed to carry out research e.g. NHS Support costs) for this study provided by a NIHR Biomedical Research Centre, NIHR Collaboration for Leadership in Health Research and Care (CLAHRC), NIHR Patient Safety Translational Research Centre or Medtech and In Vitro Diagnostic Cooperative in all study sites?

Please see information button for further details.

◯ Yes ◉ No

Please see information button for further details.

5b. Do you wish to make an application for the study to be considered for NIHR Clinical Research Network (CRN) Support and inclusion in the NIHR Clinical Research Network Portfolio?

Please see information button for further details.

◉ Yes ◯ No

The NIHR Clinical Research Network provides researchers with the practical support they need to make clinical studies happen in the NHS e.g. by providing access to the people and facilities needed to carry out research "on the ground".

If you select yes to this question, you must complete a NIHR Clinical Research Network (CRN) Portfolio Application Form (PAF) immediately after completing this project filter question and before submitting other applications. Failing to complete the PAF ahead of other applications e.g. HRA Approval, may mean that you will be unable to access NIHR CRN Support for your study.

6. Do you plan to include any participants who are children?

○ Yes ◉ No

7. Do you plan at any stage of the project to undertake intrusive research involving adults lacking capacity to consent for themselves?

○ Yes ◉ No

Answer Yes if you plan to recruit living participants aged 16 or over who lack capacity, or to retain them in the study following loss of capacity. Intrusive research means any research with the living requiring consent in law. This includes use of identifiable tissue samples or personal information, except where application is being made to the Confidentiality Advisory Group to set aside the common law duty of confidentiality in England and Wales. Please consult the guidance notes for further information on the legal frameworks for research involving adults lacking capacity in the UK.

8. Do you plan to include any participants who are prisoners or young offenders in the custody of HM Prison Service or who are offenders supervised by the probation service in England or Wales?

○ Yes ◉ No

9. Is the study or any part of it being undertaken as an educational project?

◉ Yes ○ No

Please describe briefly the involvement of the student(s):
The student is undertaking a research project for his PhD. A Director of Studies is assigned to oversee the project

9a. Is the project being undertaken in part fulfilment of a PhD or other doctorate?

◉ Yes ○ No

10. Will this research be financially supported by the United States Department of Health and Human Services or any of its divisions, agencies or programs?

○ Yes ◉ No

11. Will identifiable patient data be accessed outside the care team without prior consent at any stage of the project (including identification of potential participants)?

○ Yes ◉ No

Integrated Research Application System
Application Form for Other clinical trial or investigation

The Chief Investigator should complete this form. Guidance on the questions is available wherever you see this symbol displayed. We recommend reading the guidance first. The complete guidance and a glossary are available by selecting Help.

Please define any terms or acronyms that might not be familar to lay reviewers of the application.

Short title and version number: (maximum 70 characters - this will be inserted as header on all forms)
Pilot:High-Protein Mediterranean Diet, Exercise and Sarcopenia V.1

PART A: Core study information

1. ADMINISTRATIVE DETAILS

A1. Full title of the research:

A high-PRotein Mediterranean diet and Resistance Exercise for cardiac rehabilitation: a pilot randomised controlled trial

A2-1. Educational projects

Name and contact details of student(s):

Student 1

	Title	Forename/Initials	Surname
	Mr	Richard	Kirwan
Address	34 Hawarden Av.		
	Liverpool		
Post Code	L17 2AL		
E-mail	rkirwan@gmail.com		
Telephone	07565427663		
Fax			

Give details of the educational course or degree for which this research is being undertaken:

Name and level of course/ degree:
PhD Clinical Nutrition

Name of educational establishment:
Liverpool John Moores University

Name and contact details of academic supervisor(s):

Academic supervisor 1

	Title	Forename/Initials	Surname
	Dr	Fatima	Perez de Heredia
Address	Room 234, James Parsons Building		

	Liverpool John Moores University
Post Code	L3 3AF
E-mail	f.perezdeherediabenedicte@ljmu.ac.uk
Telephone	01512312003
Fax	

Academic supervisor 2

	Title	Forename/Initials	Surname
	Dr	Ian G	Davies
Address	IM Marsh Campus		
	Barkhill Road		
	Liverpool		
Post Code	L17 6BD		
E-mail	I.G.Davies@ljmu.ac.uk		
Telephone	0151 231 5290		
Fax			

Academic supervisor 3

	Title	Forename/Initials	Surname
	Dr	Tom	Butler
Address	University of Chester		
	Parkgate Road		
	Chester		
Post Code	CH1 4BJ		
E-mail	t.butler@chester.ac.uk		
Telephone	01244 511660		
Fax			

Please state which academic supervisor(s) has responsibility for which student(s):
Please click "Save now" before completing this table. This will ensure that all of the student and academic supervisor details are shown correctly.

Student(s)	Academic supervisor(s)
Student 1 Mr Richard Kirwan	☑ Dr Fatima Perez de Heredia ☑ Dr Ian G Davies ☑ Dr Tom Butler

A copy of a current CV for the student and the academic supervisor (maximum 2 pages of A4) must be submitted with the application.

A2-2. Who will act as Chief Investigator for this study?

- ○ Student
- ◉ Academic supervisor
- ○ Other

A3-1. Chief Investigator:

	Title	Forename/Initials	Surname
	Dr	Fatima	Perez de Heredia
Post	Lecturer		
Qualifications	BSc Biological Sciences, University of Murcia (2000) Post-graduate Certificate in Education, University of Murcia (2003) PhD Biology, University of Murcia (2007) Post-graduate Certificate in Higher Education, Liverpool John Moores University (2017)		
ORCID ID	0000 0002 2537 3327		
Employer	Liverpool John Moores University		
Work Address	James Parsons Bld, Byrom St Byrom Street Campus Liverpool		
Post Code	L3 3AF		
Work E-mail	F.PerezDeHerediaBenedicte@ljmu.ac.uk		
* Personal E-mail			
Work Telephone	01512312003		
* Personal Telephone/Mobile			
Fax			

** This information is optional. It will not be placed in the public domain or disclosed to any other third party without prior consent.*
A copy of a current CV (maximum 2 pages of A4) for the Chief Investigator must be submitted with the application.

A4. Who is the contact on behalf of the sponsor for all correspondence relating to applications for this project?
This contact will receive copies of all correspondence from REC and HRA/R&D reviewers that is sent to the CI.

	Title	Forename/Initials	Surname
	Dr	Dave	Harriss
Address	Research Innovation Services Exchange Station Tithebarn Street, Liverpool		
Post Code	L2 2QP		
E-mail	sponsor@ljmu.ac.uk		
Telephone	0151 231 2121		
Fax			

A5-1. Research reference numbers. *Please give any relevant references for your study:*

Applicant's/organisation's own reference number, e.g. R & D (if available):	N/A
Sponsor's/protocol number:	N/A
Protocol Version:	V.1
Protocol Date:	21/08/2019
Funder's reference number (enter the reference number or state not applicable):	N/A
Project website:	N/A

Registry reference number(s):

The UK Policy Framework for Health and Social Care Research sets out the principle of making information about research publicly available. Furthermore: Article 19 of the World Medical Association Declaration of Helsinki adopted in 2008 states that "every clinical trial must be registered on a publicly accessible database before recruitment of the first subject"; and the International Committee of Medical Journal Editors (ICMJE) will consider a clinical trial for publication only if it has been registered in an appropriate registry. Please see guidance for more information.

International Standard Randomised Controlled Trial Number (ISRCTN):

ClinicalTrials.gov Identifier (NCT number):

Additional reference number(s):

Ref.Number Description	Reference Number
N/A	N/A

A5-2. Is this application linked to a previous study or another current application?

◯ Yes ◉ No

Please give brief details and reference numbers.

2. OVERVIEW OF THE RESEARCH

To provide all the information required by review bodies and research information systems, we ask a number of specific questions. This section invites you to give an overview using language comprehensible to lay reviewers and members of the public. Please read the guidance notes for advice on this section.

A6-1. Summary of the study. *Please provide a brief summary of the research (maximum 300 words) using language easily understood by lay reviewers and members of the public. Where the research is reviewed by a REC within the UK Health Departments' Research Ethics Service, this summary will be published on the Health Research Authority (HRA) website following the ethical review. Please refer to the question specific guidance for this question.*

The study aims to investigate whether a high-protein Mediterranean diet, alone or in combination with resistance exercise, can increase muscle mass in cardiac rehabilitation patients, and reduce the risk of future cardiac events.

Cardiovascular risk can be increased by different factors, such as obesity (an excess of body fat), or sarcopenia, which is a gradual loss of muscle mass that happens as we age. Sometimes both conditions can occur simultaneously, and the individual may appear to have a normal body weight, but have disproportionately low levels of muscle mass and high levels of body fat; this is known as sarcopenic obesity, and it is associated with greater risk of heart disease. Increasing the proportion of muscle mass to body fat in people with sarcopenic obesity may reduce the risk of future heart disease.

We will work with cardiac rehabilitation patients, recruited through the NHS. In the facilities of Liverpool John Moores University in Byrom Street campus, we will assess the presence of sarcopenic obesity, measuring body composition by dual energy X-ray absorptiometry (DXA), bio-impedance, and anthropometric measures (height, weight, waist circumference). DXA uses X-rays to create an image of the body which clearly shows bone, fat and lean muscle mass, while bio-impedance uses a low intensity electric current to estimate body fat.

Participants identified as having sarcopenic obesity will begin a lifestyle intervention in one of 4 different groups:

1-control: they will follow the usual cardiac rehabilitation dietary and exercise advice,
2-diet group: will follow a high-protein Mediterranean-style diet,
3-exercise group: will perform resistance exercise regularly,
4-diet and exercise group: will follow the experimental diet and the resistance exercise training.

Before and after the intervention, we will measure muscle and body fat levels, muscle strength through simple function tests, and markers of cardiovascular health, including cholesterol sub-fractions, triglycerides, fasting glucose and insulin, and inflammatory proteins.

A6-2. Summary of main issues. *Please summarise the main ethical, legal, or management issues arising from your study and say how you have addressed them.*

Not all studies raise significant issues. Some studies may have straightforward ethical or other issues that can be identified and managed routinely. Others may present significant issues requiring further consideration by a REC, R&D office or other review body (as appropriate to the issue). Studies that present a minimal risk to participants may raise complex organisational or legal issues. You should try to consider all the types of issues that the different reviewers may need to consider.

This research project has been designed by the student and academic supervisory team detailed in this form. Whilst every care has been taken to minimise risks, the study will entail the use of ionising radiation from dual energy x-ray absorptiometry (DXA) scanner. The DXA scanner allows for the most accurate measurements of body fat and muscle mass, which is an integral aspect of the study, and will also ensure the validity of the results. The levels of ionising radiation emitted by the scanner are very small, and below the amount of daily background radiation to which everyone is exposed; therefore, the risk of harm to the participants is negligible. Lastly, as an imaging technique, the DXA scan is safer and cheaper than the available alternatives of standard X-ray and CT scans. The participants' involvement will be limited to one (for patients taking part in phase 1 only) and three scans (for patients taking part in phase 2 as well), in order to reduce the exposure to ionising radiation as much as possible.

As the participants will have experienced a cardiac event prior to be prescribed rehabilitation, they may have a lower tolerance for exercise. To reduce the risks posed by an exercise regime, participants will undergo an exercise tolerance test at the beginning of the study to better adapt the exercise regime to their abilities. Exercise programs will also be developed by suitably qualified cardiac rehabilitation exercise instructors.

The high-protein diet will not pose any concern as high-protein diets have been proven to safe, provided the individual has normal kidney function. For that reason, kidney disease or malfunction will be an exclusion criteria for the trial.

All participants will receive a participant information sheet, and asked to sign a consent form, once they have understood and agreed with the conditions of participation in the study.

Data will be anonymised to maintain confidentiality, and all personal data will be kept securely, and only the research team will have access to them.

3. PURPOSE AND DESIGN OF THE RESEARCH

A7. Select the appropriate methodology description for this research. *Please tick all that apply*:

- ☐ Case series/ case note review
- ☐ Case control
- ☐ Cohort observation
- ☐ Controlled trial without randomisation
- ☑ Cross-sectional study
- ☐ Database analysis
- ☐ Epidemiology
- ☑ Feasibility/ pilot study
- ☐ Laboratory study
- ☐ Metanalysis
- ☐ Qualitative research
- ☐ Questionnaire, interview or observation study
- ☑ Randomised controlled trial
- ☐ Other (please specify)

A10. What is the principal research question/objective? *Please put this in language comprehensible to a lay person.*

Is it possible to increase muscle mass and strength with a high-protein, Mediterranean style diet and resistance exercise, in high risk cardiac rehabilitation patients?

Our main objective is to investigate the feasibility of an intervention based on a high-protein Mediterranean-style diet and resistance exercise in cardiac rehabilitation patients with sarcopenic obesity (low proportion of muscle mass and higher of body fat). We will investigate if the intervention can increase lean body mass and muscle strength.

A11. What are the secondary research questions/objectives if applicable? *Please put this in language comprehensible to a lay person.*

We believe that the intervention will help reduce markers of heart disease and frailty. We will investigate whether changes in lean body mass and strength are linked to changes in markers of cardiovascular and metabolic health (e.g., blood lipids, insulin sensitivity). The ultimate goal is to develop a cardiac rehabilitation-based intervention to decrease the risk of future cardiovascular events.

A12. What is the scientific justification for the research? *Please put this in language comprehensible to a lay person.*

Cardiovascular disease (CVD) in the UK is responsible for 1 in 4 deaths every year (>150,000 people), with coronary heart disease (CHD) being the most common type. In addition, the economic cost of CVD is considerable; the annual cost of healthcare for CVD alone in the UK is £9 billion with a further £10 billion lost from the economy due to premature deaths, disability, and productivity loses.

Mortality figures due to CHD have been reduced recently, in part due to increased provision of cardiac rehabilitation (CR), a therapeutic approach based on exercise training to improve cardiac function, plus advice regarding smoking cessation, dietary changes and weight loss. Most of the evidence on the benefit of CR examines the links between exercise and CV morbidity and mortality, but evidence from secondary prevention of CVD (prevention from a second cardiac event after an initial event) suggests Mediterranean diet-based approaches are the most adequate to treat these patients.

Regarding body weight, we find an “obesity paradox” in the cardiac population, where increased mortality has been linked to normal body weights, masking a high body fat content due to low lean body mass (LBM). Sarcopenia is the progressive loss of lean mass during ageing, particularly muscle mass; it results from multiple possible causes, including chronic disease, inflammation, insulin resistance, nutritional deficiencies and muscle disuse. Patients presenting low LBM and abdominal fat accumulation, known as sarcopenic obesity (SO), are at greater risk of CVD, and this is exacerbated in CR. This increased risk of CVD in SO is considered to be a consequence, at least in part, of increased levels of proinflammatory cytokines produced in visceral fat, which can result in damage and dysfunction of the vascular endothelium; this in turn can induce the secretion of further cytokines and growth factors, leading to a pro-coagulant state in the endothelium, smooth muscle cell proliferation, and increased migration of leukocytes, causing further inflammation, a thickening of the artery wall, and the development of atherosclerotic plaques. The increase in proinflammatory cytokines may also contribute to the progression of SO through their association with reduced muscle mass and strength, further compounding the condition.

Increasing relative LBM content, rather than simply promoting weight loss, may be an appropriate target in CR patients. Higher protein intakes (1.2-1.5 g/kg/body weight) combined with resistance training can positively influence muscle mass, and have also been shown to promote greater improvements in body composition due a reduction in total body fat mass. Of particular relevance is the presence of anabolic resistance in older adults which can result in a reduced muscle protein synthetic (MPS) response to the ingestion of amino acids. Overcoming this anabolic resistance may play a key role in increasing LBM in SO subjects and potential strategies may include supplementation with leucine, an amino acid which induces a particularly potent MPS response, or larger doses of total protein, which will contain higher amounts of leucine. This may be particularly important in the post-exercised state, due to the increased MPS response to the presence of amino acids after a bout of resistance exercise.

In the light of the relevant literature, our project will assess the feasibility and efficacy of combining the cardio-protective properties of Mediterranean style diets, with higher protein intakes and exercise training, in order to overcome anabolic resistance, improve LBM and reduce markers of cardiovascular risk in cardiac rehabilitation patients with sarcopenic obesity.

In addition, our project will be hugely beneficial to the student Richard Kirwan, by expanding his knowledge and skills regarding a broad spectrum of methodologies for data collection and analysis. It will also provide a sound scientific basis for further postgraduate research.

A13. Please summarise your design and methodology. *It should be clear exactly what will happen to the research participant, how many times and in what order. Please complete this section in language comprehensible to the lay person. Do not simply reproduce or refer to the protocol. Further guidance is available in the guidance notes.*

Phase 1: Cross-sectional Study and Focus Groups.

We will assess body composition in CR patients, by dual-X-ray densitometry (DXA), bio-impedance and anthropometric measures (height, weight and waist circumference) to calculate the body shape index. We will also take measures of muscle strength (handgrip). These measurements will allow us to estimate the prevalence of SO in CR patients. Sample size provided by power calculations is 240 patients.

We will conduct a systematic review of dietary methods to increase muscle protein synthesis in adults, in order to inform decisions on the appropriate dietary strategy for Phase 2.

We will invite participants to attend focus groups (n = 10-12). We will gather their feedback on food recipes and the resistance exercise training protocol. Furthermore, willingness to be involved and randomised to a particular intervention arm will be assessed by a survey conducted among patients attending CR clinics. A description of the study protocol will be provided. The CR participants will be asked open and closed questions, which will provide an indication of the rate of recruitment that can be expected.

Phase 2: Pilot 2x2 Factorial (diet x exercise) Randomised Controlled Trial (RCT, 12 week).

Patients identified as presenting SO will be randomly assigned to one of four groups (n=10-15/group):
1) control: this group will follow the standard lifestyle guidelines advised on standard cardiac rehabilitation (CONT);
2) high-protein Mediterranean diet group: will be prescribed a Mediterranean-style diet (adapted to their preferences and circumstances) with increased protein content (HPMD)(protein intake will be based on 1.2-1.6g/kg of ideal body weight for height);
3) exercise group: will be prescribed resistance training (EX);
4) diet and exercise group: will be prescribed both high-protein Mediterranean-style diet (as previously described) and resistance training and (HPMD+EX).

As there is currently no standard definition to classify SO, CR patients will be selected for participation according to the results from Phase 1 cross-sectional study.

We will take baseline and endpoint measurements of:
- body composition (DXA and bio-impedance) and anthropometric measures (height, weight and waist circumference);
- functional test of muscle strength (handgrip strength);
- markers of cardiometabolic risk in blood (cholesterol subfractions, triglycerides, fasting glucose, HOMA-IR, IL-1β, IL-6, TNFα, C-reactive protein);
- dietary intake via 4-day records and Mediterranean Diet Score questionnaires.

We will maintain frequent contact with participants to ensure continued adherence to diet and to monitor progress through simple anthropometric measures.

We will continue conducting focus groups with participants to gather feedback regarding follow-up, adherence/compliance, acceptability, affordability and easiness of preparation of meals, and the exercise training protocol. PPI will be highly valuable as well for preparing funding applications.

A14-1. In which aspects of the research process have you actively involved, or will you involve, patients, service users, and/or their carers, or members of the public?

- [x] Design of the research
- [x] Management of the research
- [x] Undertaking the research
- [ ] Analysis of results
- [x] Dissemination of findings
- [ ] None of the above

Give details of involvement, or if none please justify the absence of involvement.
The research team have already met with the Service Users Research Endeavour (SURE) group of the Liverpool Heart and Chest Hospital. SURE includes former or existing patients, carers, members of the public, and clinicians. They provided advice regarding the participant information documents, and approved the study.

We will continue with specific focus groups of patients in CR. The PPI groups will participate and feed back on the different aspects of the research, including:

- feedback on recruitment,
- tasting of high-protein foods,
- feedback on recipes, appeal of the meals, how they fit with the Merseyside culture,
- involvement and support of other members of the family (especially those in charge of purchasing and cooking food),
- familiarity session(s) with resistance training, exploring ability to perform exercises and personalising the training system,
- preferences to undertake resistance exercise (e.g. in a supervised or unsupervised setting),
- preferred follow-up frequency and method (e.g. face to face, phone, etc.),
- issues with dietary and exercise adherence,
- help shape the protocol and ensure the project is participant-friendly and sensitive by assisting with ethical considerations,
- discuss research findings and help with lay dissemination.

Participants will be invited to work as partners to a trial management group and on future grant applications.

All PPI activities will be recorded and evaluated, with aims to present at conferences and publish in appropriate peer reviewed journals.

4. RISKS AND ETHICAL ISSUES

RESEARCH PARTICIPANTS

A15. What is the sample group or cohort to be studied in this research?

Select all that apply:

- [ ] Blood
- [ ] Cancer
- [x] Cardiovascular
- [ ] Congenital Disorders
- [ ] Dementias and Neurodegenerative Diseases
- [ ] Diabetes
- [ ] Ear
- [ ] Eye
- [ ] Generic Health Relevance
- [ ] Infection
- [ ] Inflammatory and Immune System
- [ ] Injuries and Accidents
- [ ] Mental Health
- [ ] Metabolic and Endocrine
- [ ] Musculoskeletal
- [ ] Neurological
- [ ] Oral and Gastrointestinal
- [ ] Paediatrics
- [ ] Renal and Urogenital
- [ ] Reproductive Health and Childbirth
- [ ] Respiratory
- [ ] Skin
- [ ] Stroke

Gender:	Male and female participants
Lower age limit: 40	Years
Upper age limit: 100	Years

A17-1. Please list the principal inclusion criteria (list the most important, max 5000 characters).

Participants will have been referred to and passed through an NHS cardiac rehabilitation program.

A17-2. Please list the principal exclusion criteria (list the most important, max 5000 characters).

Individuals cannot take part in this project if:
- They are under 18 years old
- They are pregnant
- They have a pacemaker or automatic defibrillator
- They have had a hip fracture or hip replacement
- They have a current renal deficiency or malfunction
- They have been advised by a medical professional not to participate in moderate to high intensity exercise

If a participant has had any of the following then they cannot undergo the scan and the session would have to be rearranged:
- An X-ray using a contrast material, such as barium, within 7 days of the proposed DXA scan
- Nuclear medicine studies within 3 days of the proposed DXA scan

RESEARCH PROCEDURES, RISKS AND BENEFITS

A18. Give details of all non-clinical intervention(s) or procedure(s) that will be received by participants as part of the research protocol. *These include seeking consent, interviews, non-clinical observations and use of questionnaires.*

Please complete the columns for each intervention/procedure as follows:

1. Total number of interventions/procedures to be received by each participant as part of the research protocol.
2. If this intervention/procedure would be routinely given to participants as part of their care outside the research, how many of the total would be routine?
3. Average time taken per intervention/procedure (minutes, hours or days)
4. Details of who will conduct the intervention/procedure, and where it will take place.

Intervention or procedure	1	2	3	4
Seeking informed consent	1	0	5 minutes	Informed consent will be obtained by the PhD student and other members of the research team, and will take place in a dedicated room within the University campus.
Questionnaire (socio-economic details including occupation, education, marital status etc)	1	0	5 minutes	Questionnaires will be issued by the PhD student and other members of the research team, and will take place in a dedicated room within the University campus.
4-day food diary (only participants in phase 2)	2	0	10 minutes	Questionnaires will be issued by the PhD student and other members of the research team, and will take place in a dedicated room within the University campus.
Dietary & Exercise Adherence Surveys (only participants in phase 2)	12	0	5 minutes	Surveys will be completed via phone call to the participants at their preferred times. These will be conducted by the PhD student and other members of the research team.
Mediterranean Diet Score Questionnaire (only participants in phase 2)	2	0	5 minutes	Questionnaires will be issued by the PhD student and other members of the research team, and will take place in a dedicated room within the University campus.

A19. Give details of any clinical intervention(s) or procedure(s) to be received by participants as part of the research protocol. *These include uses of medicinal products or devices, other medical treatments or assessments, mental health interventions, imaging investigations and taking samples of human biological material. Include procedures which might be received as routine clinical care outside of the research.*

Please complete the columns for each intervention/procedure as follows:

1. Total number of interventions/procedures to be received by each participant as part of the research protocol.
2. If this intervention/procedure would be routinely given to participants as part of their care outside the research, how many of the total would be routine?
3. Average time taken per intervention/procedure (minutes, hours or days).
4. Details of who will conduct the intervention/procedure, and where it will take place.

Intervention or procedure	1	2	3	4
Scans using the dual energy X-ray absorptiometry (DXA) scanner	1-3	0	30 minutes	The scans will be conducted by a collaborator on this project, who is a trained DXA operator and a designated Radiation Protection Supervisor for the University
Body composition measurement by bio-impedance	1-3	0	5 minutes	This will be conducted by the PhD student and other members of the research team, and will take place in a dedicated room within the University campus
Anthropometric measurements	1-3	0	10 minutes	These measurements will be taken by the PhD student and other members of the research team, and will take place in a dedicated room within the University campus
Blood sample collection	2	0	10 minutes	These measurements will be taken by the PhD student and other members of the research team, and will take place in a dedicated room within the University campus
Resistance training	36	0	40 minutes	Resistance exercise will be completed by the participants in their homes and/or at their community centres, using resistance bands and following an agreed upon resistance training program.
Grip strength test	3	0	1 minute	Grip strength will be measured by the PhD student and other members of the research team, and will take place in a dedicated room within the University campus

A20. Will you withhold an intervention or procedure, which would normally be considered a part of routine care?

○ Yes ◉ No

A21. How long do you expect each participant to be in the study in total?

Participants in phase 1 (cross-sectional study) will be in the study for an estimated time of 45 minutes. If they are involved in the focus groups, this will add another 20 minutes to their participation.

Participants in phase 2 (RCT) will be in the study for an additional period of 12 weeks.

After this, the participant's obligation is complete. Further communication with the research team will only take place should the participant choose to get in contact to request further information regarding the project, or to withdraw.

A22. What are the potential risks and burdens for research participants and how will you minimise them?

For all studies, describe any potential adverse effects, pain, discomfort, distress, intrusion, inconvenience or changes to lifestyle. Only describe risks or burdens that could occur as a result of participation in the research. Say what steps would be taken to minimise risks and burdens as far as possible.

Participants will be scanned by a DXA scanner, which will involve use of small doses of ionising radiation, though this

is a minimal amount compared to other medical imaging techniques, such as standard X-ray and CT scans. The DXA scan would be equal to approximately 2 microsieverts (μSv); this is compared to 20 microsieverts for a chest X-ray and 10,000 microsieverts for a CT scan. It is also less than a transcontinental flight (40 μSv) and one day of natural background radiation (8 μSv).
Great care will be taken to ensure vulnerable people (for example, pregnant women) will not be permitted to take part, both during the initial screening and recruitment, and also during the scans where mandatory exclusion criteria are checked before the scan can begin.
The study will limit the involvement of each participant to three scans, and they will be fully informed as to the exposure they will have, should they choose to participate. The radiation exposure is so low that no shielding of the room or of
the person conducting the scans is necessary; therefore, the anticipated risk to the participant is negligible.

The participants are being asked to be involved in a study which has a central focus on body weight and composition, which can be a very sensitive subject. As the participants will be willing volunteers for the study, it is expected that only people who are comfortable with this issue will be involved. Additionally, the participants can request for their bio-impedance and anthropometric measurements to be taken by a member of the research team of the same gender. They can also request a chaperone to be present during the procedure. Privacy and confidentially will be ensured when taking measurements, and all data will be anonymised upon collection and storage.

In addition, participants will be reminded that they can withdraw and remove themselves from the situation (and the study) at any time.

For the DXA scans, questionnaires and anthropometric measurements, it is not anticipated that there will be significant inconvenience for those who choose to take part in this project, as their time involvement is minimal at 45 minutes per visit, and we will do our best to accommodate their visit times to their preferences and availability (within the limits of the research requirements).

Participants will be made aware of the requirements of the dietary intervention while seeking consent, and it is expected that those who are comfortable with this dietary change will be involved. The dietary intervention will not use difficult to source ingredients and will be tailored to suit the tastes of the participants. Various recipes and food substitutions will be provided to participants at the beginning of the protocol. The high-protein diet of the protocol would only be potentially detrimental to individuals who have renal deficiency, and therefore this is one of the studies exclusion criteria. Participants will also receive regular contact from the PhD student regarding their adherence, and will also use a smart-phone based app daily to improve adherence.

Participants will be made aware of the requirements of the exercise intervention while seeking consent, and it is expected that those who are comfortable with this change in physical activity will be involved. All participants will have received exercise tolerance tests as part of their NHS cardiac rehabilitation and will be cleared for the exercise protocol. They will also receive instruction from a collaborator suitably trained in exercise for cardiac rehabilitation regarding how to safely complete the exercise protocol.

The involvement of the participants and their data will be kept confidential, with no personal identifying data being present on questionnaires, forms or DXA scans. The unique code that can identify a participant will be kept on a secure system that can only be accessed by the research team. Therefore it is not expected that there will be a significant risk of a confidentiality breach.

A23. Will interviews/ questionnaires or group discussions include topics that might be sensitive, embarrassing or upsetting, or is it possible that criminal or other disclosures requiring action could occur during the study?

○ Yes ◉ No

A24. What is the potential for benefit to research participants?

The participants will be able to take with them their scans, should they choose, and have highlighted to them any indicators of potential health risk, such as low bone mineral density and fracture risk, that the DXA may reveal.

Participants will receive the results of their blood tests and have highlighted to them any indicators of potential health risk, such as levels of cholesterol, triglycerides and glucose outside normal ranges.

It is expected that the diet and exercise interventions will benefit the participants by improving their body composition, increasing their muscle strength, and reducing their risk of future cardiovascular events.

A25. What arrangements are being made for continued provision of the intervention for participants, if appropriate, once the research has finished? *May apply to any clinical intervention, including a drug, medical device, mental health intervention, complementary therapy, physiotherapy, dietary manipulation, lifestyle change, etc.*

As the diet and exercise interventions will be designed to be as easily integrated into the participants' lifestyles as possible, they may choose to continue with the protocol upon completion of the trial.

Changes in participants' health markers (body composition, cholesterol, triglycerides, etc.) will be revealed to the participants upon completion of the study, and may encourage participants to continue with the lifestyle change.

A26. What are the potential risks for the researchers themselves? *(if any)*

No risks are foreseen with this study; however, to ensure the safety of the researchers themselves, all interactions with the participants will be carried out by phone, at the community centres, and in the University campus.

RECRUITMENT AND INFORMED CONSENT

In this section we ask you to describe the recruitment procedures for the study. Please give separate details for different study groups where appropriate.

A27-1. How will potential participants, records or samples be identified? Who will carry this out and what resources will be used?*For example, identification may involve a disease register, computerised search of social care or GP records, or review of medical records. Indicate whether this will be done by the direct care team or by researchers acting under arrangements with the responsible care organisation(s).*

Potential participants will be initially identified by our contacts within collaborating NHS hospitals, such as (but not exclusively) the Liverpool Heart & Chest Hospital Cardiac Rehabilitation Department.

All participants will be issued with a unique identifying code by the PhD student. This code will be recorded on the questionnaires they are required to complete, as well as on the forms for data collection and the images that will be produced by the DXA scanner.

This will ensure all participant details are confidential, but the research team will be able to trace samples and data back to the participant, should they wish to withdraw from the study and their data not to be used.

Participants' signed consents and any document that can identify them will be kept securely in locked file cabinets, only accessed by the research team. The participant identifying codes and all electronic information and databases will be stored securely and encrypted in password protected computers within the University server, with access restricted to the research team.

A27-2. Will the identification of potential participants involve reviewing or screening the identifiable personal information of patients, service users or any other person?

(●) Yes ( ) No

Please give details below:
Potential participants will be identified initially by members of the patients clinical care teams at collaborating NHS hospitals.

A27-3. Describe what measures will be taken to ensure there is no breach of any duty of confidentiality owed to patients, service users or any other person in the process of identifying potential participants.*Indicate what steps have been or will be taken to inform patients and service users of the potential use of their records for this purpose. Describe the arrangements to ensure that the wishes of patients and service users regarding access to their records are respected. Please consult the guidance notes on this topic.*

Only members of the clinical care team will have access to patient and disease registers used to identify potential participants. Explicit verbal consent from potential participants will be sought by a member of the clinical care team in order for them to check whether they meet the inclusion criteria or make the initial approach to potential participants.

Participants will be provided with transparency information about the legal basis and all details of processing

personal data.

It can be the case that clinical data will be cross-analysed with data collected during phase 1 of the study. Should this happen, these data will only be requested by the research team after the participants have given their signed consent to participate in the study and have their clinical data included in the analysis.
Data will always be handled anonymously and used for the sole purpose of research. Storage and access will follow the same procedures described in section A27-1.

A27-4. Will researchers or individuals other than the direct care team have access to identifiable personal information of any potential participants?

(●) Yes ( ) No

A27-5. Has prior consent been obtained or will it be obtained for access to identifiable personal information?

(●) Yes ( ) No

If Yes, please give details below.

As explained in 27-3, participants who have consented to take part in phase 1 of the study will be asked whether they consent for the clinical team to share specific clinical information that can be relevant to the study (for example, blood levels of markers of cardiometabolic risk to be cross-analysed with body composition measurements).

A28. Will any participants be recruited by publicity through posters, leaflets, adverts or websites?

( ) Yes (●) No

A29. How and by whom will potential participants first be approached?

Potential participants will first be approached by a member of their cardiac rehabilitation team to register their interest in the project. They will then be contacted by the PhD student who will provide further information by way of the participant information sheet and additional relevant documentation, and they will have the opportunity to ask any questions they might have. This opportunity will be extended to all subsequent meetings.

A30-1. Will you obtain informed consent from or on behalf of research participants?

(●) Yes ( ) No

If you will be obtaining consent from adult participants, please give details of who will take consent and how it will be done, with details of any steps to provide information (a written information sheet, videos, or interactive material). Arrangements for adults unable to consent for themselves should be described separately in Part B Section 6, and for children in Part B Section 7.

If you plan to seek informed consent from vulnerable groups, say how you will ensure that consent is voluntary and fully informed.

Informed consent will be taken by the lead researcher and will follow the distribution of a participant information sheet, which will contain all the details necessary for participants to make a fully informed decision on whether or not they wish to take part.
Once they have read and understood this information, they will be asked to sign a participant consent form, which will include some final declarations regarding the information relayed to them.

If you are not obtaining consent, please explain why not.

Please enclose a copy of the information sheet(s) and consent form(s).

A30-2. Will you record informed consent (or advice from consultees) in writing?

◉ Yes ○ No

A31. How long will you allow potential participants to decide whether or not to take part?

Once a participant registers interest in taking part, they will be given a copy (printed or electronic) of the participant information sheet, along with a calendar of dates providing details of when they can meet with the researcher and take part in the study. The dates will range from between one and eight weeks, dependent on uptake from that point, which will give them at least one week to reflect on the project and ask questions.

A32. Will you recruit any participants who are involved in current research or have recently been involved in any research prior to recruitment?

○ Yes
◉ No
○ Not Known

A33-1. What arrangements have been made for persons who might not adequately understand verbal explanations or written information given in English, or who have special communication needs?*(e.g. translation, use of interpreters)*

The participants will be required to read and understand the recruitment documents and then to get in touch with the research team to register their interest. It is anticipated that only those with a sufficient level of understanding will follow this process through to completion.
We lack the necessary resources to provide interpreters or translation, and understanding of written information will be crucial for adequate compliance with the study, so should the situation arise that a potential participant has not been able to understand the verbal explanations or written communication, then they will be excluded from the study.

A34. What arrangements will you make to ensure participants receive any information that becomes available during the course of the research that may be relevant to their continued participation?

Participants will receive information about the study as it becomes available via email (preferably) or text, and participants will be required to acknowledge receipt and understanding of the communication. Should acknowledgement not be forthcoming, participants will be contacted by phone by the PhD student.

A35. What steps would you take if a participant, who has given informed consent, loses capacity to consent during the study? *Tick one option only.*

◉ The participant and all identifiable data or tissue collected would be withdrawn from the study. Data or tissue which is not identifiable to the research team may be retained.
○ The participant would be withdrawn from the study. Identifiable data or tissue already collected with consent would be retained and used in the study. No further data or tissue would be collected or any other research procedures carried out on or in relation to the participant.
○ The participant would continue to be included in the study.
○ Not applicable – informed consent will not be sought from any participants in this research.
○ Not applicable – it is not practicable for the research team to monitor capacity and continued capacity will be assumed.

Further details:

CONFIDENTIALITY

In this section, personal data means any data relating to a participant who could potentially be identified. It includes pseudonymised data capable of being linked to a participant through a unique code number.

Storage and use of personal data during the study

A36. Will you be undertaking any of the following activities at any stage (including in the identification of potential participants)?*(Tick as appropriate)*

- [x] Access to medical records by those outside the direct healthcare team
- [ ] Access to social care records by those outside the direct social care team
- [x] Electronic transfer by magnetic or optical media, email or computer networks
- [ ] Sharing of personal data with other organisations
- [ ] Export of personal data outside the EEA
- [x] Use of personal addresses, postcodes, faxes, emails or telephone numbers
- [ ] Publication of direct quotations from respondents
- [ ] Publication of data that might allow identification of individuals
- [ ] Use of audio/visual recording devices
- [x] Storage of personal data on any of the following:
  - [x] Manual files (includes paper or film)
  - [ ] NHS computers
  - [ ] Social Care Service computers
  - [ ] Home or other personal computers
  - [x] University computers
  - [ ] Private company computers
  - [ ] Laptop computers

Further details:
All physical copies of documents containing personal data that can be used to identify participants (e.g. consent forms) will be stored securely and locked in filing cabinets within University premises, only accessed by the lead researcher and the PhD student in accordance with LJMU policies.
Other physical copies of documents not containing information that can lead to identifying participants (e.g. questionnaires) will be stored securely in a locked office within University premises.
Electronic copies of documents containing personal information that can identify participants will be stored on a password-protected University computer within a domain that is private to the lead researcher; all files will be encrypted and will only be accessible by the lead researcher and PhD student.
Electronic files containing the study results (databases) will be stored in the University OneDrive system accounts of the lead researcher and the PhD student, so they can access these data from their personal computers, for the sole purpose of statistical analysis of results. The University OneDrive system is encrypted and maintained by the University IT services, ensuring security, privacy and back-up of the files. All files accessed in this way will be encrypted, and will not contain information that can be used to identify participants.
Individual information will never be published. Should any qualitative research publication be produced (e.g. from focus groups), we will never use quotes that may allow identification of participants.
All personal information will be deleted following the individuals' participation in the study, and only completely anonymised databases of results will be retain for the purpose of publication and dissemination of results.

A37. Please describe the physical security arrangements for storage of personal data during the study?

Physical copies of documents will be stored in the lead researcher's office, which is locked, in a key-locked filing cabinet.
Electronic documents will be stored in LJMU computers. All computers within the University allow for an individual to log on to their own personal domain, which is password protected, as well as having various anti-spam and anti-hack software.
Any personal participant information will be coded and stored within the lead researchers secure personal domain. University computers are maintain on a daily (security) and weekly (backup) basis. This guarantees the privacy and stability of the documents stored in them.

A38. How will you ensure the confidentiality of personal data?*Please provide a general statement of the policy and procedures for ensuring confidentiality, e.g. anonymisation or pseudonymisation of data.*

This research project will comply fully with the Liverpool John Moores University Data Protection policy, which was derived from the Data Protection Act 1998. In order to ensure confidentiality, all data will be anonymised, with any personal identifiable data being held securely and only accessible by the research team.
Documents containing personal data that can be used to identify participants and those containing the coding keys will not be stored together.
All data will be processed in accordance with the eight principles of Data Protection. The University staff involved in this project are required to undertake annual training regarding data protection and will ensure that the policy is adhered to.

A40. Who will have access to participants' personal data during the study? *Where access is by individuals outside the direct care team, please justify and say whether consent will be sought.*

Access to personal data that can be used to identify participants will only be available for the PhD student undertaking the research and the lead researcher.
Other personal data contained in databases will be accessible as well to the research collaborators listed in this application form.

Storage and use of data after the end of the study

A41. Where will the data generated by the study be analysed and by whom?

Anonymised data will be analysed by the research team (PhD student, academic supervisors, and research collaborators)
Analysis of databases will take place in the University computers of the PhD student and the lead researcher. Results of the analysis will be shared for supervision and discussion with the other members of the research team.

A42. Who will have control of and act as the custodian for the data generated by the study?

	Title Forename/Initials Surname
	Dr Fatima Perez de heredia
Post	Senior Lecturer
Qualifications	FHEA, PG Cert in Higher Education, LJMU (2017) PhD Biology, University of Murcia (2007) Post-graduate Certificate in Education, University of Murcia (2003) BSc Biological Sciences, University of Murcia (2000)
Work Address	Room 234, James Parsons Building Liverpool John Moores University
Post Code	L3 3AF
Work Email	f.perezdeherediabenedicte@ljmu.ac.uk
Work Telephone	01512312003
Fax	

A43. How long will personal data be stored or accessed after the study has ended?

- ◯ Less than 3 months
- ◯ 3 – 6 months
- ◯ 6 – 12 months
- ◯ 12 months – 3 years

◉ Over 3 years

If longer than 12 months, please justify:
Data generated for any clinical research sponsored by the Liverpool John Moores University needs to kept for a minimum of 10 years. The data obtained during the study will contain information on numerous health markers as they relate to the interventions. The immediate outcomes of the investigation are body composition and muscle strength, as well as cardiometabolic markers, but data collected will include as well valuable information on factors to determine adherence and compliance, participant feedback, lifestyle (dietary) habits, etc. We need to take into account that further analysis of data may be relevant in the light of novel scientific and medical discoveries.
The expected volume of variables to be collected and of the resulting databases will require careful and accurate analysis, and that will require years. Any personal data collected that is not fundamental to the research can be destroyed earlier; however Information consent forms may need to be kept for the whole archiving period.

A44. For how long will you store research data generated by the study?

Years: 20
Months:

A45. Please give details of the long term arrangements for storage of research data after the study has ended.*Say where data will be stored, who will have access and the arrangements to ensure security.*

Following the completion of the research project, the data will belong to the University who will be the custodian.
Physical copies of documents containing personal data will be destroyed and disposed off in a secure and confidential manner, according to data protection regulations.
Electronic files containing personal information that can identify participants will be erased from the University computers and system.
Electronic files containing anonymised data will be stored on a secure password-protected computer and the academic supervisor for this project will have the responsibility for maintaining the integrity of the security of the data.

INCENTIVES AND PAYMENTS

A46. Will research participants receive any payments, reimbursement of expenses or any other benefits or incentives for taking part in this research?

◉ Yes ○ No

If Yes, please give details. For monetary payments, indicate how much and on what basis this has been determined.
Participants may be reimbursed for their travel expenses to and from the University campus.
Participants may also be provided with certain food ingredients necessary for the diet interventions, depending on available resources.

A47. Will individual researchers receive any personal payment over and above normal salary, or any other benefits or incentives, for taking part in this research?

○ Yes ◉ No

A48. Does the Chief Investigator or any other investigator/collaborator have any direct personal involvement (e.g. financial, share holding, personal relationship etc.) in the organisations sponsoring or funding the research that may give rise to a possible conflict of interest?

○ Yes ◉ No

NOTIFICATION OF OTHER PROFESSIONALS

A49-1. Will you inform the participants' General Practitioners (and/or any other health or care professional responsible for their care) that they are taking part in the study?

(●) Yes ( ) No

If Yes, please enclose a copy of the information sheet/letter for the GP/health professional with a version number and date.

A49-2. Will you seek permission from the research participants to inform their GP or other health/ care professional?

(●) Yes ( ) No

It should be made clear in the participant's information sheet if the GP/health professional will be informed.

PUBLICATION AND DISSEMINATION

A50-1. Will the research be registered on a public database?

The UK Policy Framework for Health and Social Care Research sets out the principle of making information about research publicly available. Furthermore: Article 19 of the World Medical Association Declaration of Helsinki adopted in 2008 states that "every clinical trial must be registered on a publicly accessible database before recruitment of the first subject"; and the International Committee of Medical Journal Editors (ICMJE) will consider a clinical trial for publication only if it has been registered in an appropriate registry. Please see guidance for more information.

(●) Yes ( ) No

Please give details, or justify if not registering the research.
Research will be registered on ClinicalTrials.gov

Please ensure that you have entered registry reference number(s) in question A5-1.

A51. How do you intend to report and disseminate the results of the study? *Tick as appropriate:*

- [x] Peer reviewed scientific journals
- [x] Internal report
- [x] Conference presentation
- [x] Publication on website
- [ ] Other publication
- [ ] Submission to regulatory authorities
- [ ] Access to raw data and right to publish freely by all investigators in study or by Independent Steering Committee on behalf of all investigators
- [ ] No plans to report or disseminate the results
- [ ] Other (please specify)

A52. If you will be using identifiable personal data, how will you ensure that anonymity will be maintained when publishing the results?

No identifiable personal data will be published. Demographics such as gender, age and BMI will be grouped wherever possible to ensure the combination of an individual persons data will could not lead to an identification. The figures reported will relate to values of body composition, anthropometric and cardiometabolic measurements representative of the study sample, and will therefore not be possible to link them with any individuals.

A53. Will you inform participants of the results?

(●) Yes ( ) No

Please give details of how you will inform participants or justify if not doing so.
During the research project debrief, participants will be invited to contact the researcher to request information regarding the outcome of the project and a summary of the results and conclusion.

5. Scientific and Statistical Review

A54-1. How has the scientific quality of the research been assessed? *Tick as appropriate:*

- ☐ Independent external review
- ☐ Review within a company
- ☑ Review within a multi−centre research group
- ☐ Review within the Chief Investigator's institution or host organisation
- ☑ Review within the research team
- ☑ Review by educational supervisor
- ☐ Other

Justify and describe the review process and outcome. If the review has been undertaken but not seen by the researcher, give details of the body which has undertaken the review:
The study proposal was reviewed and approved by LJMU's Research Committee.
Details of the study were finalised by collaboration of the supervisory team, and the specific methodology was further developed with the participation of the PhD student. The research methods to be used are validated and established techniques commonly used in clinical research.
The study has then been presented at and approved by the Service Users Research Endeavour (SURE) group of the Liverpool Heart and Chest Hospital, and by the Research and Innovation Committee of the same hospital. Both committees provided valuable feedback.

For all studies except non-doctoral student research, please enclose a copy of any available scientific critique reports, together with any related correspondence.

For non-doctoral student research, please enclose a copy of the assessment from your educational supervisor/ institution.

A56. How have the statistical aspects of the research been reviewed? *Tick as appropriate:*

- ☐ Review by independent statistician commissioned by funder or sponsor
- ☐ Other review by independent statistician
- ☐ Review by company statistician
- ☐ Review by a statistician within the Chief Investigator's institution
- ☐ Review by a statistician within the research team or multi−centre group
- ☑ Review by educational supervisor
- ☐ Other review by individual with relevant statistical expertise
- ☐ No review necessary as only frequencies and associations will be assessed – details of statistical input not required

In all cases please give details below of the individual responsible for reviewing the statistical aspects. If advice has been provided in confidence, give details of the department and institution concerned.

	Title	Forename/Initials	Surname
	Dr	Fatima	Perez de Heredia
Department	School of Natural Sciences and Psychology		
Institution	Liverpool John Moores University		
Work Address	Room 234, James Parsons Building		
	Liverpool John Moores University		

Post Code	L3 3AF
Telephone	01512312003
Fax	
Mobile	
E-mail	f.perezdeherediabenedicte@ljmu.ac.uk

Please enclose a copy of any available comments or reports from a statistician.

A57. What is the primary outcome measure for the study?

Changes in body composition, specifically lean body mass, as determined by dual energy x-ray absorptiometry (DXA) scanner.

A58. What are the secondary outcome measures?*(if any)*

i) Muscle strength as determined by hand grip strength.
ii) Body fat content as determined by DXA.
iii) Anthropometric measures (height, weight and waist circumference)
iv) Measurements of cardiometabolic risk markers:
- cholesterol sub-fractions
- triglycerides
- fasting glucose
- HOMA-IR
- pro-inflammatory markers (e.g. IL-1β, IL-6, TNFα, C-reactive protein)
- adipokines and myokines
v) feasibility of the intervention(e.g. recruitment, adherence/attrition, compliance)

A59. What is the sample size for the research? *How many participants/samples/data records do you plan to study in total? If there is more than one group, please give further details below.*

Total UK sample size: 240
Total international sample size (including UK): 240
Total in European Economic Area:

Further details:
A power calculation indicates an adequate sample size of 240 participants for the cross-sectional stage of the study (screening for sarcopenic obesity).
For the second stage (feasibility study and pilot intervention), we aim to recruit 10-15 participants per intervention group (40-60 participants), who will be invited from the participants in the cross-sectional stage.

A60. How was the sample size decided upon? *If a formal sample size calculation was used, indicate how this was done, giving sufficient information to justify and reproduce the calculation.*

For the cross-sectional stage, sample size was calculated using an online calculator, based on an estimated prevalence of sarcopenic obesity of 20% with precision at 5% and confidence at 95%.

Online calculator: http://sampsize.sourceforge.net/iface/

The sample size for the feasibility study was intentionally kept small to allow ease of recruitment and management of participants before development into a fully powered trial.

A61-1. Will participants be allocated to groups at random?

(●) Yes ( ) No

If yes, please give details of the intended method of randomisation:
Participants will be allocated to groups randomly using the online randomisation tool, Research Randomizer https://www.randomizer.org/

A62. Please describe the methods of analysis (statistical or other appropriate methods, e.g. for qualitative research) by which the data will be evaluated to meet the study objectives.

Associations between variables of body composition, anthropometry, and muscle strength in the cross-sectional study will be analysed by correlation and logistic regression tests. Effects of demographic variables (e.g. gender, age, SES) will be assessed by means of regression analysis.
The intervention study will have a 2x2 factorial design (diet x exercise), and outcomes will be analysed by means of the mixed model with repeated measures (before and after intervention) and two-way ANOVA (diet x exercise).

6. MANAGEMENT OF THE RESEARCH

A63. Other key investigators/collaborators. *Please include all grant co−applicants, protocol co−authors and other key members of the Chief Investigator's team, including non-doctoral student researchers.*

	Title	Forename/Initials	Surname
	Dr	Ian	Davies
Post	Reader in Nutritional Science		
Qualifications	Postgraduate Certificate in Learning and Teaching in HE, LJMU (2008) PhD, University of Surrey (2004) MPhil, LJMU (1998) BSc Biochemistry, University of Liverpool (1995)		
Employer	Liverpool John Moores University		
Work Address	IM Marsh Campus Barkhill Rd.		
Post Code	L17 6BD		
Telephone	0151 231 5290		
Fax			
Mobile			
Work Email	I.G.Davies@ljmu.ac.uk		

	Title	Forename/Initials	Surname
	Dr	Tom	Butler
Post	Senior Lecturer in Nutrition & Dietetics		
Qualifications	Postgraduate Certificate in Learning and Teaching in Higher Education, University of Chester (2017) Postgraduate Diploma in Nutrition and Dietetics, University of Chester (2015) PhD Clinical Biosciences Institute, University of Hull (2012) Postgraduate Certificate in Research Training, University of Hull (2010) BSc (Hons) Human Biology (2008)		
Employer	University of Chester		
Work Address	University of Chester Parkgate Road Chester		
Post Code	CH1 4BJ		
Telephone	01244 511660		
Fax			
Mobile			
Work Email	t.butler@chester.ac.uk		

A64. Details of research sponsor(s)

A64-1. Sponsor

Lead Sponsor

Status:
- ○ NHS or HSC care organisation
- ◉ Academic
- ○ Pharmaceutical industry
- ○ Medical device industry
- ○ Local Authority
- ○ Other social care provider (including voluntary sector or private organisation)
- ○ Other

Commercial status: Non-Commercial

If Other, please specify:

Contact person

Name of organisation	Liverpool John Moores University
Given name	Dave
Family name	Harriss
Address	Research Innovation Services, Exchange Station, Tithebarn Street
Town/city	Liverpool
Post code	L2 2QP
Country	UNITED KINGDOM
Telephone	0151 231 212
Fax	
E-mail	sponsor@ljmu.ac.uk

A65. Has external funding for the research been secured?

Please tick at least one check box.

- ☑ Funding secured from one or more funders
- ☐ External funding application to one or more funders in progress
- ☐ No application for external funding will be made

What type of research project is this?

- ◉ Standalone project
- ○ Project that is part of a programme grant
- ○ Project that is part of a Centre grant
- ○ Project that is part of a fellowship/ personal award/ research training award
- ○ Other

Other – please state:

Please give details of funding applications.

Organisation	NHS Liverpool Clinical Commissioning Group
Address	2 Renshaw Street
Post Code	L1 2SA
Telephone	0151 296 7000
Fax	
Mobile	
Email	enquiries@liverpoolccg.nhs.uk

Funding Application Status: ○ Secured ◉ In progress

Date Funding decision expected: 30/09/2019

Amount: 27,166

Duration

Years: 0

Months: 6

If applicable, please specify the programme/ funding stream:

What is the funding stream/ programme for this research project?

Research Capability Funding (RCF) 2019/20

A66. Has responsibility for any specific research activities or procedures been delegated to a subcontractor (other than a co-sponsor listed in A64-1) ? *Please give details of subcontractors if applicable.*

○ Yes ◉ No

A67. Has this or a similar application been previously rejected by a Research Ethics Committee in the UK or another country?

○ Yes ◉ No

Please provide a copy of the unfavourable opinion letter(s). You should explain in your answer to question A6-2 how the reasons for the unfavourable opinion have been addressed in this application.

A68-1. Give details of the lead NHS R&D contact for this research:

	Title	Forename/Initials	Surname
		Victoria	Wilkinson
Organisation	Liverpool Heart & Chest Hospital		
Address	Research Unit		
	Liverpool Heart & Chest Hospital		
	Thomas Drive		
Post Code	L14 3PE		

Work Email	vicky.wilkinson@lhch.nhs.uk
Telephone	0151 600 1467
Fax	
Mobile	

Details can be obtained from the NHS R&D Forum website: http://www.rdforum.nhs.uk

A68-2. Select Local Clinical Research Network for NHS Organisation identified in A68-1:

North West Coast

For more information, please refer to the question specific guidance.

A69-1. How long do you expect the study to last in the UK?

Planned start date: 06/01/2020
Planned end date: 06/01/2021
Total duration:
Years: 1 Months: 0 Days: 1

A70.

Definition of the end of trial, and justification in the case where it is not the last visit of the last subject undergoing the trial

The study will be complete when all samples (blood, anthropometric, strength and DXA data) have been fully taken from all participants completing the 12-week intervention, i.e. at the endpoint interview.
Participants may be contacted 6 months later for a voluntary follow up questionnaire.
Relevant consent forms will be obtained for each participant, allowing for the samples to be stored for at least 20 years.

A71-1. Is this study?

- (●) Single centre
- ( ) Multicentre

A71-2. Where will the research take place? *(Tick as appropriate)*

- [x] England
- [ ] Scotland
- [ ] Wales
- [ ] Northern Ireland
- [ ] Other countries in European Economic Area

Total UK sites in study 1

Does this trial involve countries outside the EU?

( ) Yes (●) No

A72. Which organisations in the UK will host the research?*Please indicate the type of organisation by ticking the box and give approximate numbers if known:*

☑ NHS organisations in England	1
☐ NHS organisations in Wales	
☐ NHS organisations in Scotland	
☐ HSC organisations in Northern Ireland	
☐ GP practices in England	
☐ GP practices in Wales	
☐ GP practices in Scotland	
☐ GP practices in Northern Ireland	
☐ Joint health and social care agencies (eg community mental health teams)	
☐ Local authorities	
☐ Phase 1 trial units	
☐ Prison establishments	
☐ Probation areas	
☐ Independent (private or voluntary sector) organisations	
☑ Educational establishments	1
☐ Independent research units	
☐ Other (give details)	
Total UK sites in study:	2

A73-1. Will potential participants be identified through any organisations other than the research sites listed above?

○ Yes ◉ No

A74. What arrangements are in place for monitoring and auditing the conduct of the research?

Annual reports will be submitted to LJMU Doctoral Academy and will be presented at the Research and Innovation Committee of the Liverpool Heart and Chest Hospital.

LJMU may perform checks as part of their quality programme.

A Trial Oversight Committee (TOC) will be formed, consisting of the academic researchers, Liverpool Heart and Chest Hospital clinical leads, and Community Centre leads. Equally, there will be a Trial Steering Group (TSG), consisting of patients engaging with cardiac rehabilitation (phase 3, patient group), the lead researcher and co-investigators, and any staff employed on the trial. The TSG will be responsible for reviewing the protocol design and ethical considerations such as participant information, and valuable feedback on patient 'friendliness' and acceptability will be documented and fed back to the TOC. Any changes made will be reported and documented.

A75-1. What arrangements will be made to review interim safety and efficacy data from the trial? Will a formal data monitoring committee or equivalent body be convened?

There is no predicted high morbidity or mortality as a consequence of the intervention, or any real health risk.
The TOC and TSG described above will monitor the feasibility and efficacy of the study in the light of the data collected and the results obtained.

If a formal DMC is to be convened, please forward details of the membership and standard operating procedures to the Research Ethics Committee when available. The REC should also be notified of DMC recommendations and receive summary reports of interim analyses.

A75-2. What are the criteria for electively stopping the trial or other research prematurely?

We do not foresee any medium or high risk to the participants as a result of the intervention proposed; however, the target population is intrinsically of higher risk and frailty, and for that reason participants will be monitored regularly. Frequent follow-up phone calls will be made to gather information of their adherence to the intervention and well-being, and a mid-point meeting will be held to promote engagement and to collect anthropometric measurements. This frequent contact will provide immediate information of any changes in the health status of a participant that could suggest deterioration, so that the participant can be referred to their GP doctor for a check up.
If the participant is not deemed fit to continue in the study, we will explain the situation and invite them to withdraw.

A76. Insurance/ indemnity to meet potential legal liabilities

***Note:** in this question to NHS indemnity schemes include equivalent schemes provided by Health and Social Care (HSC) in Northern Ireland*

A76-1. What arrangements will be made for insurance and/or indemnity to meet the potential legal liability of the sponsor(s) for harm to participants arising from the management of the research? *Please tick box(es) as applicable.*

Note: Where a NHS organisation has agreed to act as sponsor or co-sponsor, indemnity is provided through NHS schemes. Indicate if this applies (there is no need to provide documentary evidence). For all other sponsors, please describe the arrangements and provide evidence.

☐ NHS indemnity scheme will apply (NHS sponsors only)

☑ Other insurance or indemnity arrangements will apply (give details below)

LJMU has arranged Public Liability insurance and/or Clinical Trials insurance (delete as required) to cover the legal liability of the University as Research Sponsor in the eventuality of harm to a research participant arising from management of the research by the University and the activities here are included within that coverage.
This does not in any way affect an NHS Trust's responsibility for any clinical negligence on the part of its staff (including the Trust's responsibility for LJMU employees/students acting in connection with their NHS honorary appointments). (Delete this 2nd paragraph if not applicable e.g. if your research takes place on University premises and/or involves no clinical intervention by the NHS).

Please enclose a copy of relevant documents.

A76-2. What arrangements will be made for insurance and/ or indemnity to meet the potential legal liability of the sponsor(s) or employer(s) for harm to participants arising from the design of the research? *Please tick box(es) as applicable.*

Note: Where researchers with substantive NHS employment contracts have designed the research, indemnity is provided through NHS schemes. Indicate if this applies (there is no need to provide documentary evidence). For other protocol authors (e.g. company employees, university members), please describe the arrangements and provide evidence.

☐ NHS indemnity scheme will apply (protocol authors with NHS contracts only)

☑ Other insurance or indemnity arrangements will apply (give details below)

LJMU holds Professional Indemnity insurance and/or Clinical Trials insurance (delete as required) to cover the legal liability of the University as Research Sponsor and/or as the employer of staff/students engaged in the research, for harm to participants arising from the design of the research, where the research protocol was designed by the University.

Please enclose a copy of relevant documents.

A76-3. What arrangements will be made for insurance and/ or indemnity to meet the potential legal liability of investigators/collaborators arising from harm to participants in the conduct of the research?

Note: Where the participants are NHS patients, indemnity is provided through the NHS schemes or through professional indemnity. Indicate if this applies to the whole study (there is no need to provide documentary evidence). Where non-NHS sites are to be included in the research, including private practices, please describe the arrangements which will be made at

these sites and provide evidence.

☐ NHS indemnity scheme or professional indemnity will apply (participants recruited at NHS sites only)

☑ Research includes non-NHS sites (give details of insurance/ indemnity arrangements for these sites below)

LJMU's Public Liability and Professional Indemnity insurance policies and/or Clinical Trials insurance (delete as required) provide an indemnity to our employees and students for their potential liability for harm to participants during the conduct of the research and the activities here are included within that coverage.

Evidence of insurance cover is available to download at
https://www2.ljmu.ac.uk/fin/secured/finance_allJMU/116465.htm

Please enclose a copy of relevant documents.

A77. Has the sponsor(s) made arrangements for payment of compensation in the event of harm to the research participants where no legal liability arises?

○ Yes ◉ No

Please enclose a copy of relevant documents.

A78. Could the research lead to the development of a new product/process or the generation of intellectual property?

○ Yes ◉ No ○ Not sure

A79. Please select the level of commercial participation in this project.

○ None
◉ Industry funding, but not industry sponsored
○ Industry funding and industry sponsored
○ Industry sponsored, but not industry funded

A80. Please select the main subject area of research. Additional sub-topics may be selected, if required

☑ Age and Ageing
☐ Anaesthetics
☐ Cancer (includes malignant haematology
☑ Cardiovascular
☐ Clinical
☐ Critical Care
☐ Dementias and Neurodegenerative Diseases
☐ Dermatology
☐ Diabetes
☐ Ear, Nose and Throat
☐ Gastrointestinal
☐ Genetics
☐ Health Services Research
☐ Hepatology
☐ Immunology and Inflammation

- [ ] Infectious Disease and Microbiology
- [ ] Injuries and Accidents
- [ ] Medicines for Children (does not include Paediatrics)
- [ ] Mental Health
- [ ] Metabolic and Endocrine
- [x] Musculoskeletal (Rheumatoid Arthritis is a separate category)
- [ ] Nervous System Disorders
- [ ] Non-malignant Haematology
- [ ] Ophthalmology
- [ ] Oral and Dental
- [ ] Paediatrics (does not include Medicines for Children)
- [ ] Primary Care
- [ ] Public Health Research
- [ ] Renal
- [ ] Reproductive Health and Childbirth
- [ ] Respiratory
- [ ] Rheumatoid Arthritis
- [ ] Stroke
- [ ] Surgery
- [ ] Urogenital

PART B: Section 3 – Exposure to ionising radiation

Complete sub-sections A and/or B as applicable with input from relevant experts. It is advisable to discuss the proposed research at an early stage with (a) a Medical Physics Expert and (b) a Clinical Radiation Expert, who will carry out the required assessments for sub-sections C and D. The lead MPE can also facilitate the completion of sub-sections A and/or B if necessary.

1. Does the study involve exposure to radioactive materials?

○ Yes ◉ No

To update the response above, go to the Project Filter Question 2 'Does the study involve exposure to radioactive materials?' and select an option.

2. Does the study involve other diagnostic or therapeutic ionising radiation?

◉ Yes ○ No

A. Radioactive materials

Details of radioactive materials

B. Other ionising radiation

B1. Details of other ionising radiation

Give details by completing the table below:

Procedure	No of procedures	Estimated procedure dose *(use national Diagnostic Reference Levels where available)*
Dual-X-ray densitometry (DXA)	3	0.66 microsieverts (μSv)

C. Dose and risk assessment

C1. What is the total participant dose from all the exposures in A1 and/or B1, and what component of this is the additional dose over and above standard practice? What are the risks associated with these two doses (total and additional)?

The dose and risk assessment should be set out below. This should be prepared by a Medical Physics Expert (MPE) who is a registered clinical scientist registered with the Health Professions Council and has expertise relevant to the planned exposures. Where the study involves different types of exposure (for example, both radioactive materials and other ionising radiation, or more than one imaging method), advice may need to be sought from other MPEs with relevant expertise. The lead MPE should produce a combined assessment for the ethics committee, giving the names of any other MPEs who have contributed to the assessment. Further guidance is available by clicking on the information button.

Participants will be aged 18 years or over.

It is estimated that each participant in this project will receive between 1 to 3 DXA scans, and that each scan will result in an effective dose of approximately 0.66 μSv to the participant. So at a maximum, a participant will receive an effective dose of approximately 2 μSv in total.

This level of dose is extremely small. Assuming a total population risk factor of fatal cancer of 5.5 x 10-2 Sv-1 for fatal cancer leads to a risk factor of 2 x 10-6 x 5.5 x 10-2 = 1.1 x 10-7 or 1 in 9,100,000 additional risk of fatal cancer. The risk of 1 in 9,100,000 is approximately equivalent to the risk of death by traveling just 3 miles in a motorcar on the

public highway.
In total, it is anticipated that the entire project will include no more than 250 volunteers. This is considered to be a small detriment to a small cohort of healthy volunteers when compared to the benefits of this study to medical and sport science.

A Diagnostic Reference Level (DRL) will be set at 2 µSv effective dose for one individual participant. This is a low level of dose and is approximately equivalent to 0.5 days' worth of natural background radiation.

In addition to the DRL, a Dose Constraint of 6 µSv will be set for each individual volunteer. This is approximately two orders of magnitude below the annual public dose limit as specified in the Ionising Radiation Regulations 2017.

Referral criteria (IRMER 2017 Regulation 6 (5)(a)) will be established for the acceptance of suitable volunteers. Details of all exposures to individual volunteers will be recorded in the project records and held indefinitely.

A set of written IRMER 2017 procedures (Regulation 6(1)) will be established as will an exposure protocol (Regulation 6 (4)).

Prior to any exposures, all volunteers will be given a briefing during which the procedure and the risks from radiation will be fully explained.

The MPE and RPA for the project will be Professor Pete Cole (RPA2000 RPA Certificate of Competence No. 1739 and RPA2000 MPE List No. 20). A list of all IRMER duty holders (Practitioners and Operators) will be maintained. They will receive suitable and sufficient training commensurate with the syllabus specified in the regulations in addition to applications specialist training on the operation of the x-ray equipment. A CRE has been formally appointed.

Special attention must be paid to pregnant/potentially pregnant women or those who are breast feeding, or other potentially vulnerable groups.

C2. Declaration by lead Medical Physics Expert

I am satisfied that the information in sub-sections A and/or B and the assessment in sub-section C provide a reasonable estimate of the ionising radiation exposure planned in this research and the associated risks.

Signature:…….. Date:

C3. Details of person acting as lead Medical Physics Expert

	Title	Forename/Initials	Surname
	Professor	Pete	Cole
Post	Radiation Protection Officer		
Details of clinical scientist registration with the Health Professions Council:			
Registration no	N/R		
Organisation	University of Liverpool		
Address	Oliver Lodge Bldg.		
	Oxford St.		
	Liverpool		
Post Code	L69 7ZE		
Telephone	01517943467		
Fax			
Mobile	07973247821		
Email	pcole@liverpool.ac.uk		

D. Clinical assessment

This sub-section should be completed by a Clinical Radiation Expert (CRE) who is a registered doctor or dentist with clinical

expertise relevant to the planned exposures. The assessment should cover potential exposure at all research sites, taking account of possible variation in normal clinical practice. Where the study involves different types of exposure (for example, both radiotherapy and other ionising radiation), advice may need to be sought from other CREs with relevant expertise. The lead CRE should produce a combined assessment for the ethics committee, giving the names of any other CREs who have contributed to the assessment. The guidance notes give advice to Chief Investigators on who can act as lead Clinical Radiation Expert (CRE) and advice for the CRE on the assessment of exposures having regard to IRMER.

Special attention must be paid to pregnant/potentially pregnant women or those who are breast feeding, or other potentially vulnerable groups.

D1. Will the exposure exceed the exposure that might be received as part of normal care at any proposed research site?

◯ Yes ◉ No

D3. Declaration by lead Clinical Radiation Expert

I am satisfied that the exposure to ionising radiation planned in this research study (as defined in A1 and/or B1) is reasonable and that the risks are adequately described in the participant information sheet for the study.

Signature:…….. Date:

D4. Details of lead Clinical Radiation Expert

	Title	Forename/Initials	Surname
	Dr	MAROUSA	NTOUSKOU

Post	Consultant Radiologist
Details of professional registration	◉ General Medical Council ◯ General Dental Council
Registration no	7485185
Organisation	Liverpool Heart And Chest Hospital
Address	Thomas Dr. Liverpool
Post Code	L14 3PE
Telephone	01516001689
Fax	
Mobile	07986725589
Email	marousa.ntouskou@lhch.nhs.uk

Employers responsible for radiation facilities at research sites must have written procedures to meet the requirements of the Ionising Radiation (Medical Exposure) Regulations 2000 (IRMER). R & D offices for NHS sites will seek confirmation from local radiation experts that local IRMER authorisation procedures have been followed. Where the local Medical Physics Expert or IRMER Practitioner disagrees with the assessments made in this Section and/or the care organisation is unable to adhere to the protocol, this should be discussed with the Chief Investigator and the lead experts for the study. Any necessary variation in the protocol or participant information sheet at particular sites should be notified to the main REC as a substantial amendment and an ethical opinion sought.

Part B: Section 5 – Use of newly obtained human tissue(or other human biological materials) for research purposes

1. What types of human tissue or other biological material will be included in the study?

Blood samples will be collected from all participants in the feasibility study. This will happen twice, before and after the intervention.

2. Who will collect the samples?

The PhD student, Richard Kirwan, has received phlebotomy training at Liverpool John Moores University and will collect all blood samples.

3. Who will the samples be removed from?

- [x] Living donors
- [ ] The deceased

4. Will informed consent be obtained from living donors for use of the samples? *Please tick as appropriate*

In this research?

(•) Yes ( ) No

In future research?

(•) Yes ( ) No ( ) Not applicable

6. Will any tissues or cells be used for human application or to carry out testing for human application in this research?

(•) Yes ( ) No

Please give further details:
White blood cells will be stored for future analysis of potential changes in epigenetic markers that can be linked to the intervention proposed (depending on availability of funding/resources)

7. Please explain what licensing arrangements apply to the procurement, processing, distribution or import of the tissues and cells to be used in the research.

Please consult the guidance notes on this question. If either you or a collaborating organisation requires a licence from the Human Tissue Authority under the Quality and Safety Regulations, please confirm that the licence has been obtained or applied for.

LJMU holds a Human Tissue Act licence to collect, store and conduct research on human tissue samples. The lead researcher received induction training in the HTA 2004 by University's research officers, and herself and the PhD student both hold a certificate of electronic training in the HTA 2004 (MRC training course). They will receive additional training tailored to the specific requirements of the current study. Training will be provided by the Research Officer responsible for HTA 2004 in the Faculty of Science of LJMU.
Collection, transport, coding, storage, analysis and disposal of samples will be conducted following the principles and regulations of the HTA 2004.

If applicable, a copy of the HTA licence should be enclosed or provided when available.

8. Will the samples be stored: *[Tick as appropriate]*

In fully anonymised form? *(link to donor broken)*

( ) Yes (•) No

In linked anonymised form? *(linked to stored tissue but donor not identifiable to researchers)*
◉ Yes ○ No

If Yes, say who will have access to the code and personal information about the donor.
Only the research team will have access to the code and personal information of the donor.

In a form in which the donor could be identifiable to researchers?
◉ Yes ○ No

If Yes, please justify.
The donor will not be identifiable to individuals outside of the research team. However, members of the research team will be able to identify the donor to relate blood samples to other physiological measurements and data from questionnaires.

9. What types of test or analysis will be carried out on the samples?

Measurements of cardiometabolic risk markers including:
1) cholesterol sub-fractions
2) triglycerides
3) fasting glucose
4) HOMA-IR
5) pro-inflammatory markers (e.g. IL-1β, IL-6, TNFα, C-reactive protein)
6) adipokines and myokines

10. Will the research involve the analysis or use of human DNA in the samples?

◉ Yes ○ No

11. Is it possible that the research could produce findings of clinical significance for donors or their relatives?

◉ Yes ○ No

12. If so, will arrangements be made to notify the individuals concerned?

◉ Yes ○ No ○ Not applicable

If No, please justify. If Yes, say what arrangements will be made and give details of the support or counselling service.
Feedback of any significant findings will be conveyed to the participant via a member of their care team along with any recommendations for changes in treatment, should they be necessary.
Should participants have indicated that they do not wish to receive feedback of clinical significance, the decision regarding any further action will remain with members of their care team.

13. Give details of where the samples will be stored, who will have access and the custodial arrangements.

The samples will be stored anonymously in freezers in the Life Science Building at the Byrom Street campus of Liverpool John Moores University (L3 3AF).
The University holds a HTA license.
Access to the samples will be limited to the research team only.

14. What will happen to the samples at the end of the research? *Please tick all that apply and give further details.*

☐ Transfer to research tissue bank

(If the bank is in England, Wales or Northern Ireland the institution will require a licence from the Human Tissue

Authority to store relevant material for possible further research.)

☐ Storage by research team pending ethical approval for use in another project

(Unless the researcher's institution holds a storage licence from the Human Tissue Authority, or the tissue is stored in Scotland, or it is not relevant material, a further application for ethical review should be submitted before the end of this project.)

☐ Storage by research team as part of a new research tissue bank

(The institution will require a licence from the Human Tissue Authority if the bank will be storing relevant material in England, Wales or Northern Ireland. A separate application for ethical review of the tissue bank may also be submitted.)

☐ Storage by research team of biological material which is not "relevant material" for the purposes of the Human Tissue Act

☑ Disposal in accordance with the Human Tissue Authority's Code of Practice

☐ Other

☐ Not yet known

Please give further details of the proposed arrangements:

We fully expect all samples to be used during analysis. If there are any samples left over then they will be disposed.

PART C: Overview of research sites

Please enter details of the host organisations (Local Authority, NHS or other) in the UK that will be responsible for the research sites. *For further information please refer to guidance.*

Investigator identifier	Research site		Investigator Name	
IN1	○ NHS/HSC Site ● Non-NHS/HSC Site		Forename	Fatima
			Middle name	
			Family name	Perez de Heredia
	Institution name	Liverpool John Moores University	Email	f.perezdeherediabenedicte@ljmu.ac.uk
	Department name	School of Natural Science and Psychology	Qualification (MD...)	PhD, FHEA
	Street address	Byrom Street	Country	UNITED KINGDOM
	Town/city	Liverpool		
	Post Code	L3 3AF		
	Country	UNITED KINGDOM		
IN2	● NHS/HSC Site ○ Non-NHS/HSC Site		Forename	Joseph
			Middle name	David
			Family name	Mills
			Email	Joseph.Mills@lhch.nhs.uk
	Organisation name	LIVERPOOL HEART AND CHEST HOSPITAL NHS FOUNDATION TRUST	Qualification (MD...)	MD
	Address	THOMAS DRIVE	Country	UNITED KINGDOM
		LIVERPOOL MERSEYSIDE		
	Post Code	L14 3PE		
	Country	ENGLAND		
IN3	● NHS/HSC Site ○ Non-NHS/HSC Site		Forename	Joseph
			Middle name	David
			Family name	Mills
			Email	Joseph.Mills@lhch.nhs.uk
	Organisation name	COMMUNITY CVD SERVICE	Qualification (MD...)	MD
	Address	THOMAS DRIVE	Country	UNITED KINGDOM
		LIVERPOOL		
	Post Code	L14 3PE		
	Country	ENGLAND		

Research Protocol for IRAS application - LJMU sponsored research

FULL/LONG TITLE OF THE STUDY:
A high-PRotein Mediterranean diet and resistance Exercise for cardiac rehabilitation: a pilot randomised controlled trial

SHORT STUDY TITLE/ACRONYM

The PRiME Study

RESEARCH REFERENCE NUMBERS

IRAS Number	256927
Sponsor reference number	
ClinicalTrials.gov number	
REC reference number	

This protocol has regard for the HRA guidance

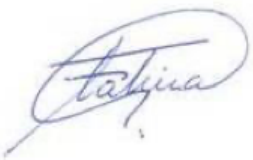
SIGNATURE PAGE

The undersigned confirm that the following protocol has been agreed and accepted and that the Chief Investigator agrees to adhere to the signed LJMU's Sponsorship CI declaration.

I agree to ensure that the confidential information contained in this document will not be used for any other purpose other than the evaluation or conduct of the investigation without the prior written consent of the Sponsor

I also confirm that I will make the findings of the study publically available through publication or other dissemination tools without any unnecessary delay and that an honest accurate and transparent account of the study will be given; and that any discrepancies from the study as planned in this protocol will be explained.

Chief Investigator:



Signature: Date: 30/09/2019

Name: (please print): Fatima Perez de Heredia

SPONSOR STATEMENT:

Where LJMU takes on the sponsor role for protocol development oversight, the signing of the IRAS form by the sponsor will serve as confirmation of approval of this protocol.

CONFIDENTIALITY STATEMENT

This document contains confidential information that must not be disclosed to anyone other than the Sponsor, the Investigator Team, HRA, host organisation, and members of the Research Ethics Committee, unless authorised to do so.

TABLE OF CONTENTS

KEY STUDY CONTACTS

Chief Investigator:	Dr Fatima Perez de Heredia Benedicte, 01512312003 F.PerezDeHerediaBenedicte@ljmu.ac.uk
Investigators:	Richard Kirwan, 07565427663, r.p.kirwan@2018.ljmu.ac.uk Dr Ian Davies, 0151 231 5290, I.G.Davies@ljmu.ac.uk Dr Tom Butler, 01244 511660, t.butler@chester.ac.uk (University of Chester) Prof Gregory Lip, gregory.lip@liverpool.ac.uk (University of Liverpool) Prof Dick Thijssen, 0151 904 6246, D.Thijssen@ljmu.ac.uk (Liverpool John Moores University) Dr Joseph Mills, 0151 600 1991, joseph.mills@lhch.nhs.uk (Medical Director for Advanced Life Support, Liverpool Heart & Chest Hospital NHS Trust)
Sponsor:	Liverpool John Moores University
Funder:	Liverpool Clinical Commissioning Group Research Capability Funding has been approved. 0151 296 7000, enquiries@liverpoolccg.nhs.uk
Chief Investigator Signature:	

There are no potential conflicts of interest.

TRAINING / CPD

Name of investigator (add new row if required)	Date completed LJMU REC training	Date completed LJMU research integrity training	Date completed research governance training	Date completed HTA training (or NA)	Date completed other training (add new column if required)
Dr Fatima Perez de Heredia				15/11/2017, to be refreshed before starting sample collection	
Dr Ian Davies					
Dr Tom Butler					
Prof Gregory Lip					
Prof Dick Thijssen					
Dr Joseph Mills					
Richard Kirwan	08/10/2018			01/11/2018, to be refreshed before starting sample collection	

STUDY SUMMARY

Study Title	A high-PRotein Mediterranean diet and resistance Exercise for cardiac rehabilitation: a pilot randomised controlled trial	
Internal ref. no. / short title	PRiME	
Proposed start date	January 2020	
Proposed end date	January 2021	
Countries in which the study will take place	England	
Lead NHS trust & R&D contact	Liverpool Heart & Chest Hospital, Research & Innovation Team (Michael Noorzadeh), 01516001158, michael.noorzadeh@lhch.nhs.uk	
Study Design	2x2 Factorial Randomized Controlled Diet & Exercise Intervention	
Study Participants	Participants in phase-3 & 4 cardiac rehabilitation	
Planned Sample Size	Cross-sectional analysis n=240 Intervention n = 60 (4 groups of 10-15 individuals)	
Planned Study Period	12 months (3 months for each patient but 7 months allowed for staggered recruitment)	
	Objectives	**Outcome Measures**
Primary	Determination of the feasibility of the intervention and its applicability in a fully powered RCT	Standard deviation of the key secondary outcome measures, willingness of participants to be randomised, number of eligible participants within the CR population, follow-up rates, response rates to questionnaires, acceptability of nutritional and exercise protocols, adherence/compliance rates, time and finances needed to implement the intervention..
Secondary	Reduction in the sarcopenic obesity index Improvement in cardiometabolic risk markers	Body composition measured by DXA and anthropometry. Changes in cardiometabolic risk markers (e.g. lipid profile, fasting glucose, HbA1c).

FUNDING AND SUPPORT IN KIND

Please provide details of how the study is being funded, both internally and externally.

FUNDER(S)	**FINANCIAL AND NON FINANCIALSUPPORT GIVEN**
LCCG RCF	£ 27,166

ROLE OF STUDY SPONSOR AND FUNDER

The research will be carried out by researchers from LJMU (the study sponsor), in collaboration with Liverpool Heart and Chest Hospital (both institutional members of the Liverpool Centre for Cardiovascular Science). Funding comes from LJMU and the LCCG RCF. The researchers are not receiving any payments other than their usual salaries.

Any support in the form of food products to be used in the intervention will be obtained under the explicit agreement that the supplying organization will have absolutely no role in study design, conduct, data analysis and interpretation or manuscript writing. The final decision regarding any of these aspects of the study will remain with the research team.

ROLES AND RESPONSIBILITIES OF STUDY MANAGEMENT COMMITTEES/GROUPS AND INDIVIDUALS

Trial Oversight Committee (TOC): it will consist of the academic investigators, Liverpool Heart and Chest clinical leads, and Community centre leads. The TOC will supervise the study in its entirety.

Trial Steering Group (TSG): it will consist of patients engaging with cardiac rehabilitation (CR) (phase 3, patient group), the principal investigator, co-investigators, and staff employed on the trial. The TSG will be responsible for reviewing the protocol design and ethical considerations, such as participant information, and valuable feedback on patient 'friendliness' and acceptability will be documented and fed back to the TOC. Any changes made will be reported and documented.

Liverpool Heart & Chest Hospital, Service Users Research Endeavour (SURE) group: This is a Patient & Public Involvement Group whose membership comprises former or existing patients, carers or members of the public. The SURE group works alongside LHCH Trust's Research Committee to appraise, monitor and complement research projects from a service user's point of view, from the quality and clarity of the documentation to the feasibility of a patients' involvement in a study.

Liverpool Heart & Chest Hospital, Research & Innovation Team: to improve the quality, relevance, and focus of research and to ensure all the study follows the guidelines set for research by the Department of Health.

PROTOCOL CONTRIBUTORS

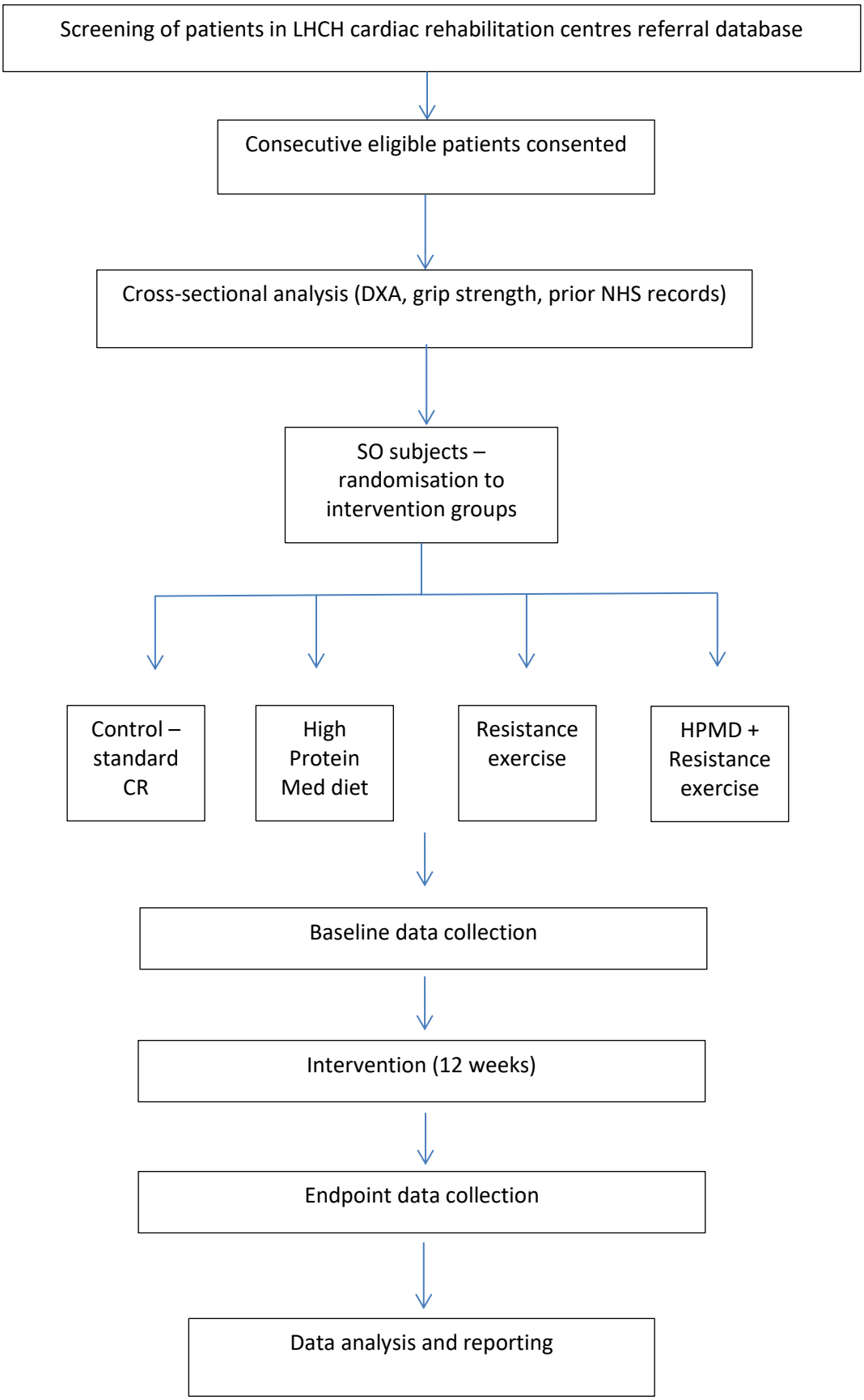
The study proposal was reviewed and approved by LJMU's Research Committee.

Details of the study were finalised by collaboration of the supervisory team, and the specific methodology was further developed with the participation of the PhD student. The research methods to be used are validated and established techniques commonly used in clinical research.

The research team have already met with the Service Users Research Endeavour (SURE) group of the Liverpool Heart and Chest Hospital and with the Research and Innovation Committee of the LHCH. Feedback was received during both meetings, and the study was approved by the SURE Group and the R&I Committee.

We will continue the work with specific focus groups of patients in CR. The PPI groups will participate and feed back on the different aspects of the research.

STUDY FLOW CHART



ABBREVIATIONS

CI	Chief Investigator
GP	General Practitioner
HRA	Health Research Authority
ICF	Informed Consent Form
LJMU REG	LJMU Research Ethics and Governance
LHCH	Liverpool Heart & Chest Hospital
NHS	National Health Service
NRES	National Research Ethics Service
PIS	Participant/ Patient Information Sheet
R&D	NHS Trust R&D Department
REC	Research Ethics Committee
SOP	Standard Operating Procedure
CVD	Cardiovascular disease
CHD	Coronary Heart Disease
CR	Cardiac Rehabilitation
CM	Cardiometabolic
SO	Sarcopenic Obesity
MPS	Muscle Protein Synthesis
LBM	Lean Body Mass
DXA	Dual-energy X-ray absorptiometry
HPMD	High Protein Mediterranean DIet
BACPR	British Association for Cardiac Prevention & Rehabilitation

1 BACKGROUND

Cardiovascular disease (CVD) in the UK is behind 1 in 4 deaths/year (>150,000 people), with coronary heart disease (CHD) being the most common type [1]. In addition to the loss of life, the economic cost of CVD is considerable; in 2015 healthcare for CVD alone in the UK amounted to £10.9 billion with a further £7.7 billion lost from the economy due to productivity loses [2].

Mortality figures due to CHD have been reduced recently, in part due to increased provision of cardiac rehabilitation (CR), a therapeutic approach based on exercise training to improve cardiac function, plus advice regarding smoking cessation, dietary changes and weight loss [3].

2 RATIONALE

Most of the evidence on the benefit of CR examines the links between exercise and CV morbidity and mortality [4]; however, there is considerable evidence showing improvements in markers of cardiovascular risk through different dietary strategies [5]. Of particular relevance, results from studies on both the primary

and secondary prevention of CVD suggest Mediterranean diet-based approaches are the most adequate to treat these patients [6-13].

Obesity, and in particular, visceral adiposity, is associated with cardiometabolic (CM) risk markers (e.g., high cholesterol and triglycerides levels, HbA1c, etc.) [14-16], however, an "obesity paradox" appears to exist in the cardiac population, where increased mortality has been linked to low body mass index (BMI) [17, 18]. However, low lean body mass (LBM) is the likely driver of this phenomenon due to sarcopenia, a progressive loss of LBM associated with aging [19]. Patients with a combination of low LBM and abdominal distribution of body fat, known as sarcopenic obesity (SO), are at greater risk of CVD, exacerbated in CR [20-23]. Thus, increasing relative LBM content, rather than simply promoting weight loss, may be an appropriate target in CR patients.

One particular barrier to maintaining or accruing LBM is the presence of anabolic resistance in older adults, which can result in a reduced muscle protein synthetic (MPS) response to both exercise and the ingestion of currently recommended intakes of protein [24]. Protein intakes above currently recommended levels (1.0-1.5 g/kg/BW) [25] combined with sufficiently intense resistance training [26] can overcome this anabolic resistance and positively influence muscle mass, ultimately leading to greater improvement in body composition, when accompanied by a reduction in total body fat mass [27].

3 RESEARCH QUESTION

We aim to determine the prevalence of sarcopenic obesity in a CR population and how this body composition relates to markers of cardiometabolic health. As there is no consensus definition of SO, multiple definitions, along with their relationship with CM risk markers, will be investigated.

We aim to investigate to what extent a high-protein Mediterranean-style diet and resistance exercise, alone and in combination, can augment LBM. Furthermore, we will ascertain whether the above interventions improve markers of cardiometabolic health.

Therefore, we will conduct a feasibility study with embedded pilot to obtain preliminary data on the practical and clinical considerations and cost-effectiveness of the proposed interventions, in preparation for an appropriately powered randomised controlled trial for increasing lean mass and improving cardiometabolic risk markers in patients with SO.

4 OBJECTIVES AND OUTCOME MEASURES

Quantitative research

Objectives	**Outcome Measures**	**Timepoint(s) of evaluation of this outcome measure (if applicable)**
Primary Objective To investigate to what extent a high-protein Mediterranean-style diet and resistance exercise, alone and in combination, can augment LBM and muscle strength	Increases in LBM will be measured using DXA and anthropometry. Increases in strength will be measured with grip strength dynamometer	To be performed within 1 week prior to and within 1 week after completion of the 12 week intervention
Secondary Objectives To ascertain whether the above intervention improves markers of cardiometabolic health.	Cardiometabolic risk markers (blood glucose, lipid profile etc) will be measured using blood samples along with measurements of blood pressure	Blood pressure will be measured in the cross-sectional stage, and before

		and after the intervention Blood will be drawn 1 week prior to and 1 week after completion of the 12 week intervention

Qualitative research

Aim/Research Questions	**Objectives**
Primary To determine the feasibility, practical and clinical considerations and cost-effectiveness of the proposed interventions, in preparation for an appropriately powered randomised controlled trial for increasing lean mass and improving cardiometabolic risk markers in patients with SO.	Participants will be asked to provide feedback on: - recruitment, - tasting of high-protein foods, - recipes, appeal of the meals, how they fit with the Merseyside culture, - involvement and support of other members of the family (especially those in charge of purchasing and cooking food), - familiarity session(s) with resistance training, exploring ability to perform exercises and personalising the training system, - preferences to undertake resistance exercise (e.g. in a supervised or unsupervised setting), - preferred follow-up frequency and method (e.g. face to face, phone, etc.), - issues with dietary and exercise adherence, Participants will also: - help shape the protocol and ensure the project is participant-friendly and sensitive by assisting with ethical considerations, - discuss research findings and help with lay dissemination.

5 STUDY DESIGN AND METHODS OF DATA COLLECTION AND DATA ANALYSIS

A single-centre, cross-sectional analysis of the prevalence of SO in CR patients followed by a single-centre, 2x2 factorial, randomised, open label controlled trial.

5.1 RANDOMISATION AND BLINDING

All participants will be assigned to their group by computer randomisation by the PhD student. Due to the nature of the intervention, blinding will not be possible.

5.2 BASELINE ASSESSMENTS

STAGE 1: Screening and focus group
To be carried out at LJMU Byrom St campus (L3 3AF) by the PhD student. The visit is expected to last 30 minutes and will entail:
i) Assessing body composition. This will be done in three different ways.

- Firstly, we will measure participant height using a stadiometer and waist circumference using a tape measure.
- Secondly, we will use a bioelectrical impedance analysis scale to measure lean body mass, total body fat mass and visceral fat mass.
- Thirdly, we will perform a DXA (Dual-energy X-ray Absorptiometry) scan for 15-20 minutes which allows us to create an image of the distribution of lean body mass and fat mass.

ii) Taking blood pressure

- Following standard protocol, participants will be asked to sit for 5 minutes before we take blood pressure; we will measure it three times to ensure an accurate reading.

iii) Grip strength test

- Will be tested using a device that one squeezes as hard as possible (grip strength dynamometer). We will do this three times to ensure an accurate reading.

(iv) Mediterranean Diet Score

- Participants will fill in a brief 13-question questionnaire regarding the frequency of their intake of certain foods

Participants will also be invited to attend a focus group to share views and opinions about food habits and preferences, and about physical activity. This will be carried out at participants community CR centre and will take 15-20 minutes

5.3 INTERVENTIONS (IF APPLICABLE)

Based on the results from the first visit, approximately 60 patients with lower proportions of muscle mass to fat mass will be asked to take part in the diet and exercise intervention, which will last 12 weeks. Participants at this stage will be allocated to one of four groups: 1) standard CR; 2) CR plus personalised advice to follow a high-protein, healthier diet; 3) CR plus resistance exercise; 4) CR plus diet and resistance exercise.

Personalised dietary advice: if allocated to groups 2 or 4, we will ask participants to make changes to their diet to adapt it to a high-protein, Mediterranean-style diet. Research shows that Mediterranean-style diets can reduce cardiovascular risk, and although there are different versions of this type of diet, they all have in common:

- eating more fruit and vegetables,
- reducing commercial pastries, and replacing refined carbohydrate foods (white bread, white rice, white pasta) by wholegrains (wholegrain bread, rice and pasta),
- replacing butter and margarine by olive oil as the main culinary fat,
- reducing fatty meat and replacing by lean meat, fish, and legumes (peas, beans, lentils), and by high-protein, low fat foods, such as low-fat dairy (participants will be provided with 2 high-protein yoghurts to eat each day).

Participants will receive personalised guidance to help follow the new diet in the form of sessions at their community CR centre along with guide books and recipe guides. The goal is to make small, easy changes to their current eating habits, so the diet will be easy to follow. All foods included will be affordable and easy to find in local supermarkets (shopping guides will be provided), and we will provide suggestions and recipes to prepare food.
If allocated to groups 1 or 3, participants will be asked to follow the diet recommendations given during phase 3 of CR.

Resistance exercise: if allocated to groups 3 or 4, participants will be asked to perform resistance exercise. This involves weights or weight machines aimed at building muscle strength. Participants will be shown how to do the exercises by qualified instructors (BACPR cardiac rehabilitation certified) in the community centre where they carry out their current phase 3 cardiac rehabilitation. All exercises have been deemed safe for cardiac rehabilitation patients and an instructor will be available at all exercise sessions should they need assistance. Participants will be required to attend 3 sessions per week and each session is expected to last approximately 45 minutes.

If allocated to groups 1 or 2, participants will be asked to continue with the standard, aerobic-style exercise (treadmills, rowing machines, elliptical trainers) they have used in phase 3 of CR. This will also require 3 sessions per week.

5.4 SUBSEQUENT VISITS

ALL stage 2 participants will have to attend two more appointments at LJMU. The second visit will be just before beginning the intervention, and the third immediately after completing the intervention. Visits are expected to last 45-60 minutes and will entail:
i) Measures of body composition, blood pressure and grip strength, as described above.

ii) A venous blood sample

- We will take about 8 teaspoons of blood. Particpants will need to fast for at least 12 hours prior to your appointment (although water is encouraged), as otherwise this may affect the result of the test.

iii) Interviews

- At the final visit we will also conduct a brief interview to ask about your experiences with the diet and/or exercise regime allocated to you, and to check whether you have experienced any so-called adverse events over the study.

iv) Food diaries (prior to lab appointments)

- Prior to second and third lab visits, participants will given a template of a four-day food diary, which they will need to complete the week before their lab appointment (on three working days plus one weekend or festive day, non-consecutive whenever possible), and bring with them on the morning of the visit. Participants will be contacted the day before to remind them of their appointment and what they must bring.

(v) Mediterranean Diet Score

- Participants will fill in a brief 13-question questionnaire regarding the frequency of their intake of certain foods

5.5 STUDY SETTING

This is a single centre study with all data collection taking place in LJMU, Byrom St. campus. All exercise will be carried out at the participants community CR centre, supervised by trained members of the community CR program.

5.6 STATISTICS AND ANALYSIS

Cross-sectional study: from the results of body composition analysis, the prevalence of SO will be analysed in the sample. Differences in prevalence between genders, and between cardiac conditions (e.g. arrhythmia, myocardial infarction, coronary artery disease etc.) will be assessed by Chi-squared tests. We will use Pearson or Spearman correlations (according to normality) to study the associations between body composition variables, markers of strength and performance, and biomarkers of cardiovascular risk (based on patient data requested from LHCH) and multiple regression to assess the predictive capacity of indicators of sarcopenic obesity and muscle strength on cardiovascular risk.

Pilot study: comparisons between intervention groups for all outcome measures will be performed by means of mixed model ANOVA, to account for inter-subject (differences between treatments) and intra-subject (differences between baseline and endpoint) variability. Since a sample size calculation will not be conducted for this component, interpretation of the results will be largely descriptive and focused on confidence limits around parameter estimates.
Statistical significance will be set at $p<0.05$, and all analyses will be conducted using IBM SPSS Statistics v25 (SPSS Inc., Chicago, IL).

6 PARTICIPANT RECRUITMENT

6.1 STUDY PARTICIPANTS

Participants will be recruited from Liverpool Heart & Chest Hospital Cardiac Rehabilitation unit. Participants in the cross-sectional study will be admissions to phase 3 CR. Participants in the intervention trial will have recently completed phase 3 CR, will have been deemed as cardiac stable, and will be willing to participate in phase 4 CR (Activity for Life). There will be no age restriction for participants.

For the cross sectional study, sample size has been estimated as n=240, based on a prevalence of SO of 20% with precision at 5% and confidence at 95%, and using the online calculator at http://sampsize.sourceforge.net/iface/.

The sample size for the feasibility study has been arbitrarily kept small (10-15 participants per intervention group) to allow ease of recruitment and management of participants before development into a fully powered randomised controlled trial.

6.2 INCLUSION CRITERIA

Phase 1:

- Informed consent given
- Referral to cardiac rehabilitation program
- Ability to attend screening at Liverpool John Moores University

Phase 2:

- Informed consent given
- Meeting selected criteria to define sarcopenic obesity (dependent on analysis from phase 1)
- Ability to attend screening at Liverpool John Moores University
- Cardiac function deemed stable after phase 3 cardiac rehabilitation

6.3 EXCLUSION CRITERIA

- Inability to perform resistance exercise (determined by primary care team)
- Renal dysfunction
- Inability/unwillingness to digest/consume dairy products
- Admission to CR due to congenital or drug/alcohol-abuse induced cardiac events
- Pregnancy

6.4 RECRUITMENT TECHNIQUE

Participants will be approached by clinicians at LHCH, who will invite them to participate and refer them to our research team for more information. Please refer to 6.5 for further details.

6.5 PARTICIPANT IDENTIFICATION

Eligible participants will be recruited from patients recently referred to cardiac rehabilitation (Liverpool Heart & Chest Hospital Cardiac Rehabilitation Unit), which is where we will start our study. In collaboration with the LHCH Cardiac Rehabilitation Unit, our research team will have access to clinical records in order to contact patients through members of the CR team. Eligible participants will first be identified by the lead clinician in the study.

Eligible participants will be approached by members of the Knowsley Community Cardiovascular Services team, part of Liverpool Heart & Chest Hospital Cardiac Rehabilitation unit. Participants will be informed of the research and provided with a participation information sheet and asked to reply with their interest after 24 hours. They will then be contacted by the PhD student.

6.6 SCREENING AND ELIGIBILITY ASSESSMENT

The maximum period from screening in phase 1 to the recruitment for the intervention (phase 2) will depend on the participants stage of CR phase 3. As phase 3 normally last 8 weeks, and assuming a participant has just started CR phase 3 there may be up to 8 weeks from the first set of screening visit to second visit (baseline) measurements.

6.7 INFORMED CONSENT

The participant must personally sign and date the latest approved version of the Informed Consent form before any study specific procedures are performed.
Written and verbal versions of the Participant Information and Informed Consent will be presented to the participants detailing no less than: the exact nature of the study; what it will involve for the participant; the implications and constraints of the protocol; the known side effects and any risks involved in taking part. It will be clearly stated that the participant is free to withdraw from the study at any time for any reason without prejudice to future care, without affecting their legal rights, and with no obligation to give the reason for withdrawal.
The participant will be allowed as much time as wished to consider the information, and the opportunity to question the Investigator, their GP or other independent parties to decide whether they will participate in the study. Written Informed Consent will then be obtained by means of participant dated signature and dated signature of the person who presented and obtained the Informed Consent. The person who obtained the consent must be suitably qualified and experienced, and have been authorised to do so by the Chief/Principal Investigator. A copy of the signed Informed Consent will be given to the participant. The original signed form will be retained at the study site.

6.8 DISCONTINUATION/WITHDRAWAL OF PARTICIPANTS FROM STUDY

Each participant has the right to withdraw from the study at any time. In addition, the Investigator may discontinue a participant from the study at any time if the Investigator considers it necessary for any reason including:

- Pregnancy
- Ineligibility (either arising during the study or retrospectively having been overlooked at screening)
- Significant protocol deviation
- Significant non-compliance with treatment regimen or study requirements
- Withdrawal of Consent
- Loss to follow up

Should a participant wish to withdraw from the study, their anonymised data will be retained for 20 years. Their data may still be included in the analysis if they withdraw after completing the intervention, and for reasons that do not affect the study outcomes. If the participant wants their data to be removed from the study, they may contact us and request such.

Withdrawn participants may be replaced should sufficient time and resources be available.

The reason for withdrawal by researcher (and by participant, if this information is volunteered) will be recorded in a study file.

7 DATA MANAGEMENT

7.1 ACCESS TO DATA

Direct access will be granted to the research team, authorised representatives from the Sponsor and host institution for monitoring and/or audit of the study to ensure compliance with regulations.

7.2 DATA RECORDING AND RECORD KEEPING

The information provided as part of the study will be anonymised. We will use codes to label, trace and identify your samples, records and questionnaires; we will never use any personal data from which participants can be identified, such as name, initials, or date of birth.
Documents that include personal data (such as the consent form, or your contact details) will be stored on password-protected computers and locked filing cabinets at LJMU, accessed only by the research team. They will be destroyed upon completion of the study, unless they would like a copy of the final study results; in that case, we will retain their names, phone numbers and email addresses, so we can contact them later. The results should be available approximately 1 year after the study is completed; participant personal data will be then deleted.
Research data will be stored in databases using the codes as identifiers, never personal data, and they will be kept in password-protected computers only accessed by the research team. Samples will be booked into a database and receive a laboratory code, with only those involved in the research having access. All data and samples will be destroyed after 20 years.
The names of participants will never be published in any communication of results and findings.

7.3 SAMPLE HANDLING

Blood samples will be processed and stored according to current UK regulations and rules of good research practice (Human Tissue Act 2004), and will be kept for a maximum of 20 years. Briefly, blood samples will be processed immediately after collection, and the serum will be stored in a secure freezer in the Life Sciences Building at LJMU's Byrom St campus. All samples will be stored pseudo-anonymously; this means that all identifiable information will be removed and replaced by a code to allow the research team to trace the samples and match them with the other measurements (body weight, muscle mass measures, etc.). Once all participants have completed the intervention, samples will be analysed. Once the specified storage period ends, all samples will be disposed of following current UK regulations.

8 SAFETY REPORTING

Adverse Events, Adverse Reactions and Serious Adverse Events will be recorded in the participants documentation as they are brought to the attention of the researchers and if necessary will be reported to the appropriate authority (LJMU REC, LHCH, participants GP etc.) in accordance with LJMU procedures.

9 QUALITY ASSURANCE PROCEDURES

The study may be monitored, or audited in accordance with the current approved protocol, GCP, relevant regulations and standard operating procedures OF LJMU.

10 ETHICAL AND REGULATORY CONSIDERATIONS

10.1 DECLARATION OF HELSINKI

The Investigator will ensure that this study is conducted in accordance with the principles of the Declaration of Helsinki and relevant regulations

10.2 ASSESSMENT AND MANAGEMENT OF RISK

Participants will be scanned by a DXA scanner, which will involve use of small doses of ionising radiation, though this is a minimal amount compared to other medical imaging techniques, such as standard X-ray and CT scans. The DXA scan would be equal to approximately 2 microsieverts (µSv); this is compared to 20 microsieverts for a chest X-ray and 10,000 microsieverts for a CT scan. It is also less than a transcontinental flight (40 µSv) and one day of natural background radiation (8 µSv).

Great care will be taken to ensure vulnerable people (for example, pregnant women) will not be permitted to take part, both during the initial screening and recruitment, and also during the scans where mandatory exclusion criteria are checked before the scan can begin.
The study will limit the involvement of each participant to three scans, and they will be fully informed as to the exposure they will have, should they choose to participate. The radiation exposure is so low that no shielding of the room or of the person conducting the scans is necessary; therefore, the anticipated risk to the participant is negligible.

The participants are being asked to be involved in a study which has a central focus on body weight and composition, which can be a very sensitive subject. As the participants will be willing volunteers for the study, it is expected that only people who are comfortable with this issue will be involved. Additionally, the participants can request for their bio-impedance and anthropometric measurements to be taken by a member of the research team of the same gender. They can also request a chaperone to be present during the procedure. Privacy and confidentially will be ensured when taking measurements, and all data will be anonymised upon collection and storage.

In addition, participants will be reminded that they can withdraw and remove themselves from the situation (and the study) at any time.

For the DXA scans, questionnaires and anthropometric measurements, it is not anticipated that there will be significant inconvenience for those who choose to take part in this project, as their time involvement is minimal at 45 minutes per visit, and we will do our best to accommodate their visit times to their preferences and availability (within the limits of the research requirements).

Participants will be made aware of the requirements of the dietary intervention while seeking consent, and it is expected that those who are comfortable with this dietary change will be involved. The dietary intervention will not use difficult to source ingredients and will be tailored to suit the tastes of the participants. Various recipes and food substitutions will be provided to participants at the beginning of the protocol. The high-protein diet of the protocol would only be potentially detrimental to individuals who have renal deficiency, and therefore this is one of the studies exclusion criteria. Participants will also receive regular contact from the PhD student regarding their adherence, and will also use a smart-phone based app daily to improve adherence.

Participants will be made aware of the requirements of the exercise intervention while seeking consent, and it is expected that those who are comfortable with this change in physical activity will be involved. All participants will have received exercise tolerance tests as part of their NHS cardiac rehabilitation and will be cleared for the exercise protocol. They will also receive instruction from a collaborator suitably trained in exercise for cardiac rehabilitation regarding how to safely complete the exercise protocol.

The involvement of the participants and their data will be kept confidential, with no personal identifying data being present on questionnaires, forms or DXA scans. The unique code that can identify a participant will be kept on a secure system that can only be accessed by the research team. Therefore it is not expected that there will be a significant risk of a confidentiality breach.

Should the participant disclose information indicating a risk of potential harm to themselves or others, the appropriate authority will be contacted (LHCH, patient's GP, police etc,).

10.3 PARTICIPANT CONFIDENTIALITY

The data custodian will be the PhD student. All investigators and study site staff must comply with the requirements of data protection legislation with regards to the collection, storage, processing and disclosure of personal information and will uphold the Act's core principles.

Information provided as part of the study will be anonymised. We will use codes to label, trace and identify samples, records and questionnaires; we will never use any personal data from which participants can be identified, such as name, initials, or date of birth.

Documents that include personal data (such as the consent form, or contact details) will be stored on password-protected computers and locked filing cabinets at LJMU, accessed only by the research team. They will be destroyed upon completion of the study, unless participants would like a copy of the final study results; in that case, we will retain their name, phone number and email address, so we can contact them later. Their personal data will then be deleted

Research data will be stored in databases using the codes as identifiers, never personal data, and they will be kept in password-protected computers only accessed by the research team. Samples will be booked into a database and receive a laboratory code, with only those involved in the research having access. All data and samples will be destroyed after 20 years.

The names of participants will never be published in any communication of results and findings.

10.4 EXPENSES AND BENEFITS

Reasonable travel expenses for any visits additional to normal care will be reimbursed, should participants so desire and on production of receipts, or a mileage allowance provided as appropriate.

The diet and exercise chosen in this study have been shown to have a number of benefits:

- High-protein foods may increase satiety after a meal, reducing hunger.
- This may also lead to a lower calorie intake and consequential potential weight loss.
- This may lead to a loss of fat mass, improving body composition.
- Muscles may grow and become stronger, meaning everyday activities will feel easier.
- Blood sugar levels may improve due to healthier muscles helping with blood sugar control.
- Blood cholesterol and fats may improve, reducing risk of further cardiac events.

10.5 OTHER ETHICAL CONSIDERATIONS

In the unlikely event of finding any abnormalities or anything of clinical significance, the findings will be checked by a clinical specialist. If the specialist feels that the abnormality was medically important, they will discuss the implications with the participant and arrange for further investigations as necessary. Participants will not be informed unless the doctor considers the finding has clear implications for their current or future health. It is important to note that data collected are not carried out for diagnostic purposes, and therefore the data are not a substitute for a clinical appointment. Rather, the data are intended for research purposes only.

10.6 RESEARCH ETHICS COMMITTEE (REC) AND OTHER REGULATORY REVIEW & REPORTS

- Before the start of the study, a favourable opinion will be sought from from the UK Health Departments Research Ethics Service NHS REC for the study protocol, informed consent forms and other relevant documents e.g. advertisements
- Approval will be obtained from LJMU REG (and Co-Sponsors) for any amendments to, or changes of status in the study **prior to** submission to the REC that ethically approved the study and any other regulatory authorities
- All correspondence will be retained.
- The protocol, informed consent form, participant information sheet and any proposed advertising material will be submitted to the HRA for written approval.
- Annual Progress Reports will be submitted to the NHS REC which gave the favourable opinion, the HRA (hra.approval@nhs.net) and the Sponsor (Sponsor@ljmu.ac.uk) on the anniversary of NHS REC Favourable Opinion, and annually thereafter until the End of Study Declaration has been submitted to the NHS REC which gave the favourable opinion, the HRA and the Sponsor
- Upon the completion of the study an End of Study Declaration (within 90 days of the end of the study) and End of Study Report (within 12 months of the end of the study) will be submitted to the NHS REC which gave the favourable opinion and LJMU REG (sponsor@ljmu.ac.uk)

- Annual Progress Reports will be submitted to the NHS REC which gave the favourable opinion and the Sponsor (Sponsor@ljmu.ac.uk) on the anniversary of HRA approval, and annually thereafter until the End of Study Declaration has been submitted to the NHS REC which gave the favourable opinion and the Sponsor.
- Upon the completion of the study an End of Study Declaration (within 90 days of the end of the study) and End of Study Report (within 12 months of the end of the study) will be submitted to the NHS REC which gave the favourable opinion and LJMU REG (sponsor@ljmu.ac.uk)
- Early termination or suspension of the research will be reported to all relevant review bodies and the Sponsor (sponsor@ljmu.ac.uk) within 15 days.

11 SCIENTIFIC REVIEW

The study protocol went through successive stages of scientific review:

1) It was first reviewed and approved for support under the cross-faculty PhD funding scheme. The panel reviewing the proposals was formed by staff from the Faculty of Science, Faculty of Education, Health and Community, and the Institute of Health Research, LJMU.
2) The study was subsequently reviewed and approved by the Service Users Research Endeavour (SURE) Group of the Liverpool Heart and Chest Hospital.
3) Finally, our study protocol was presented at and approved by the Research and Innovation Committee of the Liverpool Heart and Chest Hospital.

All different stages of review were carried out by independent reviewers, both internal (from LJMU) and external (LHCH), meeting the requirements of Level 4 studies.

Level 1 No Peer Review Required – minimal risk (no patient contact)	**Level 2 Review by project supervisor** (student projects with either no or minor patient/participant involvement)	**Level 3 Review by departmental colleague** (Low-risk projects with minimal patient involvement)	**Level 4 External, independent peer review**
Short questionnaire studies for use among hospital staff or GPs.	Human tissue samples [anonymous to investigator]	Human tissue samples [anonymous to investigator]	Clinical trial of an investigational medicinal product

Questionnaires asking patients about the quality of hospital services.	Study administering questionnaires	Study administering questionnaires	Clinical trial of a medical device
Use of data from medical notes by clinician looking after patient.	Qualitative study	Qualitative study	Performance Evaluation of an in vitro diagnostic device
	Study limited to working with data	Study limited to working with data	Other clinical trial or clinical investigation
		Non-intimate examination techniques, e.g. blood pressure measurement.	Research Tissue Bank
			Human tissue (tissue samples and data) [newly obtained, identifiable or obtained from surplus]

12 PATIENT & PUBLIC INVOLVEMENT

The research team initially liaised with Knowsley Community Cardiovascular Service in charge of phase 4 cardiac rehabilitation, to gather information relevant to the current practice and support offered to cardiac rehabilitation patients after leaving the hospital, as well as this population specific characteristics, needs and requirements.

The research team then consulted with the Liverpool Heart & Chest Hospital, Service Users Research Endeavour (SURE) group (see ROLES AND RESPONSIBILITIES OF STUDY MANAGEMENT COMMITTEES/GROUPS AND INDIVIDUALS on page 7.

The SURE group has already provided advice regarding the participant information documents, and approved the study.

We will continue with specific focus groups of patients in CR. The PPI groups will participate and feed back on the different aspects of the research (see qualitative research in OBJECTIVES AND OUTCOME MEASURES)

All PPI activities will be recorded and evaluated, with aims to present at conferences and publish in appropriate peer reviewed journals.

13 PROTOCOL COMPLIANCE

Protocol adherence will be monitored with weekly phone call with each participant, carried out by the PhD student.
Accidental protocol deviations can happen at any time. They will be adequately documented on the relevant forms and reported to the Chief Investigator and Sponsor immediately.
Deviations from the protocol which are found to frequently recur are not acceptable, will require immediate action and could potentially be classified as a serious breach.
The Investigator may discontinue a participant from the study at any time if the Investigator considers it necessary for any reason. Such occurrences will be detailed in the individual participant study logs

14 INSURANCE

Table 11.1

The research study includes the following:	**Please state YES or NO to at least one.**
treating or preventing disease or diagnosing disease	YES
ascertaining the existence degree of or extent of a physiological condition	YES
assisting with or altering in any way the process of conception	NO
investigating or participating in methods of contraception	NO
inducing anaesthesia	NO
otherwise preventing or interfering with the normal operation of a physiological function	NO
None of the above	NO

Table 11.2

The study is limited to the following activities and will be undertaken in the UK.	**Please state YES or NO**
Questionnaires, interviews, psychological activity including CBT	YES
Venepuncture (withdrawal of blood)	YES
Muscle biopsy	YES
Measurements of physiological processes including scanning	YES
Collections of body secretions by non-invasive methods	YES
Intake of foods or nutrients or variation of diet (other than administration of drugs).	YES

LJMU has Clinical Trials insurance to cover the legal liability of the University as Research Sponsor in the eventuality of harm to a research participant arising from management and design of the research by the University and the activities here are included within that coverage.
LJMU's Clinical Trials insurance policies provide an indemnity to our employees and students for their potential liability for harm to participants during the conduct of the research and the activities here are included within that coverage.

15 CONTRACTS AND AGREEMENTS

A standard Non-Commercial Model Agreement (mNCA) will be negotiated with Liverpool Heart & Chest Hospital

A research passport (letter of access) will be applied for, for the PhD student who will undertake the research within the NHS.

16 DEFINITION OF END OF STUDY

The end of study is the date of completion of analysis of samples.

17 END OF STUDY AND ARCHIVING

The end of study is the date of the last visit of the last participant. Relevant consent forms will be obtained for each participant, allowing for the samples to be stored and data to be archived for at least 20 years.

18 ACCESS TO THE FINAL STUDY DATASET

Aim: to describe who will have access to the final dataset
The PhD student, all supervisors and the lead clinician will have access to the full dataset. The study will allow site investigators to access the full dataset if a formal request describing their plans is approved by the

steering group. Any secondary analysis will only be undertaken with the consent of the participants. All patient documentation will reflect the future use of data in research.

19 DISSEMINATION POLICY

- All the data from the study will be own and managed by the research team. Experimental data will be appropriately coded and stored in a database (MS Excel) – all information in this database will be anonymous, and individual data will not be shared, published or disseminated in any way.
- Data will be analysed with appropriate statistical tests (according to the nature and amount of the data within each variable of study). A final report will be produced to inform all parties involved in this study: Liverpool John Moores University, University of Chester, Liverpool Heart and Chest Hospital, Knowsley Community Centre, SURE Patient group, and relevant stakeholders (*e.g.*, funders, NHS). The report will be shared with the partners via email; it will be communicated using social media as well, so that other parties interested can request it.
- Data will be analysed as well for presentation in professional conferences (*e.g.*, European and International Congress on Obesity, European and International Nutrition Conference, British Association for Cardiac Prevention & Rehabilitation Conference etc) and publication in specialised journals (*e.g.*, International Journal of Obesity, American Journal of Clinical Nutrition, European Journal of Clinical Nutrition, Journal of the American College of Cardiology, Circulation, European Heart Journal etc). The research team will be responsible for the authorship and reviewing of the conference proceedings and publications. The fully anonymised dataset will be made public at the request of journals, or as a requirement by the funding bodies (*e.g.*, LJMU repository).
- Current funding from Liverpool John Moores University and the Liverpool Clinical Commissioning Group will be acknowledged in all publications, and so will any additional future funding. These funding bodies do not request publication or reviewing rights. In the event of collaborating with industry sponsors, an agreement will be signed so that the research team retain the rights over data property and publication rights.
- Participants will be informed of the final results of the study – a lay summary will be produced to share the findings with them, and they can receive copy of the final report as well, should they wish. Participants can also request a summary report on their personal results, directly to the PI and/or the PhD student who will be their main contact throughout the study.
- The study protocol will be registered publicly with clinicaltrials.gov

20 AUTHORSHIP ELIGIBILITY GUIDELINES AND ANY INTENDED USE OF PROFESSIONAL WRITERS

The Investigators will be involved in reviewing drafts of the manuscripts, abstracts, press releases and any other publications arising from the study. Authors will acknowledge that the study was funded by an LCCG RCF grant and any other grants, should application be successful. Authorship will be determined in accordance with the ICMJE guidelines and other contributors will be acknowledged.

21 REFERENCES

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22 APPENDICES

22.1 APPENDIX 1- REQUIRED DOCUMENTATION

- Research protocol or project proposal
- Letter from statistician
- Summary CV for Chief Investigator (CI)
- Participant information sheet (PIS)
- Participant consent form
- Letters of invitation to participant
- GP information sheets or letters
- Validated questionnaire
- Non-validated questionnaire
- Referee's report or other scientific critique report
- Summary, synopsis or diagram (flowchart) of protocol in non-technical language
- Covering letter on headed paper
- Letter from sponsor
- Letter from funder
- SoECAT
- Non-Commercial Model Agreement
- Evidence of Sponsor insurance or indemnity
- Summary of any applicable exclusions to sponsor insurance
- Summary CV for student
- Summary CV for supervisor (student research)
- MHRA "Notice of No Objection" Letter (Medical Devices) and relevant correspondence

22.2 APPENDIX 3 – AMENDMENT HISTORY

Amendment No.	Protocol Version No.	Date issued	Author(s) of changes	Details of Changes made

Ymchwil Iechyd a Gofal Cymru
Health and Care Research Wales

NHS
Health Research Authority

Dr Fatima Perez de Heredia
Room 234, James Parsons Building
Liverpool John Moores University
L3 3AF

Email: hra.approval@nhs.net
HCRW.approvals@wales.nhs.uk

11 December 2019

Dear Dr Perez de Heredia

Initial Assessment Letter

Study title:	**A high-PRotein Mediterranean diet and Resistance Exercise for cardiac rehabilitation: a pilot randomised controlled trial**
IRAS project ID:	**256927**
Protocol number:	**N/A**
REC reference:	**19/NW/0762**
Sponsor	**Liverpool John Moores University**

Thank you for your application for HRA and Health and Care Research Wales (HCRW) Approval. I am writing to confirm that you are now able to share the Local Information Pack with participating NHS organisations in England and Wales in order to invite them to arrange of capacity and capability to deliver your study. Please note that **the research should not begin** at any participating NHS organisations in England or Wales until HRA and HCRW Approval is issued.

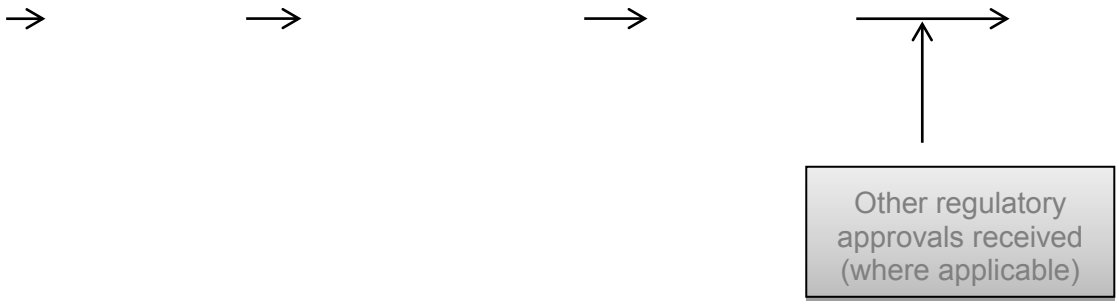
To share the Local Information Pack with participating NHS organisations in England and Wales please use the template email available on the IRAS website.

Once the Local Information Pack has been shared, please work with participating NHS organisations to arrange capacity and capability, in line with the instructions provided in the "Information to support study set up" section towards the end of this letter.

What happens next with my application for HRA and HCRW Approval?

Your application is progressing. Please find below an indication of where you are in the process (indicated by the red box).

Application submitted | Validation | **REC Review and Assessment (including issue of initial assessment letter)** | Response to queries | HRA and HCRW Approval



I am undertaking the assessment of the application and you will receive any queries following the REC meeting.

How should I work with participating NHS/HSC organisations in Northern Ireland and Scotland?
HRA and HCRW Approval does not apply to NHS/HSC organisations in Northern Ireland and Scotland.

If you indicated in your IRAS form that you have participating organisations in Northern Ireland and/or Scotland, the national coordinating function of each participating nation has been informed and provided with the initial document set. The relevant national coordinating function/s will contact you as appropriate. We will provide them the final document set and study wide governance report when available.

Please see IRAS Help for information on working with NHS/HSC organisations in Northern Ireland and Scotland.

How should I work with participating non-NHS organisations?
HRA and HCRW Approval does not apply to non-NHS organisations. You should work with your non-NHS organisations to obtain local agreement in accordance with their procedures.

Who should I contact for further information?
Please do not hesitate to contact me for assistance with this application. My contact details are below.

Your IRAS project ID is **256927.** Please quote this on all correspondence.

Yours sincerely,

Amber Ecclestone

Approvals Specialist

Email: hra.approval@nhs.net

North West - Greater Manchester East Research Ethics Committee

3rd Floor, Barlow House
4 Minshull Street
Manchester
M1 3DZ

Tel: 02071048199
Fax:

16 June 2020

Mr Richard Kirwan
34 Hawarden Av.
Liverpool
L17 2AL

Dear Mr Kirwan

Study title:	**A high-PRotein Mediterranean diet and Resistance Exercise for cardiac rehabilitation: a pilot randomised controlled trial**
REC reference:	**19/NW/0762**
Protocol number:	**N/A**
Amendment number:	**3**
Amendment date:	**06 April 2020**
IRAS project ID:	**256927**

The above amendment was reviewed by the Sub-Committee in correspondence.

Ethical opinion

The Subcommittee expressed that the P.I.S. was incomplete as the P.I.S. section “What will happen if I take part” only seemed to cover the Initial Questionnaire, while the Protocol (section 5.2) referred to a 4-part study.

The Consent form (Item 4) referred to a telephone interview, which was not mentioned in the P.I.S.

The Subcommittee suggested the applicants expand the current P.I.S. to cover all 4 parts, or produce a P.I.S. and Consent form for each part.

If a revised P.I.S. was to cover all 4 parts, then a better description should be given for each part, with an approximate time for that section to be completed.

The Hedonic Food Scale involves preparing meals and then commenting on them. The Subcommittee comment that part could take a considerable time.

The Subcommittee requires a logo from LJMU added to the top of page 1.

The applicants needed to state from when data can no longer be withdrawn, e.g. after it is anonymised and merged with other data, rather than state data which can be withdrawn at any time.

The Subcommittee also agreed that sections on Data Protection (from LJMU) and an independent contact (in LJMU) are needed.

The Subcommittee were of the opinion that the P.I.S. should state how individuals can go about participating in the study. The Subcommittee suggested a form asking for the contact details of potential participants, e.g. address if they wanted the questionnaire(s) posted or email address if they wish to do the study online.

The Researchers responded to the Subcommittee's comments and submitted an updated PIS. This was then sent back to the Subcommittee who agreed a favourable ethical opinion of the amendment on the basis described in the notice of amendment form and supporting documentation could now be issued.

Approved documents

The documents reviewed and approved at the meeting were:

Document	*Version*	*Date*
Non-validated questionnaire [Telephone Questionnaire]	1	25 March 2020
Notice of Amendment (non-CTIMP)	3	06 April 2020
Other [Research for the Future Recruitment Email]	1	25 March 2020
Other	1	25 March 2020
Other	2	25 March 2020
Participant consent form	1	25 March 2020
Participant information sheet (PIS)	1	25 March 2020
Research protocol or project proposal	1.2	25 March 2020
Participant information sheet (PIS)	1.1	11 June 2020

Membership of the Committee

The members of the Committee who took part in the review are listed on the attached sheet.

Working with NHS Care Organisations

Sponsors should ensure that they notify the R&D office for the relevant NHS care organisation of this amendment in line with the terms detailed in the categorisation email issued by the lead nation for the study.

Amendments related to COVID-19

We will update your research summary for the above study on the research summaries section of our website. During this public health emergency, it is vital that everyone can promptly identify all relevant research related to COVID-19 that is taking place globally. If you have not already done so, please register your study on a public registry as soon as possible and provide the HRA with the registration detail, which will be posted alongside other information relating to your project.

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

HRA Learning

We are pleased to welcome researchers and research staff to our HRA Learning Events and online learning opportunities– see details at: https://www.hra.nhs.uk/planning-and-improving-research/learning/

19/NW/0762:	**Please quote this number on all correspondence**

Yours sincerely

pp
Mr Simon Jones
Chair

E-mail: gmeast.rec@hra.nhs.uk

Copy to: *Mr Richard Kirwan*

North West - Greater Manchester East Research Ethics Committee

Attendance at Sub-Committee of the REC meeting on 25 May 2020

Committee Members:

Name	*Profession*	*Present*	*Notes*
Dr Michael Hollingsworth	Retired Senior Lecturer in Pharmacology	Yes	
Mr Simon Jones	Podiatrist	Yes	Chaired the meeting.

Also in attendance:

Name	*Position (or reason for attending)*
Miss Mia Cooper	Approvals Administrator

Health Research Authority

North West - Greater Manchester East Research Ethics Committee

3rd Floor, Barlow House
4 Minshull Street
Manchester
M1 3DZ

Tel: 0207 104 8009

Please note: This is the favourable opinion of the REC only and does not allow the amendment to be implemented at NHS sites in England until the outcome of the HRA assessment has been confirmed.

02 December 2020

Mr Richard Kirwan
34 Hawarden Av.
Liverpool
L17 2AL

Dear Mr Kirwan

Study title:	**A high-PRotein Mediterranean diet and Resistance Exercise for cardiac rehabilitation: a pilot randomised controlled trial**
REC reference:	**19/NW/0762**
Protocol number:	**N/A**
Amendment number:	**19LJMUSponsor092**
Amendment date:	**01 October 2020**
IRAS project ID:	**256927**

The above amendment was reviewed at the meeting of the Sub-Committee held on 27 November 2020 by the Sub-Committee in correspondence.

Ethical opinion

The members of the Committee taking part in the review gave a favourable ethical opinion of the amendment on the basis described in the notice of amendment form and supporting documentation.

Approved documents

The documents reviewed and approved at the meeting were:

Document	*Version*	*Date*
Completed Amendment Tool [Amendment_Tool_v1.2_11Jun20 signed]	v1.2	01 October 2020
Other [IRAS form Part B section 3]	n/a	15 October 2020
Participant consent form [Participant Consent Form Stage 1 V2.2 tracked changes.doc]	v2.2	25 August 2020
Participant information sheet (PIS) [Participant Information Sheet Stage 2 V3.3 tracked changes]	v3.3	25 August 2020
Research protocol or project proposal [TEM001 PRiME Research Protocol for IRAS application V1.3 tracked changes]	v1.3	25 August 2020

Membership of the Committee

The members of the Committee who took part in the review are listed on the attached sheet.

Working with NHS Care Organisations

Sponsors should ensure that they notify the R&D office for the relevant NHS care organisation of this amendment in line with the terms detailed in the categorisation email issued by the lead nation for the study.

Amendments related to COVID-19

We will update your research summary for the above study on the research summaries section of our website. During this public health emergency, it is vital that everyone can promptly identify all relevant research related to COVID-19 that is taking place globally. If you have not already done so, please register your study on a public registry as soon as possible and provide the HRA with the registration detail, which will be posted alongside other information relating to your project.

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

HRA Learning

We are pleased to welcome researchers and research staff to our HRA Learning Events and online learning opportunities– see details at: https://www.hra.nhs.uk/planning-and-improving-research/learning/

IRAS Project ID - 256927:	**Please quote this number on all correspondence**

Yours sincerely

Signed on behalf of
Mr Simon Jones
Chair

E-mail: gmeast.rec@hra.nhs.uk

Enclosures: *List of names and professions of members who took part in the review*

Copy to: *Mr Richard Kirwan*

North West - Greater Manchester East Research Ethics Committee

Attendance at Sub-Committee of the REC meeting on 27 November 2020

Committee Members:

Name	*Profession*	*Present*	*Notes*
Dr Michael Hollingsworth	Retired Senior Lecturer in Pharmacology	Yes	
Mr Simon Jones	Podiatrist	Yes	Chair

Also in attendance:

Ms Amber Ecclestone	Approvals Specialist	Yes	

Health Research Authority

North West - Greater Manchester East Research Ethics Committee

3rd Floor, Barlow House
4 Minshull Street
Manchester
M1 3DZ

Tel: 02071048199
Fax: N/A

02 March 2022

Mr Richard Kirwan
34 Hawarden Av.
Liverpool
L17 2AL

Dear Mr Kirwan

Study title:	**A high-PRotein Mediterranean diet and Resistance Exercise for cardiac rehabilitation: a pilot randomised controlled trial**
REC reference:	**19/NW/0762**
Protocol number:	**N/A**
Amendment number:	**19LJMUsponsor092**
Amendment date:	**09 February 2022**
IRAS project ID:	**256927**

The above amendment was reviewed by the Sub-Committee in correspondence.

Ethical opinion

The members of the Committee taking part in the review gave a favourable ethical opinion of the amendment on the basis described in the notice of amendment form and supporting documentation.

Approved documents

The documents reviewed and approved at the meeting were:

Document	*Version*	*Date*
Completed Amendment Tool [256927_19LJMUsponsor092_V1.0]	1.0	09 February 2022
Participant information sheet (PIS) [TC Stage 2]	v1.4	09 February 2022
Research protocol or project proposal [TC]	v1.4	09 February 2022

Membership of the Committee

The members of the Committee who took part in the review are listed on the attached sheet.

Working with NHS Care Organisations

Sponsors should ensure that they notify the R&D office for the relevant NHS care organisation of this amendment in line with the terms detailed in the categorisation email issued by the lead nation for the study.

Amendments related to COVID-19

We will update your research summary for the above study on the research summaries section of our website. During this public health emergency, it is vital that everyone can promptly identify all relevant research related to COVID-19 that is taking place globally. If you have not already done so, please register your study on a public registry as soon as possible and provide the HRA with the registration detail, which will be posted alongside other information relating to your project.

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

HRA Learning

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IRAS Project ID - 256927:	**Please quote this number on all correspondence**

Yours sincerely

pp
Chair

Mr Simon Jones

E-mail: gmeast.rec@hra.nhs.uk

Copy to: *Mr Richard Kirwan*

Health Research Authority

North West - Greater Manchester East Research Ethics Committee

Attendance at Sub-Committee of the REC meeting held via correspondence.

Committee Members:

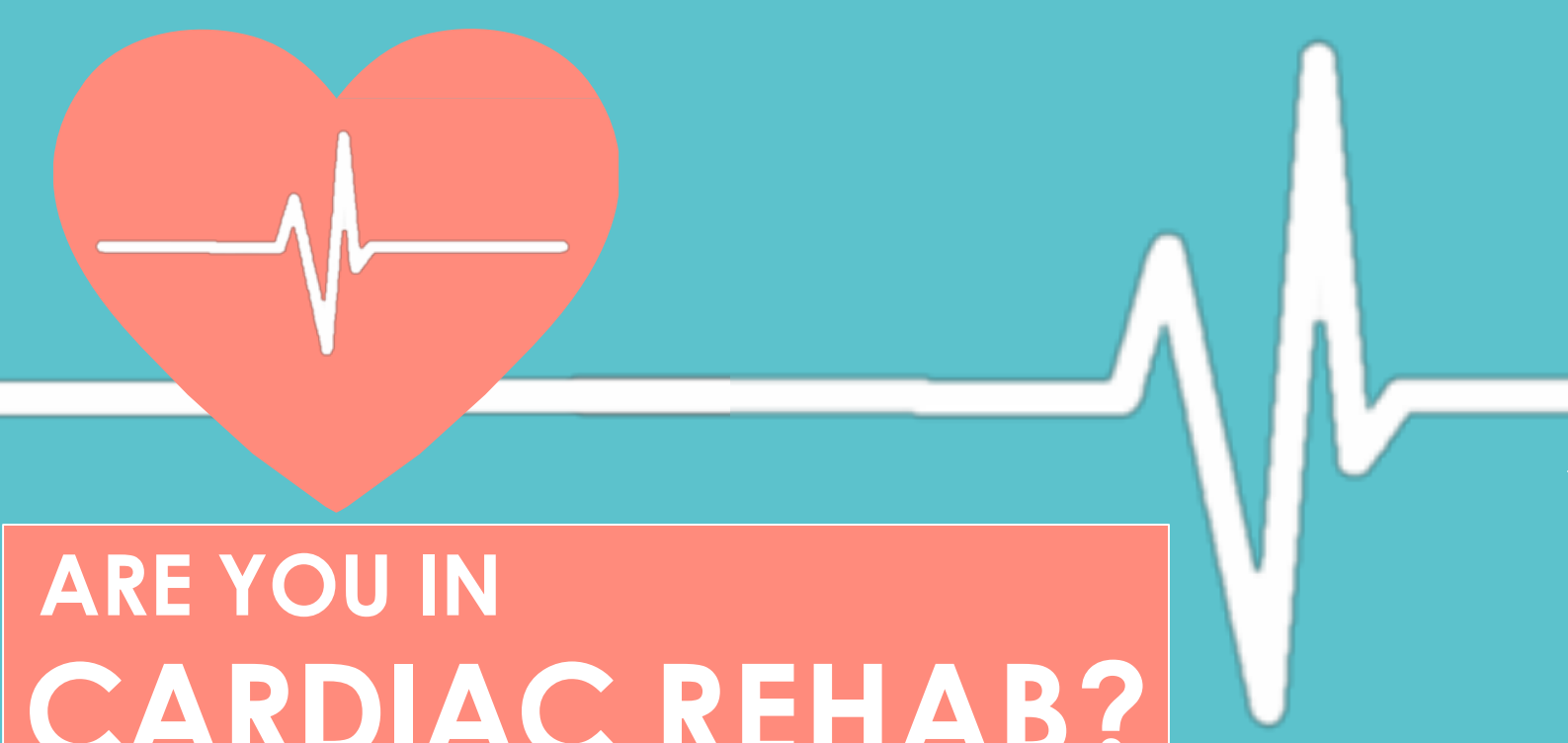
Name	*Profession*	*Present*	*Notes*
Dr Isabelle Butcher	Project Manager	Yes	Chaired the meeting.
Dr Gary Whittle	Consultant in Dental Public Health (retired)	Yes	

Also in attendance:

Name	*Position (or reason for attending)*
Miss Mia Cooper	Approvals Administrator

Appendix 3

Participant facing documents: study advertising, consent, questionnaires etc.



ARE YOU IN CARDIAC REHAB?

DO YOU WANT:

TO BE PART OF SCIENTIFIC RESEARCH (TO REDUCE CARDIAC EVENTS)

AND LEARN MORE ABOUT YOUR OWN HEALTH AND WELLBEING!

IF INTERESTED PLEASE ASK A MEMBER OF YOUR CADIAC REHAB TEAM OR EMAIL R.P.KIRWAN@2018.LJMU.AC.UK

Institute for Health Research

The PRiME Trial

Liverpool John Moores University

University of Chester

The PRiME Trial

Step 1

Want to know more?

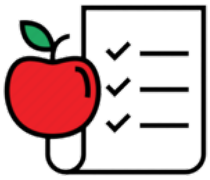
Sign a **consent form** and we'll call you with all the details!

Step 2

We'll call you to explain the study.

Then you can can **take your time to think** if you want to participate.

Step 3



We'll **arrange an appointment** for you to visit John Moores University for testing

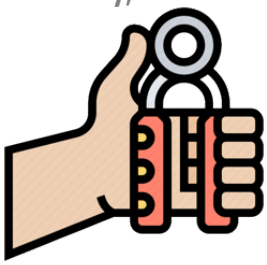
We'll give you a **food diary** to complete before your visit

Step 4

At John Moores University, we'll run some tests **(about 60 minutes)**

Muscle Size

Blood Pressure



Grip Strength

Blood Test

Questionnaire

Step 5

You'll be assigned to Group A or Group B for **12 WEEKS**

Activity for Life (Huyton & Kirkby Leisure Centres)

Group A
Normal Cardiac Rehab

Group B
Heart Healthy Diet and Resistance Exercise Group

You'll get **weekly phone check-ins** to make sure everything is ok

Step 6

Come back to John Moores University, for the same tests as Step 4 **(about 60 minutes)**

LIVERPOOL JOHN MOORES UNIVERSITY

INSTITUTE FOR HEALTH RESEARCH

Liverpool Heart and Chest Hospital NHS
NHS Trust

THOMAS DRIVE,
LIVERPOOL, L14 3PE
Telephone 01512281616

IRAS ID: 256927

Centre Number:

Study Number:

Participant Identification Number:

CONSENT FORM

Title of Project: A High-protein Mediterranean diet and resistance exercise for cardiac rehabilitation: a pilot randomised controlled trial

Name of Researcher: Fatima Perez de Heredia

Please initial box

I consent to being contacted by members of the research team regarding participation in this study. ☐

PHONE NUMBER: _____

_____	_____	_____	_____
Name of Participant	Date	Signature	Participant ID
_____	_____	_____	
Name of Person taking consent	Date	Signature	

Principal Investigator: Dr Fatima Perez de Heredia Benedicte, Liverpool John Moores University and Liverpool Centre for Cardiovascular Science.

Trust study number:

IRAS ID: 256927
Short title: The PRiME Study

TITLE: A High-protein Mediterranean diet and resistance exercise for cardiac rehabilitation: a pilot randomised controlled trial

Participant information sheet

You are being invited to take part in a research study. Before you decide whether to take part, it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully. Talk to others about the study if you wish.

Part 1 explains the purpose of the study and what will happen to you if you take part.

Part 2 gives you more detailed information about the conduct of the study.

Please ask us if there is anything that is not clear or if you would like more information. You may contact the study investigators (find their details later in this form). Take time to decide whether or not you wish to take part.

PART 1

What is the purpose of the study?

The study aims to investigate whether a high-protein cardioprotective diet, alone or in combination with resistance exercise, can increase muscle mass in cardiac rehabilitation (CR) patients, and reduce the risk of future cardiac events.

Cardiovascular risk can be increased by different factors, such as obesity (an excess of body fat), or sarcopenia, which is a gradual loss of muscle mass that happens as we age. Sometimes both conditions can occur simultaneously, and the individual may appear to have a normal body weight, but have disproportionately low levels of muscle mass and high levels of body fat; this is known as sarcopenic obesity, and it is associated with greater risk of heart disease. Increasing the proportion of muscle mass to body fat in people with sarcopenic obesity may reduce the risk of future heart disease.

Why have I been chosen?

You have been chosen for this study because you have recently been referred to CR, which is where we will start our study. In collaboration with the Knowsley Community Cardiac Services or Volair gym group, cardiac rehab participants will be informed of the study by staff members.

To participate, you will need to meet the following requirements:

- being able to perform resistance exercise (determined by primary care team),
- being able to digest and willing to consume dairy products,
- not suffering from chronic kidney disease,
- not having been admitted to CR due to congenital or drug/alcohol-induced cardiac events.
- not being pregnant

Do I have to take part?

No. Taking part in this study is entirely voluntary. If you decide to participate, you will be given this information sheet to keep, and be asked to sign a consent form. You are still free to withdraw at any time and without giving a reason. A decision to not take part or to withdraw will not affect your rights, or any future treatment or service you receive.

What will I be asked to do, if I take part? Diet and Exercise Intervention

You will be asked to take part in the diet and exercise intervention, which will last 12 weeks. You will be allocated to one of two groups: 1) standard CR; 2) CR plus personalised advice to follow a high-protein, healthier diet and resistance exercise. All participants will be assigned to their group by computer randomisation.

Personalised dietary advice: if you are allocated to group 2, we will ask you to make changes in your diet to adapt it to a high-protein, cardioprotective diet. Research shows that cardioprotective diets can reduce cardiovascular risk, and although there are different versions of this type of diet, they all have in common:

- eating more fruit and vegetables,
- reducing commercial pastries, and replacing refined carbohydrate foods (white bread, white rice, white pasta) by wholegrains (wholegrain bread, rice and pasta),
- replacing butter and margarine by olive oil in some meals and dishes,
- reducing fatty meat and replacing by lean meat, fish, and legumes (peas, beans, lentils), and by high-protein, low fat foods, such as low-fat dairy (you will be provided with vouchers for 2 high-protein yoghurts to eat each day).

You will receive personalised guidance to help you follow the new diet. The goal is to make small, easy changes to your current eating habits, so the diet will be easy to follow. All foods included will be affordable and easy to find in local supermarkets, and we will provide suggestions and recipes to prepare food.

If you are allocated to group 1, you will be asked to follow the diet recommendations given during phase 3 of CR.

Resistance exercise: if you are allocated to group 2, you will be asked to perform resistance exercise. This involves weights or weight machines aimed at building muscle strength. You will be shown how to do the exercises by qualified instructors in the community centre where you carry out your current phase 3 cardiac rehabilitation. All exercises have been deemed safe for cardiac rehabilitation patients and an instructor will be available at all exercise sessions should you need assistance. You will be required to attend 3 sessions per week and each session is expected to last approximately 45 minutes.

If you are allocated to group 1, you will be asked to continue with the standard, aerobic-style exercise (treadmills, rowing machines, elliptical trainers) you have used in phase 3 of CR.

Monitoring progress: ALL participants will have to attend two appointments at LJMU. The first visit will be just before beginning the intervention, and the second immediately after completing the intervention. Visits are expected to last 60-90 minutes and will entail:

i) Assessing your muscle mass. This will be done in three different ways.

Firstly, we will measure your height and we will use a tape measure to measure your waist circumference. Please ensure that you come wearing comfortable fitting clothing that you can

easily move up or down to take these measurements. If you prefer for a team member of the same sex to take these, please do let us know so that we can facilitate this.

Secondly, we will ask you to step onto a sophisticated body composition scale. This device uses a tiny electric current to measure your lean body mass, your body fat mass and the amount of fat surrounding your organs.

Thirdly, we will ask you to lie on a device called a DXA (dual-energy X-ray absorptiometry) for 15-20 minutes. This device works like a lower-intensity X-ray and allows us to create an image of the distribution of your lean body mass and your fat mass.

Fasting: Please ensure that you have nothing to eat or drink (other than water) for at least 12 hours prior to your appointment, as otherwise your results may be invalid. You can continue to drink water while fasting.

You must also avoid any calcium supplements for at least 24 hours before your tests.

ii) Taking your blood pressure

Following standard protocol, you will be asked to sit for 5 minutes before we take your blood pressure; we will measure it three times to ensure an accurate reading.

iii) Grip strength test

Grip strength is a good measure of overall health; we will test it using a device that you squeeze as hard as you can. We will do this three times to ensure an accurate reading.

iv) A venous blood sample

We will take about 8 teaspoons of blood. Please note that you will need to fast for at least 12 hours prior to your appointment (although water is encouraged), as otherwise this may affect the result of the test. You will also have to refrain from drinking alcohol and undertaking any strenuous exercise the night before; again, both can affect the result of the test.

We will process and store your blood samples according to current UK regulations and rules of good research practice (Human Tissue Act 2004), and will keep them for a maximum of 10 years. Briefly, blood samples will be processed immediately after collection, and the serum will be stored in a secure freezer in the Life Sciences Building at LJMU. All samples will be stored pseudo-anonymously; this means that all identifiable information (*e.g.*, your name) will be removed and replaced by a code to allow the research team to trace the samples and match them with the other measurements (body weight, muscle mass measures, etc.). Once all participants have completed the intervention, we will analyse the samples to see if there have been changes in the risk markers for heart disease such as cholesterol, triglycerides, blood sugar and insulin . All stored samples will consist of serum only, which does not contain any identifiable information. Once the specified storage period ends, all samples will be disposed of following current UK regulations.

v) Focus Groups

At the final visit we will also conduct a brief focus group to ask about your experiences with the diet and/or exercise regime allocated to you, and to check whether you have experienced any adverse events over the study. These focus groups will be audio recorded but will not contain any identifiable information such as names. These recordings will be stored for a maximum of 10 years after which they will be destroyed.

vi) Food diaries (prior to your lab appointments)

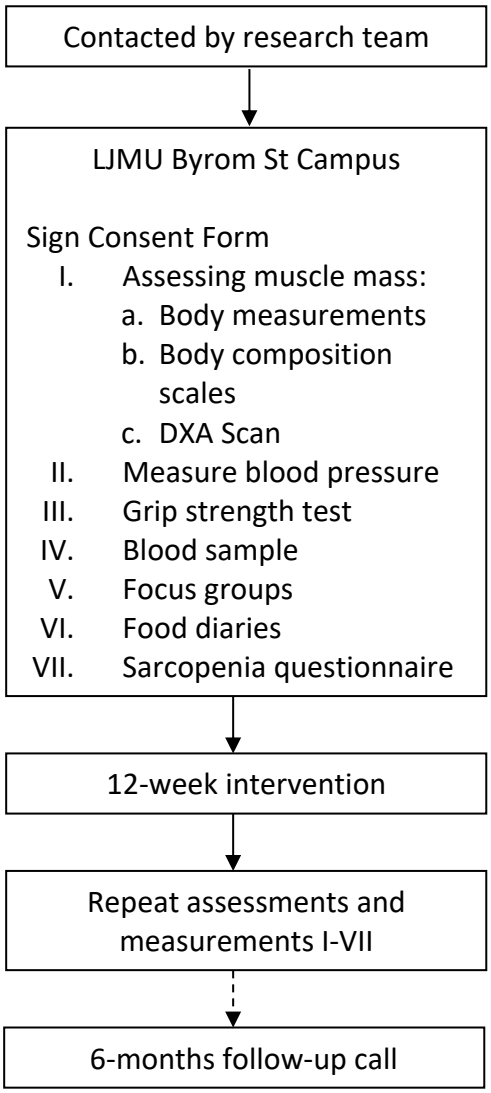
Before your first and second lab visits, at your local CR centre, we will give you a template of a four-day food diary, which you will need to complete the week before your lab appointment (on three working days plus one weekend or festive day, non-consecutive whenever possible), and bring with you on the morning of the visit.

vii) Sarcopenia questionnaire

This questionnaire asks about sarcopenia, which is a muscle weakness that comes about with ageing. It will take approximately 10 minutes.

Follow-Up Call: You may be contacted by the researchers approximately 6-months after the completion of your intervention to briefly ask you some questions about your current diet and exercise habits.

Flow Chart



What are the possible disadvantages and risks of taking part?

If you participate in the intervention, you may incur travel expenses associated with attending your meetings at LJMU. This will not be reimbursed by LJMU.

You may need to dedicate some time to reading the study materials as well as to buying ingredients and changing cooking routines (although all ingredient recommendations are easy to find in UK supermarkets and all recipes have been designed to be easy to prepare).

There are very few disadvantages to the dietary and exercise intervention; however, you may experience some of the following:

- Some mild muscle pain from the exercise training – this is perfectly normal, and as your body gets used to the training, you will experience this far less and may not feel it at all.
- You may increase the amount of fibre you are eating, and may need to use the toilet more frequently than normal. This is a healthy side effect of a high-fibre diet, but you may also feel bloated or gassy at times.
- This study will use ionising radiation to assess body fat and muscle mass with DXA scans. If you take part in stage 1, you will be scanned once, and in stage 2 you will be scanned two more times. Ionising radiation can cause cell damage that may, after many years or decades turn cancerous. About 50% of people will develop cancer at some point in their life, and taking part in this study will add only an extremely small chance of this happening to you. This study will use up to 3 x DEXA scans, all of which will be extra to those that you would have if you did not take part. The amount of radiation you will receive from one DXA scan is approximately half to one third of the radiation from a standard chest X-ray, and equivalent to the amount of natural background radiation received in any two days.
- The possible risks of blood tests are discomfort and bruising at the site where the needle goes in. These complications usually are minor and go away shortly after the tests are done.

What are the possible benefits in taking part?

Participants in the high protein diet groups will be provided with 2 high-protein yoghurts per day for the duration of the trial.

The diet and exercise chosen in this study have been shown to have a number of benefits:

- High-protein foods may leave you feeling fuller than normal after a meal. You may feel less hungry between meals, and this may help prevent you from over-eating other foods.
- This also means that you might eat less than you usually do, leading to a lower calorie intake and consequential potential weight loss.
- You may lose fat mass, improving your body composition.
- Exercise is also known to provide numerous health benefits and to improve quality of life.

What happens when the research study stops?

After all participants have completed their part of the research study, we will analyse all the information collected. We will use this information to determine whether we can see a benefit to the diet and exercise intervention. We will arrange a seminar for you to find out about the overall results of the study, and provide a final report for you to take away.

What if there is a problem?

Any complaint about the way you have been dealt with during the study or any possible harm you might have suffered will be addressed. You can find more detailed information in Part 2.

You have the right to withdraw from the study at any time and without any reason, and this will not affect any of your rights. Should you wish to withdraw from the study, your anonymised data will be

retained for 10 years. Your data may still be included in the analysis if you withdraw after completing the intervention, and for reasons that do not affect the study outcomes. If you want your data to be removed from the study, please contact us (see contact details below).

Will my taking part in this study be kept confidential?

If you decide to take part, all information collected about you during the course of the study will be anonymised and kept strictly confidential within the research team. It will not be possible to identify you from any report that is published from this study. With your permission, your GP will be informed that you are taking part in the study and there may be an exchange of information between your GP and the research team. Should the results of any tests result in any abnormal findings your GP will be notified via the lead clinician.

Information and samples collected from you will also be used to support other research in the future, and may be shared with other researchers although all shared samples will be anonymous and all data will be destroyed after 10 years.

What will happen to the results of the research project?

The results from this study will be part of the doctoral thesis of Richard Kirwan. They will also be presented at national and international conferences, and submitted to peer-reviewed journals in the fields of nutrition and health. Results will always be presented and published in a collective, anonymous fashion; no participants can be identified.

Who has reviewed this study?

This study has been reviewed and supported as a sponsor by LJMU, and by the Liverpool Heart and Chest Hospital Research & Innovation Committee. Ethical approval has been granted by the NHS Research Ethics Committee (Reference number: 19/NW/0762).

What do I do now?

If you would like to participate in the study simply inform a member of the Knowsley Community Cardiac Services or Volair gym team and we will be in touch shortly with further details. You will then need to read and sign a participant consent form in the presence of a member of our research team at your first visit to LJMU.

Contact details:

1. Dr Joseph Mills — Tel 0151 600 1991
2. Richard Kirwan — Tel 07565427663
   email: R.P.Kirwan@2018.ljmu.ac.uk
3. Dr Fatima Perez de Heredia — Tel 0151 231 2003
   email: F.PerezDeHerediaBenedicte@ljmu.ac.uk

This completes Part 1 of the Information Sheet. If the information in Part 1 has interested you and you are considering participation, please continue to read the additional information in Part 2 before making your decision.

PART 2

What if there is a problem?

If you have a concern about any aspect of this study, you should ask to speak with the principal researchers, Dr Fatima Perez de Heredia and **Richard Kirwan,** who will do their best to answer your questions **on the telephone number provided above**. These researchers will also contact you regularly during the study to ensure your wellbeing.

If you have concerns about any aspect of the way you have been approached or treated during the course of the study, you may direct complaints to an independent LJMU representative:

Dr Dave Harriss
Research Governance Manager
Research Innovation Services
Exchange Station
Tithebarn Street
Liverpool
L2 2QP
Email: Sponsor@ljmu.ac.uk
Phone: 0151 231 2121

LJMU has Clinical Trials insurance to cover the legal liability of the University as Research Sponsor in the eventuality of harm to a research participant arising from management and design of the research by the University and the activities mentioned here are included within that coverage.

LJMU's Clinical Trials insurance policies provide an indemnity to our employees and students for their potential liability for harm to participants during the conduct of the research and the activities mentioned here are included within that coverage.

How will we use information about you?
We will need to use information from you and from your medical records for this research project.

This information will include your:
- Name
- Contact details

People will use this information to do the research or to check your records to make sure that the research is being done properly.

People who do not need to know who you are will not be able to see your name or contact details. Your data will have a code number instead.

We will keep all information about you safe and secure.

Once we have finished the study, we will keep some of the data so we can check the results. We will write our reports in a way that no-one can work out that you took part in the study.

What are your choices about how your information is used?
- You can stop being part of the study at any time, without giving a reason, but we will keep information about you that we already have.
- We need to manage your records in specific ways for the research to be reliable. This means that we won't be able to let you see or change the data we hold about you.

- If you agree to take part in this study, you will have the option to take part in future research using your data saved from this study.

Where can you find out more about how your information is used?
You can find out more about how we use your information

- at www.hra.nhs.uk/information-about-patients/
- our leaflet available from www.hra.nhs.uk/patientdataandresearch
- by asking one of the research team
- by sending an email to r.p.kirwan@2018.ljmu.ac.uk or
- by ringing us on 07565427663.

What happens to my research data after the study?
Researchers must make sure they write the reports about the study in a way that no-one can work out that you took part in the study.

Once they have finished the study, the research team will keep the research data for several years, in case they need to check it. You can ask about who will keep it, whether it includes your name, and how long they will keep it.

Usually your hospital or GP where you are taking part in the study will keep a copy of the research data along with your name. The organisation running the research will usually only keep a coded copy of your research data, without your name included. This is kept so the results can be checked.

If you agree to take part in a research study, you may get the choice to give your research data from this study for future research. Sometimes this future research may use research data that has had your name and NHS number removed. Or it may use research data that could show who you are. You will be told what options there are. You will get details if your research data will be joined up with other information about you or your health, such as from your GP or social services.

Once your details like your name or NHS number have been removed, other researchers won't be able to contact you to ask you about future research.

Any information that could show who you are will be held safely with strict limits on who can access it.

You may also have the choice for the hospital or researchers to keep your contact details and some of your health information, so they can invite you to take part in future clinical trials or other studies. Your data will not be used to sell you anything. It will not be given to other organisations or companies except for research.

Will the use of my data meet GDPR rules?
GDPR stands for the General Data Protection Regulation. In the UK we follow the GDPR rules and have a law called the Data Protection Act. All research using patient data must follow UK laws and rules.

Universities, NHS organisations and companies may use patient data to do research to make health and care better.

When companies do research to develop new treatments, they need to be able to prove that they need to use patient data for the research, and that they need to do the research to develop new treatments. In legal terms this means that they have a 'legitimate interest' in using patient data.

Universities and the NHS are funded from taxes and they are expected to do research as part of their job. They still need to be able to prove that they need to use patient data for the research. In legal terms this means that they use patient data as part of 'a task in the public interest'.

If they could do the research without using patient data they would not be allowed to get your data.

Researchers must show that their research takes account of the views of patients and ordinary members of the public. They must also show how they protect the privacy of the people who take part. An NHS research ethics committee checks this before the research starts.

What if I don't want my patient data used for research?
You will have a choice about taking part in a clinical trial testing a treatment. If you choose not to take part, that is fine.

In most cases you will also have a choice about your patient data being used for other types of research. There are two cases where this might not happen:

When the research is using anonymous information. Because it's anonymous, the research team don't know whose data it is and can't ask you.
When it would not be possible for the research team to ask everyone. This would usually be because of the number of people who would have to be contacted. Sometimes it will be because the research could be biased if some people chose not to agree. In this case a special NHS group will check that the reasons are valid. You can opt-out of your data being used for this sort of research. You can ask your GP about opting-out, or you can find out more.

Who can I contact if I have a complaint?
If you want to complain about how researchers have handled your information, you should contact the research team. If you are not happy after that, you can contact the Data Protection Officer. The research team can give you details of the right Data Protection Officer.

If you are not happy with their response or believe they are processing your data in a way that is not right or lawful, you can complain to the Information Commissioner's Office (ICO) (www.ico.org.uk or 0303 123 1113).

What will happen to the results at the end of the research study?
The results of this study will not be known until approximately 1 year after the last participant has completed their involvement in the trial.

At the end of the study, the researchers will produce a summary report on the overall findings. As mentioned in Part 1, we will arrange a seminar for you to find out about the overall results of the study, and provide a final report for you to take away.

The results will be part of the doctoral thesis of Richard Kirwan, and will also be presented at national and international conferences, and submitted to peer-reviewed journals in the fields of nutrition and health. No personal information would be included in any publication.

If you are interested in the results of this study or wish to receive a copy of the manuscript, please contact the research team.

Who is organising and funding the research?

The research will be carried out by researchers from LJMU, in collaboration with Liverpool Heart and Chest Hospital (both institutional members of the Liverpool Centre for Cardiovascular Science). Funding comes from LJMU and Liverpool Clinical Commissioning Group Research Capability Funding .The researchers are not receiving any payments other than their usual salaries.

Who has reviewed the study?
LJMU and the Research & Innovation Committee for the Liverpool Heart & Chest Hospital reviewed the study. Ethical approval has been granted by the NHS (Reference number: 256927)

Thank you very much for considering taking part in our research. Please discuss this information with your family and friends if you wish

LIVERPOOL JOHN MOORES UNIVERSITY

Liverpool Heart and Chest Hospital NHS
NHS Trust

THOMAS DRIVE,
LIVERPOOL, L14 3PE
Telephone 01512281616

IRAS ID: 256927

Centre Number:

Study Number:

Participant Identification Number:

CONSENT FORM

Title of Project: A High-protein Mediterranean diet and resistance exercise for cardiac rehabilitation: a pilot randomised controlled trial

Name of Researcher: Fatima Perez de Heredia

Please initial box

1. I confirm that I have read the information sheet dated..... (version.....) for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily. ☐
2. I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason, without my medical care or legal rights being affected. ☐
3. I understand that relevant sections of my medical notes and data collected during the study, may be looked at by researchers from Liverpool John Moores University, from regulatory authorities or from the NHS Trust, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records. ☐
4. I understand that the information and samples collected from me will be used to support other research in the future, and may be shared anonymously with other researchers and that all samples and data will be destroyed after 20 years. ☐
5. I agree to my General Practitioner being informed of my participation in the study. I agree to my General Practitioner being involved in the study, including any necessary exchange of information about me between my GP and the research team. ☐
6. I agree to take part in the above study. ☐

Name of Participant	Date	Signature	Participant ID
Name of Person taking consent	Date	Signature	

When completed: 1 for participant; 1 for researcher site file; 1 to be kept in medical notes.

Liverpool Heart and Chest Hospital NHS
NHS Trust

THOMAS DRIVE,
LIVERPOOL
L14 3PE
Tel: 0151 228 1616
Homepage: http://www.lhch.nhs.uk;
Email:webmaster2@ctc.nhs.uk

Study Number:

Patient Identification Number for this trial (CRF No.):

Principal Investigators: Fatima Perez de Heredia (LJMU), Richard Kirwan (LJMU), Ian Davies (LJMU), Tom Butler (University of Chester)

Title of Project: A high-protein Mediterranean diet and resistance exercise for cardiac rehabilitation: a pilot randomised controlled trial

Dear Doctor,

Re: Your Patient – ……..

Date of Birth : ……..

NHS reference number : ……..

Address : ……

Your patient has kindly agreed to participate in the above research project, a single-centre, cross-sectional analysis of the prevalence of Sarcopenic Obesity and a single-centre, randomised, open label controlled trial. Your patient will take part in a cross-sectional analysis of the levels of sarcopenic obesity amongst cardiac rehabilitation patients. Should you're patient meet the certain definitions of sarcopenic obesity they may be invited to participate in a further feasibility study in phase 4 cardiac rehabilitation. This would investigate the effects of a high-protein diet and resistance exercise on lean body mass and cardiometabolic risk markers.

Yours sincerely,

Fatima Perez de Heredia
(Lead Investigator)

Personal Information

Patient ID: _____

Date of Birth (dd/mm/yyyy): _____

Biological Gender: _____

Ethnicity (please circle one):	Caucasian
	North African/Middle Eastern
	Sub-Saharan African
	South Asian
	East Asian
	Other (please specify) _____
Marital Status (please circle one):	Single
	Married/Cohabiting
	Divorced/Separated/Widowed
Education (please circle one):	No qualifications
	GCE 'O' levels, CSE, GCSE
	GCE 'A' level or equivalent
	Further education (e.g., HNC, HND)
	Degree or equivalent
	Postgraduate degree
Occupation (please circle one):	Retired
	Employed
	Unemployed
On a scale of 1-10 how physically active would you consider the majority of work you have done during your life:	1 = very inactive (desk work)
	10 = very active (manual/construction work) _____
On a scale of 1-10 how much time would you spend each week on leisure time physical activity/exercise during your life:	1 = very inactive (less than 30 minutes per week)
	10 = very active (more than 7 hours per week) _____
Current Smoker (please circle one):	Yes
	No

MEDITERRANEAN DIET SCORE TOOL

A Mediterranean dietary pattern ('Med diet') is typically one based on whole or minimally processed foods. It's rich in protective foods (fruits, vegetables, legumes, wholegrains, fish and olive oil) and low in adverse dietary factors (fast food, sugar-sweetened beverages, refined grain products and processed or energy-dense foods) with moderate red meat and alcohol intake.

Evidence shows **overall dietary pattern** (reflected in TOTAL SCORE) as well as **individual components** reflect risk; a higher score is associated with lower risk of CVD and all-cause mortality (BMJ 2008;337:a1344). During rehabilitation patient scores should ideally rise in response to dietary advice and support.

This tool can be used by health professionals with appropriate nutritional knowledge and competencies, such as Registered Dietitians (NICE, 2007, 2013). It can be used as both an *audit tool* and *as part of a dietary assessment* at baseline, end of programme and 1 year follow-up, along with assessment and advice for weight management, salt intake and eating behaviours. For information on complete requirements for dietary assessments and advice, please refer to the latest NICE/Joint British Societies guidelines (BACPR, 2012. The BACPR Standards and Core Components for Cardiovascular Disease Prevention and Rehabilitation, 2nd Ed.).

	Question	Yes	No	Nutritional issue to discuss in response
1.	Is olive oil the main culinary fat used?			**Choosing Healthier Fats** Olive oil is high in monounsaturated fat. Using unsaturated fats instead of saturated fats in cooking and preparing food is advisable.
2.	Are ≥ 4 tablespoons of olive oil used each day?			**Healthy fats are better than very low fat** Med diet is more beneficial than a very low fat diet in prevention of CVD. So replacing saturated with unsaturated fat is better than replacing it with carbohydrates or protein.
3.	Are ≥ 2 servings (of 200g each) of vegetables eaten each day?			**Eat plenty of fruits and vegetables** Eating a wide variety of fruit and vegetables every day helps ensure adequate intake of many vitamins, minerals, phytochemicals and fibre. Studies have shown that eating plenty of these foods is protective for CVD and cancer.
4.	Are ≥ 3 servings of fruit (of 80g each) eaten each day?			
5.	Is < 1 serving (100-150g) of red meat/ hamburgers/ other meat products eaten each day?			**Choose lean meats and consider cooking methods** Red and processed meats are high in saturated fat, can be high in salt and are best replaced with white meat or fish or vegetarian sources of protein. Grill or roast without fat, casserole or stir fry.
6.	Is < 1 serving (12g) of butter, margarine or cream eaten each day?			**Keep saturated fat low** These foods are high in saturated fat which can increase your blood cholesterol level. Choose plant-based or reduced-fat alternatives.
7.	Is < 1 serving (330ml) of sweet or sugar sweetened carbonated beverages consumed each day?			Excessive consumption of sugar-sweetened beverages can worsen many risk factors for CVD: keep consumption to < 1/day.
8.	Are ≥ 3 glasses (of 125ml) of wine consumed each week?			**Moderate alcohol intake with meals** While this does have some protective effect but ***there is no evidence that non-drinkers should take up drinking alcohol.***
9.	Are ≥ 3 servings (of 150g) of legumes consumed each week?			**Include soluble fibre** These foods are high in soluble fibre and other useful nutrients. Regular consumption is advisable for raised cholesterol.
10.	Are ≥ 3 servings of fish (100-150g) or seafood (200g) eaten each week?			**Eat more oily and white fish** Oily fish is an excellent source of essential omega-3 fats. White fish is very low in saturated fat.
11.	Is < 3 servings of commercial sweets/pastries eaten each week?			**Eat less processed food** These foods are usually high in saturated fat, salt or sugar and often contain trans fats. Replacing these with healthy snacks such as fruit or unsalted nuts is beneficial.
12.	Is ≥ 1 serving (of 30g) of nuts consumed each week?			**Snack on modest servings of unsalted nuts** Nuts are rich in unsaturated fat, phytosterols, fibre, vitamin E and iron, **e.g. walnuts, almonds, hazelnuts**
13.	Is chicken, turkey or rabbit routinely eaten instead of veal, pork, hamburger or sausage?			'White meat' choices are lower in saturated fat. Remove the skin and consider your cooking method.
14.	Are pasta, vegetable or rice dishes flavoured with garlic, tomato, leek or onion eaten ≥ twice a week?			Using a tomato and garlic or onion or leek-based sauce regularly is a key feature of the Med diet.
TOTAL SCORE (total no. of 'yes' answers)				

LIVERPOOL JOHN MOORES UNIVERSITY

Liverpool Heart and Chest Hospital NHS
NHS Trust

PRiME

4-day FOOD AND DRINK DIARY

For office use only	
Participant ID	Visit

Contents

Instructions

PLEASE READ THROUGH THESE PAGES BEFORE STARTING YOUR DIARY

We would like you to keep this diary of everything you eat and drink over 4 days. Please include all food consumed at home and outside the home e.g. work, university or restaurants.

When to fill in the diary

Please record your eating as you go, **not from memory** at the end of the day. Use written notes on a pad if you forget to take your diary with you. Each diary day covers a 24hr period, so please include any food or drinks that you may have had during the night. Remember to include foods and drinks between meals (snacks) including water. This way it will only take a few minutes each time you complete parts of the diary.

Day and Date

Please write down the day and date at the top of the page each time you start a new day of recording. Please include one weekend day.

Time Slots

Please note the time of each eating occasion into the space provided. For easy use each day is divided into sections, from the first thing in the morning to late evening and through the night.

Where and with whom?

For each eating occasion, please tell us what **room or part of the house** you were in when you ate, e.g. kitchen, living room. If you ate at your work canteen, a restaurant, fast food chain or your car, write that location down. We would also like to know **who you share your meals with**, e.g. whether you ate alone or with others. If you ate with others please describe their relationship to you e.g. partner, children, colleagues, or friends. We would also like to know **when you ate at a table** and **when you were watching television whilst eating**. For those occasions where you were **not** at a table or watching TV please write 'Not at table' or 'No TV' rather than leaving it blank.

What do you eat?

Please describe the food you eat in as much detail as possible. Be as specific as you can. Pages 3-10 will help with the sort of detail we need, like **cooking methods** (fried, grilled, baked etc) and any **additions** (fats, sugar/sweeteners, sauces, pepper etc).

- **Homemade dishes**

If you have eaten any **homemade dishes** e.g. chicken casserole, please record the name of the recipe, ingredients with amounts (including water or other fluids) for the whole recipe, the number of people the recipe serves, and the cooking method. Write this down in the recipe section at the end of the record day. Record how much of the whole recipe you have eaten in the portion size column (see examples in this booklet).
If you used a recipe provided in one of the CALIBER study menu plans, please just record [NAME OF RECIPE (CALIBER)], e.g. 'Chunky chilli con carne (CALIBER)'

- **Take-aways and eating out**

If you have eaten **take-aways** or **made up dishes not prepared at home** such as at a restaurant or a friend's house, please record as much detail about the ingredients as you can e.g. vegetable curry containing chickpeas, aubergine, onion and tomato.

- **Brand name**

Please note the **brand name** (if known). Most packed foods will list a brand name, e.g. Bird's eye, Hovis, or Supermarket own brands.

- ***Labels/Wrappers***

Labels are an important source of information for us. It helps us a great deal if you collect labels from all **ready meals,** and labels from **foods of lesser known brands** and bring them with you to your next lab appointment.
Or you could take a photo of the label and product name on your phone and email it to t.harrison@2015.ljmu.ac.uk . Please state the date and eating occasion (breakfast, lunch, dinner, snack) when sending your photos. Thank you.

Portion sizes

Examples for how to describe the **quantity** or **portion size** you had of a particular food or drink are shown further on in this booklet.
For foods, quantity can be described using:
• **household measures**, e.g. one teaspoon (tsp) of sugar, two thick slices of bread, 4 tablespoons (tbsp) of peas, ½ cup of gravy. Be careful when describing amounts in spoons that you are referring to the correct spoon size.
• **weights from labels**, e.g. 4oz steak, 420g tin of baked beans, 125g pot of yoghurt
• **number of items**, e.g. 4 fish fingers, 2 pieces of chicken nuggets, 1 regular size jam filled doughnut
• **picture examples** for specific foods can be found in this booklet.

For drinks, quantity can be described using:
• the **size of glass, cup etc** (e.g. large glass) or the **volume** (e.g. 300ml). Examples of typical drinks containers are on pages 15-16.
• **volumes from labels** (e.g. 330ml can of fizzy drink).
We would like to know the **amount that was actually eaten** which means taking **leftovers** into account. You can do this in two ways:
1. Record what was served and make notes of what was not eaten e.g. 3 tbsp of peas, only 2 tbsp eaten; 1 large sausage roll, ate only ½
2. Only record the amount actually eaten i.e. 2 tbsp of peas, ½ a large sausage roll

Was it a typical day?

After each day of recording you will be prompted to tell us whether this was a typical day or whether there were any reasons why you ate and drank more or less than usual.

Please let us know if you would like an example of a completed food diary.

Examples and advice on food descriptions

Food/Drink	*Description & Preparation*	*Portion size or quantity*
Bacon	Back, middle, streaky; smoked or un-smoked; fat eaten; dry-fried or fried in oil/fat (type used) or grilled rashers	Number of rashers
Baked beans	Standard, reduced salt or reduced sugar	Spoons, weight of tin
Beefburger (hamburger)	Home-made (ingredients), from a packet or take-away; fried (type of oil/fat), microwaved or grilled; economy; with or without bread roll, with or without salad e.g. lettuce, tomato	Large or small, ounces or in grams if info on package
Beer	What sort e.g. stout, bitter, lager; draught, canned, bottled; % alcohol or low-alcohol or home-made	Number of pints or half pints, size of can or bottle
Biscuits	What sort e.g. cheese, wafer, crispbread, sweet, chocolate (fully or half coated), shortbread, home-made	Number, size (standard or mini variety)
Bread (see also sandwiches)	Wholemeal, granary, white or brown; currant, fruit, malt; large or small loaf; sliced or unsliced loaf	Number of slices; thick, medium or thin slices
Bread rolls	Wholemeal, white or brown; alone or with filling; crusty or soft	Size, number of rolls
Breakfast cereal (see also porridge)	What sort e.g. Kellogg's cornflakes; any added fruit and/or nuts; Muesli – with added fruit, no added sugar/salt variety	Spoons or picture 1
Buns and pastries	What sort e.g. iced, currant or plain, jam, custard, fruit, cream; type of pastry; homemade or bought	Size, number
Butter, margarine & fat spreads	Give full product name	Thick/average/thin spread; spoons
Cake	What sort: fruit (rich), sponge, fresh cream, iced, chocolate coated; type of filling e.g. buttercream, jam	Individual or size of slice, packet weight, picture 10

Food/Drink	Description & Preparation	Portion size or quantity
Cereal bars	What sort; with fruit/nuts, coated with chocolate/yoghurt; fortified with vitamins/minerals	Weight/size of bar; from multipack
Cheese	Type e.g. cheddar, cream, cottage, soft; low fat	Picture 9, or number of slices, number of spoons
Chips	Fresh, frozen, oven, microwave, take-away (where from); thick/straight/crinkle/fine cut; type of oil/fat used for cooking	Picture 4, as A, B, or C or 2 x B, etc
Chocolate(s)	What sort e.g. plain, milk, white, fancy, diabetic; type of filling;	Weight/size of bar
Coffee	With milk (see section on milk); half milk/half water; all milk; ground/filter, instant; decaffeinated. If café/takeaway, was it cappuccino, latte etc	Cups or mugs, size of takeaway e.g. small. medium
Cook-in sauces	What sort; pasta, Indian, Chinese, Mexican; tomato, white or cheese based; does meat or veg come in sauce; jar or can	Spoons, size of can or jar
Cream	Single, whipped, double or clotted; dairy or non-dairy; low-fat; fresh, UHT/Longlife; imitation cream e.g. Elmlea	Spoons
Crisps	What sort e.g. potato, corn, wheat, maize, vegetable etc; low-fat or low-salt; premium variety e.g. Kettle chips, Walker's Sensations	Packet weight, standard or from multipack
Custard	Pouring custard or egg custard; made with powder and milk/sugar, instant, ready to serve (tinned or carton); low fat, sugar free	Spoons
Egg	Boiled, poached, fried, scrambled, omelette (with or without filling); type of oil/fat, milk added	Number of eggs, large, medium or small
Fish (including canned)	What sort e.g. cod, tuna; fried (type of oil/fat), grilled, poached (water or milk) or steamed; with batter or breadcrumbs; canned in oil, brine or tomato sauce	Size of can or spoons (for canned fish) or picture 7 for battered fish

Food/Drink	Description & Preparation	Portion size or quantity
Fish cakes & fish fingers	Type of fish; plain or battered or in breadcrumbs; fried, grilled, baked or microwaved; economy	Size, number, packet weight
Fruit - fresh	What sort; eaten with or without skin	Small, medium or large
Fruit - stewed/canned	What sort; sweetened or unsweetened; in fruit juice or syrup; juice or syrup eaten	Spoons, weight of can
Fruit – juice (pure)	What sort e.g. apple, orange; sweetened or unsweetened; pasteurised or UHT/Longlife; freshly squeezed; added vitamins/minerals, omega 3	Glass (size or volume) or carton size
Ice cream	Flavour; dairy or non-dairy alternatives e.g. soya; luxury/premium	Spoons/ scoops
Jam, honey	What sort; low-sugar/diabetic; shop bought/brand or homemade	Spoons, heaped or level, or thin or thick spread
Marmalade	Type; low-sugar; thick cut; shop bought/brand or homemade	Spoons, heaped or level, or thin or thick spread
Meat (see also bacon, burgers & sausages)	What sort; cut of meat e.g. chop, breast, minced; lean or fatty; fat removed or eaten; skin removed or eaten; how cooked; with or without gravy	Large/small/medium, spoons, or picture 6 for stew portion
Milk	What sort; whole, semi-skimmed, skimmed or 1% fat; fresh, sterilized, UHT, dried; soya milk (sweetened/unsweetened), goats' milk, rice milk, oat milk; flavoured; fortified with added vitamins and/or minerals	Pints, glass (size or volume) or cup. On cereal: *damp/normal/ drowned*. In tea/coffee: *a little/some/a lot*

Food/Drink	*Description & Preparation*	*Portion size or quantity*
Nuts	What sort; dry roasted, ordinary salted, honey roasted; unsalted	Packet weight, handful
Pie (sweet or savoury)	What sort/filling; one pastry crust or two; type of pastry	Individual or slice, or picture 8
Pizza	Thin base/deep pan or French bread; topping e.g. meat, fish, veg; stuffed crust	Individual, slice, fraction of large pizza e.g. ¼
Porridge	Made with oats or cornmeal or instant oat cereal; made with milk and/or water; added sugar, honey, syrup or salt; with milk or cream	Bowls, spoons
Potatoes (see also chips)	Old or new; baked, boiled, roast (type of oil/fat); skin eaten; mashed (with butter/spread and with or without milk); fried/chips (type of oil/fat); instant; any additions e.g. butter	Mash – spoons, number of half or whole potatoes, small or large potatoes
Pudding	What sort; e.g. steamed sponge; with fruit; mousse; instant desserts; milk puddings	Spoons, picture 10 for slice of sponge
Rice	What sort; e.g. basmati, easy cook, long or short grain; white or brown; boiled or fried (type of oil/fat)	Spoons or picture 2
Salad	Ingredients; if with dressing what sort (oil and vinegar, mayonnaise)	Amount of each component
Sandwiches and rolls	Type of bread/roll (see Bread & Rolls); butter or margarine; type of filling; including salad, mayonnaise, pickle etc. If shop-bought, where from?	Number of rolls or slices of bread; amount of butter/margarine (on both slices?); amount of filling
Sauce – cold (including mayonnaise)	Tomato ketchup, brown sauce, soy sauce, salad cream, mayonnaise; low fat;	Spoons

Food/Drink	Description & Preparation	Portion size or quantity
Sauce – hot (see also cook-in sauces)	What sort; savoury or sweet; thick or thin; for gravy - made with granules, stock cube, dripping or meat juices	Spoons
Sausages	What sort; e.g. beef, pork; fried (type of oil/fat) or grilled; low fat	Large or small, number
Sausage rolls	Type of pastry	Size - jumbo, standard, mini
Scone	Fruit, sweet, plain, cheese; type of flour; homemade	Small, medium or large
Savoury snacks - in packet	What sort: e.g. Cheddars, cheese straws, Twiglets, Pretzels	Size (standard or mini variety), packet weight
Smoothies	If homemade give recipe. If shop-bought, what does it contain e.g. fruit, milk/yoghurt, fruit juice	Glass or bottle (size or volume)
Soft drinks – squash/ concentrate/cordial	Flavour; no added sugar/low calorie/sugar free; "high" juice; fortified with added vitamins and/or minerals	Glass (size or volume)
Soft drinks – carbonated/fizzy	Flavour; diet/low-calorie; canned or bottled; cola – caffeine free	Glass, can or bottle (size or volume)
Soft drinks – ready to drink	Flavour; no added sugar/low calorie/sugar free; real fruit juice? If so, how much?; fortified with added vitamins and/or minerals	Glass, carton or bottle (size or volume)
Soup	What sort; cream or clear; fresh/chilled, canned, instant or vending machine. If home-made, give recipe	Spoons, bowl or mug
Spaghetti, other pasta	What sort; fresh/chilled or dried; white, wholemeal; canned in sauce; type of filling if ravioli, cannelloni etc	Spoons (or how much dry pasta) or picture 3

Food/Drink	*Description & Preparation*	*Portion size or quantity*
Spirits	What sort: e.g. whisky, gin, vodka, rum	Measures as in pub
Sugar	Added to cereals, tea, coffee, fruit, etc; what sort; e.g. white, brown, demerara	Heaped or level teaspoons
Sweets	What sort: e.g. toffees, boiled sweets, diabetic, sugar-free	Number, packet weight
Tea	With/without milk (see section on milk); decaffeinated, herb	Mugs or cups
Vegetables (not including potatoes)	What sort; how cooked/raw; additions e.g. butter, other fat or sauce	Spoons, number of florets or sprouts, weight from tins or packet
Wine, sherry, port	White, red; sweet, dry; % alcohol or low-alcohol	Glass (size or volume)
Yoghurt (inc drinking yoghurt), fromage frais	What sort: e.g. natural/plain or flavoured; creamy, Greek, low-fat, very low fat/diet, soya; with fruit pieces or fruit flavoured; twinpot; fortified with added vitamins and/or minerals; longlife/UHT; probiotic	Pot size or spoons
Home-made dishes	Please say what the dish is called (record recipe or details of dish if you can in the section provided) and how many persons it serves	Spoons – heaped or level, number, size
Ready-made meals	Full description of product; does it contain any accompaniments e.g. rice, vegetables, sauces; chilled or frozen; microwaved, oven cooked, boil-in-the-bag; low fat, healthy eating range. Enclose label and ingredients list if possible in your plastic bag	Packet weight (if didn't eat whole packet describe portion consumed)
Take-away food or food eaten out	Please say what the dish is called and give main ingredients if you can. Give name of a chain restaurant e.g. McDonalds	Spoons, portion size e.g. small/medium/large

Pictures for food portion size guidance

Use the pictures to help you indicate the size of the portion you have eaten.
Write on the food record the picture number and size A, B or C nearest to your own helping.

Remember that the pictures are much smaller than life size.
The actual size of the dinner plate is 10 inches (25cm), the side plate, 7 inches (18cm), and the bowl, 6.3 inches (16cm).

1. Breakfast cereals

2. Rice

3. Spaghetti

4. Chips

5. Broccoli/ cauliflower

6. Stew /curry

7. Battered fish

8. Quiche / Pie

9. Cheese

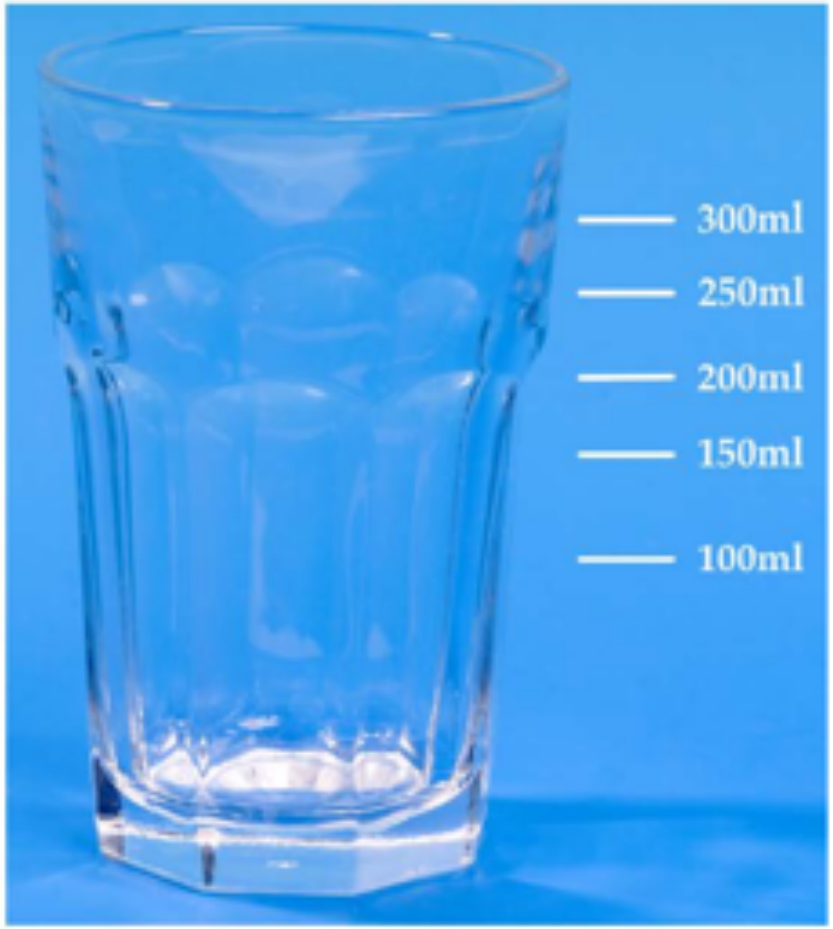
10. Sponge cake

Drink volume guidance - Typical quantities of drinks in various containers measured in millimetres (ml)

	Small glass	Average (medium) glass	Large glass	Vending cup	Cup	Mug
Soft drinks	150	200	300			
Wine	125	175	250			
Hot drinks				170	190	260

Glasses come in different shapes and sizes. On the next page is a life size glass showing approximate volumes. You can use this to estimate how much you have consumed.

Life Size Glass



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The 4-day diary

Day 1:		Date:		
Time	Where? With whom? TV on? At table?	Food/Drink description & preparation	Brand name	Portion size and quantity eaten (e.g. half of it/all of it etc.)
6am to 9am				
9am to 12 noon				

Time	Where? With whom? TV on? At table?	Food/Drink description & preparation	Brand name	Portion size and quantity <u>eaten</u> (e.g. half of it/all of it etc.)
12 noon to 2pm				
2pm to 5pm				

Time	Where? With whom? TV on? At table?	Food/Drink description & preparation	Brand name	Portion size and quantity <u>eaten</u> (e.g. half of it/all of it etc.)
5pm to 8pm				
8pm to 10pm				
10pm to 6am				

Was the amount of **food** that you had today about what you usually have, less than usual, or more than usual?

Yes, usual ☐ No, **less** than usual ☐ No, **more** than usual ☐

Please tell us why you had less than usual

Please tell us why you had more than usual

Was the amount you had to **drink** today, including water, tea, coffee and soft drinks [and alcohol], about what you usually have, less than usual, or more than usual?

Yes, usual ☐ No, **less** than usual ☐ No, **more** than usual ☐

Please tell us why you had less than usual

Please tell us why you had more than usual

Did you **finish all the food and drink** that you recorded in the diary today?

Yes ☐ No ☐

If no, please **go back to the diary and make a note of any leftovers**

Please record on the next pages any recipes or (if not already described) ingredients of made up dishes or take-away dishes.

Write in recipes or ingredients of made up dishes or take-away dishes			
NAME OF DISH:		***Serves:***	
Ingredients	***Amount***	***Ingredients***	***Amount***
Brief description of cooking method			

Write in recipes or ingredients of made up dishes or take-away dishes			
NAME OF DISH:		***Serves:***	
Ingredients	***Amount***	***Ingredients***	***Amount***
Brief description of cooking method			

Day 2:		**Date:**		
Time	**Where? With whom? TV on? At table?**	**Food/Drink description & preparation**	**Brand name**	**Portion size and quantity <u>eaten</u> (e.g. half of it/all of it etc.)**
6am to 9am				
9am to 12 noon				

Time	Where? With whom? TV on? At table?	Food/Drink description & preparation	Brand name	Portion size and quantity <u>eaten</u> (e.g. half of it/all of it etc.)
12 noon to 2pm				
2pm to 5pm				

Time	Where? With whom? TV on? At table?	Food/Drink description & preparation	Brand name	Portion size and quantity <u>eaten</u> (e.g. half of it/all of it etc.)
5pm to 8pm				
8pm to 10pm				
10pm to 6am				

Was the amount of **food** that you had today about what you usually have, less than usual, or more than usual?

Yes, usual ☐ No, **less** than usual ☐ No, **more** than usual ☐

Please tell us why you had less than usual

Please tell us why you had more than usual

Was the amount you had to **drink** today, including water, tea, coffee and soft drinks [and alcohol], about what you usually have, less than usual, or more than usual?

Yes, usual ☐ No, **less** than usual ☐ No, **more** than usual ☐

Please tell us why you had less than usual

Please tell us why you had more than usual

Did you **finish all the food and drink** that you recorded in the diary today?

Yes ☐ No ☐

If no, please **go back to the diary and make a note of any leftovers**

Please record on the next pages any recipes or (if not already described) ingredients of made up dishes or take-away dishes.

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Ingredients	***Amount***	***Ingredients***	***Amount***
Brief description of cooking method			

Write in recipes or ingredients of made up dishes or take-away dishes			
NAME OF DISH:		***Serves:***	
Ingredients	***Amount***	***Ingredients***	***Amount***
Brief description of cooking method			

Day 3:		Date:		
Time	Where? With whom? TV on? At table?	Food/Drink description & preparation	Brand name	Portion size and quantity eaten (e.g. half of it/all of it etc.)
6am to 9am				
9am to 12 noon				

Time	Where? With whom? TV on? At table?	Food/Drink description & preparation	Brand name	Portion size and quantity <u>eaten</u> (e.g. half of it/all of it etc.)
12 noon to 2pm				
2pm to 5pm				

Time	Where? With whom? TV on? At table?	Food/Drink description & preparation	Brand name	Portion size and quantity <u>eaten</u> (e.g. half of it/all of it etc.)
5pm to 8pm				
8pm to 10pm				
10pm to 6am				

Was the amount of **food** that you had today about what you usually have, less than usual, or more than usual?

Yes, usual ☐ No, **less** than usual ☐ No, **more** than usual ☐

Please tell us why you had less than usual

Please tell us why you had more than usual

Was the amount you had to **drink** today, including water, tea, coffee and soft drinks [and alcohol], about what you usually have, less than usual, or more than usual?

Yes, usual ☐ No, **less** than usual ☐ No, **more** than usual ☐

Please tell us why you had less than usual

Please tell us why you had more than usual

Did you **finish all the food and drink** that you recorded in the diary today?

Yes ☐ No ☐

If no, please **go back to the diary and make a note of any leftovers**

Please record on the next pages any recipes or (if not already described) ingredients of made up dishes or take-away dishes.

Write in recipes or ingredients of made up dishes or take-away dishes			
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Ingredients	***Amount***	***Ingredients***	***Amount***
Brief description of cooking method			

Write in recipes or ingredients of made up dishes or take-away dishes			
NAME OF DISH:		***Serves:***	
Ingredients	***Amount***	***Ingredients***	***Amount***
Brief description of cooking method			

Day 4:		Date:		
Time	Where? With whom? TV on? At table?	Food/Drink description & preparation	Brand name	Portion size and quantity eaten (e.g. half of it/all of it etc.)
6am to 9am				
9am to 12 noon				

Time	Where? With whom? TV on? At table?	Food/Drink description & preparation	Brand name	Portion size and quantity <u>eaten</u> (e.g. half of it/all of it etc.)
12 noon to 2pm				
2pm to 5pm				

Time	Where? With whom? TV on? At table?	Food/Drink description & preparation	Brand name	Portion size and quantity <u>eaten</u> (e.g. half of it/all of it etc.)
5pm to 8pm				
8pm to 10pm				
10pm to 6am				

Was the amount of **food** that you had today about what you usually have, less than usual, or more than usual?

Yes, usual ☐ No, **less** than usual ☐ No, **more** than usual ☐

Please tell us why you had less than usual

Please tell us why you had more than usual

Was the amount you had to **drink** today, including water, tea, coffee and soft drinks [and alcohol], about what you usually have, less than usual, or more than usual?

Yes, usual ☐ No, **less** than usual ☐ No, **more** than usual ☐

Please tell us why you had less than usual

Please tell us why you had more than usual

Did you **finish all the food and drink** that you recorded in the diary today?

Yes ☐ No ☐

If no, please **go back to the diary and make a note of any leftovers**

Please record on the next pages any recipes or (if not already described) ingredients of made up dishes or take-away dishes.

Write in recipes or ingredients of made up dishes or take-away dishes			
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Ingredients	***Amount***	***Ingredients***	***Amount***
Brief description of cooking method			

Write in recipes or ingredients of made up dishes or take-away dishes			
NAME OF DISH:		***Serves:***	
Ingredients	***Amount***	***Ingredients***	***Amount***
Brief description of cooking method			

Questionnaire | Time : ±10 min

SarQoL
Sarcopenia and Quality of Life

Quality of life in sarcopenia

This questionnaire asks about sarcopenia, which is a muscle weakness that comes about with ageing. Sarcopenia can affect your daily life. This survey will enable us to find out if the state of your muscles currently affects your quality of life.

Please choose the most appropriate response for each question. The questionnaire should take you approximately 10 minutes to complete.

1. Do you currently feel you have a reduction in:

	A lot	Some	A little	None
The strength in your arms?	☐	☐	☐	☐
The strength in your legs?	☐	☐	☐	☐
Your muscle mass?	☐	☐	☐	☐
Your energy?	☐	☐	☐	☐
Your physical capabilities?	☐	☐	☐	☐
Your general flexibility?	☐	☐	☐	☐

2. Do you have pain in your muscles?

- ☐ Often
- ☐ Sometimes
- ☐ Rarely
- ☐ Never

3. When undertaking light physical activities (walking slowly, doing the ironing, dusting, washing-up, DIY, watering the garden, etc.), do you:

	Often	Occasionally	Rarely	Never	I do not undertake these types of physical activities
Have difficulty?	☐	☐	☐	☐	☐
Get tired?	☐	☐	☐	☐	☐
Experience pain?	☐	☐	☐	☐	☐

4. When undertaking moderate physical activities (fast walking, cleaning windows, hoovering, washing the car, pulling up weeds in the garden, etc.), do you:

	Often	Occasionally	Rarely	Never	I do not undertake these types of physical activities
Have difficulty?	☐	☐	☐	☐	☐
Get tired?	☐	☐	☐	☐	☐
Experience pain?	☐	☐	☐	☐	☐

5. When undertaking intense physical activities (running, hiking, lifting heavy objects, moving furniture, digging the garden, etc.), do you:

	Often	Occasionally	Rarely	Never	I do not undertake these types of physical activities
Have difficulty?	☐	☐	☐	☐	☐
Get tired?	☐	☐	☐	☐	☐
Experience pain?	☐	☐	☐	☐	☐

6. Do you currently feel old?

- ☐ Yes, very
- ☐ Yes, somewhat
- ☐ Yes, a little
- ☐ No, not at all

7. If yes to question 6, what gives you that impression?

(Choose as many answers as you like)

- ☐ I become unwell easily
- ☐ I take many medications
- ☐ I feel a weakness in my muscles
- ☐ I have problems with my memory
- ☐ I've had to face the death of several people close to me
- ☐ I do not have much energy, I am often tired
- ☐ My eyesight is poor
- ☐ Other:

8. Do you feel physically weak?

- ☐ Yes, completely
- ☐ Yes, somewhat
- ☐ Yes, a little
- ☐ No, not at all

9. Do you feel you are limited in:

	A lot	Some	A little	None
The length of time you can walk for?	☐	☐	☐	☐
How often you go out walking?	☐	☐	☐	☐
The distance you can walk?	☐	☐	☐	☐
The speed at which you can walk?	☐	☐	☐	☐
The length of your steps?	☐	☐	☐	☐

10. When you are walking:

	Often	Occasionally	Rarely	Never	I am unable to walk
Do you feel very tired?	☐	☐	☐	☐	☐
Do you need to sit down regularly to recover?	☐	☐	☐	☐	☐
Do you have difficulty crossing roads quickly enough?	☐	☐	☐	☐	☐
Do you have difficulties with uneven surfaces?	☐	☐	☐	☐	☐

11. Do you have problems with your balance?

- ☐ Often
- ☐ Occasionally
- ☐ Rarely
- ☐ Never

12. How often do you fall?

- ☐ Very often
- ☐ Occasionally
- ☐ Rarely
- ☐ Never

13. Do you think that your physical appearance has changed?

- ☐ Yes, very
- ☐ Yes, somewhat
- ☐ Yes, a little
- ☐ No, not at all

14. If yes to question 13, in what way? (Choose as many answers as you like)

- ☐ Change in your weight (you've put on weight or you've lost weight)
- ☐ Appearance of wrinkles
- ☐ Loss of height
- ☐ Loss of muscle mass
- ☐ Hair loss
- ☐ Getting white or grey hair
- ☐ Other: _____

15. If yes to question 13, are you upset by this change?

- ☐ Yes, very
- ☐ Yes, somewhat
- ☐ Yes, a little
- ☐ No, not at all

16. Do you feel frail?

- ☐ Very much so
- ☐ A little
- ☐ Not at all

17. Do you currently have difficulty in undertaking any of the following daily activities:

	Unable to do	Great difficulty	A little difficulty	No difficulty	Not applicable
Climbing a flight of stairs?	☐	☐	☐	☐	☐
Climbing several flights of stairs?	☐	☐	☐	☐	☐
Going up one or several steps without holding on to the banister?	☐	☐	☐	☐	☐
Squatting or kneeling?	☐	☐	☐	☐	☐
Stooping or leaning down to pick up an object off the floor?	☐	☐	☐	☐	☐
Getting up from the floor without holding on to anything?	☐	☐	☐	☐	☐
Getting out of a low chair without armrests?	☐	☐	☐	☐	☐
Moving, generally, from a sitting position to a standing position?	☐	☐	☐	☐	☐
Carrying heavy objects (large bags full of shopping, saucepan filled with water, etc.)?	☐	☐	☐	☐	☐
Opening a bottle or a jar?	☐	☐	☐	☐	☐
Using public transport?	☐	☐	☐	☐	☐
Getting in or out of a car?	☐	☐	☐	☐	☐
Doing your shopping?	☐	☐	☐	☐	☐
Doing the housework (making the bed, hoovering, doing the ironing, washing the dishes, etc.)?	☐	☐	☐	☐	☐

18. Does your muscle weakness limit your movement?

- ☐ Yes, a lot
- ☐ Yes, somewhat
- ☐ Yes, a little
- ☐ No, not at all

19. If yes to question 18, for what reasons? (Choose as many answers as you like)

- ☐ Fear of pain
- ☐ Fear that you might not be able to
- ☐ Fear of feeling tired after these activities
- ☐ Fear of falling
- ☐ Other: _____

20. Does your muscle weakness limit your sex life?

- ☐ I am not sexually active
- ☐ Yes, completely
- ☐ Yes, somewhat
- ☐ Yes, a little
- ☐ No, not at all

21. How has your participation in physical activities/sport changed?

- ☐ Increased
- ☐ Decreased
- ☐ Unchanged
- ☐ I have never participated in physical activities or sports

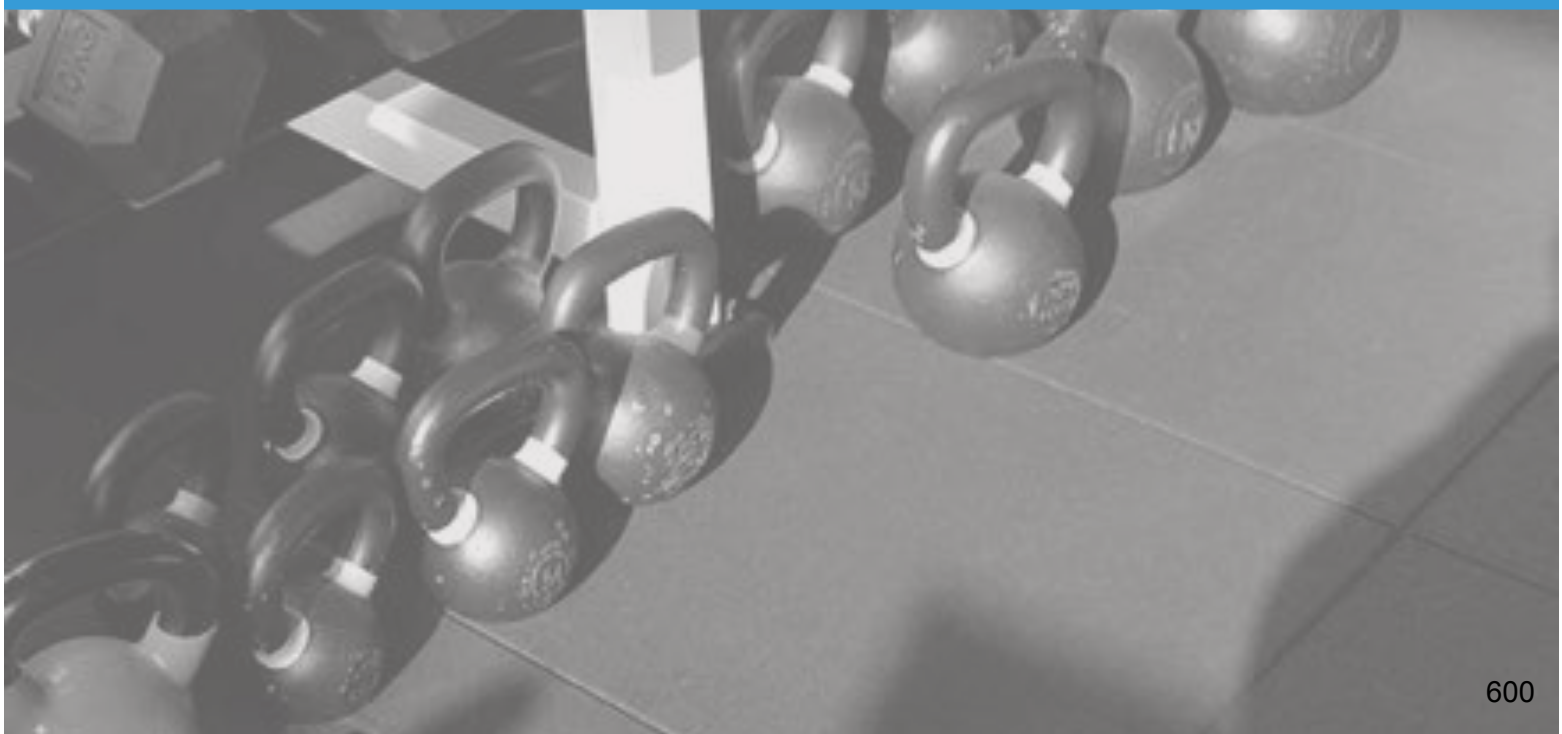
22. How has your participation in leisure activities (going out to eat, gardening, doing DIY, shooting/fishing, senior citizens clubs, playing bridge, going for a walk, etc.) changed?

- ☐ Increased
- ☐ Decreased
- ☐ Unchanged
- ☐ I have never participated in leisure activities

The PRiME Trial

Food and Exercise for a Healthier Heart!

EXERCISE GUIDE



Contact Information

Richard Kirwan *BSc MSc AFHEA*
Liverpool John Moores University
School of Natural Sciences and Psychology

Email: r.p.kirwan@2018.ljmu.ac.uk
Mobile: 07 565 427 663

This study was approved by the NHS Research Ethics Committee on DAY MONTH YEAR (Ref. XXX). If you any concerns regarding your involvement in this research, please discuss these with the researcher in the first instance. If you wish to make a formal complaint, please contact the Patient Complaints Manager at Liverpool Heart & Chest Hospital on 01516001257 and your communication will be dealt with as appropriate.

Contents

QUICK GUIDE

For the next twelve weeks you have been allocated to the exercise arm of the PRiME trial, which means that you will be asked to follow a resistance exercise program.

We will undertake a variety of assessments with you when you come to see us in our laboratory at the Tom Reilly Building at the Bryrom St. campus on two occasions, including blood samples, body composition, grip strength and dietary intake.

These guidance notes will give you some basic information on the exercise and type of exercises you will perform over the next 12 weeks.

In this booklet, you will also find the names and contact details of the people involved in this research.

THE EXERCISE

This guide will help you to better understand the exercise program and the type of exercises you will be doing over the next 12 weeks.

This is a RESISTANCE exercise program which uses free-weights (barbells and dumbbells) and weight machines to help increase strength and muscle quality. We believe this type of exercise may also be beneficial for people in cardiac rehabilitation.

SAFETY

All the exercises in this program have been deemed suitable for cardiac rehabilitation patients by our cardiac rehabilitation exercise experts.

You will receive full instruction in all the exercises you will do in this program from the cardiac rehabilitation certified trainers at your gym who have been working with you during your Phase 3 cardiac rehab. They will be present in the gym to support your training and to monitor your wellbeing during the training program.

Should you have any questions about your training program or exercise technique please feel free to speak with your trainers.

SHOULD YOU EVER FEEL UNWELL, LIGHT HEADED, OUT OF BREATH ETC. PLEASE LET YOUR TRAINERS KNOW IMMEDIATELY!

YOUR PROGRAM

For the next 12 weeks, you will complete a:

- full-body resistance (strength) training session
- 3 times a week

WHAT IS FULL BODY TRAINING?

Full-body training simply means that you will train a variety of muscle groups (chest, back, arms and legs) in one session.

HOW MANY EXERCISES WILL I DO?

Each session will consist of 6 different exercise including upper body pushes and pulls and lower body movements. These exercises may be different on each day of the week but each day will repeat weekly.

This means that if you train every Monday, you will do the same set of exercises every Monday for the 12 weeks of your program. This way you will know what to expect at every session.

WHAT ARE SETS AND REPS?

You will hear and read the terms “sets and reps” regularly during this program. These are terms used to describe the number of times you perform an exercise.

- A rep is the number of times you perform a specific exercise (lifting a weight once is “1 rep”)
- A set is a cycle of reps that you complete.

For example, suppose you complete 15 reps of a chest press. You would say you've completed "one set of 15 reps." A set can be any number of reps, so if you complete 10 reps of a bench press, you would say you've completed "one set of 10 reps," and if you complete just five reps, then that would be "one set of five reps."

HOW MUCH WEIGHT SHOULD I USE?

At your first session, the trainers at your gym will teach you how to do the exercises and they will also tell you how much weight you should start with, depending on how much weight you can lift relatively comfortably.

Throughout the program you should lift until **"near failure"** meaning until you can't lift the weight again without cheating on the movement. Your trainer will explain this fully but this is very, very important. To get the most out of this training you really need to train hard.

HOW MANY SETS AND REPS SHOULD I DO?

For each exercise you will perform:

- 1-4 sets of
- 8-12 reps

You will intially start doing only 1-2 sets of each exercise. Your first session will be to establish what your starting weights should be for each exxercise. The number of sets will increase weekly until you can do 4 sets and will remain at 4 sets for the remaining sessions.

WILL MY WEIGHTS, SETS AND REPS STAY THE SAME?

No! The objective of this program is to increase the weight gradually over time as your muscles get used to the weights you lift, with the objective of gaining strength over time.

For example, when you start, you may use 20kg on chest press.

- Your first week, you will do 1-2 sets of 8-12 reps
- Once you can do both sets for 12 reps, you will add another set
- When you can do all 3 sets for 12 reps, you will add another set
- When you can do all 4 sets for 12 reps, you will increase the weight on the machine to the next level (for example 25kg)
- The reps you can do with each set will probably drop but you won't increase the weight again until you can do all 4 sets for 12 reps

A & B ROUTINES

Your training program will consist of 2 separate routines (A & B) with two different sets of exercises. You will alternate these 2 routines every time you are in the gym. Your routine will look a little like this:

	DAY 1	DAY 2	DAY 3
WEEK 1	A	B	A
WEEK 2	B	A	B

This alternating routine will allow you to use a variety of exercises and fully develop your strength over the course of the training program.

ROUTINE A	ROUTINE B
Leg Press Deadlift Machine	Leg Extension Leg Curl
Chest Press Machine Row	Chest Fly Cable Row
Dumbbell Shoulder Press Pronated Lat Pull-down	Shoulder Press Machine Supinated Lat Pull-down

-------- Exercises in dotted boxes are superset pairs

HOW TO DO YOUR ROUTINE

There are three ways you can do your routine:

1. One at a time: doing all sets of one exercise until complete and then moving onto the next exercise
2. Supersets of two exercises: doing two exercises (a superset pair), one after another until all sets are complete (THIS MAY BE THE FASTEST WAY TO DO ALL THE EXERCISES)
3. As a circuit: doing all exercises, one after another, one set at a time

For example, each number in the following chart represents a different exercise and shows how you can do them in different orders.

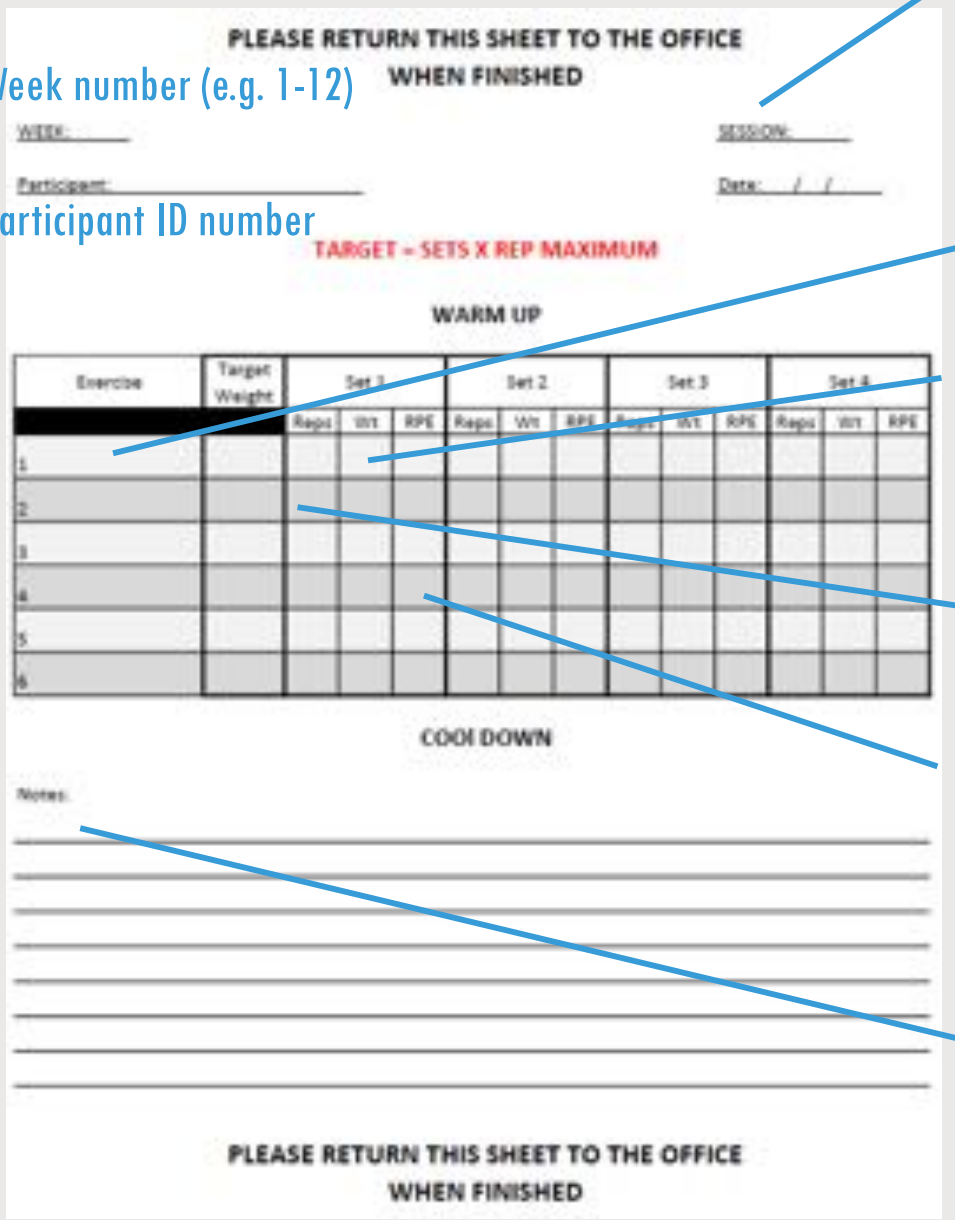
ONE AT A TIME	SUPERSET	CIRCUIT
1	1	1
1	2	2
1	1	3
1	2	4
2	1	5
2	2	6
2	1	1
2	2	2
3	3	3
3	4	4
3	3	5

HOW TO RECORD YOUR PROGRESS

Every gym session you do you will need to record the weight you lift and the amount of sets and reps you do.

THESE NUMBERS ARE INCREDIBLY IMPORTANT AND HAVE A LARGE EFFECT ON THE RESULTS OF THIS STUDY.

At the start of every session you will get a piece of paper from the trainer at the gym, similar to the image below, and can fill it in during your training session. Once finished, please hand it pack to the trainer.



PLEASE RETURN THIS SHEET TO THE OFFICE WHEN FINISHED

WEEK: _____ SESSION: _____

Participant: _____ Date: / /

TARGET = SETS X REP MAXIMUM

WARM UP

Exercise	Target Weight	Set 1			Set 2			Set 3			Set 4		
		Reps	Wt	RPE	Reps	Wt	RPE	Reps	Wt	RPE	Reps	Wt	RPE
1													
2													
3													
4													
5													
6													

COOL DOWN

Notes:

PLEASE RETURN THIS SHEET TO THE OFFICE WHEN FINISHED

Week number (e.g. 1-12)

Weekly session number (e.g. 1,2 or 3)

Participant ID number

Write exercise name here

Write weight used in this column (this should be the same for all sets)

Write reps achieved in this column

Write relative perceived exertion here (how hard was the set on a scale of 1-10)

Any notes from this session e.g. any pain or discomfort, lack of sleep from night before etc.

LIVERPOOL JOHN MOORES UNIVERSITY

Liverpool Heart and Chest Hospital NHS
NHS Trust

PLEASE RETURN THIS SHEET TO THE OFFICE WHEN FINISHED

WEEK: _____ SESSION: _____

Participant: _____ Date: / /

TARGET = SETS X REP MAXIMUM

WARM UP

Exercise	Target Weight	Set 1			Set 2			Set 3			Set 4		
		Reps	Wt	RPE	Reps	Wt	RPE	Reps	Wt	RPE	Reps	Wt	RPE
1													
2													
3													
4													
5													
6													

COOl DOWN

Notes:

PLEASE RETURN THIS SHEET TO THE OFFICE WHEN FINISHED

The PRiME Trial

Food and Exercise for a Healthier Heart!

GUIDE BOOK

Contact Information

Richard Kirwan *BSc MSc AFHEA*
Liverpool John Moores University
School of Natural Sciences and Psychology

E: r.p.kirwan@2018.ljmu.ac.uk
M: 07 565 427 663

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Contents

Contents

QUICK GUIDE

For the next twelve weeks you have been allocated to Group B of the PRiME trial, which means that you will be asked to follow a high protein, cardioprotective eating plan.

We will undertake a variety of assessments with you when you come to see us in our laboratory at the Tom Reilly Building at the Bryrom St. campus on two occasions, including blood samples, body composition, grip strength and dietary intake.

These guidance notes will give you further information on which types of food to eat more or less of with this plan and how to read food labels and become aware of the nutritional content of out-of-home cooked meals to support your efforts to stick to this eating plan.

In this booklet, you will also find the names and contact details of the people involved in this research.

PART 1: INTRODUCTION

Hello and welcome to the PRiME trial! We're very happy to have you with us!

This booklet has been designed to be your guide over the next twelve weeks while you are part of our study group. It will support you to consume healthy food whether you are cooking at home, buying ready-made meals or eating out. You can enjoy delicious, healthy meals in all of these situations.

We will also want to give you information on why we're doing our study and what to expect during your visits to the university. You'll also learn about what will happen when the study has finished and we have analysed the results.

This means that not only will you be helping very important research but you can also find out how you and your body did over the course of these twelve weeks and how your body composition, blood profile, and strength might have been affected.

We hope that you will find your time on the study interesting, inspiring, motivating and delicious.

WHAT DO WE WANT TO FIND OUT?

As someone who is attending cardiac rehabilitation (cardiac rehab) you have learned that you can help to reduce your risk of having further heart and circulation related problems by making changes to your food and exercise. With our research we want to improve cardiac rehab and make the food and exercise recommendations more specific and more effective.

You have been selected for this study because you have a specific body composition; lower muscle mass and higher fat mass which may put you at a slightly greater risk of heart issues. Firstly, we want to find out if we can increase your muscle mass through food and exercise. Then we want to see if that will improve other important markers of heart health including cholesterol, blood lipids and sugar and grip strength.

Different foods and different types of exercise can have very different effects on health and that's why this research is so important. We want to know what type of food and exercise will have the greatest effect on improving the health of people in cardiac rehab. Once we know that we can potentially make changes to the current cardiac rehab guidelines in the UK allowing everyone in cardiac rehab to benefit from this information.

Thank you for helping us with this important research.

WHAT TO EXPECT DURING YOUR VISITS TO THE LAB

We will ask you to come to the Tom Reilly Building in Liverpool John Moores University, Byrom St. campus (L3 3AF) for two visits (Please see Page 38 for directions):

1. right before you start your food and exercise guidelines and...
2. 12 weeks later, at then end of the study period.

Each visit is expected to last between 60 and 90 minutes and will include:

A venous blood sample

We will take about 8 teaspoons of blood.

Please note that you will have to have fasted for at least 12 hours before your appointment or your blood profile will be different from than normal – painting a wrong picture of how the food and exercise is working for you.
You will also have to avoid drinking alcohol or doing any strenuous exercise the night before. Again both can have an impact on your blood profile! We will analyse this blood sample at the end of the study to see how any risk factors for heart disease might have changed over the course of twelve weeks.

Assessing your body composition

This will be done in a number of different ways.

Firstly, we will use a tape measure to measure your waist circumference, hip circumference, thigh circumference, calf circumference and neck circumference as these are all sites on the human body that can give us clues about the overall distribution of body fat. Please ensure that you bring a pair of shorts with you to these visits as we will ask you to change into these before we take these measurements. If you prefer for a team member of the same sex to take these, please do let us know so that we can do this for you.

Secondly, we will ask you to step onto body composition scales (far bigger than the common bathroom ones) and measure your lean body mass, your body fat mass and the amount of fat surrounding your organs.

Finally, we will ask you to lie down on the table of a special device called a DXA for 15 minutes. This device uses a small amount of radiation to measure the body fat, muscle and bone in your body and actually creates an image of what this looks like.

Taking your blood pressure

As blood pressure has been found to be an important factor in cardiometabolic health, we will assess your blood pressure every time you come to see us in our labs. Following standard protocol, we will take your blood pressure three times at each appointment and calculate the average of these three.

Measuring your strength

Muscle strength is a good indicator of general health and we can use it to determine the effects of your food and exercise program. We will measure it in two ways:

- Grip Strength: we will ask you to hold a grip testing machine and squeeze it as hard as you can. We will do this three times and calculate the average of these three.

- Back Strength: we will ask you to step on a special matt that has a cable and handle attached to it. We will then ask you to bend over, grip the cable and then stand up erect to pull the cable as hard as you can. We will do this three times and calculate the average of these three.

Going through a couple of brief questionnaires with you and conducting one final interview

During your first and your final visit we will go through a check list to see which types of foods you have consumed over the previous four weeks.

We will also conduct a brief interview to check whether you have experienced any so-called adverse events over the study period whilst using the food and exercise program.

During your final visit we will also ask to stay with us for a little longer to conduct a brief interview with you asking you about your experiences with your food and exercise program.

BEFORE YOUR LAB APPOINTMENTS - FOOD DIARY

Just before your first and last visit to the lab, we will give you a template for a three-day food diary. You can pick this up at the gym where you do your cardiac rehab. You will need to complete this for three days before your lab appointment (two weekdays and a one weekend day).

This will not take long to complete. Please bring this food diary with you to your lab appointment and pass it on to our research team.

DURING THE STUDY

During the 12 weeks of the study period, every two weeks you will receive a brief phone call from one of the investigators. This will be to see how you are getting on with your food and exercise program, to answer any questions you might have and to give advice about how to better stick to your food and exercise program.

STUDY FLOW CHART

WEEK BEFORE FIRST LAB VISIT

- Complete 3-day food diary

FIRST LAB VISIT

- Provide blood sample
- Undergo body composition assessment
- Take blood pressure
- Measure strength

DURING 12 WEEK STUDY

- Follow food recommendations
- Follow exercise program 3 days per week
- Speak with investigators every 2 weeks

WEEK BEFORE LAST LAB VISIT

- Complete 3-day food diary

FINAL LAB VISIT

- Provide blood sample
- Undergo body composition assessment
- Take blood pressure
- Measure strength
- Complete interview on food and exercise experience and report adverse effects

YOUR APPOINTMENT SCHEDULE

Please write the dates of your visits here
once you have been told by the research team

COLLECT FIRST FOOD DIARY TEMPLATE FROM GYM	
FIRST LAB VISIT	
COLLECT LAST FOOD DIARY TEMPLATE FROM GYM	
FINAL LAB VISIT	

PART 2: THE FOOD

This guide will help you to follow what is known as a high-protein, cardioprotective eating plan for the next twelve weeks.

This eating plan has certain features that have been shown in other studies to be beneficial for heart and muscle health. We have summarised those features here.

	EAT MORE	EAT LESS
DAILY	• Olive Oil (4 or tablespoons/day) • Vegetables (5 or more servings/day) • Fruit (3 or more servings/day) • Lean meat (instead of fatty or processed meat)	• Sugar-sweetened drinks (less than 1 serving/day) • Butter, margarine or cream (less than 1 serving/day) • Fatty and processed meat (less than 1 serving/day)
WEEKLY	• Legumes (3 or more servings)/week) • Fish or Shellfish (3 or more servings/week) • Nuts (3 or more servings/week) • Tomato-based sauces with Garlic/Onion (2 or more servings/week)	• Bakery products, sweets, pastries or ice-cream (less than 3 servings/week)

EAT MORE

OLIVE OIL

WHY?

Olive oil is a very healthy fat which has been shown to benefit heart health. You can use it in cooking and for dressing salads and vegetables.

HOW MUCH?

<u>Aim for 4 tablespoons a day</u>

You can do this by making olive oil your main cooking oil, adding it to salads or pouring it over cooked vegetables.

Cooking Oil

Olive Oil

Dressing Salads

4 tablespoons a day

Roasting Vegetables

EAT MORE

LEAN PROTEIN

WHY?

Protein is needed for the growth of muscle and recovery from exercise. Lean proteins are full of nutrients and also keep you feeling fuller, longer

HOW MUCH?

<u>Aim for 3-4 large servings per day</u>

-lean beef (fillet steak, stewing steak, round steak, 5% lean mince)
-lean pork (pork loin, 5% pork mince)
-chicken/turkey breast, 5% mince
-fish (cod, haddock, whiting, , sardines, plaice, mackerel, salmon, trout, tuna)
-low fat dairy (fat-free greek yoghurt, quark cheese, cottage cheese)
-eggs

3-4 eggs

120-140g Lean (<5% fat) beef or pork

250-300g Low Fat Yoghurts/Cheese

130-150g fish
*3 servings per week

100-130g Poultry (Chicken/Turkey Breast)

*BUYING "REDUCED PRICE" MEATS (YELLOW STICKERS) IN SUPERMARKETS AND FREEZING THEM CAN BE A VERY EFFECTIVE WAY TO SAVE MONEY ON MEAT PRODUCTS!

EAT MORE

VEGETABLES

WHY?

Vegetables are full of vitamins, minerals and other substances that we need to be healthy. They keep our digestive system healthier and keep us feeling full for longer

HOW MUCH?

<u>Aim for 5 servings a day</u>
(1 serving is half a cup)
Have at least two servings (1 cup) with each meal. Raw vegetables are fantastic too.
Aim to eat many different coloured vegetables during the week.
<u>Frozen and canned vegetables are perfect too.</u>
**Starchy vegetables like potatoes, sweet potatoes and sweet corn don't count.*

EAT MORE

FRUIT

WHY?

Fruits are also full of vitamins, minerals and important nutrients. They keep us feeling full for longer and are useful for dealing with cravings for sweets.

HOW MUCH?

<u>Aim for 3 servings a day</u>

(1 serving is half a cup or 1 small-medium fruit)

Have a serving fruit with each meal as a dessert or as a snack between meals.

Frozen fruit is perfect but **tinned fruit in syrup is not.*

EAT MORE

LEGUMES (Beans/Peas /Lentils)

WHY?

Legumes are a great source of plant protein, vitamins, minerals and fibre and has many health benefits especially for your digestive system.

HOW MUCH?

<u>Aim for 3 servings a week</u>

(1 serving is 150g /1 cup /half a tin)

You can eat legumes as a side dish, add them to soups and stews.

Tinned and frozen legumes are great and convenient

EAT MORE

NUTS

WHY?

Nuts are a good source of healthy fats, vitamins, minerals and fibre which can all benefit heart health.

HOW MUCH?

<u>Aim for 3 servings a week</u>

(1 serving is 30g or 1 small handful)

Raw nuts are best but roasted are ok.

Try to avoid nuts that are heavily salted or coated in sugar/chocolate

** Be careful not to overeat them.*

EAT MORE

TOMATO-BASED SAUCES

WHY?

Tomatoes contain a high amount of antioxidant nutrients which are particularly easy to absorb when it is cooked into a sauce especially with onions/garlic/herbs.

HOW MUCH?

<u>Aim for 2 servings a week</u>

(1 serving is half a cup)

Supermarket jars of Italian-style tomato sauces are convenient and great ways to prepare meat and vegetables.

** Avoid sauces with added cheese or cream*

EAT MORE

FISH & SEAFOOD

WHY?

Fish & seafood are high in protein which helps with muscle growth. Oily fish also contains good fats which can improve heart health

HOW MUCH?

<u>Aim for 3 servings a week</u>
(1 serving is 100-150g of fish or 200g of shellfish)
<u>Frozen and canned fish and seafood are cheap and convenient too.</u>
Tinned fish in tomato sauce also counts as one of your servings of tomato-based sauce .
** Avoid breaded and deep fried fish (Baked is ok)*

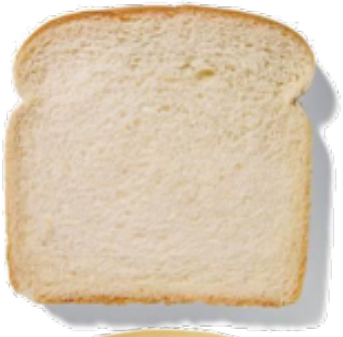
WHOLEGRAIN SWAPS

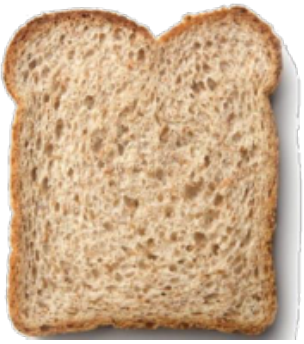
Refined Carbohydrates

Unrefined/Wholegrain Carbohydrates

WHY?

Unrefined carbohydrates and wholegrains are higher in fibre, vitamins and minerals and have been shown to improve heart health





EAT LESS OF THESE

Fatty & Processed Meats

Less than once/day

Butter, Margarine or Cream

Less than once/day

Commercial Pastries, Sweets & Ice-cream

Less than 3 times/week

Sugar Sweetened Drinks

Less than once/day

EAT THESE INSTEAD

Below 5% Fat Alternatives

Olive Oil & Olive Oil Spray

Fruit & Low-Fat Dairy Desserts

Water, Tea, Coffee & Sweeteners

*BUYING "REDUCED PRICE" MEATS (YELLOW STICKERS) IN SUPERMARKETS AND FREEZING THEM CAN BE A VERY EFFECTIVE WAY TO SAVE MONEY ON MEAT PRODUCTS!

A QUICK GUIDE TO PROTEIN AMOUNTS

You may have realised that we are putting a big emphasis on protein in this study and that's because we think it's very important for heart health.

We would like you to **aim for 40g of protein with each meal** and you can do that with a mix of different protein sources.

The following pages will show you how much protein is in common portions of food.

FOOD/PROTEIN (g)		FOOD/PROTEIN (g)	
	7g		4g
1 individual pot of fat-free GREEK style yoghurt (125g) **ALDI**		1 individual pot of light probiotic yoghurt (100g) **DANONE ACTIVIA**	
	7g		20g
1 individual pot of light yoghurt (175g) **MULLER LIGHT**		1 individual pot of fruit-flavoured Quark (200g) **ARLA**	

FOOD/PROTEIN (g)

8g

1 glass semi-skimmed milk
(250ml)
ALL BRANDS

7g

1 slice reduced-fat cheddar cheese
(25g)
TESCO

7g

1 large egg
(60g)
ALL BRANDS

FOOD/PROTEIN (g)

25g

1 bottle Protein chocolate
flavoured milk (480ml)
ARLA

9g

1/3 of a tub fat-free cottage cheese
(100g)
TESCO

11g

1/3 of a tub fat-free GREEK yoghurt
(166g)
ALDI

FOOD/PROTEIN (g)

10g

1 SKINNY pork sausage
(60g)
ALDI

35g

1 fillet steak
(170g)
ALL BRANDS

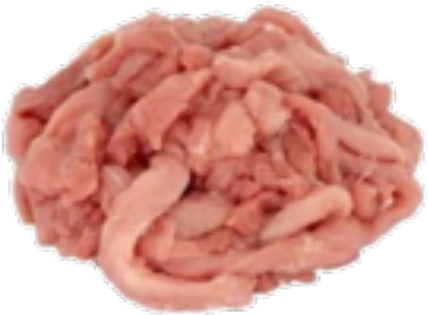
23g

1 medium salmon fillet
(94g)
ALL BRANDS

FOOD/PROTEIN (g)

36g

1 small chicken breast
(150g)
ALL BRANDS



41g

1 serving lean pork stir fry
(130g)
ALL BRANDS

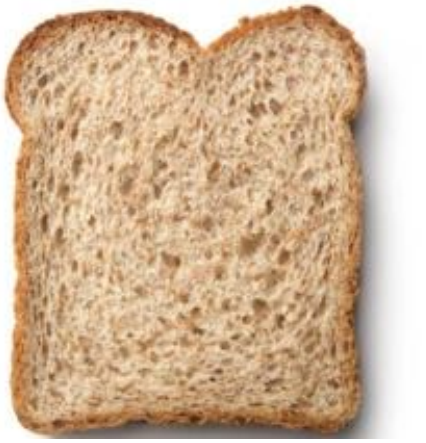
25g

1 medium cod fillet
(140g)
ALL BRANDS

FOOD/PROTEIN (g)

23g

1 small tin wild Alaskan Salmon (105g)
JOHN WEST



5g

1 slice wholemeal bread (44g)
ALDI

9g

½ tin of baked beans in tomato sauce (207g)
ALL BRANDS

FOOD/PROTEIN (g)

20g

1 small tin sardines in tomato sauce (120g)
ALL BRANDS

3g

1 serving porridge oats (30g)
ALL BRANDS

7g

1 serving of almonds/peanuts (30g)
ALL BRANDS

BUILDING A HIGH PROTEIN MEAL

You can combine multiple sources of protein together in a meal to help you get about 40g of protein. You can also add, wholegrains, legumes, fruit and vegetables to get a more complete meal.

Here are a few examples of how you can do that when eating at home.

Breakfast

Porridge made with milk, topped with banana & almonds and a yoghurt

1 serving porridge oats (30g)

3g

+

1 glass semi-skimmed milk (250ml)

8g

+

1 individual pot of fruit-flavoured Quark (200g)

20g

+

1 serving of almonds/peanuts (30g)

7g

+

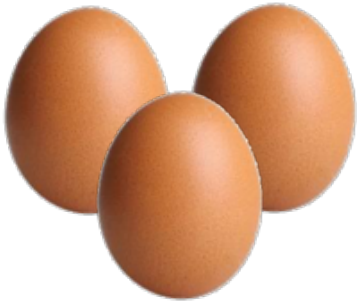
1 small banana

=

38g of Protein

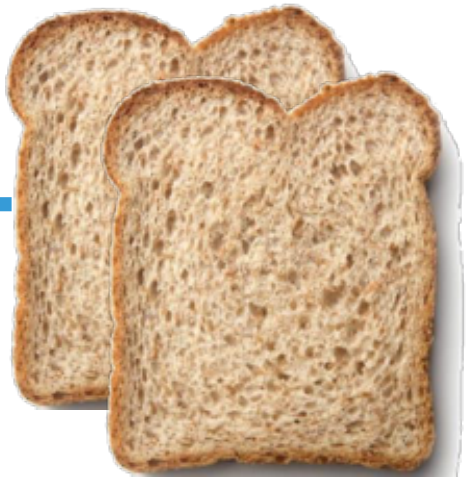
Breakfast

Scrambled eggs and beans on toast with tomatoes grilled in olive oil



3 large eggs
(60g each)

21g



2 slices wholemeal bread
(44g each)

10g

½ tin of baked beans in tomato sauce (207g)

9g

2 grilled tomatoes (with olive oil)

40g of Protein

Lunch

Salmon salad dressed with olive oil with fruit and yoghurt for dessert

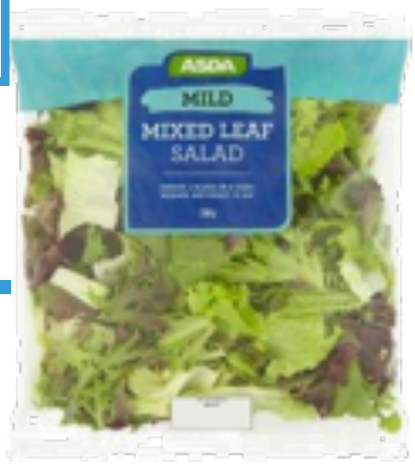
1 small tin Salmon (105g)

23g

+

1 grated carrot

+



1/3 bag of mixed leaves

+

1 tbsp. olive oil as dressing

+

1 medium apple

+

1 individual pot of fruit-flavoured Quark (200g)

20g

= 43g of Protein

Lunch

Chicken salad sandwich with nuts, yoghurt and fruit for dessert

1 pack, tesco chicken salad sandwich

23g

+

1 serving of almonds/peanuts (30g)

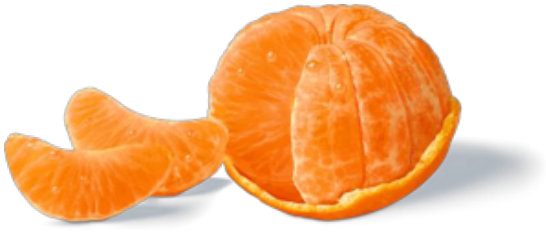
7g

+

1 individual pot of light yoghurt (175g)

7g

+



1 medium orange

=

37g of Protein

Lunch

Ready-made soup with added cooked chicken and spinach and yoghurt for dessert

½ pot Tomato & Lentil Soup (300g)

8g

+

½ pack Cooked Chicken Breast (120g)

26g

+

1 large handful Spinach (50g)

+

1 single serve pot fat-free GREEK yoghurt (125g)

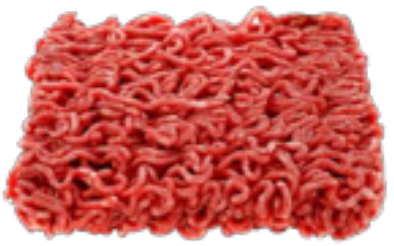
7g

=

41g of Protein

Dinner

Beef, vegetable & bean bolognaise on pasta with far-free Greek yoghurt & fruit



1 portion lean mince beef (125g)

26g

+

2 servings of mixed vegetables (200g)

+

½ tin mixed beans (123g)

8g

+

1 portion wholegrain pasta (50g)

6g

+

¼ jar of tomato pasta sauce (125g)

+

1/3 of a tub fat-free GREEK yoghurt (166g)

11g

+

1 portion of Strawberries (100g)

=

51g of Protein

EASY WAYS TO ADD MORE FRUIT & VEG TO YOUR MEALS

Eating more fruit and veg is one of the easiest ways to improve your health.

If you're not used to eating a lot of fruit and veg, here are a few tips to help you add more to your daily meals and snacks.

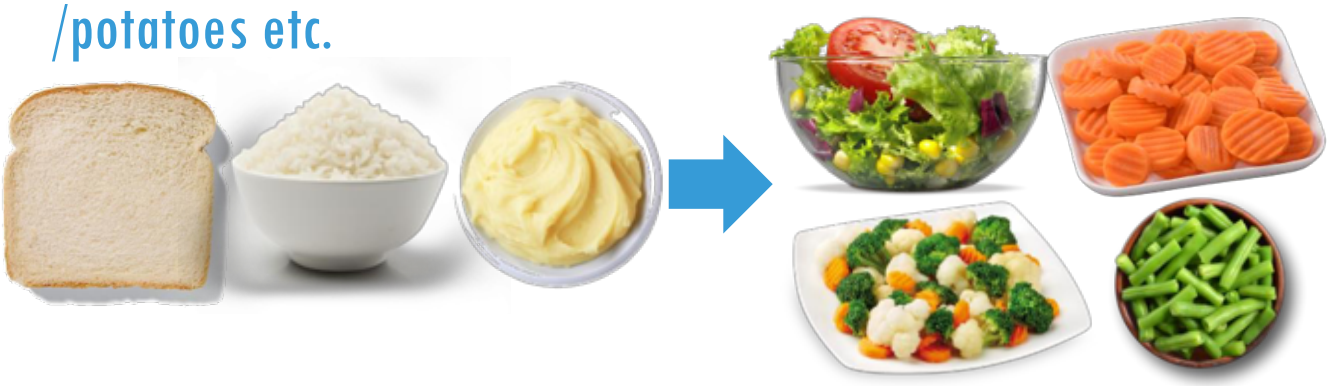
- Eat vegetable based soups. Ready-made soups pots are a great option. Cheap and very convenient.

- Add extra veg like spinach (which doesn't take long to cook) to soups and stews.

- Try veggie noodles instead of or mixed with regular noodles. These are very common in some supermarkets now.

- Add finely chopped tomatoes, peppers, onions, mushrooms, spinach or other veg to scrambled eggs and omelettes.

- Swap vegetables for some of your bread/ pasta /rice /potatoes etc.



- Add finely chopped vegetables to pasta sauces. Mixing them in a blender can be very quick and easy.

- Aim for a "salad a day". A salad isn't just leaves… you can add lots of your favourite vegetables (raw or cooked), pickles, seasonings and meat (AVOID: premade pasta/potato/egg salads and coleslaws with lots of mayo)

Leaves of choice: Spinach, Lettuce, Lambs Lettuce, Kale, Cress

Your favourite vegetables

Add pickles for flavour

Olive oil, vinegar, seasoning

Meat, Eggs, Fish, Cheese

What does a typical day look like?

Breakfast

Porridge made with semi-skimmed milk, topped with a banana and some nuts, a pot of protein yoghurt and some coffee.

Lunch

Chicken salad sandwich with a pot of protein yoghurt, a mandarin orange and a bottle of sugar free soft drink.

Dinner

Tomato and lentil soup cooked with wholegrain pasta, a chicken breast cooked in olive oil and frozen vegetables dressed in olive oil and seasoning.

Snacks

(if hungry)

Humus with vegetable sticks or some fresh fruit of choice

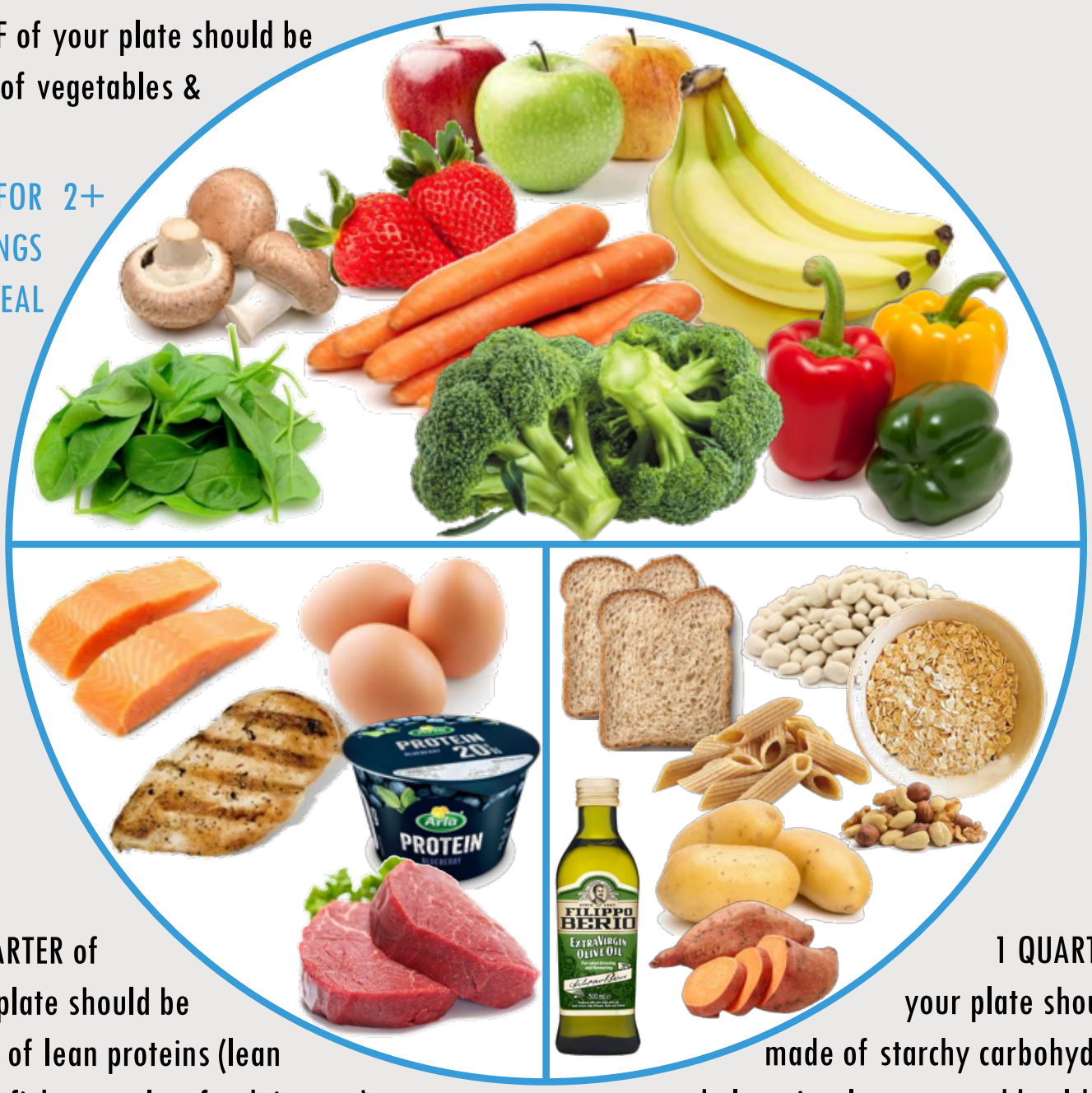
CREATING YOUR HEALTHY PLATE

Sometimes it can be useful to see what a healthy, balanced meal can look like on a plate. You can use this plate to help you build your own healthy meals.

FILL YOUR PLATE LIKE THIS

1 HALF of your plate should be made of vegetables & fruit

AIM FOR 2+ SERVINGS PER MEAL



1 QUARTER of your plate should be made of lean proteins (lean meat, fish, eggs, low-fat dairy etc.)

AIM FOR 40G OF PROTEIN PER MEAL

1 QUARTER of your plate should be made of starchy carbohydrates, wholegrains, legumes and healthy oils

SUPPLEMENTARY ADVICE

Hydration

If you have been advised by your doctor to drink a specific amount fluids during the day, please follow these recommendations.

Otherwise, please aim to drink 6-8 glasses (200ml or medium-sized) of water, tea, herbal tea, coffee or sugar-free-drink per day to ensure staying hydrated. This will also help your body to deal with the increase in fibre following a healthy eating plan. If you think that plain water is too boring you can make this more interesting by adding slices of citrus fruit and/or mint or cucumber or using a small amount of sugar-free cordial. You can sweeten tea and coffee with artificial sweeteners such as Canderel (aspartame), sucralose or stevia.

Please avoid drinks that contain sugar such as soft drinks and fruit juices.

Alcohol

We do not recommend you consume alcohol while following this eating plan. However, you may encounter social situations where you might like some alcohol. In such situations a glass of red wine or a measure of spirits such as whisky, brandy, vodka and sugar-free mixers (sugar-free soft drinks, slimline tonic etc) are probably the best options.

***Beer, cider and mixed drinks with sugary sodas or alcopops are best avoided or reduced to the absolute minimum.**

Snacks

It may be useful to carry some useful snacks with you if you feel you may get hungry and not have access to better options. Some great ideas for this eating-plan include:

- Fruit
- Protein bars (very useful if you get a craving for chocolate)
- High protein yoghurt (travel pouches)
- Nuts (pack your own in small Ziploc bags to avoid overeating)

TIPS FOR EATING OUT

Eating out whilst following these recommendations can be quite straight forward depending on the type of cuisine that you choose and very often it is easy to ask for a few small changes to your meal to help it fit your goals.

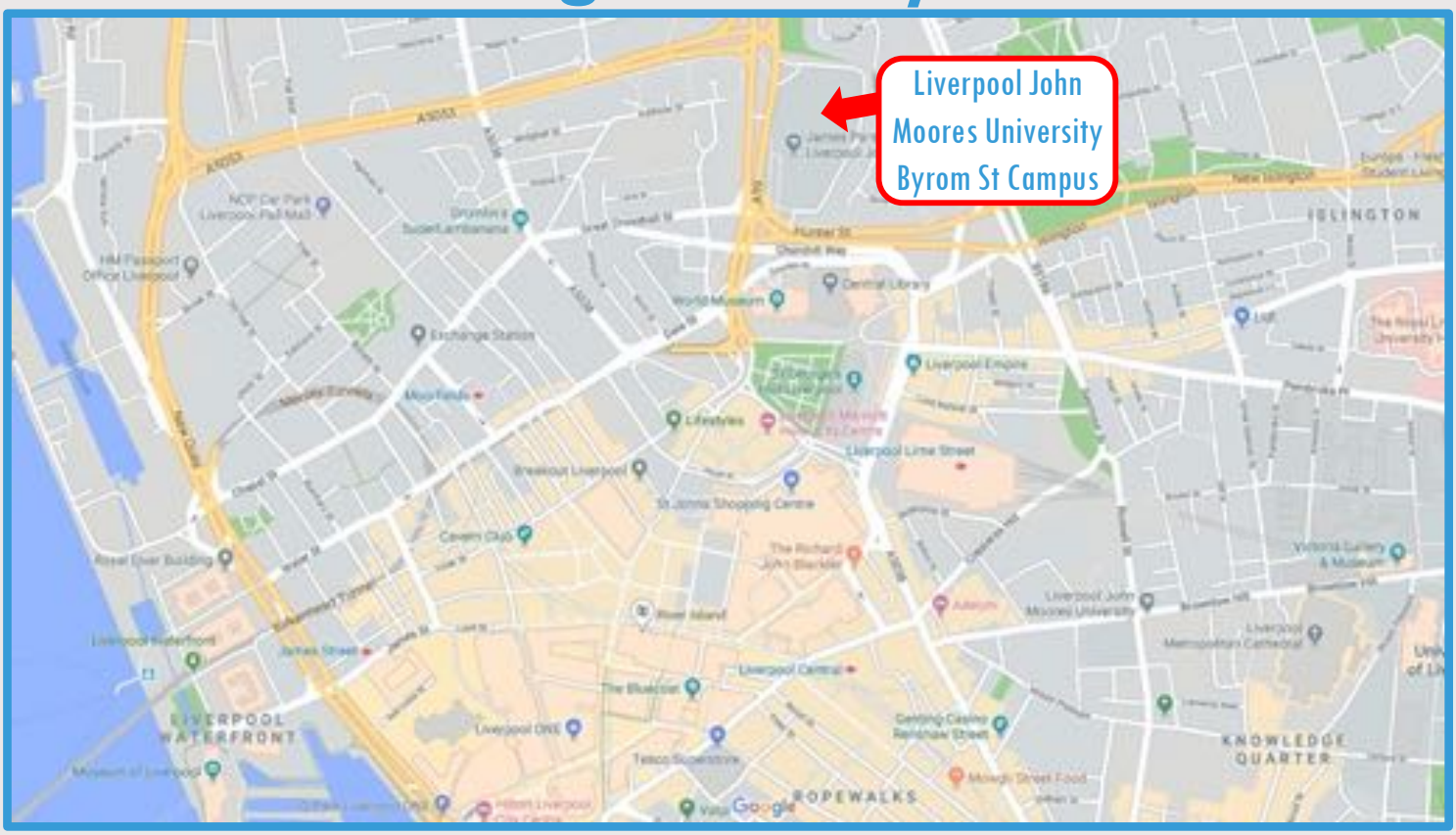
- Many restaurants and pubs now will allow you to swap your potatoes/chips for a side salad.
- Instead of breaded/battered fish or chicken have the steamed, roasted, baked or grilled versions instead.
- Cheesy and creamy sauces (e.g. carbonara, nacho cheese, pepper cream sauce, are best avoided due to the high fat content.
- Many restaurants will have vegetable side dishes you can order to add more veg to your meal. These will also help you to feel fuller for longer
- You don't need to always have a dessert but if you do want one, try opting for fruit based desserts like salads and sorbets instead of creamy puddings and ice-creams.
- If you know that you're going to a restaurant where you won't have many vegetable options or where you expect to eat a larger amount of food, you can base your meals during the day on lean proteins and vegetables to compensate in advance.
- **Buffet restaurants:** these can be tricky with so many different options but always start your meal by having a plate of salad or cooked vegetables dressed with olive oil and vinegar if available. This means you'll get your vegetables first and will feel full quicker. Try and avoid fried, and deep fried food and avoid excess sauces on your foods. Try and get plenty of protein from lower fat meats like chicken or seafood.
- **Burger restaurants:** most burger patties can be quite high in fat so opting for grilled chicken burgers can be a good option. Avoid adding extra bacon, cheese or mayonnaise which can be quite high in fat. Tomato-based sauces (ketchup, barbecue sauce) may be better options.

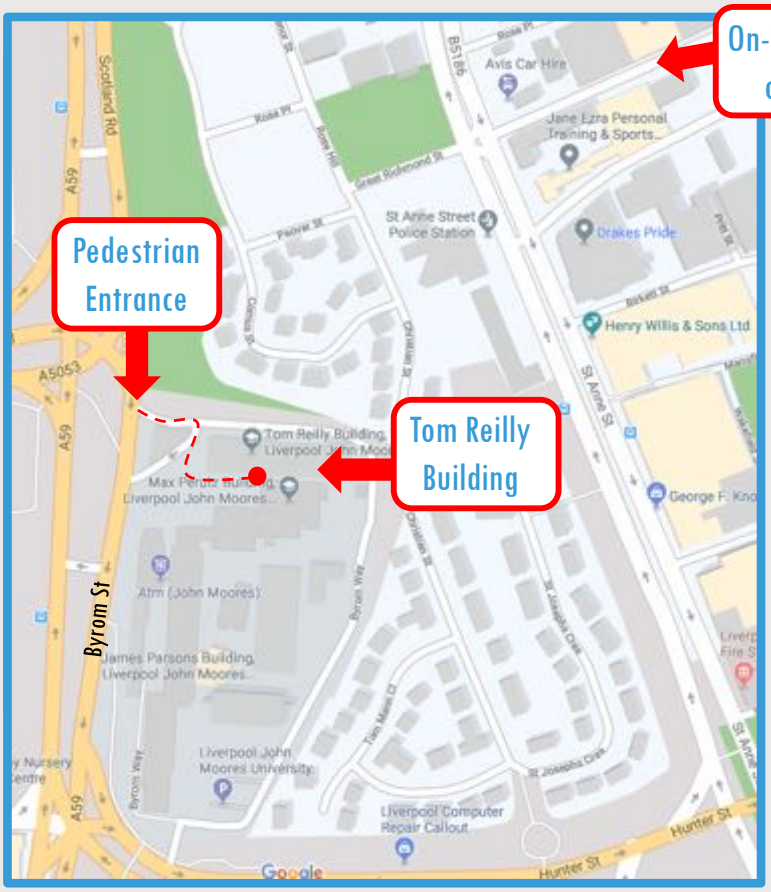
- **Italian restaurants:** Italian food can be quite useful on this eating plan due to the use of vegetables and olive oil. Aim for dishes with tomato-based sauces and avoid creamy and cheesy sauces like carbonara. You can also order extra vegetable dishes and sometimes legume dishes. Many pizza chains now offer low fat cheese and you can get lots of vegetables as toppings as well as chicken. Sausage, ham and pepperoni are best avoided.
- **Indian restaurants:** Indian food also uses lots of tomatoes, vegetables and legumes. Stick with tomato based sauces like bhuna, madras, dopiaza, jalfrezi and rogan josh and avoid creamy sauces like tikka masala, korma and pasanda. Deep fired starters like popadoms, bhaaji and samosas are best kept to a minimum. Rice and naans are fine as sides but try and reduce the amount you eat and be weary of high fat toppings like cheese or very sweet fillings.
- **Chinese restaurants:** some Chinese dishes use lots of veg and you can usually order vegetable dishes on the side such as vegetable stir fry. Avoid deep fried dishes like sweet and sour, crispy fried dishes and deep fried starters like spring rolls.
- **Carvery restaurants:** You can ask for leaner cuts of meat like pork loin or chicken/turkey. Ask for extra vegetables to replace some of the potatoes or Yorkshire puddings. Don't use too much gravy as it can add quite a lot of fat.
- **Mexican restaurants:** these can be very good options as you can fill burritos with beans, vegetables and meats like chicken and ask for less rice. Try and avoid too much sour cream.

REMEMBER

If you can't stick to this eating plan at a meal out, don't worry. Just do your best.
One meal won't ruin your progress as long as **most of the time** your food follows these guidelines!

How to get to Byrom St





PUBLIC TRANSPORT

The following transport lines have routes that pass near Byrom Street Campus LJMU

Bus: 10A, 471, 79D, 82D, 86D, X3

Train: LONDON MIDLAND, MERSEYRAIL, NORTHERN.

The PRiME Trial

Food and Exercise for a Healthier Heart!

EASY RECIPES

Contents

A WORD ON RECIPES

The idea of this recipe book is to give you an idea of all the amazing foods you can eat by following this particular way of eating.

The recipes are aimed at making it easier for you to follow this way of eating and to do that we don't want you to feel that you have to follow the recipes to the letter. One of the great things about food and cooking is that you can experiment yourself with different combinations to make a recipe that suits your taste buds.

Feel free to swap around different vegetables for vegetables, proteins for proteins and seasonings for seasonings. You could create your own kitchen masterpiece (in which case, let us know and we can pass on the recipe to others).

Above all else, enjoy the experience of cooking and the amazing food you make.

BREAKFASTS

PROTEIN PORRIDGE

PROTEIN PORRIDGE

INGREDIENTS

- 30g porridge oats
- 200ml semi-skimmed milk
- 125g fat-free quark
- 20g raisins
- 30g almonds (or nuts of choice) (chopped)
- Granulated artificial sweetener (to taste)
- Ground cinnamon or cloves to taste

MAKES 1 SERVING
32g of Protein/serving

INSTRUCTIONS

1. Add the milk, oats and raisins to a microwavable bowl and microwave on high for 2 minutes
2. Once cooked, add the quark, nuts and sweetener and cinnamon. Mix well.
3. Eat straight away.

BEANS & EGGS ON TOAST

BEANS & EGGS ON TOAST

INGREDIENTS

- 2 large eggs
- ½ a can baked beans in tomato sauce
- 1 tsp. olive oil
- 2 medium tomatoes or 4-5 cherry tomatoes (halved)
- 1 tbsp. extra virgin olive oil
- 1 slice of wholegrain bread
- Garlic powder
- Salt and black pepper

MAKES 1 SERVING
27g of Protein/serving

INSTRUCTIONS

1. Preheat the oven to 200 degrees C
2. In a bowl, mix the tomatoes, olive oil, salt, pepper and garlic powder.
3. Place on an oven tray and bake for 10 minutes until softened
4. Heat the beans in a bowl in the microwave.
5. Fry the eggs in a fry pan with olive oil.
6. Toast the bread.
7. Place the bread onto a plate, top with the beans and then the eggs.
8. Serve the roasted tomatoes on the side.

CHICKEN & VEG OMELETTE BITES

CHICKEN & VEG OMELETTE BITES

INGREDIENTS

- 6 large eggs
- 50ml semi-skimmed milk
- Salt and black pepper
- 1 tbsp. Extra virgin olive oil
- 2 medium spring onions (finely chopped)
- Half a red bell pepper (finely chopped)
- Olive oil (for greasing)
- 1 very large handful of spinach
- 2 tsp of grated parmesan cheese (optional)
- ½ tsp of dried mixed herbs
- 100g cooked chicken breast

MAKES 2 SERVINGS
35g of Protein/serving

INSTRUCTIONS

1. Preheat the oven to 190 degrees C.
2. Grease a bun tin (with 12 cups) lightly with a little olive oil. You can also use a small, roasting tray lined with baking parchment and cut the eggs later.
3. Add the olive oil to a large frying pan and over a medium heat, fry the chopped bell pepper and spring onion.
4. When the veg have softened, add the spinach and chicken and stir until soft.
5. Divide the vegetable mix evenly amongst the bun wells (or pour into the tray)
6. Put eggs, milk, salt, pepper, cheese and herbs in a separate bowl and mix well.
7. Pour the egg mixture evenly over the veg mix in the bun wells (or tray)
8. Bake until the egg is set, about 12-15 minutes. The eggs will deflate somewhat once removed from the oven and as they cool.
9. Run a knife around the edges of the omelette bites to loosen them from the bun tin or tray. Serve immediately or at room temperature.

PROTEIN SMOOTHIE

PROTEIN SMOOTHIE

INGREDIENTS

- 250g fat-free Greek yoghurt or quark
- 100g frozen blueberries or raspberries
- 1 banana
- 1 small carrot
- 1 handful of spinach leaves
- Granulated artificial sweetener to taste
- Water (if necessary to thin it out)

MAKES 1 SERVING
30g of Protein/serving

INSTRUCTIONS

1. Add all the ingredients (except the water and sweetener) to a strong blender and blend until smooth
2. Add the water and sweetener to the desired consistency and sweetness.

*Use any combination of fruits and vegetables that you like and feel free to add in spices like cinnamon or fresh herbs like mint.

MUSHROOM, SPINACH & CHEESE OMELETTE

MUSHROOM, SPINACH & CHEESE OMELETTE

INGREDIENTS

- 3 large free range eggs
- Salt and pepper to taste
- 1 tbsp. extra virgin olive oil
- ½ small onion (finely chopped)
- 3-4 large murshrooms (sliced)
- 1 handful of spinach
- 35g of grated reduced-fat cheese
- ½ tsp of dried mixed herbs

MAKES 1 SERVING
33g of Protein/serving

INSTRUCTIONS

1. Heat the oil in a large frying pan and saute the onion and mushroom until softened.
2. Add the spinach and continue to cook until softened.
3. Beat the eggs in a bowl with the seasoning and grated cheese then pour over the mushroom and spinach mixture.
4. Cook the eggs undisturbed until the edges begin to pull away from the edge of the pan and begin to set. If you cover the frying pan with a lid, it will cook much faster.
5. If you have the skills you can flip the omelette or put the fry pan under a hot grill for 2-3 minutes or until the eggs have puffed and have cooked through.
6. Cut into wedges and serve warm or at room temperature

TINNED SARDINES ON TOAST

TINNED SARDINES ON TOAST

INGREDIENTS

- 2 tins of sardines in tomato sauce (120g each)
- 3 medium tomatoes or 5-6 cherry tomatoes (halved)
- 2 tbsp. extra virgin olive oil
- 2 slices of wholegrain bread
- Salt and black pepper

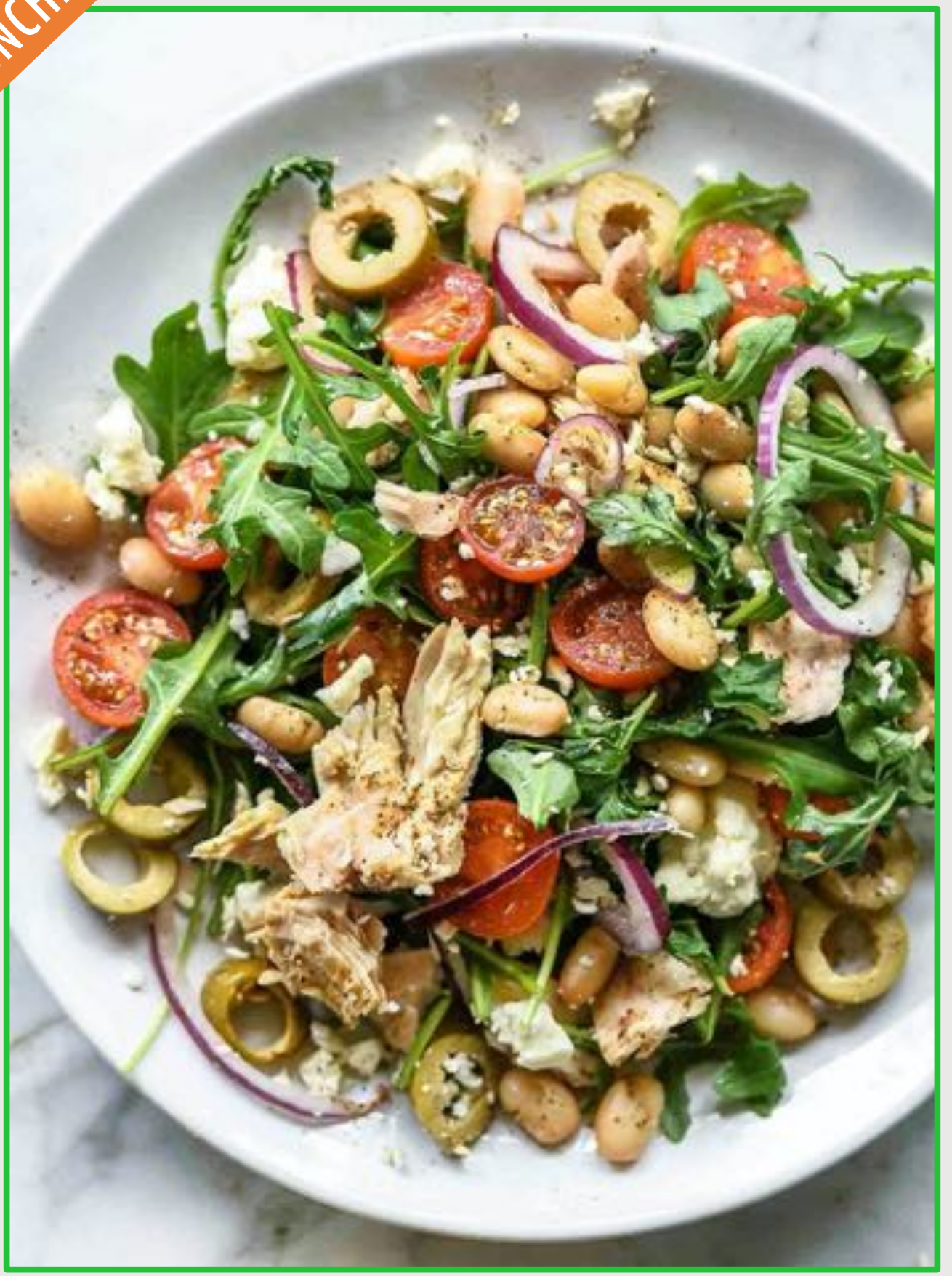
MAKES 2 SERVINGS
27g of Protein/serving

INSTRUCTIONS

1. Preheat the oven to 200 degrees C
2. In a bowl, mix the tomatoes, olive oil, salt, pepper and garlic powder.
3. Place on an oven tray and bake for 10 minutes until softened
4. 5 minutes before the tomatoes are done, place the sardines on a tray and into the oven to heat through
5. Toast the bread.
6. Place the bread onto a plate, top with the hot sardines and serve with the roasted tomatoes on the side

LUNCHES

SALMON & BEAN SALAD



SALMON & BEAN SALAD

INGREDIENTS

- 2 handfuls of mixed leaves or favourite lettuce
- 1 400g can of cannellini/butter/mixed beans rinsed and drained
- 1 large can (213g) red or pink salmon in brine
- 1/2 cup cherry tomatoes halved
- 2 pickled gherkins (sliced)
- 1 small red onion (thinly sliced)
- 2 tablespoons extra virgin olive oil
- 1/2 lemon
- Salt and black pepper

MAKES 2 SERVINGS
29g of Protein/serving

INSTRUCTIONS

1. Slice the onions and place in a bowl of warm water to soak for 5 minutes (this reduces the sharp taste of raw onion)
2. In a large bowl, combine the leaves, beans, salmon, tomatoes, pickles and red onion. Drizzle with the olive oil and the juice from the lemon. Toss to combine.
3. Season to taste with salt and black pepper.

CHICKEN & FETA SALAD

CHICKEN & FETA SALAD

INGREDIENTS

- 1/2 a cucumbers (peeled and cut into 1/2 inch slices)
- 100g small tomatoes (quartered)
- 1/2 red onion (thinly sliced)
- 2 tbsp. olives (or pickled gherkins) (sliced)
- 200g cooked chicken breast (chopped)
- 100g reduced-fat feta-style cheese (broken into large chunks)
- 2 tbsp. extra virgin olive oil
- 2 tbsp. vinegar
- 1 clove garlic (peeled and minced)
- 1 tablespoon dried oregano
- 2 teaspoons artificial sweetener
- Salt and black pepper to taste

MAKES 2 SERVINGS
30g of Protein/serving

INSTRUCTIONS

1. Slice the onions and place in a bowl of warm water to soak for 5 minutes (this reduces the sharp taste of raw onion)
2. In a large serving bowl, combine the cucumbers, tomatoes, red onion and olives.
3. In a cup or glass, combine the olive oil, red wine vinegar, garlic, oregano, sweetener and salt and pepper. Mix well until blended.
4. Pour ¾ of the dressing on the cucumber mixture and toss to coat.
5. Pour the remaining dressing on the chicken and feta and gently mix to coat.
6. Add the chicken and feta to the salad and serve.

BARBECUE BEEF & VEG WRAP

BARBECUE BEEF & VEG WRAP

INGREDIENTS

- 200g very lean beef mince
- ½ red bell pepper (finely chopped)
- ½ small onion (finely chopped)
- ½ can black beans (or favourite beans) (drained)
- Salt and black pepper
- 1 tsp dried mixed herbs
- 2 tbsp. extra virgin olive oil
- 3 tbsp. barbecue sauce
- 2 wholegrain wraps
- 1 handful of mixed salad leaves
- 2 tbsp. fat-free Greek yoghurt (optional)

MAKES 2 SERVINGS
32g of Protein/serving

INSTRUCTIONS

1. Fry the beef and chopped vegetables in the olive oil
2. When the beef is browned add the salt, pepper, mixed herbs and barbecue sauce.
3. Add half the beef mixture to each of the wraps and top each with half the salad leaves.
4. Drizzle over some of the Greek yoghurt (if using). Roll the wraps.
5. Serve immediately

EASY CHICKEN PITA

EASY CHICKEN PITA

INGREDIENTS

- 1 wholemeal pita bread
- 130g of raw chicken breast or 120g cooked chicken breast (chopped or pulled) if you buy pre-cooked chicken
- 1 tbsp. extra virgin olive oil
- Salt and black pepper
- Low-fat sauce of choice (ketchup, barbecue, sweet chilli etc.)
- Salad leaves of choice (spinach, lettuce, rocket etc.)
- Salad vegetables of choice (peppers, tomatoes, cucumber etc.) (chopped)

MAKES 1 SERVING
33g of Protein/serving

INSTRUCTIONS

1. If using raw chicken, cut into small chunks, sprinkle with salt and pepper and fry until cooked through
2. Rub a few drops of water over one side of the pita bread and place in the microwave for 20 seconds. This will make the pita puff up and be easier to open.
3. Slice open one side of the pita and fill evenly with leaves, vegetables and drizzle in the olive oil, salt and pepper.
4. Add the chicken and top with your sauces of choice. (You can also toss the chicken in your sauce of choice and microwave for 1 minute before adding it to the pita).
5. Serve immediately

VEGETABLES, SAUCES & SIDES

SPINACH & BEAN MASH

SPINACH & BEAN MASH

INGREDIENTS

- 2 tins of white beans (e.g. butter beans or cannellini) (rinsed and drained)
- 2 cloves of garlic (minced)
- 2 tbsp. olive oil
- ½ a vegetable stock cube dissolved in 100ml of hot water
- Salt and black pepper
- 2 handfuls of spinach (chopped0
- 1 spring onion (finely chopped)

MAKES 4 SERVINGS
8g of Protein/serving

INSTRUCTIONS

1. Heat the oil in a saucepan and cook the garlic and half the spring onion for 1 minute
2. Add the beans and half the stock, mix well and mash. Add more stock if necessary
3. Add the spinach, stir and allow to wilt.
4. Season with salt and pepper to taste.
5. Serve immediately topped with the remaining spring onion.

*This is a fantastic alternative to mashed potato and can be served with many other main dishes

HERBY ROAST POTATOES

HERBY ROAST POTATOES

INGREDIENTS

- 4 medium potatoes (cut into quarters)
- 2 tbsp. extra virgin olive oil
- Salt and black pepper
- Mixed herbs & garlic powder (optional)

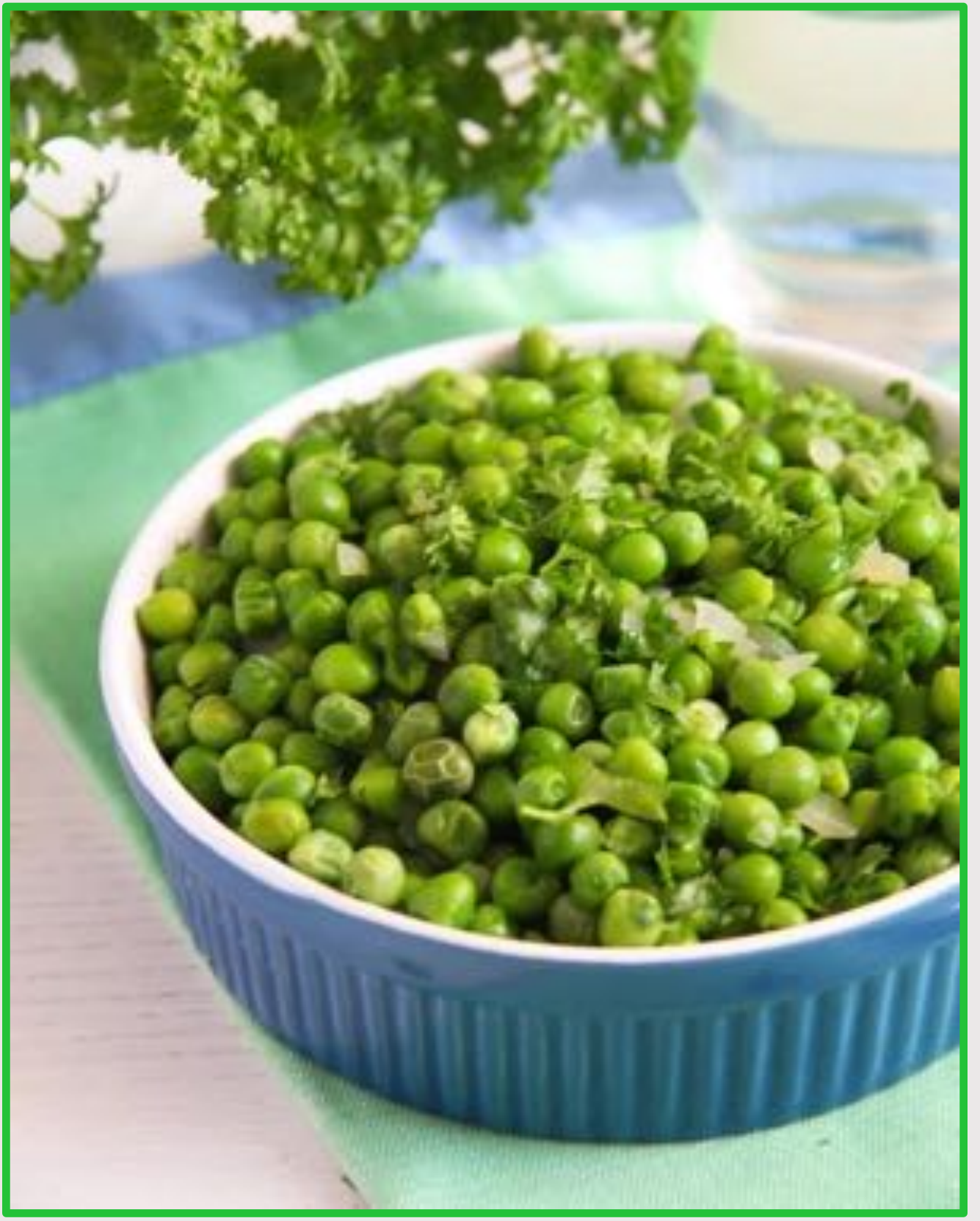
MAKES 2 SERVINGS
0g of Protein/serving

INSTRUCTIONS

1. Preheat the oven to 200 degrees C.
2. Pierce the potato quarters with a fork and place in a bowl and microwave on high for 10 minutes (or until starting to soften).
3. Once slightly softened, add the olive oil, salt, pepper and herbs & garlic if using and toss to cover.
4. Place on a baking tray, skin side down and roast in the oven for 20 minutes or until golden and crispy on the outside.
5. Serve immediately

*You can also make this recipe with sweet potato

SIMPLE PEAS & ONIONS



SIMPLE PEAS & ONIONS

INGREDIENTS

- 1 small onion
- 1 tablespoon olive oil
- 450 g frozen peas
- 60ml water
- Salt and black pepper
- Small bunch of parsley (optional)

MAKES 4 SERVINGS
7g of Protein/serving

INSTRUCTIONS

1. Chop the onion very finely. Heat the oil in a saucepan and cook the onion until translucent and softer, about 3 minutes.
2. Add the peas, water and a little salt, stir and bring to a boil. Cover the saucepan, leaving a small crack open, turn down the heat and simmer the peas for about 3-4 minutes or until cooked to your liking. Do not overcook.
3. Check the salt, add some freshly ground black pepper and sprinkle with the chopped parsley. Serve immediately.

ROAST POTATO WEDGES

ROAST POTATO WEDGES

INGREDIENTS

- 4 medium potatoes (cut into wedges)
- 2 tbsp. extra virgin olive oil
- Salt and black pepper
- Mixed herbs & spices of choice (a tasty mix can include garlic powder, cumin, oregano, Italian seasoning, onion powder, chili powder or even curry powder)

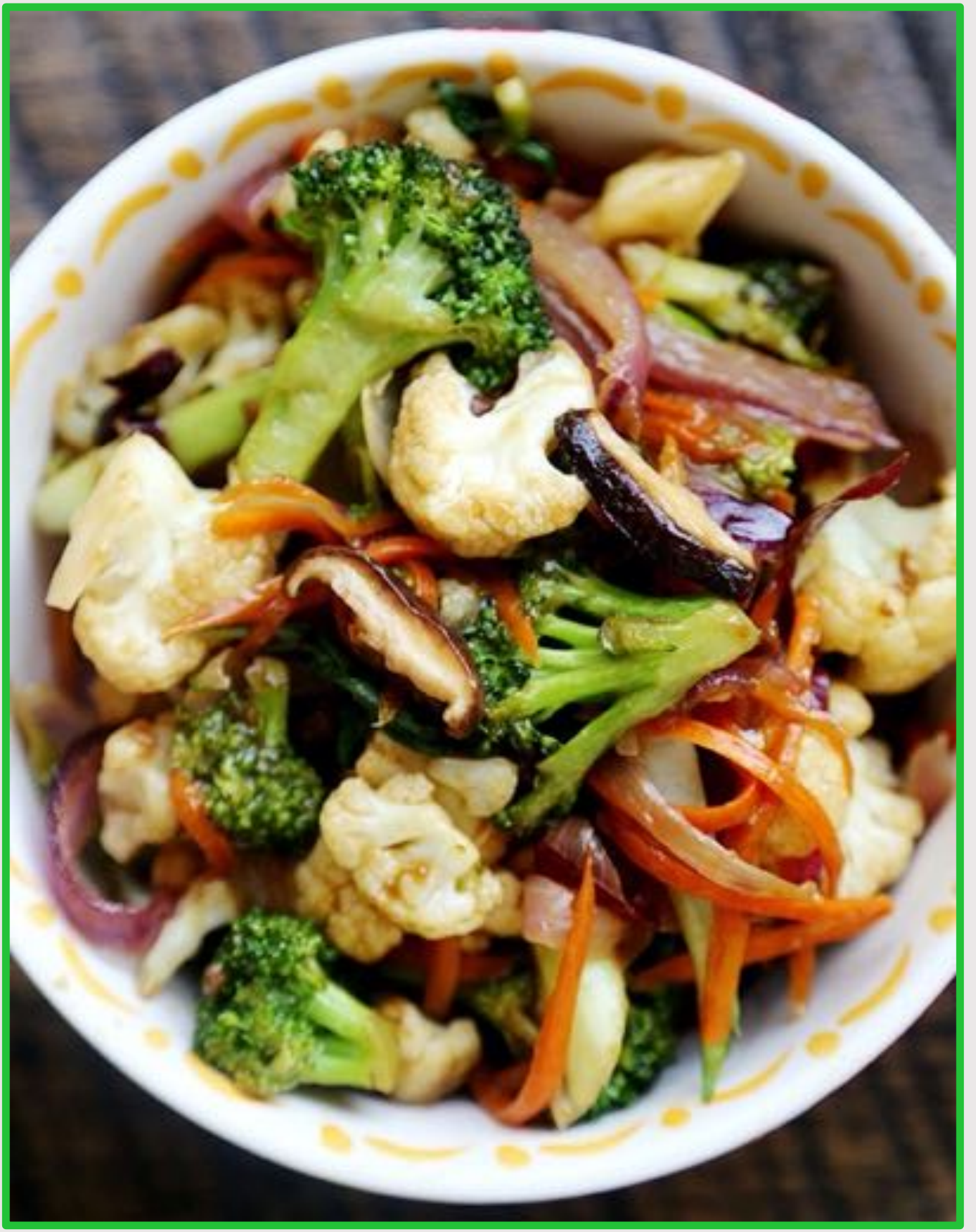
MAKES 2 SERVINGS
0g of Protein/serving

INSTRUCTIONS

1. Preheat the oven to 200 degrees C.
2. Put the potato wedges in a bowl add the olive oil and mix to coat the wedges.
3. Add the salt, pepper and herbs & spices and toss to cover.
4. Place on a baking tray, skin side down and roast in the oven for 25 minutes or until golden and crispy on the outside.
5. Serve immediately

*You can also make this recipe with sweet potato

QUICK FROZEN VEG STIR-FRY



QUICK FROZEN VEG STIR-FRY

INGREDIENTS

- 200g frozen veg (broccoli, cauliflower, carrots, peas etc.)
- 1 tablespoon olive oil
- Black pepper
- 1 tbsp. Soya sauce
- Garlic powder

MAKES 1 SERVING
0g of Protein/serving

INSTRUCTIONS

1. Put the veg into a microwave-proof bowl and microwave for about 4 minutes or until just tender.
2. Heat the olive oil in a frying pan over a medium heat, add the veg and toss well in the olive oil for 1-2 minutes.
3. Add the soya sauce, pepper and garlic powder and stir to coat the veg.
4. Continue stirring for 1 minute and serve immediately.

*This works fine with any veg and fresh veg is perfect too.

ROAST VEGETABLE AND BEAN BAKE

ROASTED VEGETABLE AND BEAN BAKE

INGREDIENTS

- 2 garlic cloves, finely chopped
- 2 aubergines, cut into 2cm pieces
- 500g courgettes, cut into 1cm slices
- 2 large onions chopped into 2cm chunks
- 3 red peppers, cut into 2cm pieces
- 400g cherry tomatoes
- 5 tbsp olive oil
- 2 x 400g tins beans (cannellini, chickpeas, black etc.) drained and rinsed
- 100g reduced fat (50%) cheddar-style cheese, grated
- 1 tbsp balsamic vinegar (optional)
- Handful of basil leaves to serve (optional)

MAKES 4 SERVINGS
16g of Protein/serving

INSTRUCTIONS

1. Heat the oven to 200° C/180° C fan. Put the onion, garlic, aubergines, courgettes, red peppers and tomatoes into 2 large roasting dishes. Drizzle over 4 tbsp oil, season with salt and pepper, then toss to coat. Roast for 40 minutes.
2. Stir the cannellini beans into the roasted veg, sprinkle over the grated cheese then roast for 5 minutes more.
3. Drizzle over the remaining 1 tbsp olive oil and the balsamic vinegar at the table. Sprinkle with the fresh basil leaves, then serve.

HEALTHY COLESLAW

HEALTHY COLESLAW

INGREDIENTS

- ½ savoy or white or red cabage (or a mix of all 3), cored and shredded
- 1 apple, cored and grated (optional)
- 2 carrots, peeled and grated
- ½ onion, peeled and finely sliced
- 100g fat-free Greek yoghurt
- Juice of half a lemon
- 2 tsp vinegar
- 2 tsp mustard of choice (wholegrain, Dijon etc) (optional)

MAKES 6 SERVINGS
1g of Protein/serving

INSTRUCTIONS

1. Mix the cabbage, apple, carrots and onion in a large bowl.
2. In a separate bowl, mix the yogurt, lemon juice, vinegar and mustard. Season, then pour over the vegetables.
3. Give everything a good stir to coat in the dressing and eat immediately, or chill until you are ready to serve.
4. Can be stored in the fridge in a well sealed container

*This sauce is delicious as a side salad, in sandwiches or on burgers

TOMATO SALSA

TOMATO SALSA

INGREDIENTS

- 3 medium sized, very ripe tomatoes
- 1 chilli pepper, seeds removed (optional)
- 1/4 medium onion (red or white), finely chopped
- Juice of half a lime
- 1 tbsp. fresh corriander roughly chopped (optional)
- Salt or to taste

MAKES 6 SERVINGS
0g of Protein/serving

INSTRUCTIONS

1. Chop the onion finely and place in a small bowl, covered with water (to help mellow the flavour)
2. Cut the tomatoes into quarters and remove the seeds. You can scoop out the seeds or cut the insides out with a knife.
3. Chop the tomatoes very finely
4. Drain off the water from the onions and add to the tomatoes in a bowl
5. Add the remaining ingredients, mix well and season to taste.

*This salsa is a great dip for thinly sliced vegetables or can be added to sandwiches, wraps and burgers

TZATZIKI SAUCE

TZATZIKI SAUCE

INGREDIENTS

- 250g Fat-free Greek yogurt
- 1 large cucumber, seeded and finely grated*
- 2 cloves garlic, minced
- 3 teaspoons dried mint (optional)
- 1 tablespoon lemon juice
- 1/2 teaspoon salt
- 1/2 teaspoon ground black pepper

MAKES 10 SERVINGS
2g of Protein/serving

INSTRUCTIONS

1. Once you have shredded the cucumber, squeeze as much of the liquid out of the cucumber as you possibly can. Otherwise, your sauce will be too watery.
2. Add all of the prepared ingredients to a bowl and mix together. Transfer to a container with a tight fitting lid.
3. Refrigerate for one to two hours for best results.

*If the sauce becomes watery after refrigerating, in spite of the squeezed cucumber, simple whisk the sauce again to incorporate the water that may have settled on top of the sauce.

This sauce is delicious on salads, in sandwiches or as a dip for vegetables

FISH & SEAFOOD DISHES

PRAWNS & VEGGIE SPAGHETTI

PRAWNS & WHOLEWHEAT SPAGHETTI

INGREDIENTS

- 300g raw frozen prawns (defrosted in fridge)
- 3 tbsp. extra virgin olive oil
- 2 large cloves garlic (pressed or minced)
- 2 handfuls of courgette noodles (or other vegetable noodle)
- Salt and black pepper
- 50g Wholewheat spaghetti
- 2 tbsps. Lemon juice
- 1 tsp. red chili flakes optional)
- 1 tbsp. grated Parmesan cheese
- Chopped parsley (optional)

MAKES 2 SERVINGS
26g of Protein/serving

INSTRUCTIONS

1. Add the prawns to a medium size bowl. Drizzle with 1 tablespoon of olive oil, add 1 clove of the minced garlic, salt and black pepper, toss and set aside.
2. Bring a large pot of salted water to a boil. Cook the spaghetti according to package directions and add the courgette noodles for the last minute. Use tongs to transfer the cooked spaghetti to a strainer and reserve the pasta water, brining it to a slow bubbling simmer.
3. In a large, high-sided frying pan, add 1 tablespoon of olive oil to the pan over medium heat. Place the shrimp in the pan and cook for 2 minutes on each side or just until opaque. Transfer the shrimp to a plate.
4. In the same pan, add 2 tbsp. of olive oil add the remaining minced clove of garlic, lemon juice, red chili flakes and 2 tbsp. reserved pasta water. Cook for 1 minute stirring once or twice.
5. Add the noodles, shrimp and 1/2 the Parmesan cheese and toss to coat.
6. Top with the remaining Parmesan cheese and parsley and serve.

HEALTHY FISH & CHIPS WITH MUSHY PEAS

HEALTHY FISH & CHIPS WITH MUSHY PEAS

INGREDIENTS

- Potato wedges (page 27) (made without the spices)
- Simple peas & onions (page 25), mashed (half the recipe)
- salt and pepper
- 1 heaped tbsp. of paprika
- 50g flour
- 1 egg
- 2 slices of slightly stale toast, grated into breadcrumbs
- 1 tbsp. herbs (fresh/dry parsley or dill), finely chopped
- 1 lemon, zest and juice
- Pinch of pepper
- 2 white fish fillets (150g each)

MAKES 2 SERVINGS
35g of Protein/serving

INSTRUCTIONS

1. Preheat the oven to 190°C
2. Prepare the potatoes as per the recipe on page 27 (don't use the spices)
3. Lightly oil another baking tray/sheet with olive oil and place in the oven
4. While the potatoes are roasting, tip the flour into a bowl. Crack the eggs into a separate bowl and beat well with a fork. In a separate bowl mix the breadcrumbs, herbs, lemon zest and the pepper together.
5. Place the fish into the flour and coat evenly. Dip the fish into the beaten egg and then into the breadcrumb mix to cover.
6. Place the fillets on the hot baking sheet and bake for 12 – 15 minutes until they look golden brown
7. While the fish and chips are baking, make the peas as per the recipe on page 25 and mash with a fork when cooked.
8. Serve up the fish, chips and mushy peas (and a side salad for extra vegetables)

Salmon Steaks & Vegetable Risotto

Salmon Steaks & Vegetable Risotto

INGREDIENTS

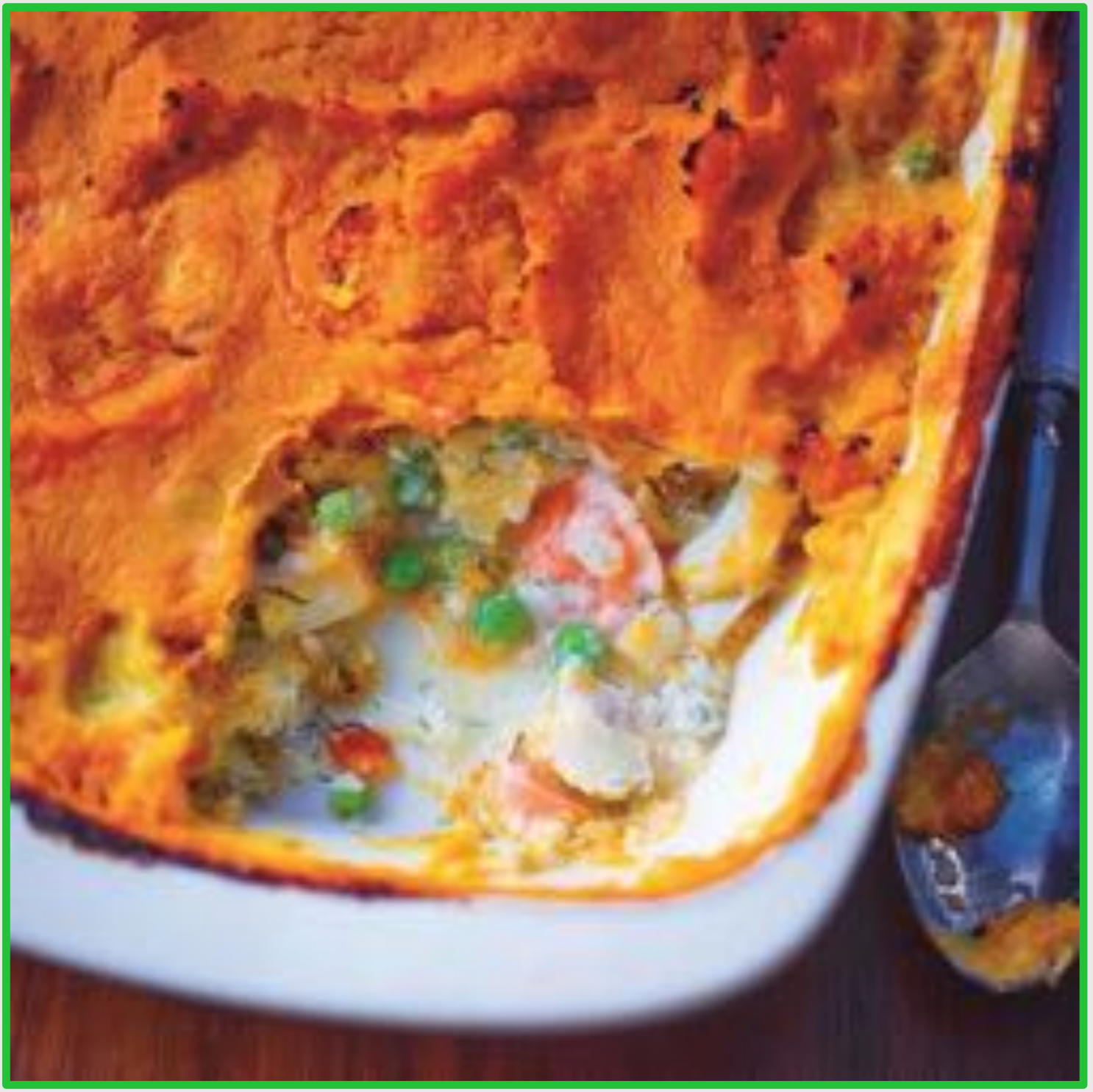
- 1 tbsp vinegar
- 1 tbsp Dijon mustard
- 1 tbsp honey
- Pinch of pepper
- 2 salmon steaks (150g each)
- 3 tbsp olive oil
- 2 leeks, chopped
- 2 medium carrots (finely chopped)
- 150g brown risotto rice
- 750ml low-salt vegetable stock
- 2 celery sticks, chopped
- Handful of fresh dill, finely chopped

MAKES 2 SERVINGS
28g of Protein/serving

INSTRUCTIONS

1. Mix the vinegar, Dijon mustard, honey and seasoning together in a bowl and brush over the salmon
2. Leave the salmon to marinate in the fridge for an hour
3. Add 2 tbsp. olive oil to the pan over a medium heat and add leeks and carrots and cook for 5 minutes, stirring frequently until the leeks have softened then turn the heat down
4. Add the rice to the pan with a good splash of stock and continue adding the stock whilst stirring every so often until the rice is creamy then add the celery. Continue for about 40 minutes
5. Add the dill and 1 tbsp. olive oil to the risotto and season to taste
6. Heat another frying pan to a high heat and lightly brush with olive oil
7. Sear the salmon steaks on each side for 3 minutes and the steaks should be ready when they flake easily when pricked with a fork
8. Serve salmon on a bed of pearl barley risotto.

FISH PIE



FISH PIE

INGREDIENTS

- 400g haddock or cod, cut into chunks
- 20g raw, frozen prawns
- 1 pack (180g) of Lightest cream cheese (Philadelphia)
- 1 tbsp. flour
- 4 medium sweet potatoes
- 1 medium onion (finely diced)
- 2 medium carrots (finely diced)
- 100g frozen peas
- 4 tbsp. olive oil
- 200ml vegetable stock
- 50g parmesan cheese (grated)
- Salt, pepper and 1 bay leaf

MAKES 4 SERVINGS
38g of Protein/serving

INSTRUCTIONS

1. Preheat the oven to 200°C
2. Peel and chop the sweet potatoes and cook in a microwave on high for 10-15 minutes or until soft (you can also steam them). Mash the potatoes and season with a little salt.
3. Heat the oil in a saucepan and cook the carrots and onions until soft.
4. In a separate bowl, mix the cream cheese and flour and then slowly and gradually add the stock until if forms a thick paste.
5. Add the fish, prawns, bay leaf and peas to the carrot and onion mix, stir well and then add the cream cheese/stock mix and parmesan and mix well. Allow to come to a gentle simmer and then remove from the heat.
6. Add the cooked fish and veg mixture to a baking dish and top with the mashed sweet potato
7. Bake in the oven for 20 minutes and the top starts to brown.
8. Serve with a side salad or some roasted vegetables

BAKED FISH

BAKED FISH PARCELS

INGREDIENTS

- 2 small whole fish (e.g. trout, mackerel etc) (gutted)
- 1 lemon cut into thin rings/slices
- 4 cloves of garlic (minced)
- Salt and pepper
- 1 tbsp. Olive oil
- Tin foil

MAKES 4 SERVINGS
30g of Protein/serving

INSTRUCTIONS

1. Preheat the oven to 200°C
2. Take two sheets of tinfoil, big enough to wrap each fish individually
3. Rub the inside and outside of the fish with olive oil, minced garlic, salt and pepper
4. Place the lemon slices in the fish cavity and place the fish on top of the foil sheets and close on top, making the shape of a tent.
5. Place the parcels on a baking tray and bake in the oven for 20 minutes
6. Serve with roasted vegetables or a side salad

MAIN MEALS

ROASTED VEG AND SAUSAGE BAKE

ROASTED VEGETABLE AND SAUSAGE BAKE

INGREDIENTS

- 370g low fat sausages (below 5% fat)
- 2 cups baby carrots (halved, if large)
- 2 cups broccoli florets
- 1 cup sliced onions
- 2 cups green beans
- 1 medium red bell pepper (seeded & cut into 1 inch pieces)
- 2 medium garlic cloves minced
- 3 tablespoons olive oil
- 2 tablespoons Italian seasoning/mized herbs
- 1/2 teaspoon table salt
- 1/2 teaspoon ground black pepper

MAKES 4 SERVINGS
16g of Protein/serving

INSTRUCTIONS

1. Preheat the oven to 200 degrees C.
2. Place the sausages on a plate and microwave for 5 minutes. This will set the sausages a little and make them easier to cut. You could also add them to boiling water for 5 minutes.
3. Cut the sausages into 1-2cm discs.
4. Place all vegetables and sausage into a bowl, drizzle with olive oil and sprinkle with Italian seasoning/mixed herbs, salt, and black pepper. Mix it all together with your hands.
5. Spread out the veggie/sausage mix on a 18x13-inch sheet pan that has been greased with a little olive oil or lined with baking parchment.
6. Bake for 20 to 25 minutes (stirring after 15 minutes) until the vegetables are cooked to fork tender.

PORK & VEGETABLE SKEWERS

PORK & VEGETABLE SKEWERS

INGREDIENTS

- 250g pork loin or loin chops (cut into large cubes) **(or any meat or fish of choice)**
- ½ a recipe of tzatziki sauce (see page XX)
- 2 tbsp. extra virgin olive oil
- Salt and black pepper
- 1 tsp. mixed herbs
- Metal skewers or wooden skewers soaked in water overnight.
- 1 red onion (quartered into 1-inch pieces)
- 5-6 mushrooms (sliced into 1/4 inch slices)
- ½ red bell pepper (cut into 1-inch pieces)

MAKES 2 SERVINGS
33g of Protein/serving

INSTRUCTIONS

1. Place the pork cubes in a freezer bag or bowl and set aside.
2. Add the tzatziki sauce and marinate the pork for 30 minutes or up to 3 hours in the refrigerator.
3. When ready set the grill to medium high heat.
4. Add the chopped vegetables to a bowl and add the olive oil, salt, pepper and mixed herbs (if using) and toss to mix well.
5. Thread the pork onto the skewers alternating with the red onion, mushrooms and red pepper until you've reached the end of the skewer, ending with pork. Discard any of the remaining marinade that had the pork in it.
6. Place the skewers under the grill and turn often so each side browns and until cooked through, about 10-15 minutes or until the pork juices run clear. Serve warm.
7. Refrigerate leftovers for up to 3 days.

CHILI CON CARNE

CHILI CON CARNE

INGREDIENTS

- 500g lean minced beef or chicken (<5% fat)
- 4 tbsp. extra virgin olive oil
- 1 onion (finely chopped)
- 1 red pepper (chopped)
- 4 small carrots (finely diced)
- 1 jar of chili con carne sauce
- 1 can of chopped tomatoes
- Salt and black pepper
- 1 large handful of spinach
- 1 400g tin of kidney beans (or beans of choice)
- 2 tbsp. grated reduced-fat cheddar cheese

MAKES 4 SERVINGS
31g of Protein/serving

INSTRUCTIONS

1. Add the chopped carrot to a microwavable bowl and pre-cook on high for 5 minutes (as carrot can take longer to cook than the other vegetables).
2. In a large frying pan or deep pot, heat the oil over a medium heat, add the onion and cook until soft
3. Add the minced meat and cook until brown, stirring constantly.
4. Add the carrots and red pepper and stir well.
5. Add the jar of chili con carne sauce, chopped tomatoes and the beans and allow to simmer. The dish is cooked now but the longer you allow it to simmer on a low heat the better the flavour.
6. When ready, season the dish with salt & pepper and add the spinach to the sauce and stir until wilted and mixed through.
7. Serve the chilli con carne over some brown rice, pasta, a baked potato or even some cooked vegetables.
8. Top with the cheese and serve.

WHOLEWHEAT SPAGHETTI BOLOGNESE

WHOLEWHEAT SPAGHETTI BOLOGNESE

INGREDIENTS

- 500g lean minced beef or chicken (<5% fat)
- 3 tbsp. extra virgin olive oil
- 1 jar of Bolognese sauce
- 4 handfuls of courgette noodles (or other vegetable noodle)
- 4 medium carrots (finely diced)
- Salt and black pepper
- 100g Wholewheat spaghetti
- 1 small onion (chopped)
- 1 large handful of spinach
- 1 400g tin of lentils (or beans of choice)
- 1 tbsp. grated Parmesan cheese

MAKES 4 SERVINGS
29g of Protein/serving

INSTRUCTIONS

1. In a frying pan, heat the oil over a medium heat, add the onion and cook until soft
2. Add the minced meat and cook until brown, stirring constantly.
3. Add the jar of tomato sauce, the lentils and the diced carrots and allow to simmer while you prepare the spaghetti.
4. Bring a large pot of salted water to a boil. Cook the spaghetti according to package directions and add the courgette noodles for the last minute. Use tongs to transfer the cooked spaghetti to a strainer.
5. Add the spinach to the pasta sauce and stir until wilted and mixed through.
6. Divide the noodles onto 4 plates and top evenly with the meat sauce.
7. Top with the Parmesan cheese and serve.

HEART HEALTHY BURGERS

HEART HEALTHY BURGERS

INGREDIENTS

- 2 low fat beef burger patties (less than 5% fat)
- 2 wholemeal bread rolls
- 2 slices reduced-fat cheddar cheese
- Low-fat sauce of choice (ketchup, barbecue, sweet chilli etc.)
- Olive oil for greasing
- Large handful of salad leaves of choice (spinach, lettuce, rocket etc.)
- 1 cup of salad vegetables of choice (peppers, tomatoes, cucumber etc.) (sliced thinly)
- Pickles (sliced) (optional)
- 2 tbsp. extra virgin olive oil

MAKES 2 SERVINGS
41g of Protein/serving

INSTRUCTIONS

1. Prepare the salad by mixing the leaves and vegetables in a bowl with the olive oil and some salt and pepper.
2. Heat a frying pan to a medium heat with a little olive oil. Add the patties and cook on each side until browned and cooked to your liking.
3. While the burgers are cooking, slice the bread roll and toast under a grill.
4. 1 minute before the burgers are finished cooking, add a slice of cheese on top and cover the frying pan with a lid to melt the cheese slightly.
5. Place the burgers on the buns, add sauce of choice, pickles and enough salad to cover the burger and top with the other half of the bun
6. Serve immediately with the remaining salad.

*This would go great with some of the coleslaw (page 32) or tomato salsa (page 34)

CHICKEN FRIED RICE

CHICKEN FRIED RICE

INGREDIENTS

- 4 tbsp. extra-virgin olive oil
- 500g chicken breasts (about 2 large breasts)
- Salt & black pepper
- 1 tbsp. sesame oil
- 1 medium onion, chopped
- 2 carrots, peeled and diced
- 3 cloves garlic, minced
- 1 tbsp. freshly minced ginger
- 4 c. cooked brown rice (or 2 microwave packs) (less if desired)
- 150g. frozen peas
- 2 large eggs, beaten
- 2 tbsp. soy sauce
- 2 green onions, thinly sliced
- 2 large handfuls of spinach

MAKES 4 SERVINGS
40g of Protein/serving

INSTRUCTIONS

1. In a medium skillet over medium heat, heat the olive oil.
2. Chop the chicken into bite-size chunks and season with salt and pepper, then add to skillet, and cook until golden and no longer pink. Remove from skillet and set aside.
3. To the same skillet, heat ½ a tablespoon sesame oil. Add the onion and carrots and cook until soft, 5 minutes. Add the garlic and ginger and cook until fragrant, 1 minute more.
4. Stir in the rice and peas and cook until warmed through, 2 minutes, stirring continuously.
5. Push rice to one side of skillet and add the remaining sesame oil to the other side. Add the eggs and stir until almost fully cooked, then fold eggs into rice. Add chicken back to skillet with soy sauce, spinach and green onions and stir to combine.

EASY CHICKEN OVEN BAKE

EASY CHICKEN OVEN BAKE

INGREDIENTS

- 500g of meat/fish of choice (lean stewing beef, pork loin, skinless chicken thighs, cod, salmon) (chopped into cubes/chunks)
- 1 500g jar of tomato-sauce (dolmio-style)
- 4 tbsp. extra virgin olive oil
- Salt and black pepper
- 2 medium carrots (chopped into small chunks)
- 1 green pepper (finely chopped)
- 1 medium onion (finely chopped)
- 1 stick celery (finely chopped)
- 100-200g of mushrooms (sliced)

MAKES 4 SERVINGS
30g of Protein/serving

INSTRUCTIONS

1. Pre-heat the oven to 180 degrees C.
2. Fry the meat in a frying pan with half the olive oil until browned on all sides but not fully cooked.
3. Add the meat or fish to an oven proof dish and add the remaining ingredients (including the olive oil). Season with salt and pepper and mix well.
4. Cover the dish with tinfoil and bake in the oven for 1 hour and thirty minutes.
5. Check the vegetables are cooked to your liking and remove dish from oven.
6. Serve immediately.

*These meals can frozen and defrosted later for a quick and easy supper

SCOUSE

SCOUSE

INGREDIENTS

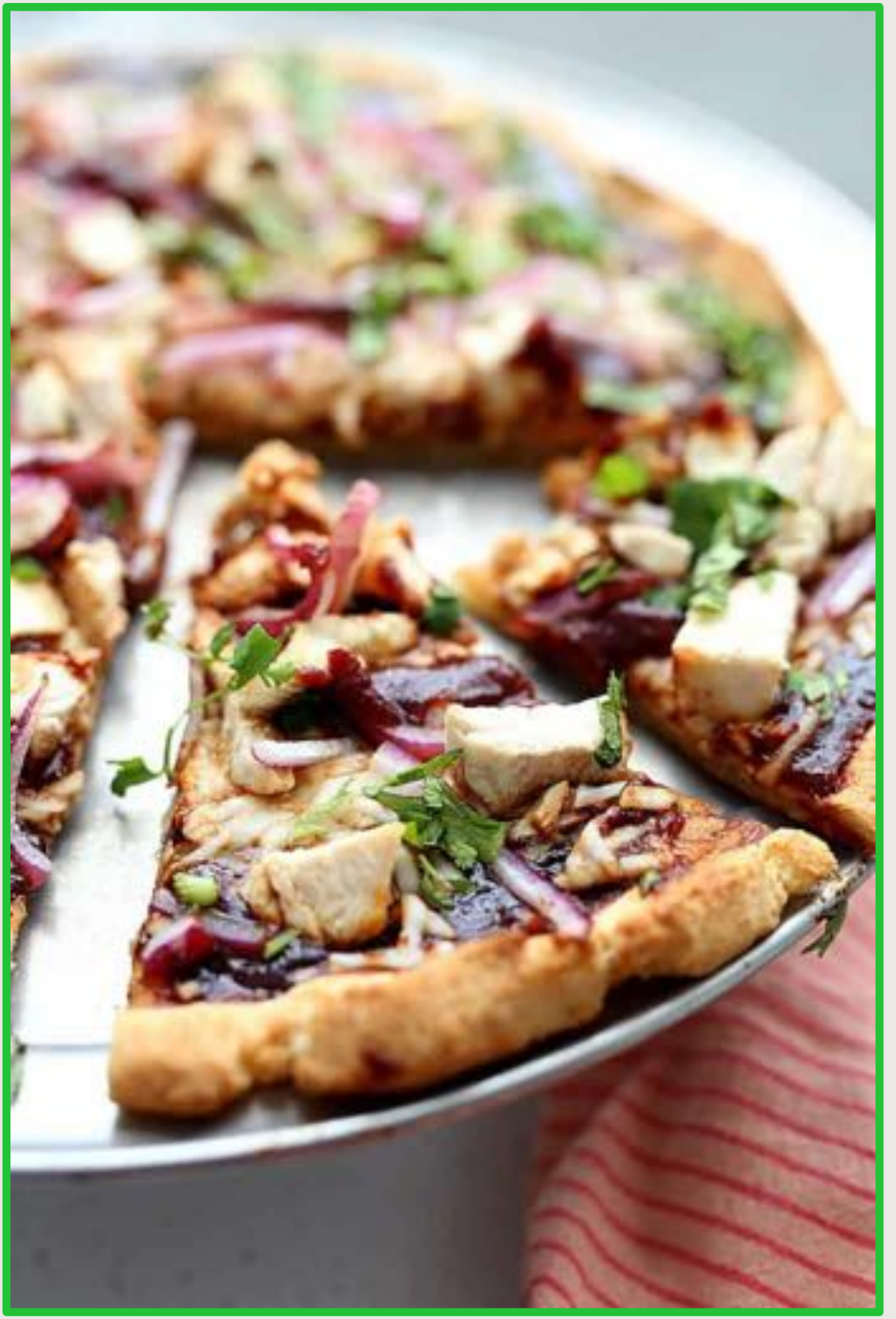
- 500g lean stewing beef (below 5%)
- 4 tbsp. olive oil
- salt and pepper
- 1 large onion, diced
- 1 beef stock cube
- 500ml hot water
- 2 bay leaves
- 4 large carrots, cut into 15mm chunks
- 6 medium potatoes, peeled and chopped into 2cm chunks
- Worcestershire sauce
- pickled red cabbage, to serve

MAKES 4 SERVINGS
27g of Protein/serving

INSTRUCTIONS

1. Dice the meat into whatever size you prefer.
2. Heat the oil in the pan to a medium temperature and add the meat. Season very well with salt and pepper. Stir until brown all over. Add the onions and stir on a medium heat for around 10 minutes; the onions must not brown.
3. Dissolve the stock cubes in the water, add to the pan and bring to the boil.
4. Add the bay leaves, stir, cover and when bubbling, reduce to medium heat. You want the stock to bubble but not violently for 1 hour.
5. Add the carrots and potatoes, turn up the heat until bubbling throughout and reduce slightly. Leave for another 45 minutes to 1 hour with the lid off the pan so it reduces.
6. The stew should reduce once the veg is added but you may need to add more water or perhaps turn the heat up to ensure the liquid is as thick as gravy.
7. Taste and season again if necessary (add 2 tbsp. of Worcestershire sauce or to taste). Serve in a bowl with the red cabbage and plenty of juice on the top.

EASY BBQ CHICKEN & VEG PIZZA



EASY BBQ CHICKEN & VEG PIZZA

INGREDIENTS

- 2 large wholemeal pitta breads (any wholemeal flat-bread will do)
- 2 tbsp. tomato paste or passata
- 2 tbsp. barbecue sauce
- 1 pack (125g) of reduced-fat mozzarella cheese (finely chopped)
- 40g reduced fat grated cheese
- Half a small onion, finely sliced
- Half a bell pepper, finely diced (or any vegetable of choice)
- 1 tbsp. olive oil
- 100g chicken breast (chopped)
- Salt, pepper and chopped basil

INSTRUCTIONS

MAKES 2 SERVINGS
36g of Protein/serving

1. Preheat the oven to 200°C
2. Heat the oil in a frying pan and quickly cook the chicken until it is cooked through. Add salt and pepper to taste and set aside.
3. Split the pittas length wise so you have two thin, pizza rounds from each pitta. Place them, inner surface facing up, on an oven tray
4. Mix the tomato paste/passata with the barbecue sauce and spread evenly over the pitta halves.
5. Sprinkle the cheeses evenly over the pitta halves, followed by the vegetables and the cooked chicken
6. Bake in the oven for about 10 minutes until the cheese has melted and started to take a golden color.
7. Serve immediately with a side salad.

CHICKEN & MUSHROOM RISOTTO

CHICKEN & MUSHROOM RISOTTO

INGREDIENTS

- 1 medium onion, peeled and chopped
- 4 tbsp olive oil
- 300g chicken breast, cut into pieces
- 2 celery stalks, finely diced
- 2 cloves of garlic, crushed
- 200g mushrooms, sliced
- ¼ cup of white wine (optional)
- 200g brown risotto (Arborio) rice
- 1L of low-salt vegetable or chicken stock
- 3 large handfuls of spinach, chopped
- 120g green beans
- ¼ cup of parmesan, grated
- 1 pack (180g) lightest cream cheese

MAKES 4 SERVINGS
33g of Protein/serving

INSTRUCTIONS

1. Fry the onion with 2 tbsp of olive oil in a wide, deep non-stick pan on a medium heat for 3-5 minutes
2. Add the chicken and fry until cooked through and meat turns white.
3. Add the celery, garlic and mushrooms and cook until soft. Add the wine (if using) and the rice to the pan
4. Add a ladle at a time of the stock to the rice & veg and continue doing so slowly until the liquid has almost evaporated/fully absorbed and continue stirring
5. Whilst waiting for the wine to absorb the liquids, steam the green beans in a separate saucepan for 3-5 minutes until tender
6. Slice green beans and add to the risotto when there is a little bit of stock left for the rice to absorb
7. When the rice is cooked through, season to liking, add the parmesan, cream cheese, spinach and 2 tbsp. of olive oil. Stir well and serve

HEALTY BANGERS & MASH

HEALTY BANGERS & MASH

INGREDIENTS

- 1 medium onion, peeled and chopped
- 400g white potatoes, cut into chunks
- 300g carrots, chopped
- 6 tbsp olive oil
- 2 onions, sliced
- 5 garlic cloves, sliced
- Salt and black pepper
- 720ml low-salt vegetable or chicken stock
- Pinch of black pepper
- Pinch of dried thyme
- 1 tbsp. tomato puree
- 8 very low-fat (below 5% fat) sausages
- 400g green beans (fresh or frozen)

MAKES 4 SERVINGS
23g of Protein/serving

INSTRUCTIONS

1. Cook potatoes and carrots in boiling water (or steam) for 20 – 25 minutes and drain well
2. Meanwhile, make the gravy in a large saucepan by adding 2 tbsp. olive oil to the pan, add the onion and the garlic and cook until golden adding a small amount of stock to prevent burning
3. Add the rest of the herbs, tomato puree the remaining stock to the onions and bring to the boil then reduce heat and simmer for 30 – 40 minutes
4. In a medium sized pan bring water to the boil and add in the green beans. Boil (or steam) for 4 minutes.
5. Mash up the potatoes, carrot, 4 tbsp. olive oil and garlic and reheat whilst constantly stirring
6. Cook the sausages in a 200° oven or 20 minutes until golden brown
7. Serve up the mash, sausages and green beans with the gravy

JERK CHICKEN WITH RICE & PEAS

JERK CHICKEN WITH RICE & PEAS

INGREDIENTS

- 400g Chicken breast
- 1 Lime
- 200g Wholegrain basmati rice
- 1 can (400ml) Light coconut milk
- Bunch of spring onions, chopped
- 3 tsp Garlic granules
- Salt and black pepper
- 1½ tsp Dried thyme
- 1 x 400g tin kidney beans (drained and rinsed
- 100ml pre-made jerk marinade/sauce

MAKES 4 SERVINGS
39g of Protein/serving

INSTRUCTIONS

1. Preheat the oven to 180°C
2. Cut each chicken breast into 2 large strips and pour the marinade over the meat, ensuring it is well coated. Cover and leave to marinate in the fridge for an hour (or overnight)
3. Put the chicken pieces in a roasting tin with the lime halves and cook for 35-40 minutes until tender and cooked through.
4. While the chicken is cooking, rinse the rice in plenty of cold water, then tip it into a large saucepan with all the remaining Ingredients: except the kidney beans. Add 165ml cold water and set over a high heat. Once the rice begins to boil, turn it down to a medium heat, cover and cook for 25 minutes.
5. Add the beans to the rice, then cover with a lid. Leave off the heat for 5 minutes until all the liquid is absorbed.
6. Squeeze the roasted lime halves over the chicken and serve with the rice & peas.
7. Serve with a side salad or some healthy coleslaw

COTTAGE PIE

COTTAGE PIE

INGREDIENTS

- 3 large potatoes, chopped into large chunks
- 4 tbsp olive oil
- 6 spring onions finely sliced
- 4 carrots, grated
- 500g lean beef/turkey/pork mince (less than 5% fat)
- 40g reduced-fat cheddar cheese (grated)
- 2 tbsp flour
- 200g frozen peas
- 200ml low-salt vegetable stock
- 6 tbsp Worcestershire sauce
- 100g mushrooms, sliced
- 1 red chilli, finely sliced

MAKES 4 SERVINGS
32g of Protein/serving

INSTRUCTIONS

1. Microwave the potatoes for 4 minutes, rest for 2 minutes and then microwave for a further 4 minutes.
2. Meanwhile, heat olive oil in a large frying pan over a high heat. Add spring onions, carrot, mushrooms and chilli. Fry for 30 seconds, stirring almost constantly.
3. Add mince and cook until brown.
4. Sprinkle in the flour. Stir the mix with the rest of the ingredients and quickly follow with the peas and the beef stock
5. Reduce the heat and stir until the sauce has thickened.
6. Remove the pan from the heat and stir through the Worcestershire sauce.
7. Mash the potato, season with salt and pepper and spoon onto the pie mix and sprinkle with the cheese
8. Place into the oven and cook until the top is golden and crispy

DESSERTS

GREEK YOGHURT "ICE CREAM" BARS

GREEK YOGHURT "ICE CREAM" BARS

INGREDIENTS

- 250g fat-free Greek yoghurt or quark
- 250mls semi skimmed milk
- 50g cocoa powder
- 2 tsp vanilla essence
- Granulated artificial sweetener of choice (to taste)
- Ice cream freezer moulds

MAKES 5 SERVINGS
6g of Protein/serving

INSTRUCTIONS

1. Add all the ingredients (except the sweetener to a bowl and mix well with a whisk.
2. Gradually add the sweetener and keep tasting until you get the right amount of sweetness.
3. Pour the mixture into moulds leaving a little space at the top for expansion in the freezer.

*Let the bars sit out at room temperature for 5-10 minutes before trying to release them from the mould. You can also run the moulds under hot water for a few seconds to release the bars.

CARROT CAKE MUFFINS

CARROT CAKE MUFFINS

INGREDIENTS

- 225g whole wheat flour
- 1 ½ teaspoons baking powder
- 1 teaspoon ground cinnamon
- ½ teaspoon baking soda
- ½ teaspoon salt
- ½ teaspoon ground ginger
- ¼ teaspoon ground nutmeg
- 2 cups peeled and grated carrots* (about 3 large or up to 6 small)
- 50g roughly chopped walnuts
- 75g raisins
- 80ml extra-virgin olive oil
- 1 cup granulated artificial sweetener
- 2 eggs
- 250g fat-free Greek yogurt
- 1 teaspoon vanilla extract

MAKES 12 SERVINGS
6g of Protein/serving

INSTRUCTIONS

1. Preheat oven to 220 degrees Celsius. Grease all 12 cups on your muffin tin with non-stick cooking.
2. In a large mixing bowl, combine the flour, baking powder, cinnamon, baking soda, salt, ginger and nutmeg. Blend well with a whisk. Add the grated carrots, raisins and chopped walnuts to the other ingredients and stir.
3. In a medium mixing bowl, combine the oil and sweetener and beat together with a whisk. Add the eggs and beat well, then add the yogurt and vanilla and mix well.
4. Pour the wet ingredients into the dry and mix with a big spoon, just until combined (a few lumps are ok). Divide the batter evenly between the 12 muffin cups. Bake muffins for 13 minutes, or until the muffins are golden on top and a toothpick inserted into a muffin comes out clean.

FRESH FRUIT & PROTEIN YOGHURT

FRESH FRUIT & PROTEIN YOGHURT

INGREDIENTS

- 250g fat-free quark, skyr or Greek yoghurt
- 30g almonds or walnuts (or nuts of choice
- Artificial sweetener (to taste)
- 1-2 cups of mixed chopped fruit (apples, bananas, oranges, kiwis, peaches, pineapple, mango, grapes, pears, raspberries, strawberries, blueberries etc.

MAKES 1 SERVING
31g of Protein/serving

INSTRUCTIONS

1. Sweeten the quark or yoghurt with artificial sweetener to taste
2. Top with the chopped fruit and nuts and serve

*You can also add half a teaspoon of vanilla essence to the yoghurt for extra flavour

THE ULTIMATE LAZY MEAL

Sometimes, you want something very quick, easy and tasty to make and you might only have 5-10 minutes to make it. In that case, this "recipe" will become your best friend.

INGREDIENTS

Take one item (or a selection) from each box.

VEGETABLES
Frozen/Pre-chopped etc.

PROTEIN
Pre-Cooked/Frozen/Pre-chopped etc.

SOUP/SAUCE
1 portion (half a container)

OPTIONAL

INSTRUCTIONS

1. Mix the chopped vegetables, protein and soup/sauce in a sauce pan or microwave bowl.
2. Heat (stove top or microwave) until cooked (5-10 minutes)
3. That's it. Serve with wholegrain bread or brown rice if hungry.

THE ULTIMATE LAZY MEAL

Add to bowl

Heat in microwave

Enjoy

SNEAK MORE VEGGIES INTO YOUR MEALS

As you can see, vegetables are a really important part of all the recipes in this guide. The tips below will help you to include more vegetables into your other meals, easily and without affecting the taste, so you can get all the health benefits of lots of vegetables, every day.

Breakfast

Frittata it.
Eggs are a great, high-protein way to start the day. Mix eggs with veggies for a healthy and hearty breakfast

Add minced broccoli or cauliflower to scrambled eggs.
This veggie addition doesn't change the texture of eggs and fits in an entire serving of veggies (at least). Steam and purée or finely grate the veg to mix with scrambled eggs.

Bake with them.
Breakfast sweets can be packed with veggies too. Try making some travel-friendly bran muffins packed with zucchini and carrots in addition to the classic raisins, walnuts, and cinnamon.

Veg out on savory oatmeal.
Classic oatmeal might be topped with brown sugar and fruit, but oats can be savory too! Cook plain oats with water and add your choice of steamed or fried veggies. Top with an egg for extra protein and season with salt, pepper, or a sprinkle of Parmesan cheese.

Try pumpkin or butternut squash pancakes or waffles.
When the frying pan is heating, throw some pumpkin or squash purée into pancake or waffle mix to fit in an extra serving of veggies (and get a fun orange tinge, too).

Pasta & Grains

Make pasta dishes go green.
When spaghetti and meatballs is on the menu, add a load of extra veggies (like spinach, peppers and mushrooms) to the dish instead of opting for a boring side salad.

Experiment with veggie noodles.
Veggie noodles are quite easy to find in big supermarkets and allow you to skip the pasta altogether. (Or use roast spaghetti squash!) Add extra veggies to the sauce for an extra dose of nutrients.

Remember herbs are leafy greens too!
Add fresh herbs to any rice, pasta, or grain dish. Or whip up a quick homemade herb pesto to add to scrambled eggs or use as a sandwich spread.

Get fancy with shepherds pie.
It's a childhood favourite, but grown ups crave it too—don't lie! When you want some, add a load of fresh veggies for a dose of extra nutrients. Spinach, tomatoes, peas, and broccoli make great additions.

Sneak them in casseroles.
Anytime that casserole dish comes out of the cupboard, get the grater out too. Finely shredded carrot or summer squash can be added to virtually any casserole without changing taste or texture!

Smoothies

Add greens to breakfast smoothies.
A handful of spinach or kale blends well with any fruit smoothie. Try by blending 250g of fat-free quark or Greek yoghurt, 1 frozen banana, 2 handfuls spinach (and 1 tablespoon peanut butter if you like).

Slurp a carrot smoothie.
Grated carrots are easy-peasy to fit into any fruit smoothie. Bonus: Because we're using all parts of the veggie, none of the fibre is lost like in juices.

Sandwiches

Sub greens for wraps.
Lettuce makes a surprisingly good stand-in for bread and tortilla wraps.

Add veggies to grilled cheese.
Melted cheese between two slices of bread doesn't have much green value. Every time the cheesy craving strikes, throw in a few layers of veggies. Spinach, corn , tomato, and red onion make great additions.

Bulk up burgers.
Everyone loves a burger so try making them healthier by adding lots of greens like spinach and lettuce, and other veggies like tomatoes, pickles, shredded carrot, onions etc…

Pizzas

Add colour

Yes, a cheesy pizza is hard to pass up. But pizzas are a great vehicle for a big pile of veggies. Practically anything works, from greens and tomato to roasted squash or root vegetables.

Prepare a pizza salad.

If pizza is for dinner, throw a salad on top for a fun meal to eat, and an easy two-in-one dinner. A favourite? Rocket and spinach salad with tomatoes and Parmesan on top of a mixed veggie pizza.

Soups & Stews

Add veggie purée to chicken soup.

Making classic chicken soup? Add a can of puréed tomatoes, squash, or spinach. It will make for a thicker soup and also sneak in some extra veggies.

Improve on ready-made soup

Add your own vegetables; frozen, sliced, pureed to ready made soup to add even more fibre, vitamins and minerals.

Spice up chili.

Add carrot, sweet potato, or butternut squash purée, peppers and broccoli to any chili or stew recipe.