Lifestyle influences on cardiometabolic health: systemic and cellular mechanisms

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Authors Declaration

I declare that the work in this thesis was carried out in accordance with the regulations of Liverpool John Moores University. Apart from the help and advice acknowledged, the work within was solely completed and carried out by the author.

Any views expressed in this thesis are those of the author and in no way represent those of Liverpool John Moores University and the School of Sport and Exercise Science.

This thesis has not been presented to any other University for examination either in the United Kingdom or overseas. No portion of the work referred to in this research project has been submitted in support of an application for another degree or qualification of this or any other university or institute of learning.

Signed

Date

Thesis Abstract

Introduction: Cardiometabolic disease (CMD) encompasses all metabolic and cardiovascular disorders including metabolic abnormalities such as dyslipidaemia, hypertension, insulin resistance, abdominal obesity and inflammation. Skeletal muscle accounts for the largest increase in insulin stimulated glucose uptake. In accordance with this, skeletal muscle mass and function has an inverse association with CMD, type 2 diabetes (T2D) and cardiovascular disease (CVD). Poor lifestyle choices can disrupt the metabolic environment causing skeletal muscle insulin resistance further exacerbating CMD and risk of T2D and CVD. Low carbohydrate (LC) diets have shown to improve markers of CMD. However, the impact of the metabolic environment on skeletal muscle insulin dynamics are yet to be investigated. Anabolic-androgenic steroid (AAS) are derived from the hormone testosterone which plays a key role in regulating skeletal muscle mass and cardiometabolic health. Paradoxically, although AAS increase muscle mass, supraphysiological levels of testosterone increase CMD and risk of CVD. Further research is warranted on markers of CMD among AAS users in addition to its impact on the metabolic environment and subsequent skeletal muscle insulin mechanisms. Therefore, this thesis aims to investigate how markers of CMD are impacted by diets differing in carbohydrate intake and anabolic-androgenic steroid use and how their subsequent metabolic environment impacts skeletal muscle cellular mechanisms.

Methods: In a randomised parallel design, willing participants followed either a VLC or high carbohydrate (HC) and provided anthropometrics, blood samples, physical activity and food diaries at 0, 4 and 8 weeks. In a cross-sectional design, resistance-trained males who use AAS and did not use (NAT) provided similar measures. Blood samples were processed for plasma and serum and analysed for markers of CMD. Mechanisms of insulin signalling were investigated using the murine C2C12 skeletal muscle cell line. Under standard cell culture procedures (Differentiating media: DM), C2C12 myoblasts and myotubes were stimulated with insulin to investigate cellular signalling and glucose uptake. To investigate the impact of diet or AAS use on insulin signalling, C2C12 myoblasts and myotubes were incubated in serum derived from participants and stimulated with insulin. The impact of human serum on C2C12 metabolism was also determined.

Results: Both diets significantly ($P < 0.05$) improved triglyceride, insulin, HOMA IR and leptin:adiponectin levels. Compared with HC, the VLC diet showed significantly ($P < 0.05$) greater improvements in blood pressure, fat mass, leptin and small dense low-density lipoprotein cholesterol (sdLDL-C) low-density lipoprotein cholesterol ratio. The VLC diet also reported trends in improving sdLDL-C, FGF21 and waist circumference ($P = 0.06$). In myoblasts, serum from both groups showed a tendency ($P = 0.07$) of increasing Akt

phosphorylation (p-AKT) but had no effect on the insulin stimulated fold-change. In myotubes, both diets showed a significant ($P < 0.01$) decrease in p-Akt but had no effect on the insulin stimulated fold-change. Insulin stimulated glucose uptake also did not significantly change with either diet. In myotubes, both diets resulted in a significant ($P < 0.05$) decrease in p-AMPK^{thr172} and which may have caused the reduction in p-Akt in myotubes.

The AAS group showed significantly ($P < 0.05$) lower levels of high-density lipoprotein cholesterol (HDL-C), apolipoprotein A1 (ApoA1) and plasminogen activator inhibitor 1. Myoblasts and myotubes significantly ($P < 0.01$) increased p-Akt^{ser473} in response to insulin stimulation in NAT and AAS conditions. No difference was observed between groups. CK activity significantly ($P < 0.01$) increased comparably between conditions yet protein abundance was significantly ($P = 0.04$) higher in AAS serum *vs.* DM after 96 hr. In myoblasts, serum significantly ($P < 0.05$) increased Akt^{ser473}, mTOR^{ser2448}, ERK1/2$^{T202/Y204}$ and P38$^{t180/Y182}$ phosphorylation. ERK and P38 phosphorylation was significantly ($P < 0.05$) higher compared to DM. In myotubes, all conditions similarly significantly ($P < 0.05$) increased p-Akt^{ser473} and p-ERK$^{T202/Y204}$ at 30 mins. MPS and the rate of MPB was similar between groups at all timepoints.

Conclusion: In summary, both a VLC and HC diet showed similar improvements in some markers of CMD. However, a VLC diet induced greater improvements in blood pressure, fat mass and sdLDL/LDL-C. Serum from both groups reduced cellular energy stress as shown by the reduction in p-AMPK which is likely to have increased insulin sensitivity at baseline.

AAS users display reduced HDL-C and ApoA1 leading to increased CVD risk. Serum derived from AAS users does not impair insulin signalling or glucose uptake compared to non-users. Serum derived from resistance trained males may enhance skeletal muscle differentiation similarly to AAS users via increased P38 and ERK activity compared with DM.

Contents

List of Figures

List of Tables

List of Equations

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II Dedication

For my parents, grandparents and family, who have always supported and been there for me, thank you, you have always worked so hard to provide me with the opportunity to fulfil my potential, I hope I have become the person you have always wanted me to be.

To Katie, thank you for your love, support and patience throughout this PhD. It hasn't always been easy, but we have shared some amazing experiences these past years and I can't wait to create many more.

III Abbreviations

AAS-p	AAS-related Polypharmacy
2NBDG	2-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino)-2-Deoxyglucose
6-PG	6-Phosphogluconate
AAS	Anabolic-Androgenic Steroid
ADP	Adenosine Diphosphate
AICAR	5-Aminoimidazole-4-Carboxamide Ribonucleotide
AMPK	AMP Activated Protein Kinase
APO	Apolipoprotein
Apo B	Apolipoprotein B
ApoA1	Apolipoprotein A1
ApoCIII	Apolipoprotein CIII
ARIC	Atherosclerosis Risk in Communities
AS160	160-kDa Substrate of Akt
ATCC	American Type Culture Collection
ATP	Adenosine Triphosphate
BCAA	Branched Chain Amino Acids
BF%	Body Fat %
BMI	Body Mass Index
BP	Blood Pressure
BSA	Bovine Serum Albumin
CE	Cholesterol Esters
CK	Creatine Kinase
CMD	Cardiometabolic Disease
CO_2	Carbon Dioxide

CON	Control
CRP	C-Reactive Protein
CS	Conditioned Human Serum
CVD	Cardiovascular Disease
DBP	Diastolic Blood Pressure
DM	Differentiating Media
DMEM	Dulbecco's Modified Minimum Essential Medium
DMSO	Dimethyl Sulphoxide
ECL	Enhanced Chemiluminescence
EDTA	Ethylenediaminetetraacetic Acid
ERFC	Emerging Risk Factors Collaboration
ERK 1/2	Extracellular Regulated Kinases 1/2
FBS	Fetal Bovine Serum
FFA	Free Fatty Acids
FFM	Fat Free Mass
FFM/I	Fat Free Mass Index
FFQ	Food Frequency Questionnaire
FGF21	Fibroblast Growth Factor 21
FSC	Forward Scatter
G6P	Glucose-6-Phosphate
G-6-PDH	Glucose-6-Phosphate Dehydrogenase
GI	Glycaemic Index
GLUT4	Glucose Transporter 4
GLUTs	Glucose Transporters
GM	Growth Media
HbA1c	Glycated Haemoglobin A1c

HC	High Carbohydrate
HDL	HDL Particle
HDL-C	High Density Lipoprotein Cholesterol
HOMA IR	Homeostatic Model of IR
HRP	Horseradish Peroxidase
HTGL	Hepatic Triglyceride Lipase
IdLDL	Intermediate Size and Density Low Density Lipoprotein
IGF-I	Insulin-like Growth Factor-I
IL-6	Interleukin-6
INS	Insulin
IQR	Interquartile Range
IR	Insulin Resistance
IRS-1	Insulin Receptor Substrate -1
ISAK	International Society for the Advancement of Kinanthropometry
JNK	cJun-N-Terminal-Kinase
LAR	Leptin/Adiponectin Ratio
lbLDL	Large and Buoyant Low-Density Lipoprotein
LC	Low Carbohydrate
LCAT	Lecithin–Cholesterol Acyltransferase
LDL	Low Density Lipoprotein
LDL-C	Low Density Lipoprotein Cholesterol
LDL-P	Low Density Lipoprotein Particle
LDL-R	Low Density Lipoprotein Receptor
L-G	L-Glutamine
Lp (a)	Lipoprotein (a)
LPL	Lipoprotein Lipase

LRP	LDL Receptor Related Protein
MAPK	Mitogen-Activated Protein Kinases
MEF2	Myocyte Enhancer Factor 2
MetS	Metabolic Syndrome
MLC	Moderately Low Carbohydrate
MPS	Muscle Protein Synthesis
MRF	Myogenic Regulatory Factors
MRF4	Myogenic Regulatory Factor 4
mTORc2	Mammalian Target of Rapamycin Complex 2
MUFA	Monosaturated Fatty Acids
MVPA	Moderate to Vigorous Intensity
Myf5	Myogenic Factor 5
MyoD	Myoblast Determination 1
NAD	Nicotinamide Adenine Dinucleotide
NADH	Nicotinamide Adenine Dinucleotide + Hydrogen
NAT	Natural
NBCS	Newborn Calf Serum
NEFA	Non-Esterified Fatty Acids
OGTT	Oral Glucose Tolerance Test
PAI-1	Plasminogen Activated Inhibitor-1
PBS	Phosphate Buffered Saline
PCOS	Polycystic Ovary Syndrome
PDK-1	3'Phosphoinositide-Dependent Kinase-1
PFA	Paraformaldehyde
PI3K	Phosphatidylinositol 3-Kinase
PIP_2	PI(4,5)-Bisphosphate

PIP_3	PI(3,4,5)-Trisphosphate
PKA	Protein Kinase A
PKC	Protein Kinase C
PS	Penicillin Streptomycin
PUFA	Polyunsaturated Fatty Acids
RCT	Randomised Controlled Trials
REC	Research Ethics Committee
RIPA	Radioimmunoprecipitation Assay
RT	Room Temperature
SBP	Systolic Blood Pressure
SC	Satellite Cells
sdLDL	Small-Dense Low-Density Lipoprotein
sdLDL-C	Small Dense Low-Density Lipoprotein-Cholesterol
SFA	Saturated Fatty Acids
SNS	Sympathetic Nervous System
SR-B1	Scavenger Receptor B1
SSC	Side Scatter
T2D	Type 2 Diabetes
TBST	TBS-Tween
TC	Total Cholesterol
TCA	Tricarboxylic Acid Cycle
TEMED	Tetramethylethylenediamine
Test C	Testosterone Cypionate
Test E	Testosterone Enanthate
TG	Triglycerides
TMT	Tris/MES Triton Buffer

TNF-α	Tumour Necrosis Factor Alpha
TRL	Triglyceride Rich Lipoprotein
TRL-C	Triglyceride Rich Lipoprotein Cholesterol
VAT	Visceral Adipose Tissue
VLDL	Very Low-Density Lipoprotein
VLC	Very Low Carbohydrate
vsdLDL	Very Small and Dense Low-Density Lipoprotein
WC	Waist Circumference
WHR	Waist to Hip Ratio

Chapter 1: General introduction and literature Review

Publication resulted from this chapter; McCullough, D., Webb, R., Enright, K.J., Lane, K.E., McVeigh, J., Stewart, C.E. & Davies, I.G. (2020). How the love of muscle can break a heart: Impact of anabolic androgenic steroids on skeletal muscle hypertrophy, metabolic and cardiovascular health. Reviews in Endocrine and Metabolic Disorders. doi.org/10.1007/s11154-020-09616-y.

1.1. General introduction

Prior to the modern era, cardiovascular disease (CVD) was evident in humans dating back as early as 3300 BC (Thompson *et al.*, 2013; Clarke *et al.*, 2014). Since then, improvements in healthcare and sanitation largely decreased human mortality rates due to infectious diseases, allowing CVD and non-communicable diseases such as diabetes and cancer to dominate the modern day causes of death worldwide (World Health Organization, 2018). All diseases of the heart and circulatory system are referred to as CVD, with type 2 diabetes (T2D), a metabolic dysfunction in which glucose uptake by tissues and organs is impaired, increasing the risk of CVD (Samuel and Shulman, 2016; British Heart Foundation, 2020). Globally, an estimated 17.8 million and 1.4 million deaths were caused by CVD and diabetes respectively in 2017, an increase of 21% in CVD and 34% in diabetes from 2007 (Roth *et al.*, 2018). In Europe, 19.9 million people are anticipated to be living with CVD and 60 million are living with diabetes (Saeedi *et al.*, 2019; Timmis *et al.*, 2020). CVD costs the UK economy £7.4 billion annually and the global economic burden of CVD is projected to reach $1,044 billion by the year 2030, nearly a $200 billion rise from 2010 (Timmis et al., 2020). Similarly, the current global cost of diabetes is estimated to be $760 billion and projected to cost $825 billion by 2030 (Williams *et al.*, 2020). Although recent healthcare advances have helped to reduce the rise in mortality rates, the very high prevalence rates of CVD and diabetes and the associated costs are widespread.

Leading an unhealthy lifestyle such as poor nutrition, drug use, lack of physical activity , and smoking can increase the risk of developing T2D and CVD (Sattar, Gill and Alazawi, 2020; Yusuf *et al.*, 2020). Associated with these diseases are the interrelated clustering of metabolic abnormalities such as high triglycerides, low high-density lipoprotein cholesterol (HDL-C), elevated blood glucose, hypertension and elevated waist circumference (WC), also referred to as metabolic syndrome (MetS) (Alberti *et al.*, 2009; Sperling *et al.*, 2015). Additionally, insulin resistance (IR), high visceral adipose tissue (VAT) and small dense low-density lipoprotein cholesterol are associated with MetS (Sperling *et al.*, 2015). This accumulation of abnormalities is often referred to as cardiometabolic disease (CMD) and highly associated with T2D and CVD (Martín-Timón, 2014; Sperling *et al.*, 2015).

Diet has a great influence on overall health and wellbeing and poor dietary patterns are associated with CMD and risk of developing T2D and CVD (Rodríguez-Monforte *et al.*, 2017; Medina-Remón *et al.*, 2018). The current UK guidelines suggest a diet high in carbohydrates (although low in sugar) and low to moderate in fat (particularly saturated fat) (PHE, 2015; 2016). These guidelines were adopted in 1983 to combat the rise in CVD in the UK (Walker,

1983). Conversely, lower carbohydrate (<20 % of energy intake) high fat diets have shown to at least perform as well as high carbohydrate diets in reducing body fat and improving metabolic health risk factors even with an increase in saturated fatty acids (SFA) intake (Bueno *et al.*, 2013; Mansoor *et al.*, 2016; Gardner *et al.*, 2018; Tay *et al.*, 2018). With such conflicting data and recommendations, further research is warranted on the effect of carbohydrates and fat on CMD and the underlying physiological and biochemical mechanisms. For example; skeletal muscle and adipose tissue can undergo drastic modifications in response to lifestyle (Beavers *et al.*, 2017) thus, investigation of such tissues may help elucidate mechanisms of disease.

Under euglycaemic-hyperinsulinaemic conditions skeletal muscle shows a pronounced increase (~80%) in glucose uptake, thus highlighting its importance in regulating whole-body glucose homeostasis (Defronzo *et al.*, 1981; Kelley *et al.*, 1988; DeFronzo and Tripathy, 2009). The human body requires approximately 4 g of glucose in the circulation and is required to sustain energy metabolism (Wasserman, 2009). When homeostasis is challenged, in response to exercise for example, glucose uptake by skeletal muscle is significantly increased; however, to maintain blood glucose levels glycogenolysis occurs within the liver to increase blood glucose and prevent hypoglycaemia (Wasserman, 2009). In contrast, a meal containing carbohydrates will rapidly increase blood glucose levels. To prevent hyperglycaemia, insulin is released by the pancreas to increase glucose uptake by the liver and muscles (Wasserman, 2009). A disruption in this precise regulatory network can have dire consequences on human health, potentially leading to MetS, T2D and subsequent CVD.

Approximately 30-40% of human body mass consists of skeletal muscle and increased levels have shown to reduce the risk of CVD (Stump *et al.*, 2006; Srikanthan, Horwich and Tseng, 2016; Tyrovolas *et al.*, 2020). Skeletal muscle tissue displays high plasticity and undergoes significant remodelling throughout the lifespan as the muscle's size and quality changes in response to ageing, hormonal changes, nutrition and physical activity levels and types (Flück and Hoppeler, 2003). Furthermore, elevated adipose tissue levels, particularly VAT, lead to increased inflammation and lipid toxicity resulting in skeletal muscle dysfunction (De Carvalho *et al.*, 2019). Although evidence has shown skeletal muscle mass and function to have an inverse association with CMD (Atlantis *et al.*, 2009; Tyrovolas *et al.*, 2020), pharmaceutical enhancement of skeletal muscle may increase the risk of CVD (Thiblin *et al.*, 2015; Goldman, Pope and Bhasin, 2019). For instance, anabolic-androgenic steroid (AAS) use has grown since the 1980s in the recreational gym user, primarily to increase muscle mass (Sagoe *et al.*, 2014). However, AAS use is an independent risk factor for premature death and cardiac events (Thiblin *et al.*, 2015).

The anabolic hormone testosterone, of which AAS are derived from, plays an integral role in regulating skeletal muscle mass and cardiometabolic health (Traish and Kypreos, 2011; Goldman, Pope and Bhasin, 2019). It is estimated that 6.4% of males and 1.6% of females use AAS to improve perceived body image (Sagoe *et al.*, 2014). Considering the relatively high prevalence rates, there is currently a lack of research on the impact on overall cardiometabolic risk and poor understanding of the cellular mechanisms driving the risk. In addition to increasing the risk of cardiovascular events, supraphysiological levels of circulating testosterone also dramatically reduce HDL-C levels, increase low-density lipoprotein cholesterol (LDL-C) levels (Achar, Rostamian and Narayan, 2010). AAS use has also been shown to be associated with elevated markers of CMD such as, increased VAT, IR and hypertension (Rasmussen *et al.*, 2017, 2018). Elevated CMD markers particularly IR and VAT lead to a perpetual cycle of dysfunctional metabolism which further exacerbates CMD (Kirk and Klein, 2009; Abdul-Ghani and DeFronzo, 2010). Understanding the effects of dysregulated metabolism on key cellular mechanisms such as insulin signalling is paramount in developing effective interventions to reduce CMD risk.

A key feature of CMD is IR which involves the inadequate response of normal insulin concentrations in adipose, muscle and liver tissues and is the precursor to T2D (Meshkani and Adeli, 2009). Within healthy insulin sensitive tissues, a highly conserved insulin signalling pathway regulates glucose uptake. Upon sensing an increase in blood glucose by the β-cells within the pancreas, insulin is secreted to increase cellular glucose uptake (Samuel and Shulman, 2016). Insulin binds to the insulin receptor and activates downstream signalling including Akt, a key mediator in insulin signalling. Activation of Akt results in glucose transporter 4 translocation to the plasma membrane allowing for glucose uptake (Karlsson *et al.*, 2005; Sakamoto and Holman, 2008). IR occurs when this signalling cascade is diminished resulting in reduced activation of key molecules, reduced cellular glucose uptake and subsequent hyperglycaemia (Samuel and Shulman, 2016). Lipotoxicity, inflammation, oxidative stress, hyperglycaemia and mitochondrial dysfunction, all features of CMD, are known to cause inhibition of the insulin signalling cascade (Boucher *et al.*, 2014). Therefore, the metabolic environment created by poor lifestyle choices such as diet and AAS use, in addition to ageing are highly responsible for IR and subsequent T2D and CVD and further research on the underpinning cellular mechanisms is required.

The development of cell culture techniques has been key in progressing the understanding of biology and has benefited society greatly in developing lifesaving treatments (Yao and Asayama, 2017). Cell culture media has been developed and modified for over 100 years to allow successful culture of biological systems such as skeletal muscle (Yao and Asayama, 2017). For example, Dulbecco's modified minimum essential medium (DMEM) is a synthetic

medium containing high concentrations of amino acids and glucose which, when supplemented with serum, allows for successful proliferation and fusion of murine C2C12 cells, which allows for the study of skeletal muscle metabolism, structure and function (Freshney, 2015; Yao and Asayama, 2017). Serum derived from animals improves cellular proliferation and survival due to it containing various components such as proteins, lipids, hormones and growth factors (Yao and Asayama, 2017). However, serum is ill-defined as its components can vary from different batches limiting the potential reproducibility of results (Freshney, 2015; Yao and Asayama, 2017). Furthermore, media development has been optimised to improve cell culture conditions but not to replicate *in vivo* conditions which may limit its relevance depending on the investigation (Freshney, 2015). To increase the mechanistic understanding of human metabolism, cellular experiments should aim to replicate the *in vivo* environment without compromising cell culture conditions.

As lifestyle factors influence the *in vivo* metabolic environment such as insulin signalling, use of serum derived from humans for cell culture use may offer a relevant cellular model to investigate cellular mechanisms associated with those lifestyles, i.e. exercise, diet or hormonal changes. Therefore, this thesis aims to investigate how markers of CMD are impacted by the lifestyle factors of diet and anabolic-androgenic steroid use and how their subsequent metabolic environment effects skeletal muscle cellular mechanisms (Figure 1.1).

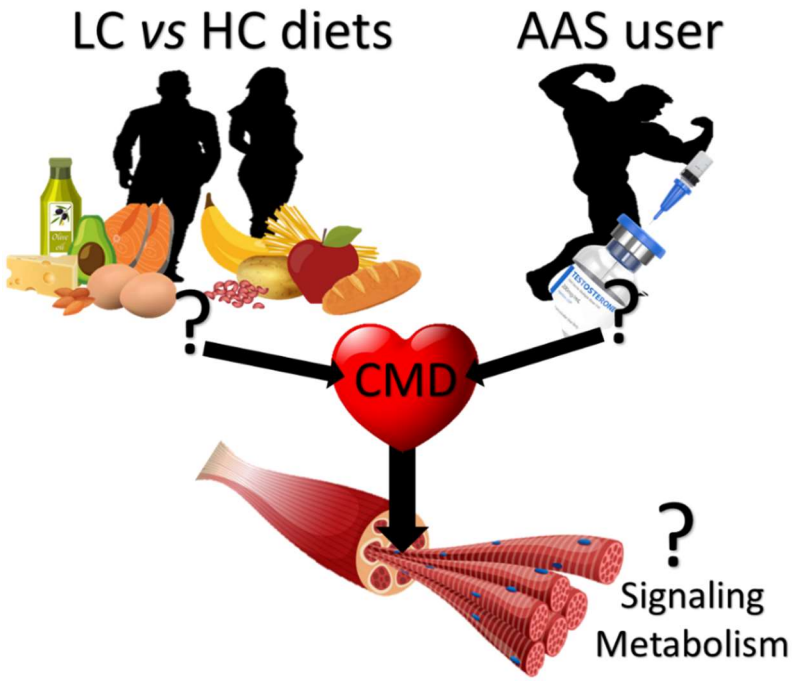


Figure 1.1. Overview of thesis goals.
AAS; Anabolic-androgenic steroids; CMD; cardiometabolic disease, HC; High carbohydrate; LC, Low carbohydrate.

1.2. Literature review

1.2.1. Skeletal muscle

1.2.1.1. Structure and function

Skeletal muscle contains 50-75% of all body proteins and is comprised of water (75%), protein (20%) and minerals, inorganics salts, fats and carbohydrates (5%) (Frontera and Ochala, 2015). The human body contains around 600 skeletal muscles and their primary functions include: locomotion, postural control, energy metabolism and generating heat (MacLaren and Morton, 2011). Skeletal muscle is a highly adaptable and organised tissue. It is surrounded by the epimysium, connective tissue that comprises of bundles of myofibres. These bundles of myofibres are surrounded by more connective tissue called the perimysium (Frontera and Ochala, 2015). Myofibres are unique in that they are multinucleated post-mitotic cells and each myofibre is surrounded by the cell membrane (sarcolemma) (Figure 1.2). The sarcolemma contains the sarcoplasm, which contains the subcellular organelles such as mitochondria and nuclei and vital energy stores such as carbohydrates (glycogen) and fats (triglycerides) (MacLaren and Morton, 2011). The largest component of the sarcoplasm are the myofibrils which are considered the contractile machinery of the muscle and contain actin (thin filament) and myosin (thick filament) proteins (MacLaren and Morton, 2011).

1.2.1.2. Regeneration, differentiation and growth

A key feature of skeletal muscle tissue is its ability to regenerate and is characterised by activation, proliferation and differentiation of satellite cells (SC) (Mukund and Subramaniam, 2020). Satellite cells are located in a specialised niche between the basal lamina and sarcolemma (Mauro, 1961; Dumont, Wang and Rudnicki, 2015). SC are tethered to binding sites within the basal lamina to relay intracellular signals from myofibres and their niche contains growth factors, chemokines and signalling molecules which all can regulate SC activity (Mukund and Subramaniam, 2020). Under resting conditions SC are quiescent and are characterised by expression of Pax7 protein (Dumont, Wang and Rudnicki, 2015). Upon activation (by injury or growth stimulus), SC become activated and express myogenic regulatory factors (MRF), myogenic factor 5 and myoblast determination 1 (MyoD) and proliferate extensively. The now activated SC or myoblast downregulate Pax7 expression while upregulating expression of further MRFs; myogenin, myocyte enhancer factor-2 and MRF4 resulting in exit of the cell cycle, differentiation and fusion to the myofibre (Figure 1.3) (Dumont, Wang and Rudnicki, 2015; Mukund and Subramaniam, 2020). During regeneration,

some of the activated SC will not commit to differentiation and go through the process of self-renewal to replenish the SC pool for future regeneration (Mukund and Subramaniam, 2020).

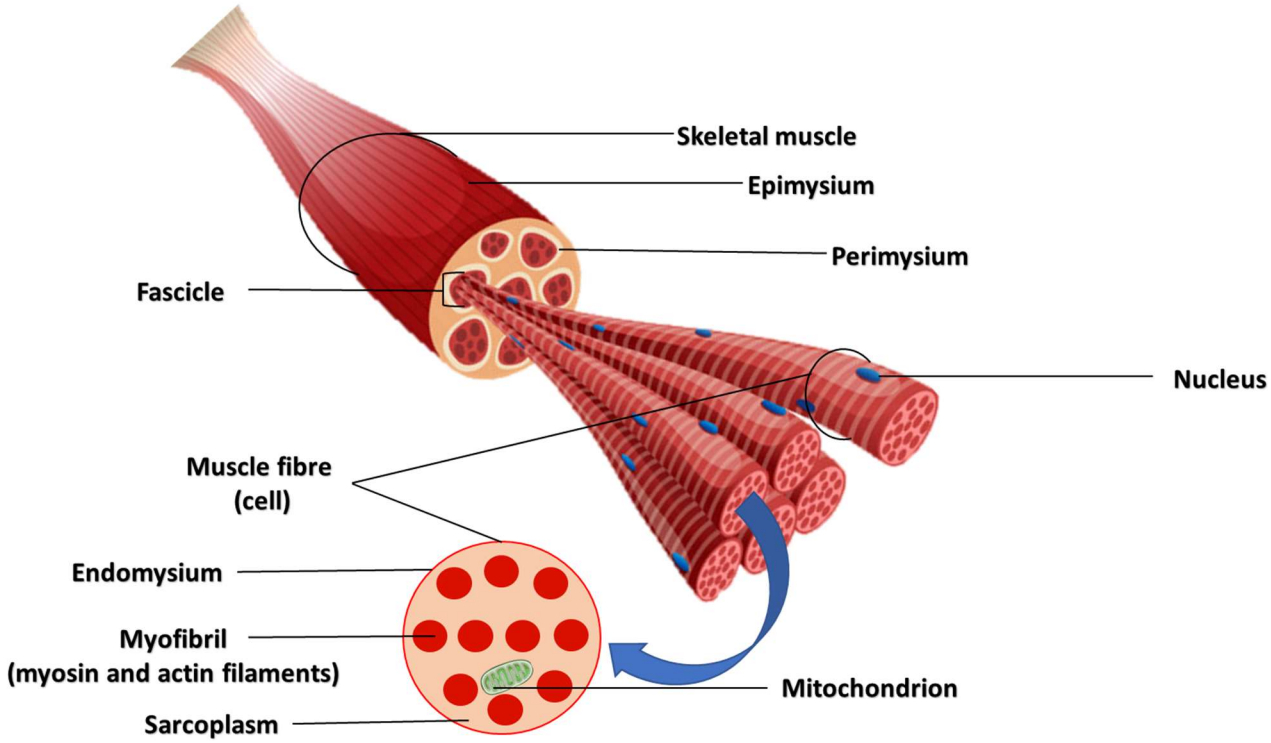


Figure 1.2. Skeletal muscle structure and components.

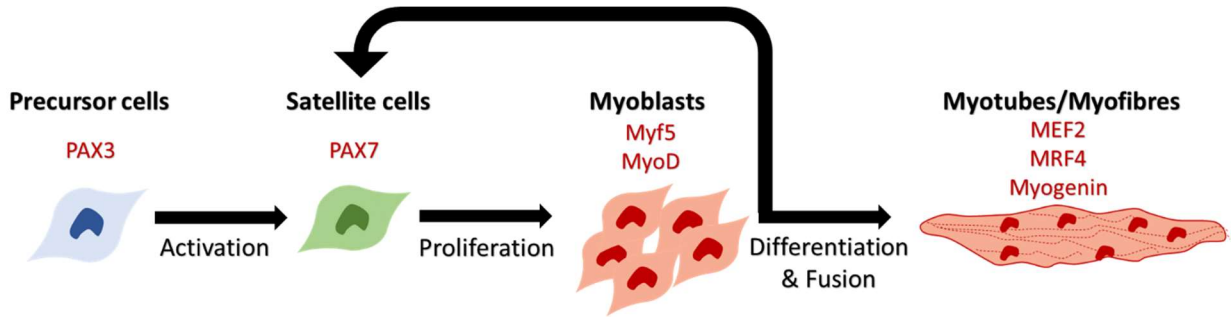


Figure 1.3. Satellite cell myogenic lineage and expression of transcription factors during skeletal muscle regeneration. *MEF2; myocyte enhancer factor 2, MRF4; myogenic regulatory factor 4*; *MyoD; Myoblast determination 1; Myf5, myogenic factor 5.*

1.2.1.3. Culture of skeletal muscle

Skeletal muscle cells have been acquired for study *in vitro* over numerous decades (Yao and Asayama, 2017). The myogenic cell line, C2 was initially derived from the thigh of 2-month old C3H mice and later re-cloned to produce the commercially available murine C2C12 cell line (Blau, Chiu and Webster, 1983; Scharner and Zammit, 2011). The C2C12 cell line is well

recognised within the literature and has been shown to be a good model to investigate skeletal muscle anabolism/catabolism, ageing, signalling and metabolism (Taylor *et al.*, 2001; Sharples *et al.*, 2011; Turner *et al.*, 2018). The production of cell lines has allowed researchers to study the same homogenous sample of cells (Yao and Asayama, 2017). There are also universal protocols specific to each cell line such as recommended media and supplements i.e. types of serum, increasing reproducibility of results globally (Yao and Asayama, 2017). However, as media and supplements were developed to enhance cell culture conditions, they may contain supraphysiological levels of nutrients, hormones or growth factors which may limit their relevance *in vivo* (Freshney, 2015). Furthermore, in addition to modifying acute cellular signalling, function and metabolism, supplements and media may alter the phenotype expression of the cell line leading to inappropriate experimental conditions. Therefore, media and supplement selection must be considered based on the investigation. For example; use of serum/plasma derived from *in vivo* with an *in vitro* model may provide a more physiologically relevant model, while also maintaining the flexibility of experimentation (Carson *et al.*, 2018; Kalampouka, van Bekhoven and Elliott, 2018; Cogan *et al.*, 2019).

Investigations studying the acute effects of conditioned serum/plasma have demonstrated the effect of nutrient availability on skeletal muscle signalling (Carson *et al.*, 2018; Cogan *et al.*, 2019). When used as a supplement for culture media, serum derived from participants (n=4) prior to and following (60 min) whey protein ingestion demonstrated significant increases in anabolic signalling in C2C12 myotubes (Carson *et al.*, 2018). However, no significant increase in muscle protein synthesis (MPS) was observed between fasted and fed serum indicating that other endocrine factors may have maximised MPS (Carson *et al.*, 2018). In a double blinded randomised crossover control trial, the impact of fasted and fed serum on glucose transporter 4 (GLUT4) translocation was investigated prior to and following the ingestion of different protein sources. Data demonstrated that cells cultured with 5% fasted human serum resulted in similar maximal increases in GLUT4 translocation as observed with 1 μM of insulin (Cogan *et al.*, 2019). Therefore, to maintain GLUT4 sensitivity during fed experiments cells were exposed to 1% serum. Following exposure of cells for 60 min with serum derived from participants who consumed hydrolysed whey protein or an isonitrogenous non-essential amino acids, GLUT4 translocation increased only in the hydrolysed whey protein group (Cogan *et al.*, 2019). These novel data highlight the impact of fasted and fed serum on acute insulin and protein dynamics of the cell. Not only can serum impact glucose and protein metabolism of skeletal muscle cells, it can also alter cell signalling pathways associated with human phenotype (Kalampouka, van Bekhoven and Elliott, 2018). For example, plasma derived from young (27 ± 2 y/o) and older (64 ± 2 y/o) adults demonstrated differential effects on acute C2C12 signalling. When cultured with older plasma, cells experienced reduced

myoblast migration in response to injury and reduced myotube diameter compared with young plasma (Kalampouka, van Bekhoven and Elliott, 2018). These results are synonymous with *in vivo* and *in vitro* data that indicates that older adults have a reduced capacity of skeletal muscle repair compared with young (Brzeszczyńska *et al.*, 2018). This highlights that the endocrine environment of human plasma or serum can impact the phenotype expressed by muscle cells and is representative of the *in vivo* phenotype. While investigations by Carson *et al*. (2018), Kalampouka, van Bekhoven and Elliott (2018) and Cogan *et al.* (2019) have demonstrated the acute effects of human serum and plasma on skeletal muscle metabolism and signalling a chronic model is yet to be investigated. A chronic model of growing and differentiating myotubes with human serum for 4-6 days may also impact *in vitro* metabolism dynamics through altering phenotype expression relevant to the *in vivo* phenotype. Such experiments warrant investigation as this may lead to a more physiologically relevant model when investigating cellular metabolism compared with traditional models using animal serum. It may also reduce the reliance on invasive measures such as muscle biopsies and animal products which is a focus of many bodies.

1.2.1.4. Skeletal muscle metabolism and health regulation

Skeletal muscle is highly metabolic due to high requirements of adenosine triphosphate (ATP) to enable force production and accounts for ~20-30% of resting energy expenditure (Zurlo *et al.*, 1990). The primary sources of energy for skeletal muscle are glycolysis and oxidative metabolism. Under euglycaemic-hyperinsulinaemic conditions skeletal muscle glucose uptake increases by ~80% (Defronzo *et al.*, 1981; Kelley *et al.*, 1988; DeFronzo and Tripathy, 2009). Skeletal muscle also plays a key role in protein metabolism and during the fasted state or when exogenous amino acids are unavailable, muscle protein is used to maintain the amino acid pool. This maintains a steady supply of amino acids for the vital organs and to be used as a precursor for gluconeogenesis to maintain euglycaemia, resulting in decreased muscle mass. With skeletal muscle contributing to such a high proportion of whole-body metabolism, any disruption in its ability to do so will negatively impact health, thus, it's not surprising that skeletal muscle has shown to regulate weight management and insulin sensitivity (Wolfe, 2006).

Obesity is primarily due to an imbalance of energy intake and energy expenditure and is widely associated with many diseases (Blüher, 2019). A major component of resting energy expenditure is skeletal muscle (Zurlo *et al.*, 1990), but its metabolic requirements can vary between individuals or within one individual across the lifespan (Manini, 2010). This variability in muscle metabolic requirements can be primarily contributed to differences in MPS due to changes in muscle mass. Muscle protein synthesis requires large amounts of energy, It is

therefore estimated that MPS costs a young athletic male ~360 kcal/d more than an elderly woman however, this can be much more in extreme cases (Wolfe, 2006). Although, increased fatness is associated with insulin resistance (IR) and the development of type 2 diabetes (T2D) (Nguyen *et al.*, 2008), the metabolic functionality of skeletal muscle also plays a direct role in regulating insulin sensitivity (Li *et al.*, 2015; Brøns and Grunnet, 2017). Deposition of triglycerides in muscle has shown to be associated with IR however, in participants with obesity without IR, muscular triglyceride deposition has not shown to be associated (Kelley *et al.*, 1999; Manco *et al.*, 2000). Therefore, an imbalance between muscle fatty acid uptake and disposal can result in an accumulation of intracellular triglycerides leading to disruption in metabolic function of muscle (Goodpaster and Sparks, 2017). Fatty acid uptake by muscle is proportional to delivery and within obese conditions, fatty acid delivery is increased however, as not all obese conditions are associated with increased muscular triglyceride deposition and IR, impaired disposal of fatty acids may be the contributing factor (Kelley *et al.*, 1999; Wolfe, 2006). Individuals with IR and T2D show impaired fatty acid oxidation resulting in reduced exercise performance (Goodpaster and Sparks, 2017). Physical inactivity, a common lifestyle factor of individuals with T2D, is a key contributor to reduced mitochondrial functionality thus causing impaired fatty acid oxidation (Turner *et al.*, 2014). Fatty acids that do not undergo oxidation are used as substrates for triglyceride and phospholipid synthesis resulting in the production of second messenger signals such as diglycerides and ceramides which have been shown to interfere with the insulin signalling cascade (Turner *et al.*, 2014; Li *et al.*, 2015; Petersen and Shulman, 2018).

As skeletal muscle tissue is highly adaptable it can undergo significant remodelling in response to physical activity, nutrition, lipotoxicity, hormonal changes and inflammation (Flück and Hoppeler, 2003; Egan and Zierath, 2013; Lipina and Hundal, 2017). Due to this, maintenance of muscle health throughout the lifespan by following a healthy lifestyle can reduce the risk of developing CMD, T2D and CVD (Strasser, Siebert and Schobersberger, 2010; Srikanthan, Horwich and Tseng, 2016; Liu *et al.*, 2019; Tyrovolas *et al.*, 2020). For example; handgrip strength, a surrogate for skeletal muscle mass/strength, has been shown to be inversely associated with systolic blood pressure (SBP) and fasting levels of triglycerides, glucose, insulin and, positively associated with high-density lipoprotein cholesterol (HDL-C) all of which are associated with CVD risk (Lawman *et al.*, 2016). A systematic review of 160 randomised control trials (RCT) in healthy participants showed exercise to significantly reduce CMD by improving levels of cardiorespiratory fitness, plasma triglycerides, HDL-C, insulin and haemoglobin a1c (HbA1c) (Lin *et al.*, 2015). Furthermore, overnutrition can result in ectopic fat accumulation resulting in skeletal muscle IR (Shulman,

2014); however, weight loss in individuals with obesity can decrease intermuscular adipose tissue and increase insulin sensitivity (Addison *et al.*, 2014; Gower and Goss, 2015).

1.2.2. Cardiometabolic disease

CMD encompasses all metabolic and cardiovascular disorders including T2D and CVD. It is typically associated with the often interconnected constellation of metabolic abnormalities such as dyslipidaemia, hypertension, IR (hyperglycaemia and hyperinsulinaemia) abdominal obesity and inflammation (Sperling *et al.*, 2015) (Figure 1.4). Specifically, increased fasting plasma triglycerides, hypertension, hyperglycaemia and abdominal obesity are referred as the medical condition, metabolic syndrome (MetS) (Alberti *et al.*, 2009). With the exception of hyperinsulinaemia and inflammation each condition is defined by specific thresholds to infer CVD risk (Table 1.1) (Arnett *et al.*, 2019; Mach *et al.*, 2020).

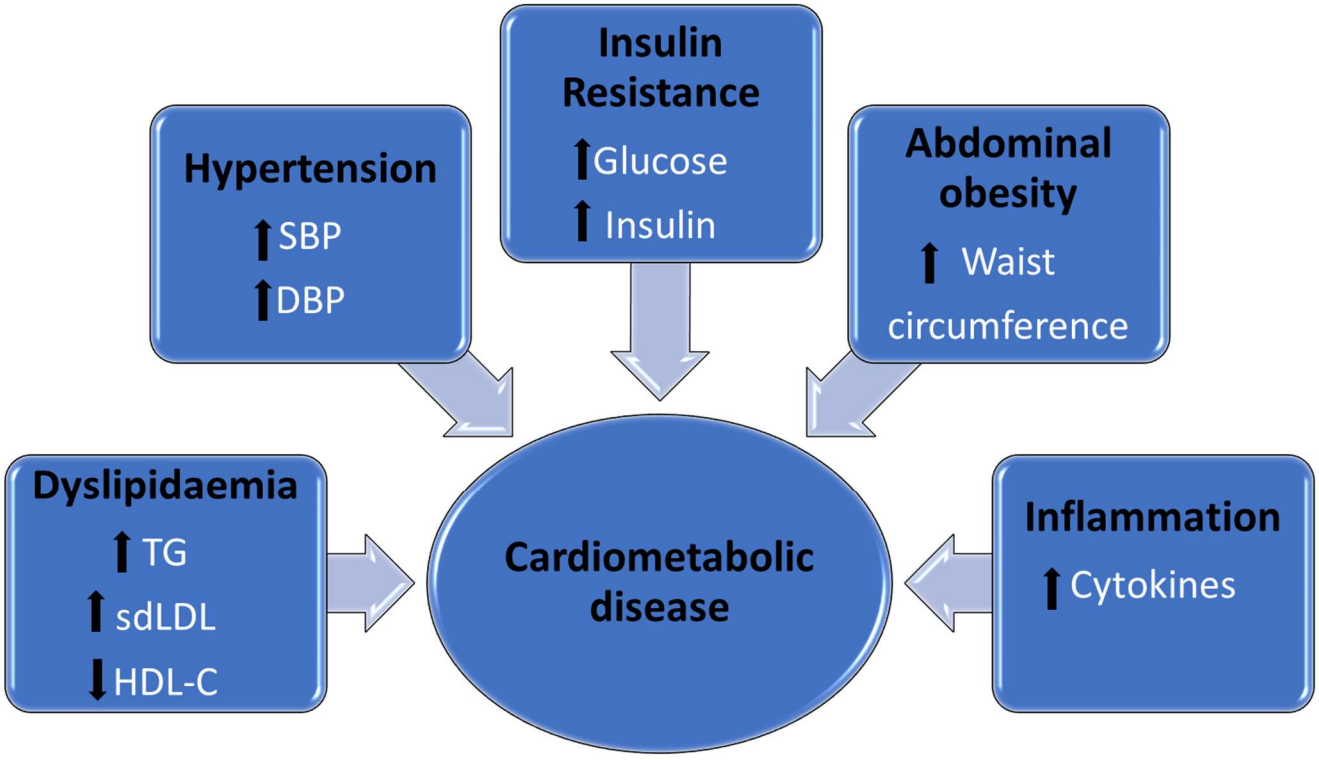


Figure 1.4. Cardiometabolic disease and metabolic abnormalities that attribute to it.
DBP; diastolic blood pressure, HDL-C; high-density lipoprotein cholesterol, SBP; systolic blood pressure, sdLDL; small-dense low-density lipoprotein, TG; triglycerides.

Table 1.1: Cardiometabolic disease thresholds.

Cardiometabolic measure	Cut off:
Fasting glucose	> 5.5 mmol/L
Fasting triglycerides	> 1.7 mmol/L
Fasting HDL-C	< 1.0 mmol/L (males), < 1.3 mmol/L (females)
Fasting LDL-C	> 3.0 mmol/L
Waist circumference:	> 102 cm (males), > 88 cm (females)
Systolic blood pressure:	> 140 mmHg
Diastolic blood pressure:	> 90 mmHg

HDL-C; high-density lipoprotein cholesterol, LDL-C; low-density lipoprotein cholesterol (Alberti *et al.*, 2009; Arnett *et al.*, 2019; Mach *et al.*, 2020)

High fasting glucose was original defined as > 6.1 mmol/L in 1997, however, to align with the population risk associated with impaired glucose tolerance (defined as 2 hr blood glucose concentration > 7.8 mmol/L), it was later changed to > 5.5 mmol/L in 2003 (Nathan *et al.*, 2007).

1.2.2.1. Dyslipidaemia

Atherogenic dyslipidaemia is underpinned by high levels of triglycerides, prevalence of small dense low-density lipoprotein (sdLDL) particles and/or low levels of HDL-C and is associated with increased CVD risk (Arnett *et al.*, 2019; Sandesara *et al.*, 2019; Mach *et al.*, 2020).

Increased triglyceride levels are reported to be associated with increased risk of CVDs as demonstrated by two large prospective cohort studies from Copenhagen (Nordestgaard *et al.*, 2007; Freiberg *et al.*, 2008). In 13,981 adults not taking lipid-lowering therapy, higher levels of non-fasting triglyceride levels were associated with CVDs and mortality after 27 years of follow-up. Compared to <1 mmol/L of triglyceride levels, participants with >5 mmol/L had an increased risk of myocardial infarction (5-fold; females, 2-fold; males), ischaemic heart disease (3-fold; females, 2-fold; males), ischaemic stroke (4-fold; females, 3-fold; males) and all-cause mortality (3-fold; females, 2-fold; males) when adjusted for medications, body mass index (BMI), age and lifestyle factors (Nordestgaard *et al.*, 2007; Freiberg *et al.*, 2008). However, the emerging risk factors collaboration (ERFC) trial which included 303,430 participants without CVD with a follow up of 2.79 million person-years showed attenuated association of triglycerides on CVD risk when accounting for HDL-C levels and no risk with the addition of non-HDL-C levels (Di Angelantonio *et al.*, 2009). Indicating that HDL-C levels may be of greater importance than triglyceride levels; however, RCTs that have increased HDL-C levels have failed to improve CVD risk and focus has shifted back to the role of

triglycerides on cardiovascular health (Sandesara *et al.*, 2019). As triglycerides are used for fuel by most cells, it is unlikely to have a causal effect on atherosclerosis. However, the cholesterol content of triglyceride-rich lipoproteins (TRL) are not easily degraded and may be more likely to cause CVD (Nordestgaard and Varbo, 2014). Like low density lipoprotein (LDL), TRL can enter the arterial intima but may lead to greater preferential retention of cholesterol-enriched remnants due to their larger size (Nordestgaard and Varbo, 2014; Duran *et al.*, 2020). TRLs can undergo direct phagocytosis by macrophages contributing to foam cell formation, inflammation and atherosclerotic plaque, unlike LDL; which requires modification by oxidation prior to phagocytosis (Nordestgaard and Varbo, 2014; Duran *et al.*, 2020). Liberation of free fatty acids and pro-inflammatory molecules due to lipoprotein lipase (LPL) activity of the TRL may also cause local injury and inflammation to blood vessels (Nordestgaard and Varbo, 2014). Therefore, high triglyceride levels may indicate as a marker for remnant lipoprotein particles rich in cholesterol that have a greater efficiency for contributing to atherosclerosis compared to LDL (Nordestgaard and Varbo, 2014) and thereby CVD risk. Increased concentrations of triglyceride rich lipoprotein cholesterol (TRL-C) associated with atherogenic dyslipidaemia is associated with a 2-fold increase in total CVD and cerebrovascular disease risk, 3-fold increased risk of myocardial infarction, and 2.5-fold increased risk of peripheral artery disease (Duran *et al.*, 2020)

Epidemiological studies have regularly validated LDL-C to be a reliable marker of CVD risk and LDL has shown to have a causal effect on CVD (Ference *et al.*, 2017). The Framingham heart study reported that men (n = 2489) and women (n = 2856) with LDL-C levels of >4.14 mmol/L had a >65% increase in CVD risk compared with LDL-C levels <3.37 mmol/L over a ten-year period (Wilson *et al.*, 1998). The Atherosclerosis Risk in Communities (ARIC) Study also showed that CVD risk increase by approximately 40% for every 1 mmol/L increment in LDL-C levels (Sharrett *et al.*, 2001). Although LDL-C was not a primary measure by the ERFC study in relation to CVD risk, higher non-HDL-C which highly correlates with LDL-C, was associated with a 50% increase in coronary heart disease risk (Di Angelantonio *et al.*, 2009). Furthermore, subsample (n = 44,234) analysis where LDL-C and non-HDL-C concentrations were both available, showed similar levels of risk in developing coronary heart disease (Di Angelantonio *et al.*, 2009). However, LDL (LDL-P) number rather than LDL-C may represent a more accurate estimation of CVD risk due to discordance between both measures (Otvos *et al.*, 2011). When discordance occurs (LDL-P > LDL-C), LDL-P shows a greater association with CVD events and markers of metabolic health compared with LDL-C (Otvos *et al.*, 2011). This discordance indicates the heterogeneity of LDL particles that vary in size, density, and lipid content (Ivanova *et al.*, 2017).

LDL can be separated into 4 groups: large and buoyant (lbLDL), intermediate size and density (idLDL), small and dense (sdLDL) and very small and dense (vsdLDL) (Ivanova *et al.*, 2017). High circulating particles of sdLDL and vsdLDL indicate a greater risk of CVD events compared to total LDL alone or lbLDL (Hoogeveen *et al.*, 2014; Ivanova *et al.*, 2017). Cholesterol is primarily synthesized in the liver and circulates around the body as very-low-density lipoprotein (VLDL) (rich in triglycerides) of which apolipoprotein B100 (ApoB) is the major apolipoprotein and is a surrogate for LDL-P (Packard *et al.*, 2000; Sniderman *et al.*, 2019). Upon interaction with lipases at various tissues, the VLDL containing triglycerides are hydrolysed, and free fatty acids are released for energy or subsequent storage as adipose tissue (Figure 1.5) (Freeman and Walford, 2015). The remaining lipoprotein is now cholesterol-rich, triglyceride poor LDL (or LDL-C). This LDL will bind to the hepatic LDL receptor to increase LDL-C clearance (Freeman and Walford, 2015). With dysregulated metabolism, as observed in CMD, there is an increase in circulating sdLDL which, has a lower affinity for the LDL receptor, therefore, having a reduced clearance rate, subsequently increasing circulating levels and CVD risk (Thongtang *et al.*, 2017). The sdLDL can also penetrate the arterial wall easier compared to lbLDL due to its small size increasing the risk of trapping ApoB depositing atherogenic cholesterol and increasing the risk of a CVD event (Sniderman *et al.*, 2019). As each sdLDL particle has less cholesterol than lbLDL particle, when cholesterol is equal there is a great number of sdLDL particles highlighting why LDL-P may estimate CVD risk compared with LDL-C. In accordance with the association of the composition of LDL particles and CVD risk, 2 main phenotypes (A and B) have been described (Diffenderfer and Schaefer, 2014). Phenotype A is characterised by the prevalence of lbLDL and idLDL whereas, phenotype B is characterised by the prevalence of sdLDL and vsdLDL (Diffenderfer and Schaefer, 2014; Ivanova *et al.*, 2017). Phenotype B has been found to be associated with low HDL-C and high TG and has also been observed in many metabolic diseases (Hoogeveen *et al.*, 2014; Fan *et al.*, 2019).

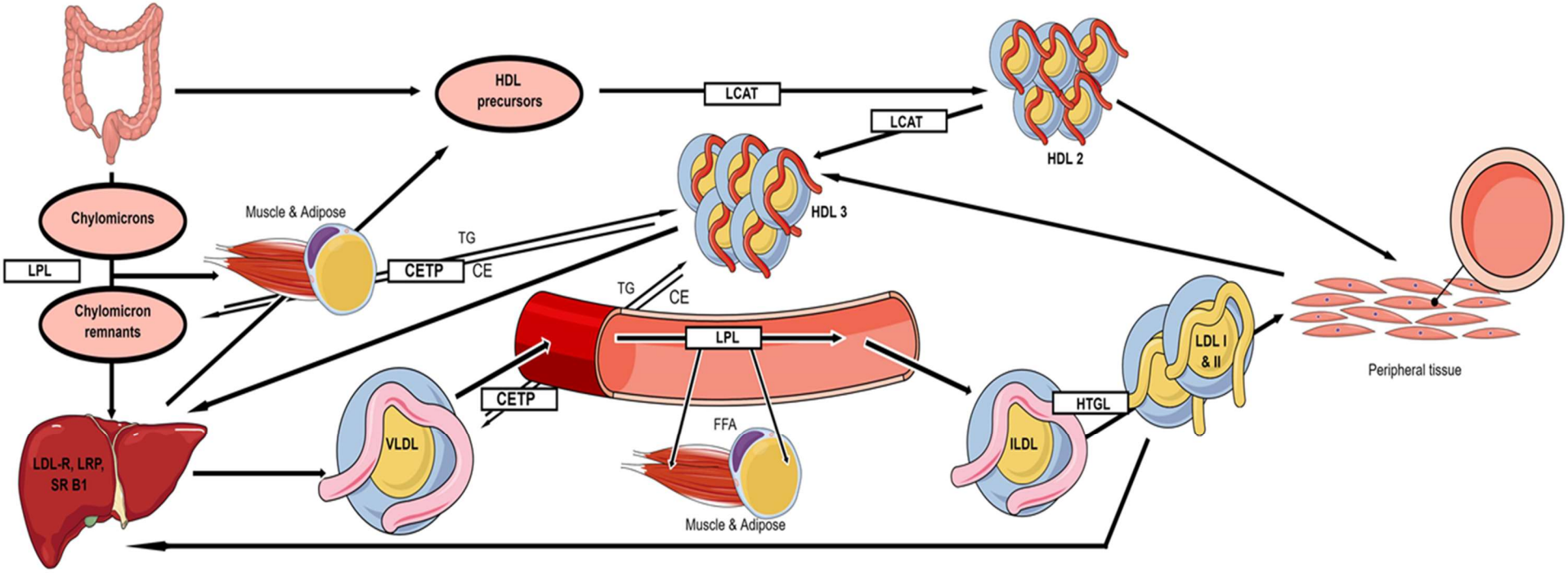


Figure 1.5. Normal lipoprotein metabolism. During normal lipoprotein metabolism, intestinally produced chylomicrons carrying dietary lipids are hydrolysed by lipoprotein lipase (LPL). Free fatty acids (FFA) are liberated and taken up by tissues. Resulting chylomicron remnants are taken up by the liver via low-density lipoprotein receptor (LDL-R) and the LDL receptor-related protein (LRP). Meanwhile, hepatically produced VLDL transport cholesterol esters (CE) and triglycerides (TG) through blood vessels, during which they undergo LPL hydrolysis, releasing FFA which are taken up by peripheral tissues. This loss of TG reduces VLDL particle size (and therefore density) and renders it cholesterol-enriched idLDL. Due to the action of HTGL, idLDL particles reduce in size again to form LDL. LDL particles have an increased propensity to deposit cholesterol in peripheral tissues; however, they primarily transport cholesterol to the liver, where they are taken up by the LDL-R. The intestine also produces precursors which contribute towards the production of HDL. Small HDL_3 particles acquire CE and TG and form larger HDL_2 particles, which with the assistance of lecithin–cholesterol acyltransferase (LCAT) subsequently exchange CE for even more TG with VLDL particles and chylomicrons, before travelling to the liver where they are taken up by scavenger receptor B1 (SR-B1) or LDL-R.

In contrast to LDL-C, HDL-C has a strong inverse association with CVD risk and measures of CMD (Rader and Hovingh, 2014). Higher levels of HDL-C have been shown to be associated with reduced CVD risk as shown by the Framingham heart, ARIC and ERFC trials (Wilson *et al.*, 1998; Sharrett *et al.*, 2001; Di Angelantonio *et al.*, 2009). HDL-C concentrations of >1.5 mmol/L showed a reduced CVD risk by approximately 35 and 40% in females and males respectively (Wilson *et al.*, 1998). However, in contrast with LDL-C lowering therapies, therapies to increase HDL-C have shown a lack of improvement in CVD risk (Katzmann and Laufs, 2019; Riaz *et al.*, 2019). A recent systematic review and meta-analysis of 31 RCTs revealed that HDL-C raising therapies (niacin, fibrates or cholesterol ester transfer protein inhibitors) did not improve CVD risk (Riaz *et al.*, 2019). Furthermore, Mendelian trials of metabolic disorders resulting in very low HDL-C levels did not result in cardiovascular disease (Rader and Hovingh, 2014). Due to this, the functionality of HDL rather than HDL-C may have a more causal relationship with CVD (Rader and Hovingh, 2014).

HDL, particularly subfraction HDL_2 transports cholesterol away from peripheral tissue, including arterial lesions, to the liver to be excreted, through a process of reverse cholesterol transport (Fuster *et al.*, 2005; Santos-Gallego *et al.*, 2011). This process of cholesterol efflux from macrophages may offer atheroprotection and has shown to be inversely associated with CVD independent of HDL-C concentrations (Khera *et al.*, 2011; Qiu *et al.*, 2017). HDL and its major apolipoprotein, apolipoprotein A1 (ApoA1), has also been observed to elicit an anti-inflammatory and antioxidant effect on the vascular system further reducing the potential of CVD (Barter *et al.*, 2004; Rader and Hovingh, 2014). Due to this, interventions that improve the functionality of HDL may be of greater importance rather than increasing HDL-C levels. Nonetheless, HDL-C is still a strong biomarker of cardiovascular health and associated with metabolic abnormalities in relation to IR (Robins *et al.*, 2011).

1.2.2.2. Insulin resistance

Insulin resistance is the precursor of the development of Type 2 diabetes (T2D), with lipid accumulation and inflammation being implicated as the primary triggers (Meshkani and Adeli, 2009; Esser *et al.*, 2014; Samuel and Shulman, 2016). IR can be measured by the hyperinsulinaemic-euglycemic clamp with IR being defined as a glucose disposal rate below 5.6 mg/kg of fat free mass+17.7/min (Tam *et al.*, 2012). IR is a key feature of CMD as it is regularly present in individuals with CVD, MetS, hypertension, obesity, polycystic ovary syndrome (PCOS), chronic low grade inflammation, testosterone deficiency and ageing (Karpe, Dickmann and Frayn, 2011; Kelly and Jones, 2013; Tangvarasittichai, 2015; Ormazabal *et al.*, 2018; Shorakae *et al.*, 2018; Arpón *et al.*, 2019).

Skeletal muscle is the largest tissue for insulin-induced glucose uptake but adipose tissue and the liver are also involved (Wilcox, 2005). A key regulator of insulin induced glucose uptake is the serine/threonine kinase Akt (Whiteman, Cho and Birnbaum, 2002). In addition to mediating insulin signalling, Akt is a multifunctional protein kinase implicated in glycogen synthesis, cell proliferation and survival, protein synthesis and inhibition of catabolic pathways (Manning and Toker, 2017). There are 3 isoforms of Akt, Akt1, 2 & 3, with Akt2 being primarily expressed in metabolic tissues and playing a key role in insulin induced glucose uptake (Garofalo *et al.*, 2003; Gonzalez and McGraw, 2009).

In healthy insulin sensitive tissue, insulin binds to the insulin receptor on the cell membrane causing its tyrosine phosphorylation of the receptor (Figure 1.6). The now activated insulin receptor causes phosphorylation of insulin receptor substrate-1 (IRS-1) on tyrosine residues, which allows the recruitment of the Type IA phosphatidylinositol -3- phosohate kinase (PI3K). PI3K catalyses the formation of PI(4,5)-bisphosphate (PIP_2) to PI(3,4,5)-trisphosphate (PIP_3) thus recruiting 3' phosphoinositide-dependent kinase-1 (PDK-1) which phosphorylates Akt at the catalytic domain Thr308 (Meshkani and Adeli, 2009; Boucher *et al.*, 2014; Samuel and Shulman, 2016; Haeusler, McGraw and Accili, 2018). Furthermore, conversion of PIP_2 to PIP_3 by insulin results in mammalian target of rapamycin complex 2 (mTORc2) activation which causes maximal activation of Akt by phosphorylation of ser473 in the hydrophobic domain (Manning and Toker, 2017). Activation of Akt initiates phosphorylation of 160-kDa substrate of Akt (AS160) which stimulates translocation of glucose transporter 4 (GLUT4) storage vesicles to fuse at the cell surface to release GLUT4 into the plasma membrane allowing cellular glucose uptake (Sakamoto and Holman, 2008; Li *et al.*, 2019).

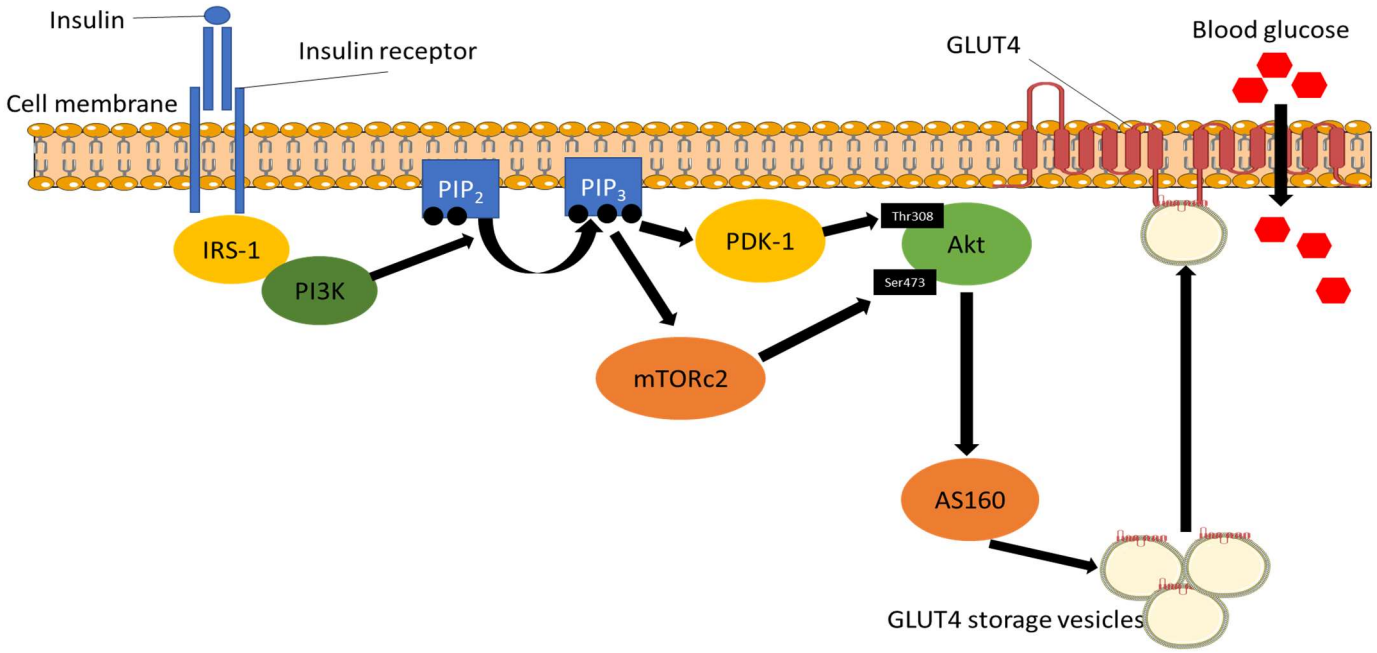


Figure 1.6. Mechanisms of Insulin stimulated cellular glucose uptake. *AS160, 160-kDa substrate of Akt; GLUT4, Glucose transporter type 4; IRS-1, Insulin receptor substrate-1; mTORc2, mammalian*

target of rapamycin complex 2; PDK-1, 3' Phosphoinositide-dependent kinase-1; PI3K, phosphatidylinositol 3' kinase; PIP_2, PI(4,5)-bisphosphate; PIP_3, PI(4,5) trisphosphate.

However, within IR tissue this signalling cascade is diminished with lipotoxicity, inflammation, oxidative stress, hyperglycaemia, hyperinsulinaemia and mitochondrial dysfunction all being implicated (Boucher *et al.*, 2014; Samuel and Shulman, 2016; Yang, Vijayakumar and Kahn, 2018). This diminished response due to metabolic disturbances is reported to occur upstream of Akt at the insulin receptor or IRS-1 resulting in reduced Akt activity, glucose uptake and protein synthesis (Figure 1.7) (Boucher *et al.*, 2014; Samuel and Shulman, 2016). Accumulation of lipid products such as diacylglycerols and ceramides, most likely from following a lifestyle consisting of poor nutrition and physical inactivity, can activate protein kinase C (PKC) isoforms which can cause serine or threonine phosphorylation of the insulin receptor thereby reducing insulin stimulated tyrosine phosphorylation (Turban and Hajduch, 2011; Yang, Vijayakumar and Kahn, 2018). Elevated free fatty acids (FFA) as a result of obesity can induce stress signalling response pathways such as cJun-N-terminal-kinase (JNK) which has shown to inhibit IRS-1 phosphorylation (Solinas and Becattini, 2017). Furthermore, pro-inflammatory cytokines such as interleukin-6 (IL-6) and tumour necrosis factor alpha (TNF-α) derived from immune cells or adipocytes is positively associated with populations of obesity and diabetes (Esser *et al.*, 2014; Liu *et al.*, 2016) and can also cause serine or threonine phosphorylation of the insulin receptor triggering IR (Boucher *et al.*, 2014). Nutrient overload is also reported to increase IR via chronic activation of ribosomal protein S6 Kinase 1 (S6K1) which inflicts serine phosphorylation of IRS-1 leading to reduced insulin sensitivity (Um, D'Alessio and Thomas, 2006; Yoon and Choi, 2016). For example, amino acids, particularly leucine, activate the mammalian target of rapamycin complex-1 (mTORc1) pathway leading to S6K1 activation (Um, D'Alessio and Thomas, 2006; Yoon and Choi, 2016). In addition to amino acids, chronic high glucose concentrations may also induce IR via Akt/mTORc1 dependent pathway. For example, in murine skeletal muscle cells (C2C12 myoblasts), high glucose concentrations are reported to induce IR and reduce Akt stimulation. However, inhibition of mTORc1/S6K1 signalling with rapamycin restored insulin-induced Akt stimulation (Leontieva, Demidenko and Blagosklonny, 2014). All the above interrelated factors that can influence insulin sensitivity are all associated with CMD and following an unhealthy lifestyle (Czech, 2017). For example, physical inactivity and poor nutrition are likely to trigger IR by favouring fat storage over fat oxidation resulting in lipid accumulation and inflammation (Eckardt, Taube and Eckel, 2011). Similarly, the same conditions are associated with abdominal obesity, hypertension and atherogenic dyslipidaemia (Esser *et al.*, 2014).

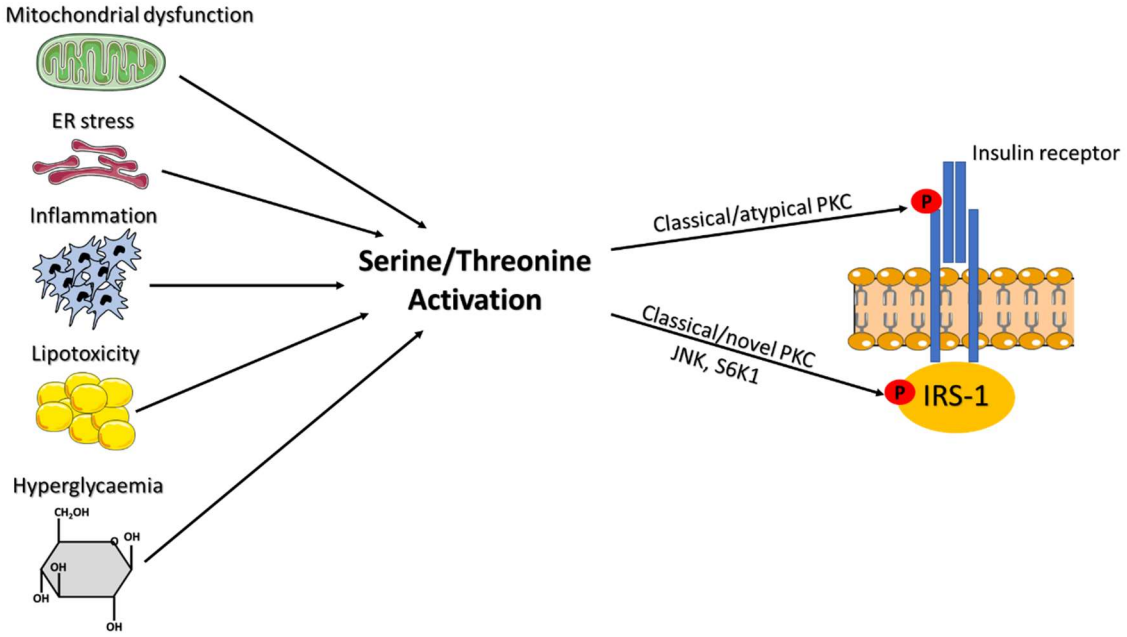


Figure 1.7. Inhibitory factors of insulin signalling via activation of serine/threonine kinases. (Adapted from Boucher *et al.* (2014))

1.2.2.3. Abdominal obesity

Accumulation of VAT is an important indicator of glucose tolerance, MetS and CVD risk, much more so than subcutaneous adipose tissue (Wajchenberg, Lé and Wajchenberg, 2000; Lee *et al.*, 2008; Preis *et al.*, 2010). VAT levels can easily be estimated by measuring WC and/or the waist to hip ratio (WHR). Cut off values in determining CVD risk are WC measures of >102 cm for men and >88 cm for women and WHR measures of >0.95 for men and >0.88 for women (Zhang *et al.*, 2008; Arnett *et al.*, 2019) and both WC and WHR have shown to be significantly associated with all-cause mortality and CVD induced mortality in a population of 44,636 women (Zhang *et al.*, 2008). Use of WC and WHR have also shown to successfully predict T2D incidence (Vazquez *et al.*, 2007). A prospective meta-regression analysis of 258,114 adults from 15 studies demonstrated that for every 1 cm increase in WC and 0.01 increase in WHR there is a 2% and 5% increase in CVD risk, respectively (De Koning *et al.*, 2007). Furthermore, when adjusted for confounding lifestyle and CMD factors no attenuation of results was observed demonstrating VAT as an independent marker of CVD (De Koning *et al.*, 2007). A recent genome-wide association study identified 102 novel loci associated with VAT and mendelian randomisation demonstrated a causal effect of VAT on hypertension, heart attack/angina, T2D and hyperlipidaemia (Karlsson *et al.*, 2019).

In healthy adipose tissue, when surplus energy is consumed, the energy is stored in subcutaneous adipose tissue. However, dysfunctional adipose tissue may not be able to expand via hyperplasia leading to lipid spillover which will be deposited in VAT and a variety

of organs including muscle tissue (Després and Lemieux, 2006; Tchernof and Després, 2013). The lipolytic rate of VAT is elevated compared to subcutaneous adipose tissue due to the increased effect of pro-lipolytic catecholamines and decreased effect of anti-lipolytic insulin (Wajchenberg, Lé and Wajchenberg, 2000; Tchernof and Després, 2013). This increases the flux of FFA to the liver, which may further increase hepatic IR and increased VLDL synthesis (Wajchenberg, Lé and Wajchenberg, 2000). An increase in VLDL with chylomicrons, increases competition for LPL allowing cholesterol ester transfer protein to exchange triglycerides with cholesterol from LDL. The now triglyceride enriched LDL has a higher affinity for hepatic lipase which hydrolyses the triglycerides producing sdLDL (Klop, Elte and Cabezas, 2013; Freeman and Walford, 2015). In addition to being involved in lipid storage and mobilisation, adipocytes are also an endocrine tissue, releasing cytokines and adipokines. An increase in VAT leads to a pro-inflammatory state as shown by an increase in C-reactive protein (CRP) and TNF-a which may further increase IR and increased risk of CVD (Park, Park and Yu, 2005; Kang *et al.*, 2016). The metabolic environment created by increased VAT, due to a poor lifestyle, has multiple health implications as shown by its effects on IR, sdLDL-C and inflammation production.

1.2.2.4. Hypertension

Hypertension is highly associated with CMD, T2D and CVD risk (Sookoian and Pirola, 2011; Sperling *et al.*, 2015). A prospective cohort study including 57,303 participants across low, middle and high income countries reported that hypertension is the largest risk factor for CVD, contributing to 22% of its population-attributable fractions (Yusuf *et al.*, 2020). This is in accordance with results observed in the Framingham Heart study where the highest tier of hypertension demonstrated the highest 10 year CVD risk in males and females (Wilson *et al.*, 1998). Obesity is highly associated with hypertension and is estimated that for every 5% increase in fat mass, there is a 20-30% risk in developing hypertension (DeMarco, Aroor and Sowers, 2014).

Progression to hypertension is multifactorial and includes a combination of genetic, environmental and lifestyle factors which can cause dysregulated vascular, renal, sympathetic nervous system and renin–angiotensin–aldosterone system (DeMarco, Aroor and Sowers, 2014). Hypertension is caused by an increase in vascular stiffness due to degenerative changes in the extracellular matrix derived from an imbalance of arterial scaffolding proteins such as elastin and collagen (Xu and Shi, 2014). Chronic low-grade inflammation, underpinned by cytokines such as CRP, TNF-α and IL-6, induced by ageing, increased VAT, T2D or an atherogenic lipid profile results in endothelial and smooth muscle cell proliferation, hypertrophy, remodelling and apoptosis (Mahmud and Feely, 2005; Petrie, Guzik and Touyz,

2018). This vascular proinflammatory state characterised by angiotensin II results in upregulation of matrix metalloproteinases leading to degradation of elastin fibres and increased collagen deposition resulting in extracellular matrix remodelling and arterial stiffening (Sun, 2015; Wang *et al.*, 2015). Obesity may also upregulate intravascular and intrarenal renin–angiotensin–aldosterone system activity triggering angiotensin II synthesis resulting in vascular stiffness (DeMarco, Aroor and Sowers, 2014). Furthermore, metabolic disorders such as T2D can cause disruption of vasodilation (nitric oxide) and vasoconstriction (endothelin) regulators resulting in hypertension (Petrie, Guzik and Touyz, 2018). An increase in adipose tissue can also result in an increase of tubular sodium absorption which causes a rise in the pressure of natriuresis curve towards higher blood pressure (BP) (DeMarco, Aroor and Sowers, 2014).

1.2.3. Impact of dietary carbohydrates and fat on CMD

While physical activity is a key lifestyle factor in the regulation of skeletal muscle health and the development of CMD, it is not the within the scope of this thesis. The focus of this thesis is dietary manipulation of carbohydrates and fats. Carbohydrates and fats are derived from dietary sources primarily to provide energy for cells which can be used immediately or stored. Carbohydrates are provided in 3 primary forms depending on their complexity due to the amount of glycosidic bonds including; monosaccharides, disaccharides and polysaccharides (Scientific Advisory Committee on Nutrition, 2015). Carbohydrates are stored as polysaccharides and therefore make up much of the carbohydrate intake from food (rice, pasta, potatoes etc) (Scientific Advisory Committee on Nutrition, 2015). Digestion of carbohydrates produces the monosaccharide glucose which will be used to produce ATP for energy via glycolysis in the cytoplasm or tricarboxylic acid cycle (TCA) in the mitochondria Additionally, carbohydrates can be stored in muscle as glycogen or can be converted to triglycerides to be stored in adipose tissue. The fate of a glucose molecule depends on the energy status of the cell and hormones such as insulin and glucagon (MacLaren and Morton, 2011; McArdle, Katch and Katch, 2015).

Lipids are essential nutrients as they form the cellular membrane and also provide the backbone of sex hormones in addition to being used for or stored as energy (Cooper, 2019). Fatty acids (FA), defined by their length and saturation can be grouped into short chain (2–6 carbon atoms), medium chain (8–12 carbon atoms), long chain (14–18 carbon atoms) and very long chain (20–26 carbon atoms) FA (Turner *et al.*, 2014). Saturated (0 double bonds), monounsaturated (1 double bonds) and polyunsaturated (>2 double bonds) FA are defined by their double bonds between carbon atoms (Scientific Advisory Committee on Nutrition, 2019). Mono (MUFA) and polyunsaturated FA (PUFA) are found in a variety of food from animal and

plant sources whereas saturated FA (SFA) are typically derived from animal products, (Lunn and Theobald, 2006). Digestion of lipids results in the production of FA which are repackaged with glycerol to form triglycerides within chylomicrons in the small intestine before being released in the blood stream. Chylomicrons carry the triglycerides to relevant tissues such as muscle or adipose tissue where it is broken down by LPL releasing FA to be carried by FA transporter proteins into the cell. FA then undergo β-oxidation within the mitochondrial matrix producing ATP and acetyl-coA for the TCA cycle or can be used to resynthesize triglycerides which can be stored in adipose tissue. Similarly to carbohydrate metabolism, this is dependent on the energy status of the cell with insulin playing a key role (MacLaren and Morton, 2011).

To combat CVD and metabolic disorders, many global bodies recommend a diet high in carbohydrates (although low in sugar) and low to moderate in fat (particularly SFA) (Public Health England, 2016; World Health Organization and World Health Organization., 2019; U.S. Department of Health and human services, 2020) due to its association with LDL-C and T2D (Scientific Advisory Committee on Nutrition, 2019). However, lower carbohydrate (<26 % of energy intake) higher fat (LC) diets have shown to at least perform as well as high carbohydrate (HC) diets in reducing body fat and improving metabolic health even with an increase in SFA intake (Figure 1.8) (Bueno *et al.*, 2013; Mansoor *et al.*, 2016; Gardner *et al.*, 2018; Tay *et al.*, 2018).

Although there is no standard definition, LC diets can be described based on the % of total energy intake (Table 1.2) (Hite, Berkowitz and Berkowitz, 2011; Gjuladin-Hellon *et al.*, 2019). A very low carbohydrate (VLC) diet may also be referred to as a ketogenic diet as it results in the increased production of ketone bodies to be used as energy in the absence of carbohydrate availability (Paoli *et al.*, 2013). The remaining energy is primarily replaced by an increase in dietary fat intake and protein intake typically increases only slightly (Paoli *et al.*, 2013; Feinman *et al.*, 2015). The increase in relative fat and protein intake usually results in greater satiety resulting in a decrease in energy intake (Feinman *et al.*, 2015).

Table 1.2. Carbohydrate intake thresholds.

Definition	% of energy intake
High carbohydrate	≥45%
Moderately low carbohydrate	≤44% to >26% (or 130 to 225 g/day)
Low carbohydrate, higher fat	10% to <26% (or 50 to 130 g/day)
Very low carbohydrate high fat	<10% or < 50g p/day

1.2.3.1. Dyslipidaemia

An increase in dietary carbohydrates can result in an increase in hepatic de novo lipogenesis resulting in in an increase in fasting triglyceride concentrations (Schwarz *et al.*, 2003). In healthy lean men, an isocaloric HC diet (65% of total energy intake) was associated with a 12% increase in de novo lipogenesis compared to a < 3% increase with a lower carbohydrate diet (46% of total energy intake). The HC diet also correlated with the increase in plasma triglycerides ($P < 0.05$, $R = 0.87$) (Schwarz *et al.*, 2003). Additionally, a recent meta-analysis reported long-term benefits on plasma triglycerides, HDL-C without effecting LDL-C in response to LC diets (Gjuladin-Hellon *et al.*, 2019). Though, short-term RCTs comparing VLC and HC diets have shown conflicting results (Volek *et al.*, 2009; Veum *et al.*, 2017). Volek *et al.* (2009) (Table 1.3) reported that a hypocaloric VLC diet in participants with obesity and dyslipidaemia resulted in significantly ($P < 0.05$) greater improvements in triglyceride, HDL-C and sdLDL-C concentrations compared with similarly hypocaloric HC diet after 12 weeks. However, in a similar RCT but with participants only presenting with obesity (N = 36), the hypocaloric VLC diet only showed significantly ($P < 0.05$) greater improvements in HDL-C levels compared with the HC diet (Veum *et al.*, 2017) (Table 1.3). Both diets showed similar improvements in triglyceride levels but the HC diet induced greater improvements in LDL-C concentrations (Veum *et al.*, 2017). However, LDL composition was not measured which has shown to improve with a VLC diet when total LDL-C remains unchanged (Volek *et al.*, 2009). The differences in results may be attributed to the populations studied as Volek *et al.* (2009) recruited males and females with obesity and dyslipidaemia; however, Veum *et al.* (2017) only recruited overweight males (Table 1.3). Furthermore, although diets were hypocaloric, both studies observed ~250 kCal per day difference between groups (the VLC group consumed less in study by Volek *et al.* (2009) and consumed more in study by Veum *et al.* (2017)) which may have influenced fat loss results, impacting CMD markers. Moreover, protein intake was significantly higher in the VLC group in study by Volek *et al.* (2009) which may also have affected results. For example, the VLC group lost greater VAT compared with the HC group in the study by Volek *et al.* (2009) but no difference was observed between groups by Veum *et al.* (2017). A 12-month RCT in participants with obesity and ~35% with MetS, reported findings similar to both Volek *et al.* (2009) and Veum *et al.* (2017) (Gardner *et al.*, 2018) (Table 1.3).

Additionally, long-term studies (12 months) have shown that participants following hypocaloric moderately low carbohydrate (MLC) diets display greater improvements in triglycerides and HDL-C concentrations compared with a calorie matched hypocaloric HC diets (Table 1.3) (Bazzano *et al.*, 2015; Gardner *et al.*, 2018). However, only Gardner *et al.* (2018) reported greater improvements in LDL-C by the HC diet whereas no difference was observed between

groups by Bazzano *et al.* (2015). Divergent LDL-C results may be due to differences in participants tested as both studies used participants who were on controlled medication which may impact results, particularly lipid lowering medication. Although not as well controlled, *ad libitum* VLC diets have also shown to improve markers of dyslipidaemia (Foster *et al.*, 2010) (Table 1.3). Compared with a hypocaloric HC diet, an *ad libitum* VLC displayed greater improvements in triglycerides, HDL-C and VLDL-C at 3 and 6 months (Foster *et al.*, 2010). However, triglycerides and VLDL-C were similar between groups at 12 and 24 months respectively (Foster *et al.*, 2010). Moreover, in the absence of weight loss a VLC diet may result in greater improvements in metabolic health (Hyde, Krauss and Volek, 2019) (Table 1.3).

In a randomised crossover design in participants following either an isocaloric VLC, MLC and a HC diet resulted in significant ($P < 0.01$) increases in fasting triglycerides and sdLDL-C levels and decreasing HDL-C concentrations with higher carbohydrate diets (Hyde *et al.*, 2019) (Table 1.3). In a similar design, a metabolic ward study in participants with obesity also reported greater reductions in triglyceride levels after 6 days of following a hypocaloric VLC ($n = 15$) diet compared with a calorie matched HC diet ($n = 16$) (Hall *et al.*, 2015) (Table 1.3). The HC diet also induced greater reductions in HDL-C and LDL-C (Hall *et al.*, 2015). However, LDL subclasses were not measured and as low HDL-C levels are associated with LDL pattern B phenotype, it is difficult to determine if there is an improvement in metabolic risk (Ivanova *et al.*, 2017). Furthermore, in free living obese participants with MetS ($n = 16$), a hypocaloric VLC diet progressing to a HC diet with incremental increases in carbohydrate every 3 weeks for a total 18 weeks showed different results (Volk *et al.*, 2014) (Table 1.3). No change was reported in LDL-C or HDL-C levels throughout; however, although the hypocaloric VLC diet significantly ($P < 0.01$) reduced triglyceride levels compared with baseline levels, increasing carbohydrates (still hypocaloric) resulted in significant ($P < 0.01$) increases in triglyceride levels (Volk *et al.*, 2014). Additionally, LC diets have been shown to consistently outperform HC diets in improving triglyceride and HDL-C concentrations as shown by several meta-analyses, although the long-term effects on LDL-C are equivocal (Bueno *et al.*, 2013; Schwingshackl and Hoffmann, 2014; Mansoor *et al.*, 2016; Gjuladin-Hellon *et al.*, 2019). LC diets therefore may reduce CVD risk by reducing de novo lipogenesis resulting in a reduction in triglyceride concentrations and synthesis of TRL-C which contribute to atherosclerotic plaque production (Nordestgaard and Varbo, 2014). The increase in HDL-C also highlights a reduction in CVD risk due to its strong inverse association (Wilson *et al.*, 1998; Sharrett *et al.*, 2001; Di Angelantonio *et al.*, 2009). Further research is warranted on the effects of a VLC diet on LDL size, particle number, and molecular composition rather than LDL-C to determine its effects on cardiovascular health.

Table 1.3. Key research papers on the LC and HC diets on CMD.

Author	Duration and design	N = (M/F)	Population	LC diet at end of intervention	Control diet at end of intervention
(Volek *et al.*, 2009)	12-weeks, RCT	40 (20/20)	BMI >25 kg/m^2, dyslipidaemia	Hypocaloric, 1504 kcal, 12% CHO, 28% PRO, 59% fat	Hypocaloric, 1478 kcal, 56% CHO, 20% PRO, 24% fat.
(Veum *et al.*, 2017)	12-weeks, RCT	44 (44/0)	BMI >29 kg/m^2, WC >98 cm	Hypocaloric, 2126 kcal, 56 g CHO, 89 g PRO, 167g fat	Hypocaloric, 2205 kcal, 281 g CHO, 92 g PRO, 72 g Fat.
(Gardner *et al.*, 2018)	1-year, RCT	609 (263/346)	BMI >28 <40 kg/m^2, 39% MetS, Controlled medication included	Hypocaloric, 1697 kcal, 30% CHO, 23% PRO, 45% Fat.	Hypocaloric, 1716 kcal, 48% CHO, 21% PRO, 29% Fat.
(Bazzano *et al.*, 2015)	1-year, RCT	148 (17/131)	BMI >30 <45 kg/m^2 Controlled medication included	Hypocaloric, 1448 kcal, 34% CHO, 24% PRO, 41% Fat	Hypocaloric, 1527 kcal, 54% CHO, 19% PRO, 30% Fat
(Foster *et al.*, 2010)	2-years, RCT	307 (99/208)	BMI >30 <40 kg/m^2	*Ad libitum* fat and protein, <20 g/day of CHO for first 12 weeks, an increase in 5 g of CHO per week thereafter.	Hypocaloric, 1500 kcal, 55% CHO, 15% PRO, 30% fat.
(Hyde *et al.*, 2019)	16-weeks, Randomised crossover design, 4-week diets with 2-week washouts between diets	16 (10/6)	BMI >30 kg/m^2, MetS	Isocaloric, 2950 kcal, LC; 45 g CHO, 150 g PRO 242 g Fat, MLC; 234 g CHO, 146 g PRO 159 g Fat HC; 420 g CHO, 144 g PRO 77 g Fat	
(Hall *et al.*, 2015)	~5-weeks, Randomised crossover design, 5-day isocaloric diet followed by 6-day hypocaloric diet with 2-4-week washouts between diets	19 (10/9)	BMI >30 kg/m^2,	5-day isocaloric diet, 2740 kcal, 50% CHO, 15% PRO, 35% Fat. Followed by 6-day hypocaloric diet, 1918 kcal, 29% CHO, 21% PRO, 50% Fat.	5-day isocaloric diet, 2740 kcal, 50% CHO, 15% PRO, 35% Fat. Followed by 6-day hypocaloric diet, 1918 kcal, 71% CHO, 21% PRO, 8% Fat.
(Volk *et al.*, 2014)	~18-weeks, single arm, hypocaloric VLC diet with incremental increases in CHO every 3 weeks	16 (12/4)	BMI; 27–50 kg/m^2, MetS	Hypocaloric: 2553 – 2509 kcal Week 1-3: VLC; 47 g CHO, 129 g PRO 209 g Fat Week 3-6: LC; 83 g CHO, 125 g PRO 193 g Fat Week 6-9: MLC; 131 g CHO, 125 g PRO 179 g Fat Week 9-12: MLC; 179 g CHO, 123 g PRO 152 g Fat Week 12-15: HC; 251 g CHO, 123 g PRO 121 g Fat Week 15-18: HC; 346 g CHO, 123 g PRO 80 g Fat	

BMI, body mass index; CHO, Carbohydrates; HC, high carbohydrate diet; LC, low carbohydrate diet; M/F, Males/Females; MetS, metabolic syndrome; MLC, moderately low carbohydrate diet; PRO, protein; RCT, randomised control trial; *VLC, very low carbohydrate diet.*

1.2.3.2. Insulin resistance

As IR and T2D involve the inadequate response of insulin to reduce post-prandial blood glucose levels, a diet lower in carbohydrates may be more suitable in reducing IR (Feinman *et al.*, 2015). In T2D participants, both a hypocaloric MLC and VLC diet for 24 weeks resulted in improvements in glycaemic control; however, the VLC diet reduced the medication required in 8/21 participants whereas the MLC reduced the medication required in only 3/29 participants (Westman *et al.*, 2008). Similar results were reported by Saslow *et al.*, (2014) where a VLC diet resulted in a 44% reduction in T2D medication whereas a MLC diet only resulted in an 11% reduction. Furthermore, both a hypocaloric VLC and HC diet resulted in similar improvements in Hba1c and weight loss but the VLC group reported more than 2-fold greater reductions in medicine (Tay *et al.*, 2018). A recent meta-analysis observed that 9 out of 11 studies reported greater reductions in T2D medication with a LC diet compared to control diets (Huntriss, Campbell and Bedwell, 2018).

Short-term weight-loss RCTs (12-weeks) have demonstrated mixed results on glucose metabolism in non-diabetics (Volek *et al.*, 2009; Veum *et al.*, 2017). In participants representing with obesity and dyslipidaemia (Table 1.3), a VLC diet induced greater improvements with fasting glucose, fasting insulin, postprandial insulin levels and homeostatic model of IR (HOMA IR) compared with a HC diet (Volek *et al.*, 2009). However, in males with obesity (Table 1.3), compared with the VLC diet, the HC diet resulted in greater improvements in fasting glucose concentrations only, but no difference was reported between groups in fasting insulin, HOMA IR, or Hba1c (Veum *et al.*, 2017). VAT is highly associated with IR; therefore, the differences in results may be due to greater reductions in VAT in the VLC group in the study by Volek *et al.* (2009), whereas changes in VAT mass were similar between groups in the study by Veum *et al.* (2017). Furthermore, partcipants following a hypocaloric MLC or HC diet for 1 year exhibited similar WC reductions and comparable decreases in fasting insulin and glucose levels (Bazzano *et al.*, 2015; Gardner *et al.*, 2018) (Table 1.3) further indicating the potential role of VAT on IR.

Independent of weight loss, Hyde *et al.* (2019) (Table 1.3) reported a small but signifcant increase in fasting glucose levels with a HC diet compared with a VLC diet but no change in fasting insulin levels. However, this led to a trend of reduced HOMA IR levels with the VLC diet (Hyde *et al.*, 2019). In contrast, after 6 days of following either a VLC or HC diet, Hall *et al.* (2015) (Table 3) reported greater improvements in fasting glucose levels with the HC diet, yet, HOMA IR and fasting insulin levels improved similarly between groups. Furthermore, a hypocaloric VLC diet signifcantly decreased fasting insulin, glucose and HOMA IR levels after 3 weeks; however, incremental increases in carbohydrate until reaching a HC (hypocaloric)

diet did not effect these measures any further (Volk *et al.*, 2014) (Table 1.3). These findings are consistent with meta-analyses that observed no difference between VLC or HC diets in fasting insulin or glucose levels (Schwingshackl and Hoffmann, 2014; Mansoor *et al.*, 2016). However, subgroup analyses highlighted that in T2D patients a VLC diet induces a greater decrease in fasting glucose levels (Schwingshackl and Hoffmann, 2014). These results indicate that neither carbohydrate nor fat quantity appear to have a direct impact on markers of IR in non-diabetics. It could also be argued that carbohydrate restriction only simulates reduced IR as a dramatic reduction in dietary carbohydrate intake will naturally reduce blood glucose levels resulting in a decline in insulin concentrations but when dietary carbohdydrates are reintroduced to the diet blood glucose and insulin levels will rise back to IR concentrations. Therefore, an increase in endogenous energy use and reduced VAT due to caloric restriction is perhaps the primary driver of improving markers of IR. This is likely to reduce circulating levels of inflammatory cytokines, FFAs and insulin levels which all can cause IR (Boucher *et al.*, 2014; Samuel and Shulman, 2016). Much research has focused on participants with obesity, further research is warranted in participants with a BMI <30kg/m^2.

1.2.3.3. Abdominal obesity

Obesity, particularly abdominal obesity is highly associated with CMD therefore, dietary strategies that reduce obesity are of great clinical importance (Després and Lemieux, 2006). Short term RCTs have reported significant reductions in VAT levels after 12 weeks of following either a hypocaloric VLC or HC diet (Volek *et al.*, 2009; Veum *et al.*, 2017) (Table 1.3). However, only Volek *et al.* (2009) reported enhanced effects of the VLC diet compared with the HC diet. Both studies report that habitiual physical activity was maintained; therefore, the difference in results is most likely due to the discrepency in caloric intake explained above (section 1.2.3.1) as negative energy balance is a key driver of fat loss (Hall and Guo, 2017). Long-term RCTs (Table 1.3) have also reported similar effects of hypocaloric MLC and HC diets on reducing WC (Bazzano *et al.*, 2015; Gardner *et al.*, 2018). However, Bazzano *et al.* (2015) reported greater reductions in WC in partcipants following the MLC diet at 3 and 6 months indicating perhaps adherence may have played role. For example, at 12 months caloric intake increased by ~200 kcal in the MLC group compared with 3 months of the diet which may have influence potential progress (Bazzano *et al.*, 2015). Additionally, Hyde *et al.* (2019) (Table 1.3) reported no change in WC or body composition when switiching between isocaloric VLC, MLC or HC diets in a randomised order highlighting a lack of effect of dietary composition when calories and protein are equal. A VLC diet may show greater improvements in VAT measures in the short term compared with HC diets; however, when diets are matched for confounding factors such as energy and protein intake, both diets induce similarly positive results on VAT levels indicating an improvement in cardiometabolic risk.

1.2.3.4. Hypertension

Obesity is a primary driver of increased BP leading to hypertension which is also a key feature of CMD (DeMarco, Aroor and Sowers, 2014; Sperling *et al.*, 2015). When associated with a reduction in fat mass, both a VLC and HC diet are reported to significantly improve SBP and diastolic blood pressure (DBP) after 12 weeks (Veum *et al.*, 2017) (Table 1.3). Similar improvements in BP are also reported after 12 months of either a MLC or HC hypocaloric diet (Bazzano *et al.*, 2015; Gardner *et al.*, 2018) (Table 1.3). A long-term *ad libitum* VLC diet which induced comparable fat loss to a hypocaloric HC diet resulted in a greater decrease in DBP at 3 and 6 months; however, this was similar between diets at 12 and 24 months (Foster *et al.*, 2010). Additionally, SBP improved comparably between diets at each timepoint (Foster *et al.*, 2010). In accordance with the impact of changes in fat mass leading to changes in BP, Hyde *et al.* (2019) observed no change in BP in adults with MetS following either a VLC, MLC or HC isocaloric diet. Similarly, after induction of fat loss with a hypocaloric VLC diet, SBP and DBP decreased (Volk *et al.*, 2014). However, progression to a HC diet with incremental increases in carbohydrate every 3 weeks did not increase BP (Volk *et al.*, 2014) (Table 1.3).

Several meta-analyses have reported mixed results on the effects of LC diets on reducing BP compared with HC diets (Bueno *et al.*, 2013; Schwingshackl and Hoffmann, 2014; Mansoor *et al.*, 2016). No difference was observed between diets by Mansoor *et al.* (2016); however, greater beneficial effects of LC diets on DBP were observed by Bueno *et al.* (2013) and Schwingshackl and Hoffmann (2014). Differing effects are most likely due to the quality of studies being reported as low (Schwingshackl and Hoffmann, 2014) and heterogenous populations being invesitgated. However, a more recent meta-analysis of 67 trials comparing 13 dietary strategies including 17,230 hypertensive and pre-hypertensive patients reported that a LC diet was the third (joint with Mediterranean diet) most effective diet at improving BP, after the Palaeolithic and Dietary Approaches to Stop Hypertension diets which were 2nd and 1st respectively (Schwingshackl *et al.*, 2019). These results indicate that a LC diet has beneficial effects on cardiovascular health by reducing hypertension, most likely due to reductions in fat mass. However, this may also be achieved by MLC or HC diets provided they induce fat loss.

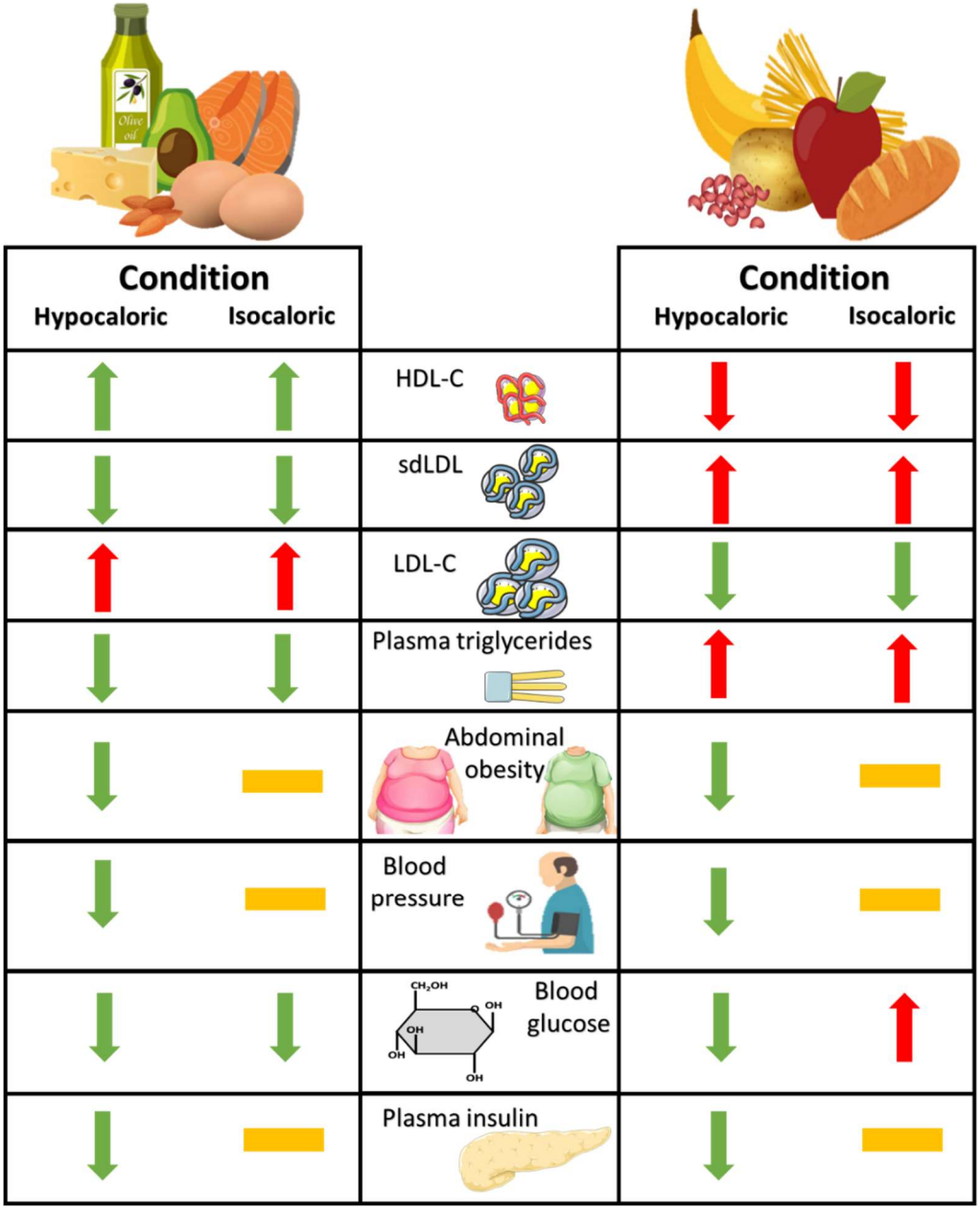


Figure 1.8. Summary of the typical effects of low carbohydrate (LC) and high carbohydrate (HC) diets on markers of cardiometabolic disease. *HDL-C; high-density lipoprotein cholesterol, LDL-C; low-density lipoprotein cholesterol, sdLDL; small-dense lipoprotein.*

1.2.4. Impact of anabolic-androgenic steroids on CMD

While dietary factors have a great impact on cardiometabolic health other lifestyle factors such as regular exercise also have a profound effect particularly on skeletal muscle (Lin *et al.*, 2015; Fabiani, Naldini and Chiavarini, 2019). The effect of exercise particularly resistance exercise, on muscle mass and strength can be augmented through pharmacological aids such as anabolic androgenic steroids (AAS) which are products of the anabolic hormone testosterone ((Bhasin *et al.*, 1996, 2001). Due to advancements in technology and pharmacology, a range

of AAS (Table 1.4) have been used by the recreational gym-user since the 1980s, primarily by young men to improve body image (Kanayama and Pope, 2018; Goldman, Pope and Bhasin, 2019). Although illegal to purchase and distribute for recreational use in many countries (ACMD, 2010; Drug Enforcement Agency, 2019; Council of Europe, 2020), it is estimated that 6.4% of males and 1.6% of females use AAS globally, with recreational sports people being the highest users (Sagoe *et al.*, 2014). While it is common for individuals to use AAS for multiple reasons, the greatest motivation to use AAS is primarily to improve body image, while competitive bodybuilding and athletic performance (non-bodybuilding) are secondary and tertiary respectively (Parkinson and Evans, 2006; Dunn, Mazanov and Sitharthan, 2009; Sagoe *et al.*, 2014; Begley, McVeigh and Hope, 2017). Individuals who use AAS are reported to be reluctant in seeking medical advice from medical professionals (Pope *et al.*, 2004), therefore, needle syringe programs are considered the primary point of care for AAS users ((van de Ven *et al.*, 2020). Current data on the use of needle syringe programmes by people who use AAS suggests that in the UK, there has been an increase from 1.88 per 1000 people to 5.72 per 1000, particularly in men aged 20-29 since 1995 (McVeigh and Begley, 2017). In Australia, this uptake of needle syringe programs by AAS users has also increased from 1-2% in 1995 to 2010 to 7% by 2012 (Dunn, Mckay and Iversen, 2014). Use of needle syringe programs may not fully illustrate the increasing prevalence of AAS use as individuals who only use oral AAS may never engage with needle syringe programs reducing their access to medical advice (van de Ven *et al.*, 2020). This trend on AAS use may be similar across the globe as it coincides with the negative impact of increasing social media use on body image perceptions (Fardouly and Vartanian, 2016; Griffiths *et al.*, 2018). Typically, an increase in muscle mass and function through resistance exercise and appropriate nutrition can facilitate reduced incidence of METS and CVD (Strasser, Siebert and Schobersberger, 2010; Tyrovolas *et al.*, 2020). However, the growing prevalence of AAS use in the recreational gym user (McVeigh and Begley, 2017) may reduce the health promoting potential of regular exercise and healthy dietary patterns as AAS use is associated with markers of CMD (Figure 1.10) and a 2-fold increase in future cardiovascular events and mortality (Achar, Rostamian and Narayan, 2010; Thiblin *et al.*, 2015).

Testosterone and its AAS derivatives increase muscle protein synthesis (MPS) and accretion, satellite cell activation and possibly decrease catabolic pathways via genomic and non-genomic mechanisms (Cheung and Grossmann, 2018). Genomic actions of AAS occur when androgens bind to the nuclear androgen receptor and translocate to the cell nucleus, binding to specific DNA sequences resulting in enhanced transcription of target anabolic genes (Parr *et al.*, 2018; Wilkenfeld, Lin and Frigo, 2018). AAS also exert non-genomic actions by binding of the membrane-located androgen receptor and additional membrane receptors such as

endothelial growth factor receptor and sex hormone-binding globulin receptor that also alter anabolic/catabolic signalling pathways (Parr *et al.*, 2018; Wilkenfeld, Lin and Frigo, 2018). Resistance exercise also increases MPS and satellite cell activation resulting in skeletal muscle hypertrophy (Egan and Zierath, 2013; Liu and Sabatini, 2020). Although testosterone administration and resistance exercise alone may increase skeletal muscle hypertrophy, the combination of both profoundly enhances this process (Bhasin *et al.*, 1996) and as a result, AAS are commonly used in conjunction with exercise to increase muscle mass and improve perceived body image.

Regular exercise is undoubtedly beneficial for mental, physical and metabolic health (Warburton, Nicol and Bredin, 2006). However, the potential benefits acquired from regular exercise may be reduced with chronic AAS use, as AAS users are at a higher risk of developing CVD, psychological disorders, neuroendocrine disorders, sex-specific disorders and a range of other disorders (Pope *et al.*, 2014; Baggish *et al.*, 2017; Bjørnebekk *et al.*, 2017; Westlye *et al.*, 2017; Goldman, Pope and Bhasin, 2019). Long term AAS use has been reported to result in premature death due to cardiovascular events; however, due to AAS use only being prevalent since the 1980s, long term longitudinal studies, on their impact, are scarce (Thiblin *et al.*, 2015). Furthermore, the direct impact of AAS use on health is difficult to determine as users reportedly use other substances to complement their AAS use while also using a variety of AAS types, doses and cycles (Table 1.4) (Sagoe *et al.*, 2015; Begley, McVeigh and Hope, 2017).

Table 1.4. List of injectable and oral AAS and typical doses used.

Injectable AAS	Typical weekly dose
Boldenone Undecanoate	200-400 mg
Drostanolone Propionate	300-450 mg
Methenolone Enanthate	200-400 mg
Nandrolone Decanoate	200-400 mg
Stanozolol	150-700 mg
Testosterone Cypionate	200-600 mg
Testosterone Enanthate	200-600 mg
Testosterone Propionate	150-300 mg
Testosterone Suspension	150-700 mg
Trenbolone Acetate	150-300 mg
Trenbolone Enanthate	200-300 mg

Trenbolone Hexahydrobenzylcarbonate	150-230 mg
Oral AAS	**Daily dose**
4-chlorodehydromethyltestosterone	20-80 mg
Fluoxymesterone	20-40 mg
Methandrostenolone, Methandienone	20-40 mg
Mesterolone	50-100 mg
Oxandrolone	20-40 mg
Oxymetholone	50-100 mg
Stanozolol	20-50 mg
Testosterone Undecanoate	80-160 mg

1.2.4.1. Dyslipidaemia

Use of AAS has shown to greatly dysregulate lipoprotein metabolism by reducing HDL-C by ≥70% and increasing LDL-C levels by >20% (Achar, Rostamian and Narayan, 2010). Testosterone has been reported to significantly decrease HDL-C, although with differential dose and time responses. Increasing doses of testosterone enanthate (Test E) for 20 weeks in resistance-trained males has reported to have an inverse dose-response relationship with HDL-C and Apo A1 but only 600 mg/wk results in significantly lower levels compared to baseline levels (Singh *et al.*, 2002). However, in contrast, 150 mg/wk for 2 weeks and a 300 mg dose of testosterone cypionate (Test C) on week 3 resulted in the largest decrease in HDL-C but no further decrease was observed with 600 mg/wk for a further 4 weeks (Kouri, Pope and Oliva, 1996). Furthermore, 3 weeks of 600 mg/wk Test E administration for clinical benefits in inactive ageing males resulted in considerable decreases in HDL-C, particularly HDL_2 (Herbst *et al.*, 2003). Although 200 mg/wk of Test E in resistance training males showed decreases in HDL-C after 6 weeks, no effect was observed on HDL_2 (Thompson *et al.*, 1989). In healthy males, 200 mg/wk of Test E administration for 12 months had a dramatic (*mean change from,.*1.15 mmol/L to 0.09 mmol/L, $P < 0.05$) reduction on fasting HDL-C levels (Meriggiola *et al.*, 1995). Interestingly, neither study observed significant deleterious changes in LDL-C or triglyceride levels in fact, Thompson *et al.* (1989) reported a decrease in LDL-C. Nandrolone administration resulted in equivocal effects on lipid metabolism. HDL-C has been reported to significantly decrease after a 200 mg starting dose of nandrolone and a further 100 mg/wk for a total of 8 weeks in male bodybuilders (Kuipers *et al.*, 1991). Although in a similar design and population, 200 mg/wk of nandrolone for 8 weeks resulted in no change in HDL-C (Hartgens *et al.*, 2004). In healthy adults, 100 mg/wk for 6 weeks resulted in no change in

HDL-C in a clinical setting (Glazer and Suchman, 1994). No effect was observed on LDL-C, triglycerides, Apo A1 or Apo B levels in the above studies (Kuipers *et al.*, 1991; Glazer and Suchman, 1994; Hartgens *et al.*, 2004). Nandrolone administration (200 mg/wk) for 6 months in ageing males undergoing haemodialysis reduced HDL-C and increased Apo B levels but had no effect on triglycerides or Apo A1. (Teruel *et al.*, 1997). In post-menopausal women, clinical administration of 50 mg/wk of nandrolone for 3 weeks decreased HDL-C and Apo A1 levels (Lippi *et al.*, 1997). In male bodybuilders, 42 mg/wk of oral stanozolol administration has shown to reduce HDL-C, ApoA1 and triglycerides after 6 weeks while also increasing LDL-C (Thompson *et al.*, 1989). In healthy males, one intramuscular injection of 50 mg of stanozolol resulted in a reduction and increase in HDL-C and LDL-C levels respectively 28 days later (Small *et al.*, 1982). Both returned to baseline levels after 56 days (Small *et al.*, 1982). Similar results have been observed in postmenopausal females with osteoporosis, as 42 mg/wk oral stanozolol resulted in reductions in HDL-C and ApoA1 levels after 2 weeks in a clinical setting and was maintained until the end of the treatment at 6 weeks along with an increase in LDL-C levels (Taggart *et al.*, 1982). No change was observed in triglyceride levels (Taggart *et al.*, 1982).

Differences in study designs, populations and lack of dietary control in some studies have resulted in differing responses in lipid metabolism with AAS administration. Nonetheless, increasing doses of testosterone has a large negative impact on HDL-C with no adverse effect on other lipid markers. Although inconsistent, the negative effects of nandrolone administration are primarily observed on HDL-C levels; however, nandrolone does appear to consistently reduce lipoprotein(a) (Lp (a)) levels (Lippi *et al.*, 1997; Teruel *et al.*, 1997; Hartgens *et al.*, 2004), an independent risk factor of CVD (Sharrett *et al.*, 2001; Saeedi and Frohlich, 2016), yet further research is warranted on the potential benefits of nandrolone, if any. In contrast, stanozolol administration may have a greater deleterious effect on lipid metabolism as it has shown to negatively affect LDL-C and HDL-C levels.

Individuals who use AAS for appearance and performance reasons typically do not use one type of AAS but rather administer a polypharmacy regime which may lead to different implications on lipid metabolism. AAS-related polypharmacy (AAS-p) may involve using multiple parenteral or oral types of AAS during a cycle, termed “stacking” in addition to but not limited to other image performance enhancing drugs (Zahnow *et al.*, 2020) such as growth hormone, insulin-like growth factor I (Anderson, Tamayose and Garcia, 2018). The chemical interactions of AAS-related polysubstance use may also elicit additional adverse health outcomes. Quantifying the adverse effects of these drugs is further complicated by the prevalence of adulterated products, an inevitable consequence of the illicit market (Evans-Brown, Kimergård and McVeigh, 2009).

Early studies reported that after 8 weeks of AAS-p administration, HDL-C and LDL-C

decreased by 49% and increased by 31% respectively (Lenders *et al.*, 1988). Similarly, this suppression on HDL-C, particularly HDL_2, is maintained after 14 weeks of self-administration (Hartgens *et al.*, 2004). However, Bonetti *et al*. only reported a decrease in HDL-C after 18 months (Bonetti *et al.*, 2008). The method of using self-administrating participants, results in a variety of AAS dosages, types and cycles being used which may lead to different health outcomes thereby making comparisons between studies difficult. Critically, however, although they may be less controlled, they may be more representative of the population compared to RCTs as it replicates the AAS, and AAS-related polysubstance methods used by this unique population.

A recent cross-sectional study reported similar results in which current users of AAS-p, displayed 45% lower HDL-C, and 26% and 35% higher LDL-C and triglyceride levels vs non-AAS using controls (Rasmussen *et al.*, 2017). A case study of prolonged AAS-p use in a 35-year-old male demonstrated an almost 100% decrease in HDL-C and a 100% increase in LDL-C during 5 years of AAS cycling (Li *et al.*, 2018). Similarly, in females, HDL-C is shown to be significantly depressed with chronic AAS use compared to healthy controls. While AAS use may also exhibit an increase in plasma triglycerides, data remains equivocal as this was only reported by Moffatt *et al.* (Cohen *et al.*, 1986; Moffatt, Wallace and Sady, 1990; Malarkey *et al.*, 1991). In addition to small sample sizes in female studies, the variety in AAS use, type, dose, and frequency might explain the differences in results. Although, the lipoprotein profile is undoubtedly impacted by chronic AAS-p use and therefore highlights the increased risk of future CVD incidence, due to the uncontrolled self-administration of AAS and other anabolic substances the severity in which it impacts health can be variable. Conversely, AAS-p is also reported to improve Lp (a) levels, similar to the effect of nandrolone administration alone (Hartgens *et al.*, 2004). Self-administration of AAS-p resulted in a decrease in Lp (a) after 8 weeks and was maintained after 14 weeks (Hartgens *et al.*, 2004). However, 24 months of AAS-p use did not result in a decrease (mean ± SD; 179 ± 117 vs 137 ± 80 mg/dL, $P > 0.05$) in Lp (a) (Bonetti *et al.*, 2008). Although non-significant, it may be clinically significant as Lp(a) levels >180 mg/dL are considered high risk of CVD (Mach *et al.*, 2020). The implications of AAS induced improvements in Lp(a) concerning CVD risk are unclear and warrant further investigation. Use of AAS also doesn't appear to negatively impact triglyceride levels in males as only one cross-sectional study reported higher triglycerides (although not clinically significant <1.7 mmol/L) with AAS use, yet self-administration studies showed no negative effects (Lenders *et al.*, 1988; Hartgens *et al.*, 2004; Bonetti *et al.*, 2008).

The mechanisms by which AAS negatively impact lipid metabolism are not fully understood, but the upregulated activity of hepatic triglyceride lipase (HTGL) has been implicated (Figure 1.9) (Thompson *et al.*, 1989; Herbst *et al.*, 2003). Phospholipase activity of HTGL catabolises

HDL-C and its removal from the plasma and conversion of idLDL to sdLDL (Glazer, 1991; Santamarina-Fojo *et al.*, 2004). Research of the impact of AAS use on LDL density are limited with most focusing on total LDL-C; however, one RCT investigated the short-term (3 weeks) effects of Test E administration on cholesterol associated with LDL density by density gradient ultracentrifugation (Herbst *et al.*, 2003). In older eugonadal males (mean 71 years old), clinically administered 600 mg of Test E increased sdLDL-C (Herbst *et al.*, 2003) indicating an increase in CVD risk (Hoogeveen *et al.*, 2014). Further research is warranted on the effect of AAS use on LDL density and its associated CVD risk. The type of AAS and route of administration also has an impact on the effect of HTGL activity and lipoprotein levels. Orally administered stanozolol increased HTGL activity, leading to elevated and reduced LDL-C and HDL_2-C levels respectively whereas injected Test E showed no change in HDL_2-C after 6 weeks, but decreased LDL-C (Thompson *et al.*, 1989). The slower liver clearance rate of orally administered AAS compared to injected AAS could have a greater detrimental effect on metabolic health and also increase the risk of hepatoxicity (Martin, Abu Dayyeh and Chung, 2008; Solbach *et al.*, 2015; Niedfeldt, 2018). Interestingly, the effect of AAS on the lipoprotein profile is reversible, as former users of AAS with long term discontinuation of at least one year, are reported to have healthy lipoprotein levels (Urhausen, Torsten and Wilfried, 2003; Rasmussen *et al.*, 2017). The reversible effects may be seen as early as 10 weeks of AAS cessation as shown by a case study in a 35-year-old male (Li *et al.*, 2018).

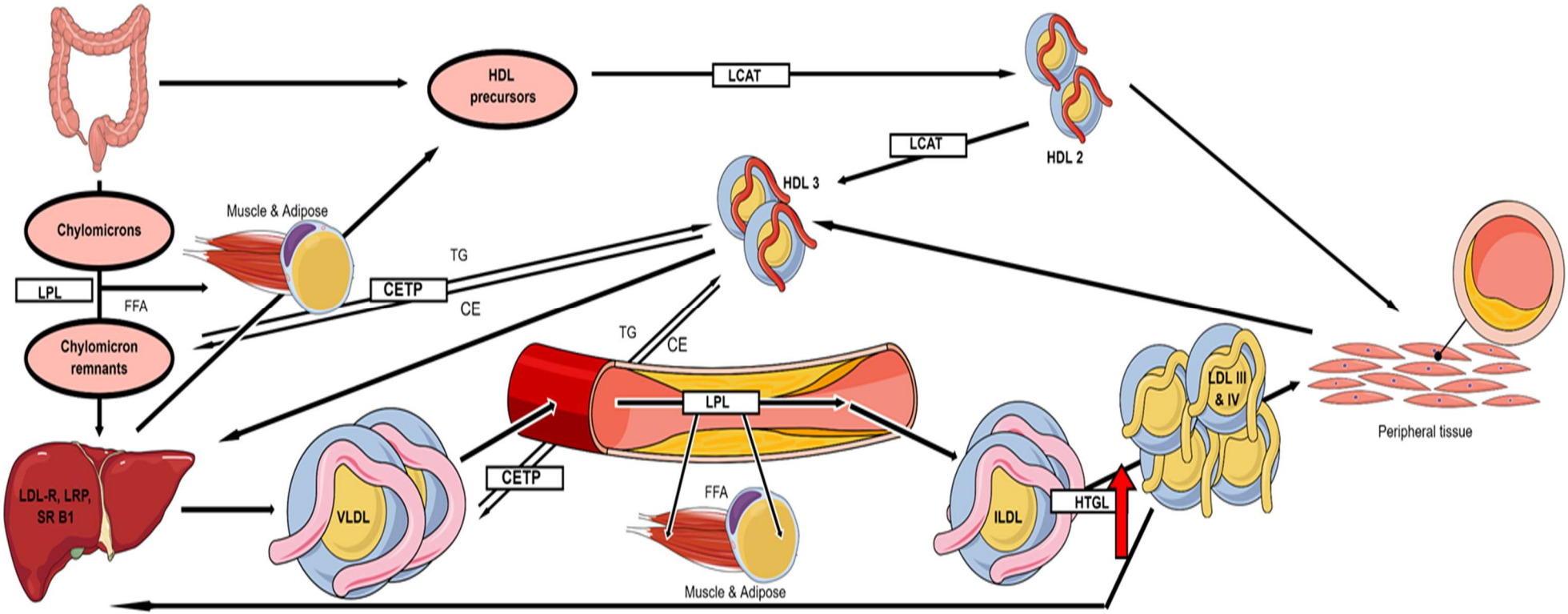


Figure 1.9. AAS related lipoprotein metabolism. During AAS-influenced lipoprotein metabolism HTGL is upregulated, most likely resulting in a preponderance of more atherogenic small, dense LDL III and IV particles, as opposed to the larger and more buoyant LDL I and II particles found in normal lipoprotein metabolism. There is also a severe decrease in the number of HDL 2 and 3 particles overall, which are generally regarded as being atheroprotective.

1.2.4.2. Insulin resistance

Acute testosterone administration has been shown to activate the PI3K/Akt pathway and GLUT4 translocation *in vitro* indicating an increase in cellular glucose uptake (Antinozzi *et al.*, 2017). However, supraphysiological levels of testosterone and nandrolone have been reported to significantly ($P < 0.05$) diminish the response of insulin-induced glucose uptake in rodents (Holmang and Bjorntorp, 1992; Frankenfeld *et al.*, 2014). Rodents also showed impairments in gluconeogenesis, most likely due to the high fasting insulin levels (Frankenfeld *et al.*, 2014). In contrast, increasing doses of testosterone (25-600 mg/wk) for 20 weeks had no effect on insulin sensitivity in resistance-trained males (Singh *et al.*, 2002). Additionally, in a double-blind crossover design, 300 mg/wk of Test E and nandrolone administration for 6 weeks did not affect glucose tolerance or fasting insulin levels in healthy males (Hobbs, Jones and Plymate, 1996). Although research is lacking, females who use AAS-p for performance are reported to display reduced insulin sensitivity (Diamond *et al.*, 1998). In healthy females, up to 12 days of methyltestosterone dosing (5 mg), reduced whole-body insulin sensitivity (Diamond *et al.*, 1998). Similarly, in postmenopausal females, 120 mg of testosterone undecanoate per week resulted in a decrease in insulin sensitivity in a clinical setting (Zang *et al.*, 2006). Hyperandrogenism in females is a significant risk factor in developing PCOS which increases the risk of developing MetS although the risk of developing CVD is currently unclear (Coviello, Legro and Dunaif, 2006; Zhao *et al.*, 2016; Fazleen, Whittaker and Mamun, 2018; Ramezani Tehrani *et al.*, 2020). Interestingly, muscle strength determined by bench press and handgrip test was shown to be higher in females with PCOS compared to healthy controls, further indicating that hyperandrogenism may be implicated in PCOS and MetS (Kogure *et al.*, 2015).

Although individual AAS use may not result in reduced insulin sensitivity in males (Hobbs, Jones and Plymate, 1996; Singh *et al.*, 2002), limited research suggests chronic AAS-p use may be detrimental to glucose metabolism as shown by Cohen *et al*. (Cohen and Hickman, 1987). Powerlifting steroid users were reported to have similar fasting glucose levels as non-using powerlifters and sedentary participants; however, they had higher fasting insulin levels that were similar to those observed in obese participants (Cohen and Hickman, 1987). An oral glucose tolerance test (OGTT) also revealed the powerlifting steroid users to have a 2-fold increase in post-glucose glycaemia compared to non-users, which was a similar increase to the obese group. Post-glucose insulinaemia in the powerlifting steroid using group was also higher compared to all groups, with it being at least 2-fold higher compared to obese participants (Cohen and Hickman, 1987). The authors only report participants use of AAS although insulin is commonly used for its anabolic potential and may have also been used by participants which may have impacted the results. More recently in males, an OGTT between

healthy controls, steroid-using bodybuilders and former steroid-using bodybuilders (mean discontinuation of 2.5 years) revealed that current and former AAS-p users had impaired glucose tolerance compared to healthy controls (Rasmussen *et al.*, 2017). Reduced insulin sensitivity in former AAS-p users, was associated with higher % body fat, which may be due to reduced testosterone levels compared to healthy controls (Mammi *et al.*, 2012; Rasmussen *et al.*, 2017).

Chronic AAS use suppresses the hypothalamic-pituitary-testicular axis resulting in reduced endogenous testosterone production (Kanayama *et al.*, 2015). Low testosterone levels reduce insulin sensitivity and increase risk of developing MetS and CVD (Kelly and Jones, 2013).

As skeletal muscle is the largest tissue for glucose disposal, increases in muscle mass should improve insulin sensitivity; paradoxically, these results indicate that chronic AAS use may cause tissue IR. This may be due to an imbalance of regulatory adipokines and cytokines from increased VAT levels and circulating lipids leading to a decreased/delayed stimulus of the PI3K/Akt signalling cascade in response to glucose ingestion, as also observed in T2D individuals (Samuel and Shulman, 2016). This dysregulated metabolism leads to a continuous cycle of VAT and IR that potentiate each other. Furthermore, chronic activation of S6K1 mediated by mTORc1, inflicts serine phosphorylation of IRS1 leading to reduced insulin sensitivity (Um, D'Alessio and Thomas, 2006; Yoon and Choi, 2016). It may be possible that chronic AAS use, leading to hyperactivation of mTORc1/S6K1 signalling may cause IR (Figure 1.9). Oestradiol has shown to be higher with AAS use compared to healthy controls and may also be a cause of IR in this population. The conversion of testosterone to oestradiol resulting in a decrease in the testosterone to oestradiol ratio has been implicated in the development of MetS in older males (Maggio *et al.*, 2010). Additionally, oestradiol is reported to bind to insulin and the insulin receptor further highlighting its potential role in inducing IR (Figure 1.10) (Root-Bernstein, Podufaly and Dillon, 2014).

Research is currently lacking on the prevalence of increased levels of VAT with AAS use, most likely due to AAS typically reducing fat and to its dysregulation of insulin sensitivity. This field of research warrants further investigation.

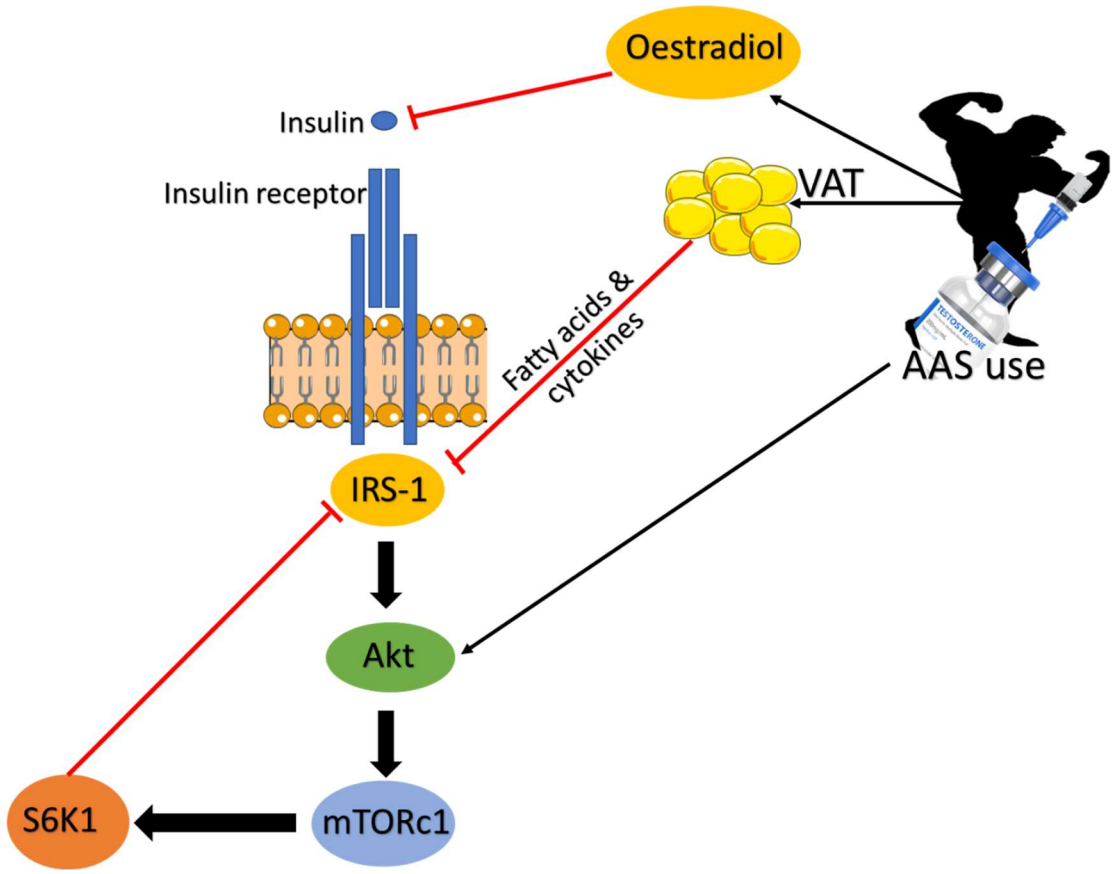


Figure 1.10. Potential mechanisms of insulin resistance with chronic anabolic steroid use.
AAS; Anabolic-androgenic steroid; IRS-1; insulin receptor substrate 1, mTORc1; mammalian target of rapamycin complex 1, S6K1; P70 S6 kinase 1, VAT; visceral adipose tissue.

1.2.4.3. Abdominal obesity

Although research is scarce on the effects of AAS on VAT a recent cross-sectional study reported current users of AAS-p to have 32% higher VAT levels (Rasmussen *et al.*, 2017). Although the AAS users had lower % body fat compared to healthy controls and former users, they had reduced adiponectin and leptin levels which are all independent predictors of IR, T2D and MetS (Hung *et al.*, 2008; Preis *et al.*, 2010; Frühbeck *et al.*, 2017; Rasmussen *et al.*, 2017; D'Elia *et al.*, 2019).

However, a RCT of the dose-response of Test E for 20 weeks showed decreases in VAT with higher doses, indicating supraphysiological doses do not increase VAT (Woodhouse *et al.*, 2004). Although cross-sectional studies cannot determine causation, it may be more representative of long term AAS and AAS-associated polysubstance use in this case. Individuals typically use a range of AAS types and other complementary drugs, doses, cycles, methods of administration and for years rather than weeks or one AAS which may explain the differences in results.

Accumulation of VAT is an important indicator of glucose tolerance, MetS and CVD risk, much more so than subcutaneous adipose tissue (Wajchenberg, Lé and Wajchenberg, 2000; Lee *et al.*, 2008; Preis *et al.*, 2010). Lipolysis is typically positively associated with IR and VAT (Morigny *et al.*, 2016); however, with AAS use, VAT was associated with lower lipolysis rates as determined by lower levels of plasma glycerol (Rasmussen *et al.*, 2017). The unusual lower lipolytic activity may be attributable to reduced activity of catecholamines due to AAS compounds such as nandrolone downregulating β3-adrenoceptor expression (Alsiö *et al.*, 2009).

1.2.4.4. Hypertension

Hypertension, as a result of chronic AAS use, is under debate due to conflicting data (Achar, Rostamian and Narayan, 2010). Although further research is required, there is some evidence to suggest that chronic AAS use in males may lead to increased BP (Urhausen, Albers and Kindermann, 2004; Achar, Rostamian and Narayan, 2010; D'Andrea *et al.*, 2018; Rasmussen *et al.*, 2018). By contrast, early studies suggested that AAS use did not have a detrimental effect on BP even with 24-hour monitoring (Lenders *et al.*, 1988; Kuipers *et al.*, 1991; Palatini *et al.*, 1996). Short-term (<8 weeks) testosterone (200 mg/wk) and nandrolone (100-200 mg/wk) administration resulted in no change in systolic or diastolic BP (Kuipers *et al.*, 1991; Chung *et al.*, 2007); however, long term controlled studies are lacking.

Lenders *et al*., reported AAS-p use to increase SBP after an average use of 5 months although the increase was not clinically relevant (118 ± 2.2 to 121 ± 2.4 mmHg) (Lenders *et al.*, 1988). Nevertheless, more recent studies have shown chronic AAS-p users to have higher SBP compared to healthy controls and former AAS-p users (D'Andrea *et al.*, 2018; Rasmussen *et al.*, 2018). These results may be clinically relevant as mean SBP was reported to be 132 mmHg and 138 mmHg in current AAS-p users (D'Andrea *et al.*, 2018; Rasmussen *et al.*, 2018). Current and former AAS users were also reported to have increased aortic stiffness. These higher levels in aortic stiffness and SBP were associated with lower mid-regional pro-atrial natriuretic peptide in AAS users (Rasmussen *et al.*, 2018). Atrial natriuretic peptides regulate vasodilation, reduce renin-angiotensin-aldosterone system activity and sympathetic nerve activity; yet, high levels of mid-regional pro-atrial natriuretic peptide are associated with hypertension and incidence of mortality (Khaleghi *et al.*, 2009; Idzikowska and Zielińska, 2018). The conflicting results regarding hypertension with AAS use may be partly due to differences in study designs. Repeated measure designs as implemented in the early studies are more indicative of causal effects compared to the most recent cross-sectional studies; however, the cross-sectional studies have larger sample sizes and potentially greater power but only association can be conferred. The lack of control on AAS type and quantity also

makes it difficult to compare findings. Nonetheless, chronic AAS use may have detrimental effects on the vasculature and consequently causing hypertension and increased risk of CVD, but more long-term controlled studies are required.

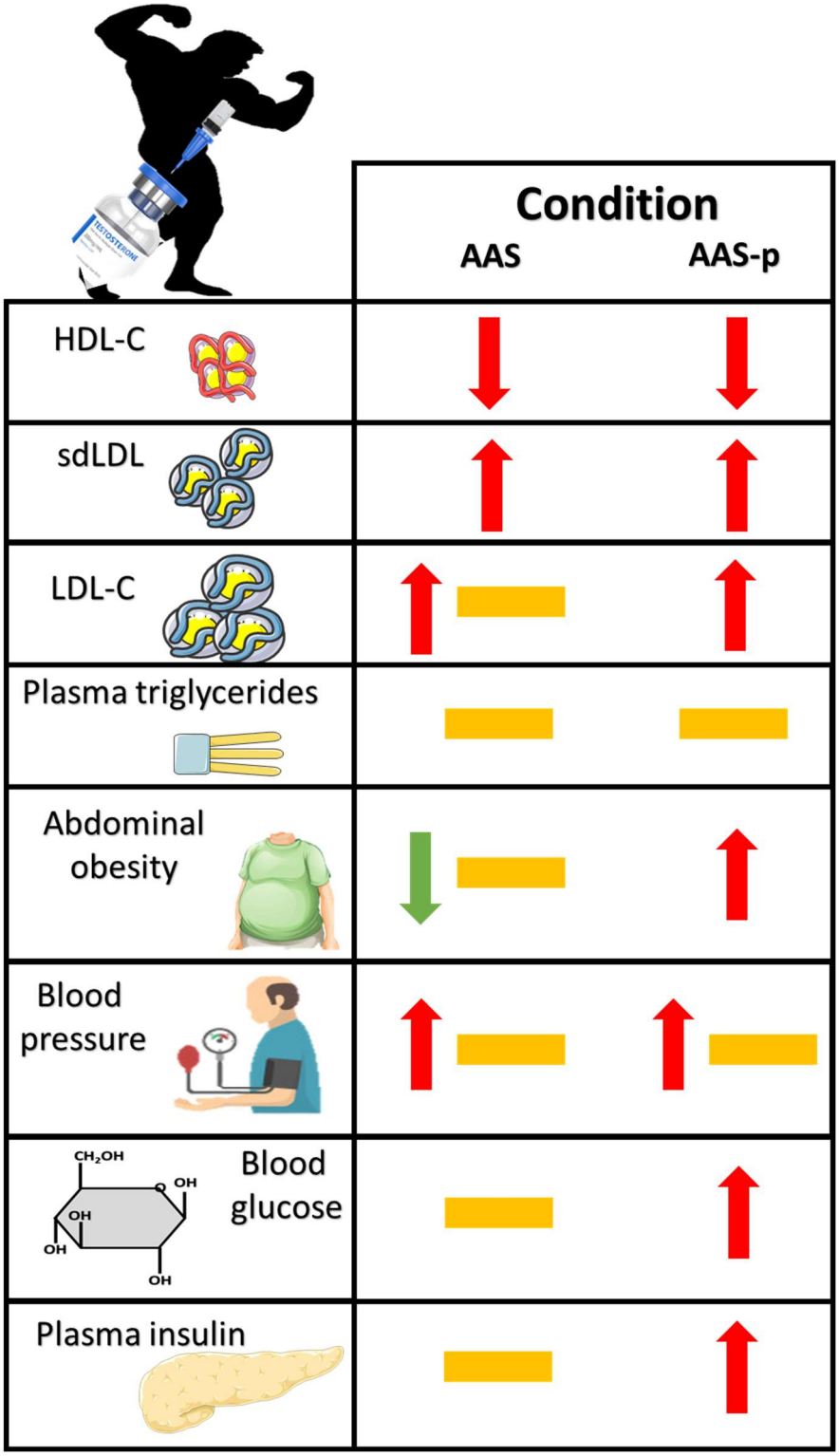


Figure 1.11. Summary of the impact of anabolic-androgenic steroid (AAS) use and AAS related polypharmacy (AAS-p) on markers of cardiometabolic disease.

1.3. Perspective and Thesis aims

The manifestation of CMD is a consequence of poor lifestyle choices resulting in increased risk of T2D and CVD. While poor dietary intake and sedentary lifestyles are in complete contrast to the lifestyle of a highly resistance trained AAS user they exhibit similar metabolic abnormalities such as dyslipidaemia. Although further research is needed, they may also share impaired skeletal muscle glucose metabolism. Skeletal muscle is a primary site of insulin stimulated glucose uptake and increase muscle mass and strength has an inverse association with CMD, T2D and CVD risk. Therefore, maintenance of skeletal muscle health and its subsequent regulation on insulin dynamics should be of great clinical importance. Research has shown that the metabolic environment plays an important role in regulating skeletal muscle insulin signalling with circulating lipids and inflammatory cytokines causing insulin resistance. This may be the case with individuals who have an elevated metabolic risk due to poor dietary intake or AAS use. LC diets have shown to improve markers of CMD; therefore, they may improve the metabolic environment and subsequent insulin signalling. However, there is currently a lack mechanistic research on how a change in the metabolic environment due to diet or AAS use impacts insulin signalling.

Therefore, the overall goal of this thesis is:

To investigate markers of CMD in response to a very low carbohydrate diet (objective 1) and among users of anabolic-androgenic steroids (objective 3). Furthermore, the subsequent impact of the metabolic environment on skeletal muscle mechanisms of insulin signalling and metabolism will also be investigated using serum derived from participants (objective 2 + 4) (Figure 1.12).

It is hypothesised that an *ad libitum* VLC diet will show greater improvements in cardiometabolic health markers compared with a HC diet (objective 1) and the greater improvements with the VLC diet will lead to better serum conditions resulting in improved *in vitro* insulin signalling and cellular metabolism relative to the HC diet objective 2). It is also hypothesised that AAS use will be associated with markers of MetS and CMD compared with matched healthy controls (objective 3) and serum derived from AAS users will lead to impaired *in vitro* insulin signalling and cellular metabolism (objective 4).

The studies included in this thesis aim to achieve the overall goal by completing the following objectives:

1. To investigate if an 8-week *ad libitum* VLC diet improves key markers of CMD in comparison with a HC diet (current UK guidelines).

2. To investigate if skeletal muscle metabolism and insulin signalling improves in response to serum from participants following VLC or HC diets.
3. To determine if key markers of CMD are upregulated among users of AAS in comparison with healthy controls.
4. To determine the impact of serum from participants who use and don't use AAS on skeletal muscle metabolism and insulin signalling.

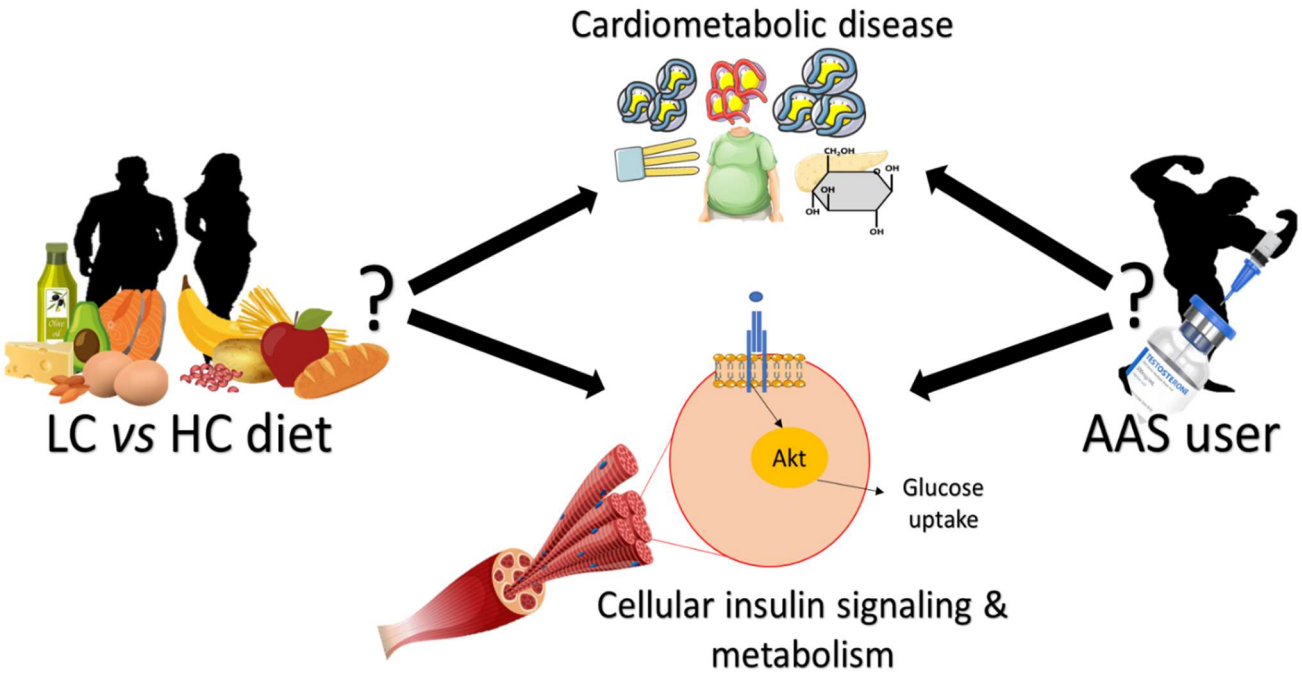


Figure 1.12. Summary of objectives investigating the effects of lifestyle factors on cardiometabolic disease and on skeletal muscle insulin signalling and metabolism.

AAS; Anabolic-androgenic steroids; CMD; cardiometabolic disease, HC; High carbohydrate; LC, Low carbohydrate.

Chapter 2: Materials and Methodology

2.1. Materials, Equipment and Specialised Software

2.1.1. Cell culture

All cell culture experiments were conducted using a Kojair Biowizard Silverline class II hood (Kojaire, Vippula, Finland). All cells were incubated in a HERAcell 150i carbon dioxide (CO_2) incubator (Thermo scientific inc. Cheshire, UK). An aspirator vacuum pump (Charles Austen Pumps Ltd, Surrey UK) was used to remove cell media and supernatant. Cell culture solutions were prepared using purified distilled water (dH_2O) through MilliQ water system (Merck, Darmstadt, Germany) or dimethyl sulphoxide (DMSO) (Sigma Aldrich, Poole UK) or phosphate buffered saline (PBS) (Sigma-Aldrich, Poole, UK). Cell images were performed using an inverted light microscope (Olympus, CKX31, Japan).

2.1.2. Cell culture reagents

Murine C2C12 cell line was purchased from American type culture collection (ATCC) (Virginia, USA). Dulbecco's Modified Eagle's medium (DMEM) was purchased from Gibco (Life Technologies, California, US) and was used for C2C12 cells. Cells were supplemented with heat inactivated horse serum (HS) or heat inactivated newborn calf serum (NBCS) and fetal bovine serum (FBS). All sera were purchased from Gibco (Life Technologies, California, US). Sera derived from human participants were heat inactivated at 56°C for 30 mins prior to cell supplementation. Media were further supplemented with L-glutamine (L-G) (2mM), penicillin streptomycin (PS) antibiotic (1 % 50 units penicillin/50 ug streptomycin). PBS purchased from Sigma-Aldrich (Poole, UK) in tablet form was used to wash the cells. PBS was reconstituted to a working concentration of 0.01 M phosphate buffer, 0.0027 M KCl, 0.137 M NaCl at a pH of 7.4 in dH_2O. Gelatin type A from porcine skin, purchased from Sigma-Aldrich (Poole, UK) was reconstituted to create a working stock of 0.2% gelatin and was used to support cell adherence and fusion. Trypsin/ Ethylenediaminetetraacetic Acid (EDTA) purchased from Sigma-Aldrich (Poole, UK) was used to detach adherent cells and was composed of 0.05% trypsin and 0.02% EDTA. To promote cell proliferation, murine C2C12 cells were grown in growth media (GM) (DMEM, 10 % FBS, 10 % NBCS, 1 % PS, 2mM L-G). To promote cell differentiation, cells were switched to differentiating media (DM) (DMEM, 2 % HS, 1 % PS, 2mM L-G). To investigate the impact of conditioned human serum (CS) on cell fusion, metabolism and intracellular signalling, DMEM was supplement with 2 % CS, 1 % PS, 2mM L-G.

Insulin (INS) solution at 10.2 mg/ml and Fibroblast growth factor 21 (FGF21) were purchased in lyophilised form from Sigma-Aldrich (Poole, UK). FGF21 was reconstituted in dH_2O at concentration of 100ug/ml. 2-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino)-2-Deoxyglucose, (2NBDG) was purchased from Thermo fisher Scientific (Waltham, MA, USA) and reconstituted in DMSO at 10 mg/ml.

2.1.3. Plasticware

Tissue culture flasks (T75, 6 well and 96 well plates) and 96 well plates for BCA and glucose uptake assays were purchased from Nunc Life Sciences, Thermo Fisher Scientific (Rosklide, Denmark). BD Falcon 96 well clear UV plates for CK assays were purchased from BD Biosciences (San Jose, CA, USA). Eppendorf tubes (0.5, 1.5 and 2 ml) were purchased from BD Biosciences (San Jose, CA, USA). Pipette tips (10, 200 and 1000 ul), Stripettes (5, 10, and 25 ml), sterile tubes (15 and 50 ml), 2 ml cryogenic vials and cell scrapers were purchased from Fisher Scientific UK (Loughborough, UK). Syringes were purchased from Terumo (Leuven, Belgium). Bottle top 0.22 µM filters (500 ml) and 0.22 µM syringe filters were purchased from Corning (Lowell, MA, USA).

2.1.4. Biochemical assays

The Clariostar plate reader was purchased from BMG LABTECH (Ortenberg, Germany) and was used to analyse BCA^{TM} protein, Creatine kinase and 2-NBDG glucose uptake. BCA protein assay reagents were purchased from Pierce (Rosklide, Denmark). Creatine Kinase assay reagents were purchased from Catachem incorporation (Conneticut, NE, USA).

2.1.5. Flow cytometry

The BD Accuri C6 flow cytometer with BD CFLOW® software (BD Biosciences, Wokingham, UK) was used for flow cytometric analyses. Flow cytometry reagents were purchased from BD (BD biosciences, San Jose, CA, USA). To detect phosphorylation of Akt^{ser473}, mTOR^{s2448}, ERK1/2$^{T202/Y204}$ and p38-MAPK$^{T180/Y182}$, the following antibodies were used for flow cytometry: anti-human/mouse phosphor-AKT (S473; APC; 675/25 nm), antihuman/ mouse phosphor-ERK1/2 (T202/Y204; Alex-afluor 488; 533/30 nm), antihuman/ mouse phosphor-mTOR (S2448; PerCP; 670/LP nm), antihuman/ mouse and phosphor-p38 MAPK (T180/Y182; PE; 585/40 nm). All were purchased from Thermo Fisher Scientific (Waltham, USA).

2.1.6. SDS-Page and Western Blotting

Mini Protean® Tetra stands with clamp kit were used for gel casting, a Powerpac™ Basic power supply was used for electrophoresis and the Tans-blot® Turbo™ Blotting system was used for protein transfer from gel to nitrocellulose membrane and were all purchased from Bio-rad (Bio-rad Laboratories, Inc. CA, USA).

2.1.7. Automated spectrophotometry

The Rx Daytona, an automated random-access clinical chemistry analyser, was purchased from Randox Laboratories (Antrim, Northern Ireland). All wash solutions, controls and calibrators were purchased from Randox Laboratories (Antrim, Northern Ireland). Reagents used to detect total cholesterol, triglycerides, low-density lipoprotein-cholesterol (LDL-C), small dense low-density lipoprotein-cholesterol (sdLDL-C), high-density lipoprotein-cholesterol (HDL-C), glucose, non-esterified fatty acids (NEFA) and Apolipoproteins (Apo) A1 and B were purchased from Randox Laboratories (Antrim, Northern Ireland).

2.1.8. Sandwich chemiluminescent immunoassay

The Evidence Investigator ™ Biochip Array technology uses sandwich chemiluminescent immunoassays to simultaneously detect multiple analytes from a single sample and was purchased from Randox Laboratories (Antrim, Northern Ireland). The metabolic syndrome I and II was purchased from Randox Laboratories (Antrim, Northern Ireland) to determine the following plasma cytokines and hormones; Ferritin, interleukin 6 (IL-6), insulin, Leptin, Plasminogen activator inhibitor-1 (PAI-1), Resistin, tumour necrosis factor- α (TNF-α), adiponectin, C-reactive protein (CRP) and cystatin C.

2.2. Cell culture and experiments

2.2.1. C2C12 Myoblast Cell Culture

Murine C2C12 myoblasts were used in all experiments and were purchased from the ATCC (Virgina USA). The C_2C_{12} cell line was originally a subclone of the C_2 cell line which is no longer commercially available (Blau et al., 1985).

C2C12 cells at 1 x 10^6 cells/ml in 2 ml cryovials were resurrected from liquid nitrogen. Cryovials were warmed quickly and once thawed cells were plated onto T75 flasks containing 14 ml of GM. T75 flasks were gelatinised prior to plating the cells with 5 ml of 0.2% gelatine and incubated at room temperature (RT) for 20 minutes before the excess gelatine was aspirated. Flasks were gently agitated to distribute the cells evenly. Cells were incubated up to 72 hr, until 80% confluent (Figure 2.1) in a humidified atmosphere of 5% CO_2 at 37 °C. Following attainment of 80% confluence, cells were washed twice with PBS to remove excess serum which inhibits the action of trypsin. To enable cell dissociation, 1 ml of trypsin was added to the cells and incubated for 5 minutes at 37 °C. Dissociation was confirmed by microscopy and trypsin was neutralised by adding 4 ml GM to the T75 flask. Cells were resuspended using a syringe and 21-gauge needle and slowly drawing up and down the cell suspension (5-10 times), to prevent cell clumping. Cell suspension was prepared for counting in a 1:1 dilution in 0.4% trypan blue stain (Bio Whittaker, Wokingham, UK). The cell suspension/trypan blue mixture was dispensed onto a Neubauer haemocytometer (Assistent, Sondheim, Germany). (For counting see section 2.2.2). When cells were counted, depending on experiment they were seeded onto 6, 12 or 96 well plates at 40,000 cells per ml. Remaining cells were frozen in liquid nitrogen for further experiments (For freezing see section 2.2.3). Upon attainment of 80% confluence, cells were washed twice with PBS and GM was replaced with DM to begin cell experiments.

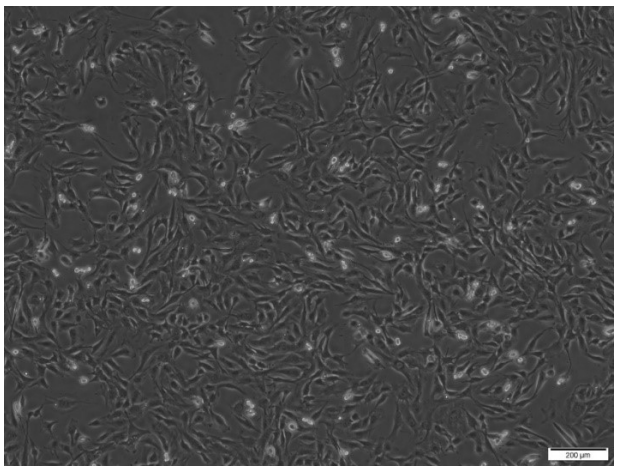


Figure 2.1. Image of C2C12 cells at 80% confluency at a scale of 200 µm.

2.2.2. Cell Counting

Ten µl 0.4% trypan blue stain and cell suspension (1:1) were transferred onto both grids of the Neubauer haemocytometer (Assistent, Sondheim, Germany). To spread the suspension evenly a glass coverslip was placed on top. Under 10x magnification, 9 sections of each grid were observed and cells within the 4 corner sections were used for counting (Figure 2.2). Only viable cells were counted, which were round and small. Non-viable cells were identified as trypan blue positive and lost membrane integrity. The average of both grids (8 sections) were used to assess the number of cells in 0.1 mm^3. The average was multiplied by 2 to account for the dilution of cells with trypan blue and then multiplied by 10,000 to equate the number of cells from 0.1 mm^3 to 1 cm^3 which is equivalent to 1 ml to provide cell concentration per ml. Cells were seeded at 40,000 cells/ml for plating and would be confluent after 48 hrs. In a 6, 12 and 96 well plate, 2, 1 and 0.125 ml of cell suspension was added to each well respectively. An even number of cells were split across all wells using the following equation:

1. Cell concentration (cells/ml) = Mean of cell count x 2 (1:1 dilution) x 10,000 (0.1 mm^3 to 1 cm^3)
2. 40,000 (cells/ml) = (40,000 ÷ cell concentration) x total volume of media required (ml)

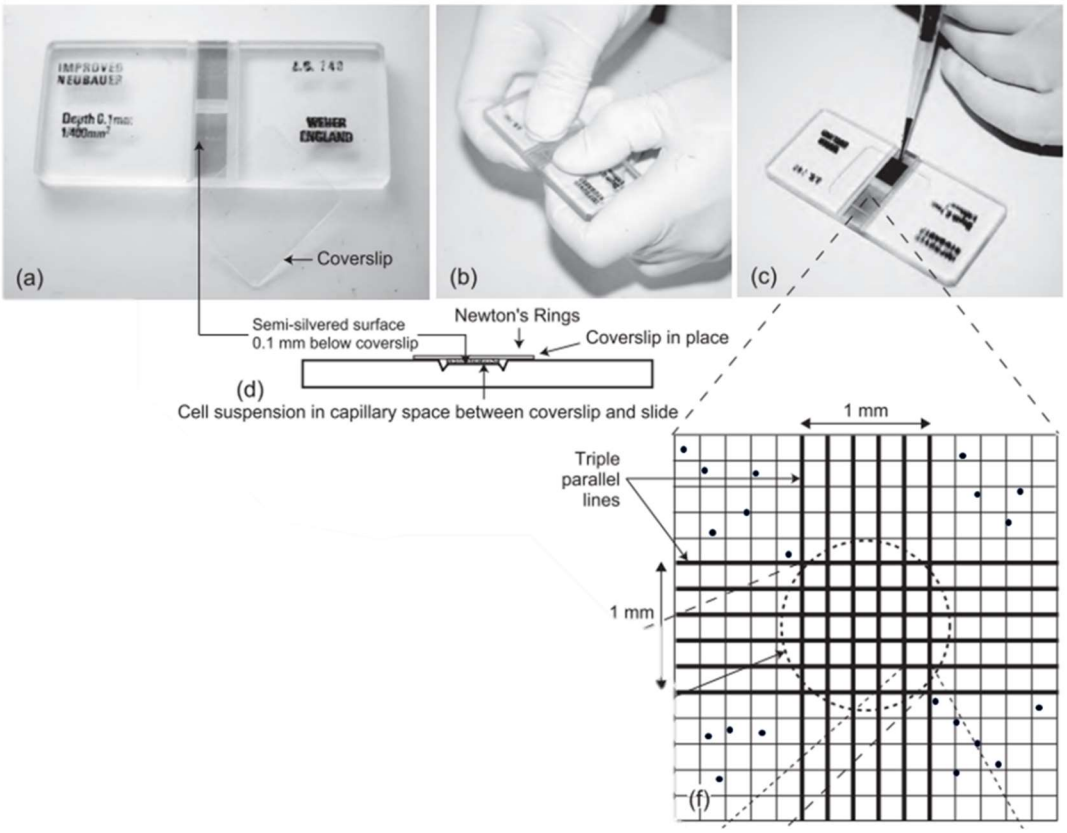


Figure 2.2. Cell counting with haemocytometer. (Adapted from Freshney, 2015). Cells (black dots) are counted in the top and bottom left and right square grids to measure cell concentration.

2.2.3. Freezing for cell storage

Remaining cells were stored in liquid nitrogen at a concentration of 1 x10^6 cells/ml in GM plus 10 % of DMSO, which was added to the cell suspension to protect against rapid freezing. DMSO protects the cell by replacing water within the cell to reduce ice crystallisation and therefore damage to the structure of the cell (Fleming and Hubel, 2006). Cell suspension was then aliquoted to 2 ml cryovials and labelled with cell line, passage number, concentration, name and date. Cells were then stored in a "Mr Frosty" (Sigma-Aldrich, Poole, UK) with 100 ml of isopropanol at -80 °C for 24 hours which delays freezing before being transferred to a cryovial box in liquid nitrogen for long-term storage.

2.2.4. Formation of myotubes

Upon attainment of 80% confluence, myoblasts were washed twice with PBS and incubated in DM at 37 °C and 5% CO_2 for up to 120 hrs to induce differentiation to myotubes. Media was topped up with 50% x well volume DM every 48 hrs. Upon attainment of myotubes at 96-120 hr cells were imaged (Figure 2.3), washed twice with PBS and processed for experimentation.

CK activity was measured at 0, 48, 96 and 120 hrs as a measure of biochemical differentiation. At each timepoint, media was aspirated, and cells were washed twice with 1 ml PBS prior to lysing with 150 µl/well 0.05 M Tris/MES Triton Buffer (TMT; 50 mM Tris MES, 1 % Triton X-100) for 5 min at RT. After 5 min, cells were scraped in all directions and lysates were transferred into 0.5 ml Eppendorf tubes. Samples were stored at -80 °C until further processing (See section 2.3.2).

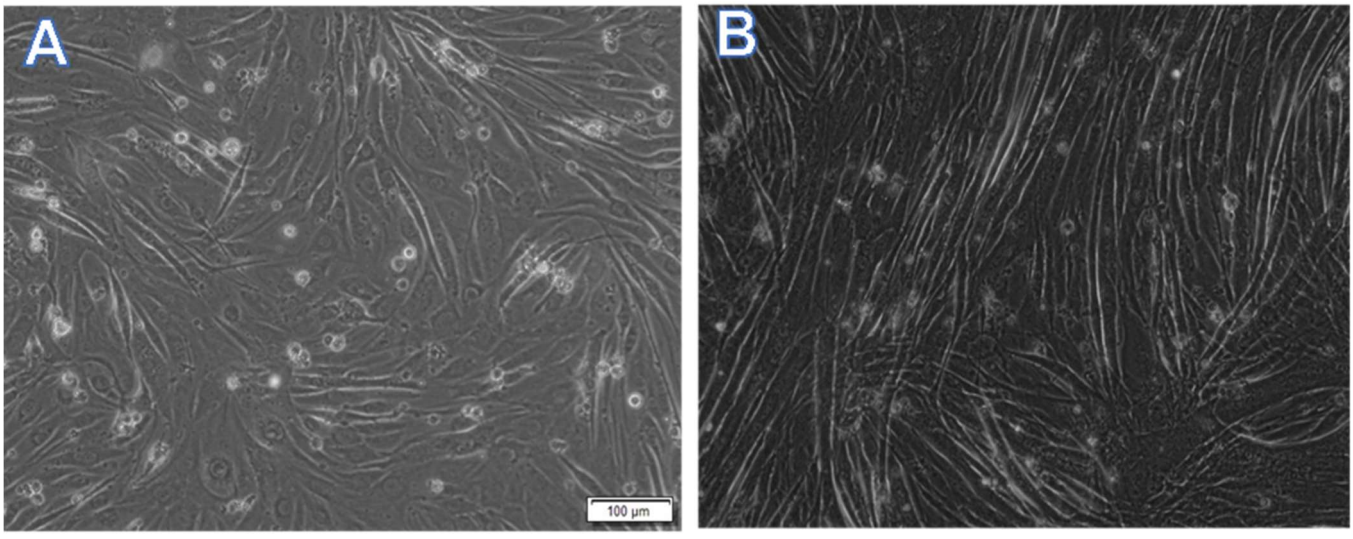


Figure 2.3. Image of cells during differentiation at A; 48 hr and B; 120 hr. Scale 100 µm.

2.2.5. Human serum

Serum derived from humans was pooled by groups and heat inactivated by incubating at 56 °C for 30 mins. The CS was aliquoted in 1 ml Eppendorf's at -80 °C for long term storage. Serum was heat inactivated to inactivate complement which is a group of proteins involved in the immune response. This is standard procedure in the growing and fusing of C2C12 cells therefore it was necessary to be consistent with all routine cell culture techniques to ensure data collected can be attributed to differences in serum metabolites/proteins associated with *in vivo* health. Use of human serum with *in vitro* experiments in this thesis is exploratory in nature therefore, serum was pooled to simplify the experiments by reducing intra and inter variability whilst also reducing the costs associated with producing replicates. For example, one experiment requires a minimum of n=3 therefore, at least 3 replicates would be required per participant increasing the use of consumables 3-fold.

For experimentation, CS was incubated for 1, 3 and 24 hours on C_2C_{12} cell myoblasts prior to stimulation with 100 nM insulin for 20 min. Pre-incubation with CS for 3 hours was most consistent and was therefore selected for insulin dosing experiments.

2.2.6. Myoblast insulin and serum signalling

Upon attainment of 80% confluence, myoblasts were washed twice with PBS. To measure the impact of CS on insulin signalling, cells were incubated for 3 hours in DM containing either 2% HS or CS. Cells were then spiked with 100 nM of insulin and incubated at 37 °C and 5% CO_2 for 20 min (Chapter 4, study 2) or 20 and 30 mins (Chapter 6, study 4).

To measure the effect of serum on cellular signalling, cells were incubated in serum free DMEM for 3 hours. After incubation cells were washed twice with PBS and incubated with DM containing 2% HS or CS for 0, 15, 30 or 60 mins. At each timepoint, cells were placed on ice, media was aspirated, and cells were washed twice with 1 ml ice-cold PBS. Cells were trypsinised for 5 mins at 37 °C and 5% CO_2 with 200 µl trypsin. After detaching of cells from wells, trypsin was neutralised with 800 µl of GM and cells were aliquoted into 2 ml Eppendorf tubes. Cells were counted prior to being centrifuged at 800 g for 5 mins at 4 °C. The supernatant was removed leaving a cell pellet. The cells were fixed with 100 µl of 2% paraformaldehyde (PFA) for 30 min prior to addition of 1 ml ice-cold 100% methanol and storage at -20 °C until further processing (See section 2.3.4).

2.2.7. Myotube insulin and serum signalling

After attaining 80% confluence, cells were incubated in DM for 96-120 hr and fully formed myotubes were washed twice with PBS and incubated for 3 hours in DM containing 2% HS or CS. Cells were spiked with 100 nM insulin and incubated at 37 °C and 5% CO_2 for 20 or 30 mins depending on experiment. After incubation, cells were immediately placed on ice and washed twice with ice cold PBS and both were processed for western blot.

Cells were placed on ice at each timepoint, media was aspirated and washed twice with 1 ml of ice-cold PBS and lysed with 150 µl per well of 1 x Radioimmunoprecipitation Assay (RIPA) buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.25% deoxycholic acid, 1% NP-40, 1 mM EDTA) (Sigma Aldrich, Poole UK). After 5 min, cells were scraped in all directions and lysates were transferred into 0.5 ml Eppendorf tubes. Samples were stored at -80 °C until further processing (See section 2.3.5).

2.2.8. Glucose uptake

To investigate myoblast glucose uptake (See section 2.4), cells were grown until 80% confluence in 96 well plates and washed twice with PBS and incubated in serum free, low glucose (5.5 mM), 1% Bovine serum albumin (BSA) DMEM for 24 hours, prior to washing twice with PBS and transferring to 0% glucose-containing DMEM plus 100µM 2-NBDG and 2% HS or CS.

To investigate glucose uptake, fully formed myotubes were washed twice with PBS and replaced with serum free, low glucose (5.5 mM), 1% BSA DMEM for 24 hours, prior to washing twice with PBS and media being replaced with 0% glucose, DM containing 100µM 2-NBDG.

Cells were then incubated at 37 °C and 5% CO_2 with 0, 10 or 100nm of insulin for 30 mins before cellular fluorescence was detected (section 2.3.1).

2.3. Principles and procedures

2.3.1. Glucose uptake

2.3.1.1. Principle

Glucose is the primary source of energy for cells and insulin-stimulated glucose uptake is essential for glucose homeostasis in muscle. Glucose that's transported into the cell via glucose transporters (GLUTs) is converted to glucose-6-phosphate (G6P) by hexokinase and is further metabolised for glycogenesis or glycolysis. 2-NBDG is a fluorescent glucose analogue which also enters the cell via GLUTs and is converted to 2-NBDG-6-phosphate by hexokinase. 2-NBDG-6-phosphate accumulates in the cell due to low intracellular phosphatase and is unable to be metabolised (Yamamoto *et al.*, 2015). Cell fluorescence is detected (λex = 460 to 490 nm, λem =530 to 550 nm) using the microplate reader. Insulin stimulated cellular uptake of 2-NBDG is expressed relative to 0 nM after subtraction of background noise.

2.3.1.2. Procedure

Cells were cultured on clear-bottom 96-well plates, prior to incubation for 24 hours in serum free, low glucose (5.5 mM), 1% BSA DMEM to reduce interference from growth factors in serum. After 24 hours, cells were washed twice with PBS and replaced with 0% glucose-containing DM with 100μM 2-NBDG. To remove background, a sample of cells contained no 2-NBDG. Cells were incubated at 37 °C and 5% CO_2 with 0, 10 or 100nm of insulin and for 30 mins. After incubation cells were placed on ice and washed 3 times with ice cold PBS. Cell fluorescence was detected (λex = 460 to 490 nm, λem =530 to 550 nm) using the microplate reader (Clariostar Ortenberg, Germany). After detection cells were washed 3 times with 100 μl PBS and lysed for 5 mins in 20 μl of RIPA buffer at RT before undergoing detection for protein content (section 2.8).

2.3.1.3. Development

Serum is reported to interfere with insulin-stimulated glucose uptake therefore, serum free media is recommended for use when investigating 2-NBDG uptake (Yamamoto *et al.*, 2015). However, to address the impact of serum on insulin stimulated glucose uptake it would not be possible to exclude the serum incubation. In line with insulin signalling experiments, cells were incubated with serum for 3 hours prior to 2-NBDG incubation. Stimulation of cells with 10 nM of insulin did not significantly increase myoblast (P = 0.20) or myotube (P = 0.17) glucose uptake.

Further tests were done which showed 100 nM of insulin to show a tendency (P = 0.06) of increasing glucose uptake in myoblasts and significantly (P = 0.04) increase glucose uptake in myotubes with co-incubation of serum and 2-NBDG (Figure 2.4). Therefore, 100 nM of insulin was chosen to investigate the effects of insulin.

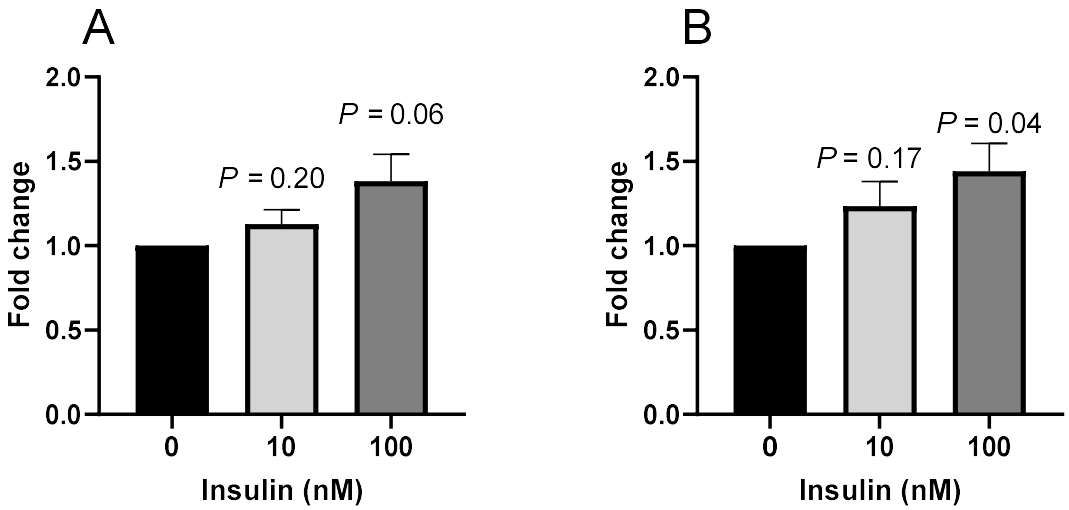
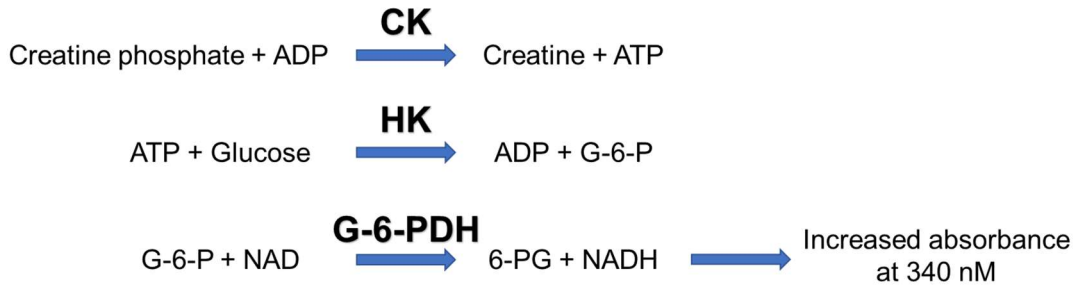


Figure 2.4. Effect of insulin on 2-NBDG glucose uptake in DM in A) myoblasts, B) myotubes. All data (n = 6 replicates in duplicate) are presented as mean ± SEM. P < 0.05 indicates significantly different to 0 nM.

2.3.2. Creatine Kinase

2.3.2.1. Principle

Creatine kinase (CK) activity is a biochemical marker of cell differentiation from myoblasts to myotubes. Previous research has validated and shown increases in CK to correlate with increases myoblast fusion (Sharples, Al-Shanti and Stewart, 2010; Sharples *et al.*, 2011; Dugdale *et al.*, 2018). CK catalyses the reaction of creatine phosphate and adenosine diphosphate (ADP) to produce creatine and adenosine triphosphate (ATP). Hexokinase, in combination with ATP phosphorylates glucose to produce G6P. In the presence of nicotinamide adenine dinucleotide (NAD), G6P is oxidised by glucose-6-phosphate dehydrogenase (G-6-PDH) to produce 6-phosphogluconate and Nicotinamide Adenine Dinucleotide + Hydrogen (NADH). This reduction of NAD to NADH increases absorbance to 340 nM and the rate of absorbency is directly related to CK activity (Equation 1).



Equation 1: Enzyme reactions involved in creatine kinase assay.

ADP; adenosine diphosphate, ATP; adenosine triphosphate, CK; Creatine kinase, G-6-P; glucose-6-phosphate NAD; Nicotinamide Adenine Dinucleotide; 6-PG: 6-Phosphogluconate; NADH; Reduced NAD.

2.3.2.2. Procedure

In duplicate, 10 µl of sample or a negative control of TMT were added to a 96 well plate. In accordance with the manufacturer's instructions (Catachem Inc, Connecticut, US) CK reagents were prepared by adding 5 ml of reagent A to 0.118 g of reagent B (Equation 2). The working reagent buffered at pH 6.7, contained 30 mmol/l creatine phosphate, 2 mmol/l ADP, 5 mmol/l AMP, 2 mmol/l NAD, 20 mmol/l N-acetyl-L-cystine, 3000 U/l HK (yeast), 2000 U/l G-6-PDH, 10 mmol/l magnesium ions, 20 mmol/l D-glucose, 10 µmol/l Di (adenosine 5') pentaphosphate and 2 mmol/l ETDA. It also contains 0.05 % sodium azide as a preservative. Using a multi-channel pipette, 200 µl working reagent was added to each well.

1. Amount of Reagent A (µl) = Total Reagent needed (200 µl) × (No of Samples)
2. Amount powder of reagent B per ml of reagent A = 0.118 g ÷ 5 = 0.0236 g
3. Amount of powder from Reagent B (g) =Amount of Reagent Bottle A (ml) × 0.0236 g

e.g. If there are 48 samples in duplicate:

Amount of Reagent Bottle A (µl) = 200 µl × 96 samples (48 in duplicate) = 19.2 ml of A

Amount of powder from Reagent B (g) = 19.2 ml × 0.0236 g = 0.453 g of B

Equation 2: Equation to determine quantity of reagents needed for creatine kinase assay.

After adding working reagent to samples, the plate was incubated in dark at 37 °C for 5 minutes. Using the Clariostar plate reader samples were measured every minute at an absorbance of 340 nM for 20 minutes total. The following equation was used to calculate CK activity (equation 3).

$\Delta A.min^{-1}$ = (Final A - Initial A) ÷ (Final Reading Time – Initial Reading time (mins))

CK activity was then determined using the following calculation:

$CK\ (U.L^{-1}) = (\Delta A.min^{-1} \times 0.21 \times 1000) \div (6.22 \times 0.01\ ml)$

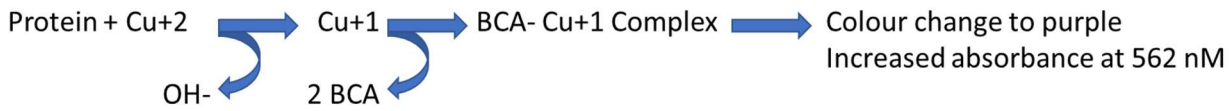
- $\Delta A.min^{-1}$ = Change in absorbance per minute at 340 nM
- 0.22 ml = Total volume (ml) (sample and reagent)
- 1000 = Conversion of units per ml to units per litre.
- 6.22 = Millimolar absorptivity of NADH at 340nM
- 0.01 ml = Sample volume (ml)

Equation 3: Calculation to determine CK activity.

2.3.3. Total protein

2.3.3.1. Principle

Total protein was determined using BCA™ assay. Protein reduces Cu^{+2} to Cu^{+1} in an alkaline environment (the biuret reaction) and produces a purple colour by bicinchoninic acid. The four amino acid residues present in protein molecules, cysteine or cystine, tyrosine, and tryptophan and number of protein bonds cause this reduction of copper. The amount of Cu^{+2} reduced is directly proportional to the amount of protein in the sample and therefore the intensity of the colour purple (Equation 4). The absorbance was measured at 562 nM.



Equation 4: Enzyme reactions involved in BCA assay.

2.3.3.2. Procedures

Protein concentration in the samples was determined against a BSA standard curve. Concentrations of 2, 1.5, 1, 0.75, 0.5, 0.25, 0.125 and 0 mg/ml of BSA were prepared by diluting with either TMT or RIPA buffer depending on experiment (Table 2.1). The BCA™ protein kit (Rockford, IL, USA) contains two reagents. At a ratio of 50:1, reagent A (sodium carbonate, sodium biocarbonate, BCA and sodium tartrate in 0.1 M sodium hydroxide) was mixed in a mixing trough with reagent B (4 % cupric acid sulphate). In a 96 well plate, 10 µl of each standard and sample were added in triplicate and duplicate respectively. Using a multi-channel pipette, 200 µl of working reagent was added to each well. The plate was incubated for 30 minutes at 37 °C and absorbance was measured at 562 nM with Clariostar plate reader.

Table 2.1. Serial dilution for BCA assay.

	BSA concentration (mg/ml)	Volume of Dilutent (µl)	Volume/source of BSA stock (µl)
A	2	0	300 of stock
B	1.5	125	375 of stock
C	1	325	325 of stock
D	0.75	175	175 of B
E	0.5	325	325 of C
F	0.25	325	325 of E
G	0.125	325	325 of F
H	0	400	0

2.3.4. Flow cytometry

2.3.4.1. Principle

Flow cytometry involves the measurement of single particles or cells in a fluid suspension using a laser. This process involves 3 systems; fluidics, optics and electronic systems (Cram, 2002). The fluid suspension is ordered into a single stream of cells that can be interrogated individually by the detection system. The fluidics system controls this process and consists of a central core in which the fluid sample is injected into and enclosed by an outer sheath fluid (Figure 2.4). A single stream of individual cells is created due to narrowing of the sheath fluid and is called hydrodynamic focusing. Hydrodynamic focusing allows cells to flow in a single file through one or more beams of focused light, called the interrogation point (Adan *et al.*, 2017). Lasers are the most common source of light in flow cytometry and produce a single wavelength of light at a specific frequency. Light that interacts with the cell, that is scattered in the forward direction 20° offset from the laser beam axis and is known as forward scatter (FSC). The FSC gives an indication of the size of the particle as larger particles refract more light than smaller particles. Light measured at 90° to the laser beam axis, is called side scatter (SSC). The SSC provides information on the complexity of the cell such as internal structures (Figure 2.5) (Adan *et al.*, 2017). Different wavelengths of fluorescence measurements detected can provide quantitative and qualitative data about fluorophore-labelled intracellular molecules. Optical filters block or transmit certain wavelengths to allow for specific detection of wavelengths. There are three major filter types; long pass filters; which allow light through above a cut-off wavelength; short pass filters permit light below a certain wavelength and band pass filters transmit light within a specified narrow range of wavelengths (termed a band width) (Cram, 2002). When a cell passes through the interrogation point it generates scattered light and fluorescence signals which results in a stream of electrons collected by the detectors. The magnitude of the electrons is proportional to the number of photons that is collected by the

photodiode or photocathode and therefore is proportional to the intensity of the scatter or fluorescence signal generated by the particle. The signal processing electronics system digitises the analogue current to quantify the signal height, area and width in numerical values (Adan *et al.*, 2017).

2.3.4.2. Procedures

To determine intracellular phosphorylation, cells were incubated with fluorophore labelled anti-phospho antibodies for Akt^{ser473}, $mTOR^{s2448}$, $ERK1/2^{T202/Y204}$ and $P38\text{-}MAPK^{T180/Y182}$. Upon interacting with light at a specific wavelength, fluorophores absorb the energy which results in excitation of the fluorophores electrons. The fluorophores excitation returns to basal levels and it re-emits the absorbed light energy at a longer wavelength and detected by the flow cytometer optics and converted to an electronic signal as described above.

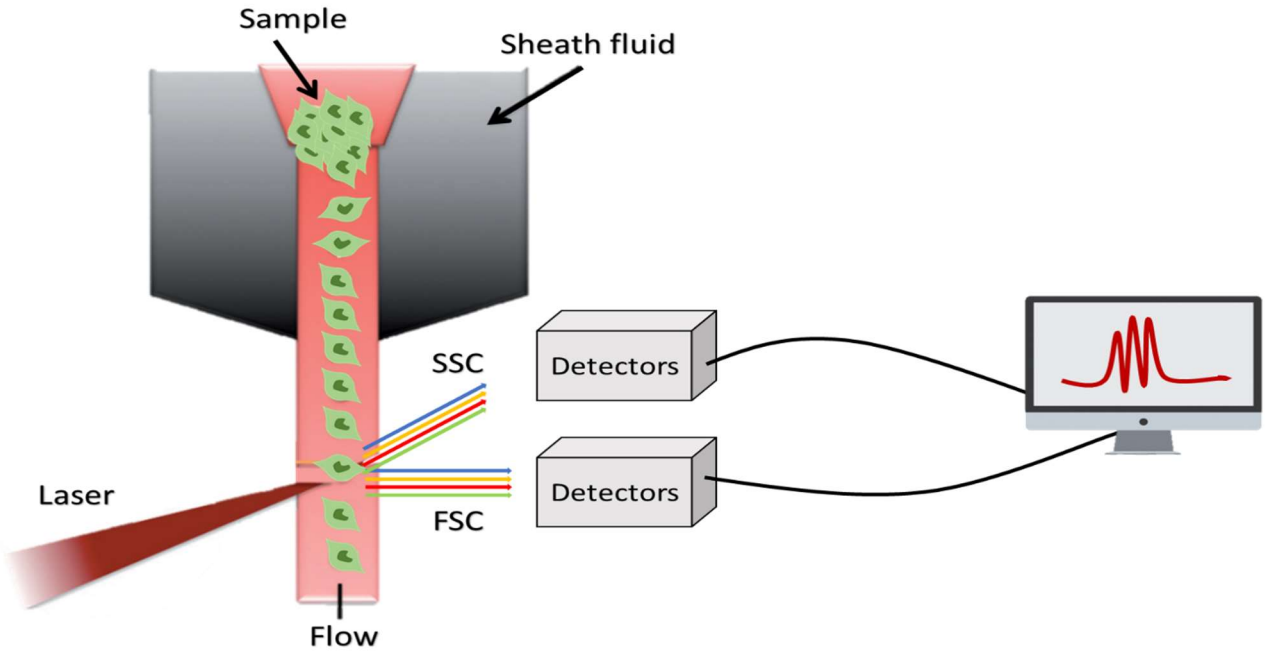


Figure 2.5. Principles of flow cytometry. A single stream of individual cells is created due to narrowing of the sheath fluid allowing cells to flow in a single file through one or more beams of focused light. Scattering of light allows for the size (FSC) and granularity (SSC) of the cell to be detected. (Adapted from Adan et al. 2017).

Intracellular phosphorylation was optimised by performing a serial dilution of each antibody for each experiment (Figure 2.6). The lowest concentration that reached 100% intracellular staining was used to minimise background to signal ratio by comparing to a negative control (no antibody and 0% staining).

Following fixation and permeabilization, cells were washed in flow buffer (PBS + 0.5 % FBS) and centrifuged at 600 g for 5 minutes at 4 °C and re-suspended at $1x10^6$ in flow buffer. Cells

were then incubated at RT for 60 mins in darkness with antibodies for anti-phospho Akt^{ser473} at 1:800 (Study 4) and at 1:200 (Study 6) dilution, mTOR^{s2448} at 1:200 dilution, ERK1/2$^{T202/Y204}$ at 1:200 dilution and p38-MAPK$^{T180/Y182}$ at 1:100 dilution. Following incubation, cells were washed 2 times by centrifuging at 600 g at 4 °C for 5 minutes and resuspending in flow buffer. Data from 1000 events were recorded by flow cytometry on a BD Accuri C6 flow cytometer with BD CFlow® Software (BD Biosciences, San Jose, CA, USA). Single populations of cells were measured by using FSC and SSC gating.

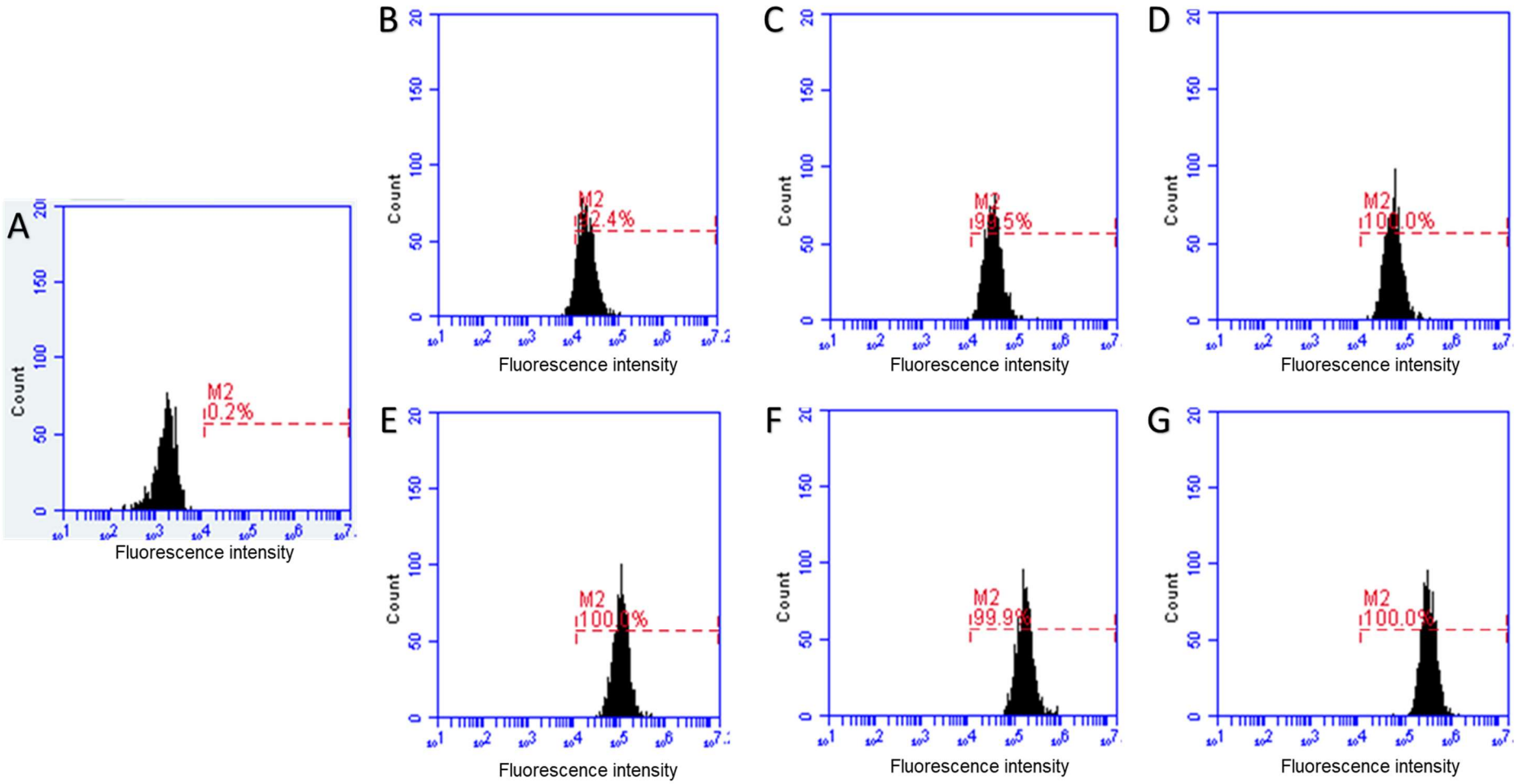


Figure 2.6. Serial dilution of phospo-Akt antibody. A) Negative control, B) 1/3200 dilution, C) 1/1600 dilution, D) 1/800, E) 1/400, F) 1/200, G) 1/100. A dilution of 1/800 resulted in the lowest fluorescence intensity and maximally stained cells.

2.3.5. SDS-PAGE and immunoblotting

2.3.5.1. Principle

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) is a discontinuous electrophoretic system and allows for separation of proteins by their molecular weight (kilo Daltons (kDa)) (Laemmli, 1970). Proteins are separated using an electrical field therefore, SDS, in addition to heat (boiling) and reducing agents, are used to disrupt the protein tertiary structure producing a linear molecule. SDS also uniformly coats the linear proteins masking their intrinsic charge resulting in a protein charge proportional to its molecular weight. The polyacrylamide gel matrix containing different bis-acrylamide concentrations alters the pore size of the matrix allowing for different molecular weight proteins to be separated. The addition of ammonium per sulphate (APS) and N, N, N', N'-Tetramethylethylenediamine (TEMED) to bis-acrylamide catalyses polymerisation. Typically, the gel is composed of a stacking gel with a lower bis-acrylamide (~5%) concentration buffered at pH 6.8 and a resolving gel (~10%) buffered to pH 8.8. This allows samples to be loaded into gel lanes and to align at the resolving gel boundary before separation.

2.3.5.2. Procedures

Following determination of protein concentration by BCA™ assay (see section 2.7), samples were resuspended in 5x Laemmli buffer (2 ml 0.5M Tris pH 6.8, 8.4ml H_2O, 3.2ml Glycerol, 3.2ml 10% SDS, 0.8 ml 2-β-mercaptoethanol (4.5%), 0.05% bromophenol blue) at 1 mg/ml. After boiling for 5 minutes at 95 ºC, 30 µg sample was loaded and electrophoresed on 5% stacking (1.7 ml 30% acrylamide 1% BIS solution, 5.7 ml H_2O, 2.5 ml 0.5 M Tris HCl pH 6.8, 100 µl 10% SDS, 50 µl 10% APS, 5 µl TEMED) and 10% resolving (6.6 ml 30% acrylamide 1% BIS solution, 8.2 ml H_2O, 5 ml 1.5 M Tris HCl pH 8.8, 200 µl 10% SDS, 100 µl 10% APS, 10 µl TEMED) gel. Stain-free 10% gels were also purchased from BioRad (Bio-Rad laboratories, Hertfordshire, UK). Samples were separated in Tris-glycine running buffer (1:10 10X Running buffer, Scientific Laboratory Supplies, Nottingham, UK) using Bio-Rad Mini-PROTEAN® Tetra vertical electrophoresis cell (Bio-Rad laboratories, Hertfordshire, UK). Voltage was set at 100 V until samples entered the resolving gel and then was increased to 150 V until the samples reached the bottom of the resolving gel. Stain-free gels were measured for total protein using fluorescent imaging (ChemiDoc™ MP imaging system, Bio-Rad Laboratories, Inc. CA, USA).

Semi-dry transfer of proteins to a nitrocellulose membrane using BioRad transfer pack was carried out. The polyacrylamide gel was stacked on a nitrocellulose membrane on top of thick filter paper with another filter paper on top of the gel (Figure 2.6). The nitrocellulose membrane and filter paper were pre-soaked in transfer buffer (600 ml H_2O, 200 ml ethanol and 200 ml 5x transfer buffer (Bio-Rad laboratories, Hertfordshire, UK)). Care was taken to avoid bubbles in the transfer stack and were removed using a roller. The sandwich was placed into a transfer cassette and pressed evenly with the cassette lid. Proteins were transferred to nitrocellulose membrane at 25 V for 10 mins. Transfer of protein was confirmed with ponceau staining of the membrane and imaged (ChemiDoc™ MP imaging system, Bio-Rad Laboratories, Inc. CA, USA).

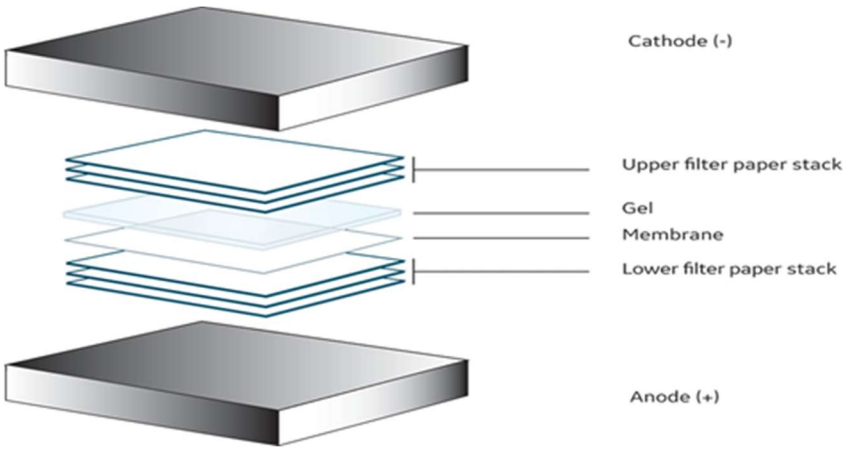


Figure 2.7. Assembly of transfer stack for successful transfer of proteins to nitrocellulose membrane from polyacrylamide gel.

Following blocking for 1-hour in 5% non-fat dried milk, membranes were incubated overnight at 4 ºC with primary antibodies (Table 2.2). After overnight incubation, membrane was washed 3 times for 5 min in TBS-Tween (TBST) (Tween-20 at 0.1%) and incubated for 1 hour in horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibody (Thermo Fisher Scientific inc, Waltham, USA) at a dilution of 1:1000 in 2.5% milk-containing TBST. All antibody incubations were as above, with the exception of puromycin which was incubated with goat anti-mouse IgG2a-specific (LI-COR Biosciences UK Ltd, Cambridge, UK) at a dilution of 1:10000 in TBST 5% BSA. Following secondary antibody incubation, membranes were washed 3 times for 5 minutes in TBST. Proteins were visualised by enhanced chemiluminescence (ECL) (Pierce™ western blotting substrate, Thermo Fisher Scientific inc, Waltham, USA) by incubating membrane in reagents at 1:1 dilution for 3 min. The membrane was then imaged by the ChemiDoc™ MP imaging system (Bio-Rad Laboratories, Inc. CA, USA).

Band densities were analysed using Image lab™ (Bio-Rad Laboratories, Inc. CA, USA.). Stain-free or ponceau stained image bands were measured for total protein levels. Bands of

targeted proteins were measured and normalised to protein concentrations as a loading control. Detected phosphorylated proteins were then normalised to its total protein expression before being compared between experimental groups.

Table 2.2. Primary antibodies and dilutions used

Antibody	Dilution	Company
Phosphorylated Akt^{ser473}	1:1000	Thermo Fisher
Total Akt	1:1000	CellSignalling
Phosphorylated $AMPK^{thr172}$	1:1000	CellSignalling
Total AMPK	1:1000	CellSignalling
Phosphorylated $P38MAPK^{t180/Y182}$	1:1000	CellSignalling
Total P38MAPK	1:1000	CellSignalling
Phosphorylated $ERK1/2^{T202/Y204}$	1:2000	CellSignalling
Total ERK1/2	1:1000	CellSignalling
Puromycin	1:20000	Millipore

2.3.6. Automated spectrophotometry

2.3.6.1. Principle

The Rx Daytona, (Randox Laboratories Ltd, UK) is an automated random-access clinical chemistry analyser. The Daytona can be considered as an automated spectrophotometer fitted with modules that automatically pipette, mix and wash samples. Spectrophotometry is based on the Beer-Lambert Law which states that the amount of light absorbed is directly proportional to the concentration of the solute in the solution and the thickness of the solution. The spectrophotometer measures the relative intensity of light absorbed and reflected by the sample solution. At a certain wavelength each solute absorbs and reflects light therefore, relative to a known concentration, an unknown concentration can be quantified by measuring its relative absorbance and reflection.

2.3.6.2. Procedure

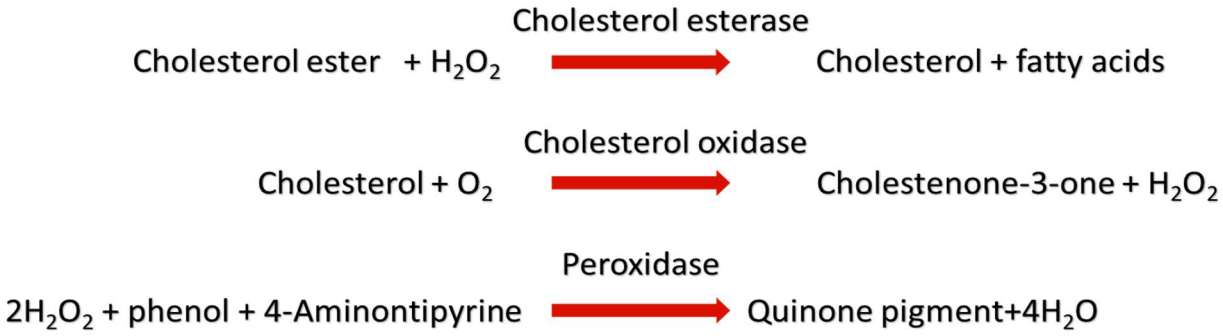
Whole blood from the antecubital vein was collected into tubes with EDTA and centrifuged at 3000 g for 15 min and 4 °C. The remaining plasma was quickly aliquoted into storage tubes and stored at -80 °C prior to Daytona analysis. Lyophilised controls and calibrators were reconstituted with H_2O. Prior to sample analysis, using the relevant calibrator kits a standard

curve for each desired analyte was created by the Daytona. After successful calibration, plasma samples and analyte controls were loaded into cuvettes which were placed into the autosampler unit in the Daytona. Plasma samples and controls were analysed for total cholesterol, triglycerides, non-esterified fatty acids, D3-hydroxybutyrate, low-density lipoprotein-cholesterol, small dense low-density lipoprotein-cholesterol, high-density lipoprotein-cholesterol, glucose, and Apolipoproteins A1 and B (Table 2.3) (Randox Laboratories, Antrim, Northern Ireland). If control values were within the desired reference ranges, the results were accepted.

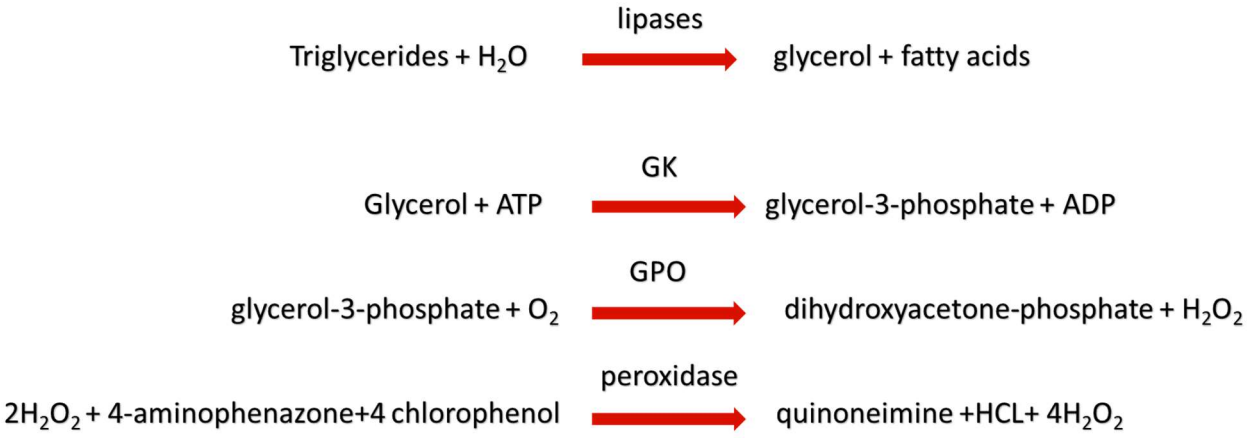
Table 2.3. Randox Daytona analytes precision.

Randox Daytona analytes	Intra assay precision (CV%)	Inter assay precision (CV%)	Principle of detection
Total cholesterol	1.67 – 3.84	1.00 – 1.39	Colorimetric (Equation 5)
Triglycerides	1.55 – 3.29	1.33 – 3.51	Colorimetric (Equation 6)
Low-density lipoprotein-cholesterol	1.14 – 2.99	1.58 – 2.50	Colorimetric (Equation 7)
Small dense low-density lipoprotein-cholesterol	<3	-	Colorimetric (Equation 8)
Glucose	1.13 – 1.76	1.52 – 1.86	Colorimetric (Equation 9)
Non-esterified fatty acids	4.74 – 4.81	4.32 – 4.51	Colorimetric (Equation 10)
D3-hydroxybutyrate	3.76 – 3.78	5.06 – 5.25	Colorimetric (Equation 11)
Apolipoprotein A1	2.67 – 4.10	3.18 – 3.22	Immunoturbidimetric immune assay*
Apolipoprotein B	1.74 – 3.08	2.04 – 3.76	Immunoturbidimetric immune assay*

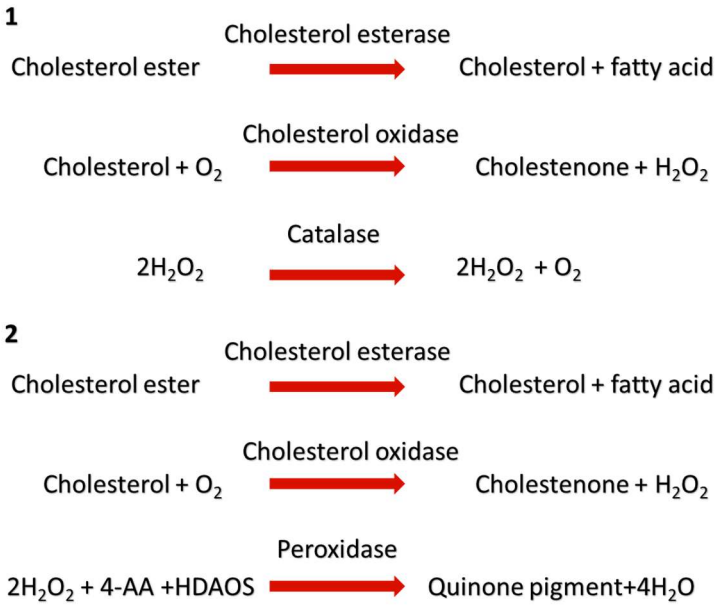
* Based on reaction of a sample containing apolipoprotein A1 or B and specific antiserum to form an insoluble complex which can be measured turbidimetrically at 340 nm.



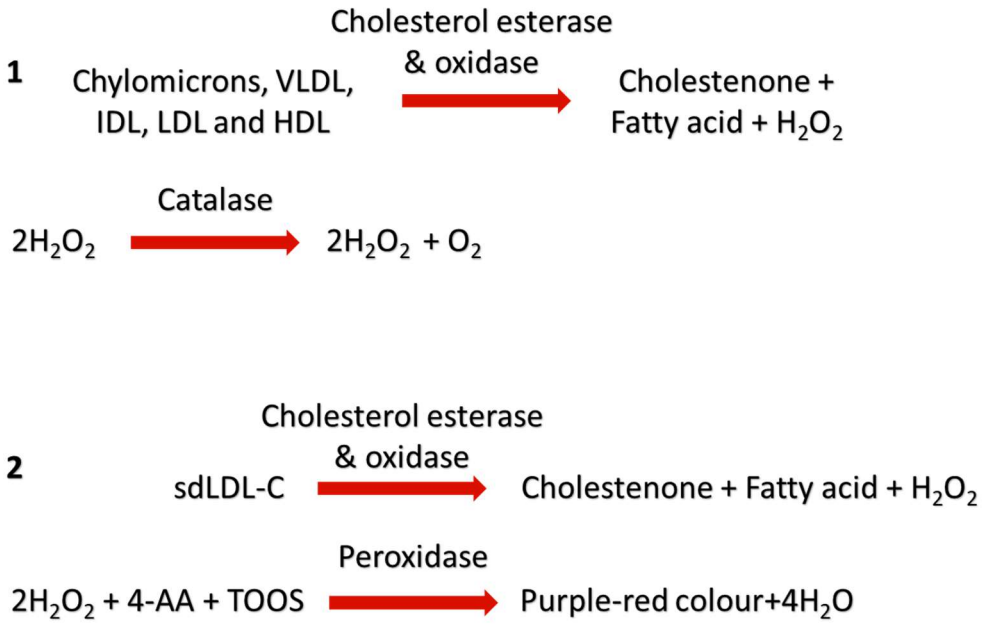
Equation 5. Colorimetric detection of total cholesterol. Enzymatic hydrolysis and oxidation of cholesterol, in the presence of phenol and peroxidase produces the indicator quinoneimine from hydrogen peroxide and 4-aminoantipyrine.



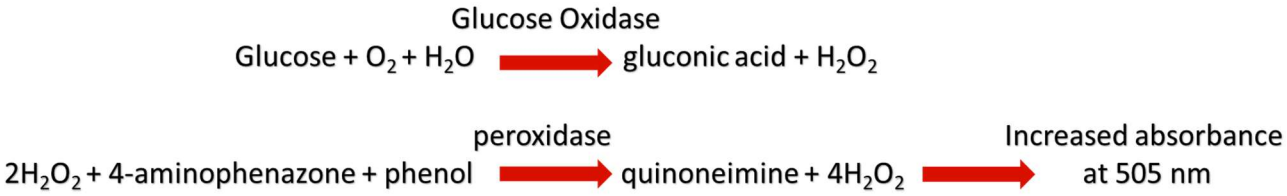
Equation 6. Colorimetric detection of triglycerides. Triglycerides are hydrolysed by lipases and the indicator quinoneimine is formed from hydrogen peroxide, 4-aminophenazone and 4 chlorophenol under the catalytic influence of peroxidase. *GK; Glycerol kinase, GPO; Glycerol phosphate.*



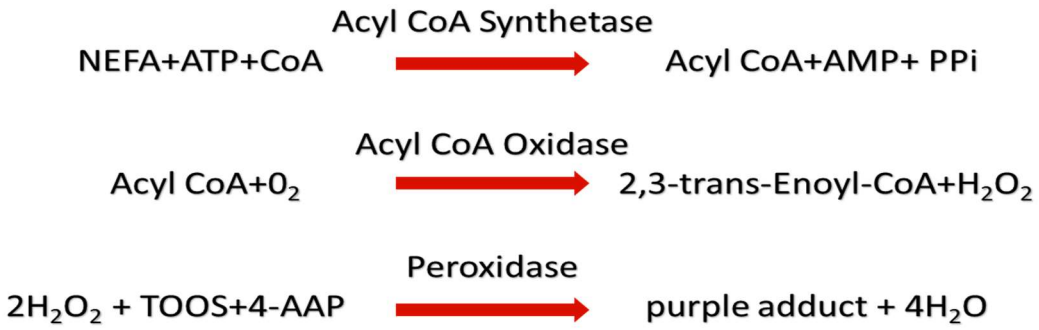
Equation 7. Colorimetric detection of low-density lipoprotein cholesterol. Step 1) Cholesterol esterase, oxidase and subsequently catalase eliminates chlyomicrons, VLDL-Cholesterol and high-density cholesterol. Step 2) low-density lipoprotein cholesterol is released by detergents and produces the indicator quinoneimine is formed under the catalytic influence of peroxidase. *4-AA; 4 – Aminoantipyrine, HDAOS; N-(2-hydroxy-3sulfopropyl)-3,5-dimethoxylanline, sodium salt.*



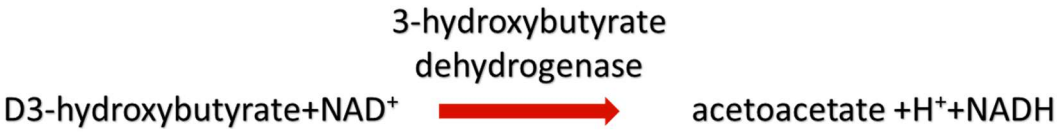
Equation 8. Colorimetric detection of small-dense low-density lipoprotein cholesterol. Step 1) Chlyomicrons, very low-density cholesterol, intermediate-density lipoprotein cholesterol, low-density lipoprotein cholesterol and high-density cholesterol are removed by surfactants and enzymes and degraded to water and oxygen by cholesterol esterase, oxidase and subsequently catalase. Step 2) small density low-density lipoprotein cholesterol is released by detergents and catalyse is inhibited by sodium azide resulting in hydrogen peroxides from chleosterol esterase and oxidase reactions and subsequently producing a purple-red clour under the catalytic influence of peroxidase. *4-AA; 4 – Aminoantipyrine, TOOS; N-Ethyl-N-(2-hydoxy-3sulfopropyl)-3-methylanline.*



Equation 9. Colorimetric detection of glucose. Oxidation of glucose by glucose oxidase produces hydrogen peroxide and in the presence of peroxidase with phenol and 4-aminophenazone, a red-violet quinoneimine dye is produced proportional to glucose concentration.



Equation 10. Colorimetric detection of non-esterified fatty acids. Acyl CoA synthetase catalyses the production of Acyl co A from non-esterified fatty acids to produce acyl CoA which is oxidized by aclyl coA oxidase to produce hydrogen peroxides subsequently producing a purple-adduct under the catalytic influence of peroxidase *4-AA; 4 – Aminoantipyrine, TOOS; N-Ethyl-N-(2-hydoxy-3sulfopropyl) m-toluidline*



Equation 11. Colorimetric detection of D3-hydoxybutyrate. D3-hydroxybutyrate is oxidised by 3-hydroxybutyrate dehydrogenase to produce acetoacetate and NADH by the reduction of NAD^+ and the change in absorbance is directly correlated with D3-hydoxybutyrate.

2.3.7. Sandwich chemiluminescent immunoassay

2.3.7.1. Principle

From a single sample, the Evidence Investigator™ Biochip Array technology (Randox Laboratories, Antrim, Northern Ireland) simultaneously detects multiple analytes. The Randox biochip contains an array of discrete test regions of immobilised antibodies specific to different biomarkers. Increased levels of targeted biomarkers in a sample will lead to increased binding of antibody labelled with HRP and thus an increase in the emitted chemiluminescent signal. Digital imaging technology detects the light signal produced from each test region on the biochip and is compared to that from a stored calibration curve. The concentration of the biomarker of interest in the sample is calculated from the calibration curve.

2.3.7.2. Procedure

Metabolic Syndrome Array I and II (Randox Laboratories, Antrim, Northern Ireland) uses a sandwich chemiluminescent immunoassay to detect markers of MetS. Metabolic Syndrome

Array I contains antibodies for Ferritin, Insulin, IL-6, Leptin, PAI-1, Resistin and TNFα simultaneously. Metabolic Syndrome Array II contains antibodies for Adiponectin, CRP and cystatin C (Table 2.4). Whole blood from the antecubital vein was collected into tubes with EDTA and centrifuged at 3000 g for 15 min and 4 °C. The remaining plasma was quickly aliquoted into storage tubes and stored at -80 °C prior to Evidence Investigator™ analysis.

Table 2.4. Metabolic syndrome array I & II assay precision.

MetS array I & II markers	Intra assay precision (CV%)	Inter assay precision (CV%)
Ferritin	5.7 - 8.2	8.2 – 10.0
Insulin	9.4 - 7.5	9.0 - 14.0
Interleukin-6	6.4 - 6.1	4.9 - 8.4
Leptin	4.6 - 7.7	6.0 - 8.7
Plasminogen activator inhibitor 1	10.5 - 10.9	13.1 - 14.3
Resistin	10.1 - 12.1	5.2 - 8.6
Tumour necrosis factor alpha	5.5 - 6.3	7.4 - 9.2
Adiponectin	8.2 - 9.6	9.7 - 12.4
Cystatin C	5.6 - 8.2	5.6 - 7.3
C-reactive protein	4.5 - 5.3	4.9 - 5.5

Lyophilised controls and calibrators were reconstituted with H_2O. Samples/calibrator/control were dispensed into each well after adding 200 µl assay dilutent (20 mM TBS, pH 7.2) (Figure 2.8). Biochips were incubated for 1 hour at 37 °C with mixing at 370 rpm. Following incubation, reagents were discarded, and biochips were washed six times using wash buffer (20 mM TBS, pH 7.4) diluted 1/31.25 in dH_2O and vigorously tapping the biochips for 15 to 20 seconds to remove fluid. After the final wash, wash solution was left in biochips for 2 min and dispensed onto lint free tissue to remove residual wash buffer. After washing, 300 µl conjugate (20 mM TBS, pH 7.5 containing HRP) was added to each well. Biochips were incubated for 1 hour at 37 °C with mixing at 370 rpm. Following incubation, wash steps were repeated six times. Following removal of wash buffer, 250 µl working signal reagent (Luminol-EV840 and Peroxide mixed at 1:1) was added to each well and incubated in darkness for 2 minutes. The biochips were placed into the Evidence Investigator™ and automatically imaged determined by the computer software. Results are processed automatically by the software and If standard curve and control values were within the desired reference ranges, the results were accepted.

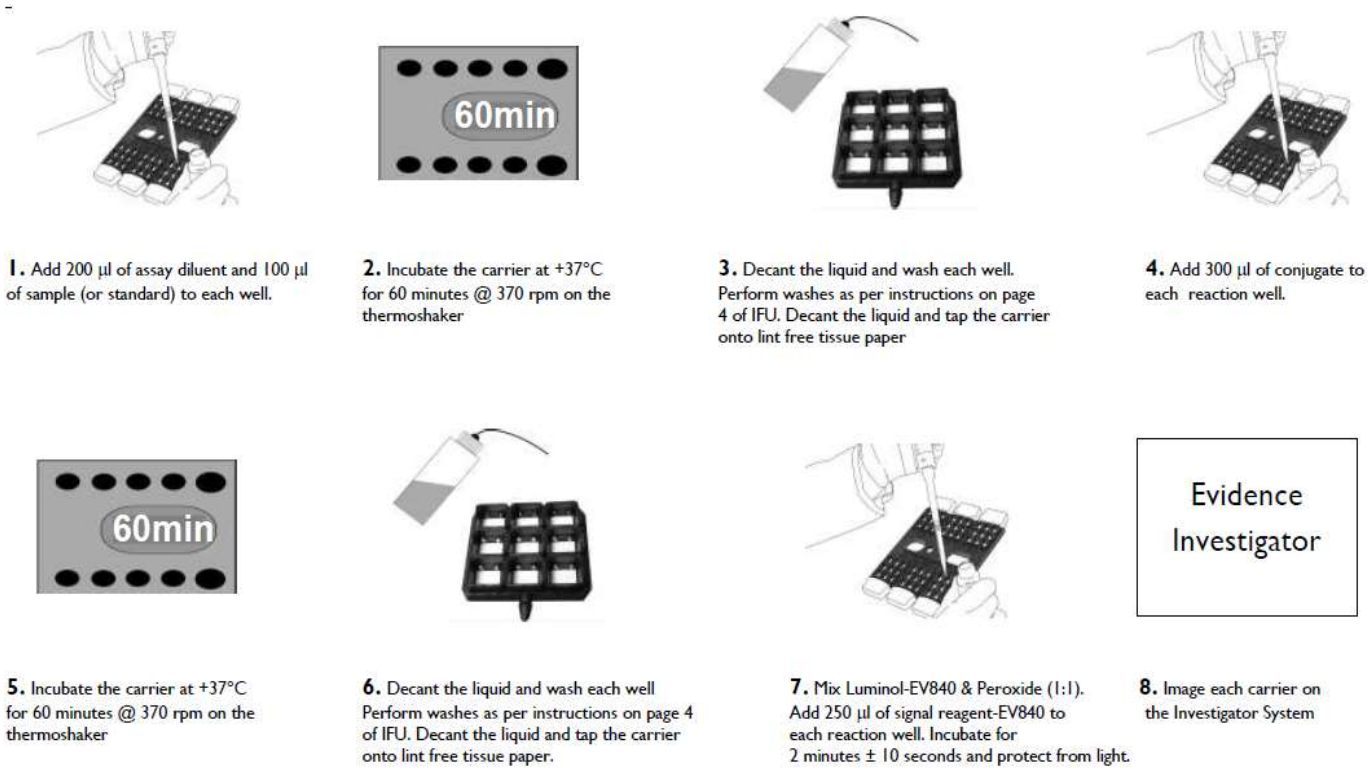


Figure 2.8. Evidence Investigator™ protocol (Randox Laboratories, Antrim, Northern Ireland).

2.3.8. Separation of LDL subclasses

2.3.8.1. Principle

Heterogeneity of LDL subclasses are associated with cardiometabolic risk, with large buoyant LDL being associated with positive metabolic health and smaller denser LDL associated with increased CVD risk (Ivanova *et al.*, 2017). Separation and analysis of LDL subclasses is therefore of clinical interest in determining cardiometabolic health. Methods of separating LDL subclasses include 3% PAGE, nuclear magnetic resonance and density gradient ultracentrifugation (Krauss and Blanche, 1992; Otvos *et al.*, 1992; Hoefner *et al.*, 2001). Separation of LDL by ultracentrifugation involves very high *g* forces (288 x 10^6 g·min) and was originally developed using high concentrations of salt however, these conditions can cause dissociation of lipids and apolipoproteins from the LDL surface (Davies, Graham and Griffin, 2003). Iodixanol, a self-generating gradient medium, is a non-ionic, iso-osmotic medium and requires relatively lower cumulative *g* force (648 x 10^5 g·min) thus making it more likely to maintain lipoprotein structural integrity and has been shown to be a validated and highly reproducible method of detecting LDL subclasses (Davies, Graham and Griffin, 2003).

2.3.8.2. Procedure

Separation of LDL subclasses was performed using the validated method of density gradient ultracentrifugation using iodixanol (Optiprep^{TM} Sigma Aldrich, Poole UK) (Davies, Graham and Griffin, 2003). Separation of lipoproteins involved using a two-step gradient of a lower layer of plasma (3 ml) at a concentration of 120 g/L in iodixanol in a 11.2 ml Beckman Optiseal centrifuge tubes and 7.9 ml of a clear Tris-buffered saline solution at a concentration of 90 g/L iodixanol of the upper layer. The upper layer was dispensed into the centrifuge tubes before 3 ml of the sample solution was carefully under-layered with a syringe and cannula. Samples were housed in a Beckman NVT65 near-vertical rotor and centrifuged at 341000$g_{(av)}$ and 16 °C for 3 h (at speed) in a Beckman Optima XL-100 ultracentrifuge, with acceleration program 5 and deceleration program 5. After centrifugation, centrifuge tubes were eluted into 0.5 ml gradient fractions, collected from the top using an Autodensiflow gradient fractionator (Labconco), coupled with a Gilson FC 204 automated fraction collector. Each fraction underwent refractometry to determine its density by the using the following formula: $p = na - b$, where a = 3.2984, b = 3.3967, n = refractive index, and p = density; based on the density, LDL subfractions were categorised as phenotype A (1.022-1.028 kg/L) and phenotype B (1.028-1.036 kg/L) (Davies, Graham and Griffin, 2003). Cholesterol was determined in each fraction using the Daytona Plus (Randox Laboratories Ltd, UK).

2.4. Statistical analysis

GraphPad Prism version 9 (California, USA) and SPSS version 25 (PASW, Chicago, IL) statistical software was used for statistical analysis. Mean ± standard deviations (which are appropriate in most circumstances) or non-parametric equivalents are reported in chapter 3 and 5 to demonstrate the variation of the mean of the populations tested. However, in chapter 4 and 6, mean ± standard error of the mean are reported to describe how representative the sample is of the population tested due to the large variation observed in the data. The alpha level for significance was set at $P < 0.05$. In most scientific settings, particularly biology, arbitrary cut-offs can cause misinterpretation of the data collected (Greenland *et al.*, 2016). Therefore, due to the exploratory nature of the studies included in this thesis, a trend in data was defined as $P = 0.05$ to < 0.1 to highlight possible areas for future research while recognising an increase in the likelihood of a type 1 error (10%) (Banerjee *et al.*, 2009; Wood *et al.*, 2014). Detailed statistical analysis and specific statistical tests carried out on data collected are presented in each relevant chapter.

Chapter 3: The Effect of Dietary Carbohydrate Manipulation on Cardiometabolic Health

3.1. Abstract

Introduction: Poor metabolic health is accompanied by the interrelated clustering of abnormalities such as obesity, hypertension, insulin resistance (IR) and dyslipidaemia referred to as cardiometabolic disease (CMD). Dietary fat intake was thought to be the main cause of CMD therefore triggering the development of many lower fat, high carbohydrate (HC) dietary recommendations; however, very low carbohydrate (<26 % of energy intake) high fat (VLC) diets have shown to at least perform as well as HC diets in improving CMD. Furthermore, few have focused on small dense low-density lipoprotein, apolipoprotein B and novel biomarkers such as fibroblast growth factor 21 and adiponectin in *ad libitum* conditions. Therefore, the aim of this study is to investigate the impact of an *ad libitum* 8-week VLC diet on markers of metabolic health compared with a HC diet in adults with a moderately elevated metabolic risk.

Methods: Males (*n*=9) and females (*n*=7) (19 – 64 years old, BMI of 18.5 – 29.9 kg/m^2) were randomly assigned to either a VLC (*n*=8) or HC (*n*=8) diet for 8 weeks. At 0, 4 and 8 weeks, anthropometrics, blood pressure, body composition (SECA mBCA 515), 4-day food diaries and fasting blood samples were collected. Total cholesterol, triglycerides, low-density lipoprotein-cholesterol (LDL-C), small dense low-density lipoprotein-cholesterol (sdLDL-C), high-density lipoprotein-cholesterol (HDL-C), glucose, apolipoproteins A1 and B and the ketone D-3 hydroxybutyrate were measure by Daytona (Randox). Ferritin, IL-6, insulin, Leptin, Plasminogen activator inhibitor-1 (PAI-1), Resistin, tumour necrosis factor- α (TNF-α), adiponectin, CRP and cystatin C were measured by Evidence investigator (Randox) FGF21 and apolipoprotein CIII was detected by ELISA analysis.

Results: The VLC group significantly (P < 0.001) decreased their carbohydrate intake and increased their fat intake at week 4 and 8. No change (P > 0.05) in dietary composition was reported in the HC group or in energy intake in either group. Both diets significantly (P < 0.05) improved triglycerides, insulin, HOMA IR levels and leptin:adiponectin levels. Compared with HC, the VLC diet showed significantly (P < 0.05) greater improvements in blood pressure, fat mass, leptin and sdLDL:LDL-C. The VLC diet also reported trends in improving sdLDL-C (P = 0.06), WC (P = 0.05) and FGF21 (P = 0.06) levels. The HC diet showed a tendency (P = 0.08) of increasing adiponectin levels.

Conclusion: These results indicate an 8-week *ad libitum* VLC diet can improve metabolic health at least as well as the current UK guidelines (HC diet) even with an increase in saturated fatty acids (SFA). Both diets improved markers of metabolic health indicating a reduction in the risk of developing metabolic diseases such as T2D and CVD. An *ad libitum* VLC diet may have greater benefits on markers of metabolic health due to a reduction in fat mass. Furthermore, the cellular mechanisms in which each diet exerts its potential benefits on metabolic health are yet to be investigated and will investigated in chapter 4.

3.2. Introduction

Poor metabolic health encompasses cardiovascular and metabolic disorders and is associated with the interrelated clustering of abnormalities such as high triglycerides, low high-density lipoprotein cholesterol (HDL-C), elevated blood glucose, hypertension and elevated waist circumference (WC) also known as metabolic syndrome (MetS) (Alberti *et al.*, 2009; Sperling *et al.*, 2015). Additionally, insulin resistance (IR), visceral adipose tissue (VAT) and small dense low-density lipoprotein cholesterol (sdLDL-C) also highly correlate with MetS (Sperling *et al.*, 2015). In the last few decades, this accumulation of abnormalities or cardiometabolic disease (CMD) has escalated, particularly in people who live with obesity (Kelli, Kassas and Lattouf, 2015) and ultimately increases the risk of developing type 2 diabetes (T2D) and cardiovascular disease (CVD) (Wilson *et al.*, 2005; Lassale *et al.*, 2018). CVD and T2D are among the top 10 diseases to cause mortality worldwide (World Health Organization, 2018b) intensifying the burden on the healthcare system. It is estimated that CVD costs the UK economy £7.4 billion annually and the global cost of CVD is estimated to reach USD 1044 billion by the year 2030, nearly a USD 200 billion rise from 2010 (Timmis *et al.*, 2020).

Obesity is commonly thought to be responsible for the rise in poor metabolic health due to its association with IR, hypertension, higher triglyceride levels and low physical activity (Strasser, 2013). However, more specifically VAT may be of greater importance in determining cardiometabolic risk as Shah *et al.*, (2014) demonstrated that VAT was associated with a greater risk of MetS independent of BMI. There are a variety of treatments to reduce risk of developing poor metabolic health, including inducing weight loss, quality of diet, increasing physical activity and pharmacological drugs (Kaur, 2014). Diet is a key modifiable risk factor in the development of MetS, T2D and CVD (Fabiani, Naldini and Chiavarini, 2019; Yusuf *et al.*, 2020). For example, a typical western diet has been implicated in the development of MetS and increased CVD risk (Rodríguez-Monforte et al., 2017; Drake et al., 2018).

The current UK guidelines suggest a diet high in carbohydrates (although low in sugar) and low to moderate in fat (particularly saturated fat) (PHE, 2015; 2016). These guidelines were adopted in 1983 to combat the rise in CVD in the UK (Walker, 1983). They were based on the Seven Countries study by Keys *et al.*, (1966) in which an association between CVD, serum cholesterol levels and energy intake provided by saturated fat (SFA) and total fat were observed. Furthermore, randomised control trials in the 1970s, showed reducing SFA reduced the risk of CVD induced mortality (Leren, 1970; Woodhill *et al.*, 1978; Hooper *et al.*, 2015) Conversely, low carbohydrate (<26 % of energy intake) high fat (LC) diets have shown to at

least perform as well as high carbohydrate (HC) diets in reducing body fat and improving metabolic health risk factors even with an increase in SFA intake (Bueno *et al.*, 2013; Mansoor *et al.*, 2016; Gardner *et al.*, 2018; Tay *et al.*, 2018). Individuals who suffer from T2D may also show greater benefits in metabolic health following a LC diet due to their inability to metabolise carbohydrates effectively; however, higher quality long-term trials are still needed (Schwingshackl and Hoffmann, 2014; Meng *et al.*, 2017). Furthermore, a LC diet has shown to have favourable effects on HDL-C, triglyceride and fasting glucose compared to a HC diet independent of weight loss (Hyde, Krauss and Volek, 2019).

Although a LC diet shows positive effects on metabolic health markers, one concern that has been commonly observed is the increase in low-density lipoprotein cholesterol (LDL-C) compared to HC diets; however, a recent meta-analysis showed after 12 months no significant differences are observed although dietary compliance maybe an issue (Gjuladin-Hellon *et al.*, 2019). Nonetheless, high LDL-C is generally considered a risk of CVD (Wadhera *et al.*, 2016) but the issue is more nuanced than previously thought. Circulating lipoprotein particles vary in their size, density, lipid and apolipoprotein composition and LDL can be separated into 4 groups: large and buoyant (lbLDL), intermediate size and density (idLDL), small and dense (sdLDL) and very small and dense (vsdLDL) (Ivanova *et al.*, 2017). High circulating particles of sdLDL and vsdLDL indicate a greater risk of CVD events compared to total LDL alone (Hoogeveen *et al.*, 2014; Ivanova *et al.*, 2017). In accordance with the association of the composition of LDL particles and CVD risk, 2 main phenotypes (A and B) have been described (Diffenderfer and Schaefer, 2014). Phenotype A is characterised by the prevalence of lbLDL and idLDL whereas, phenotype B is characterised by the prevalence of sdLDL and vsdLDL (Diffenderfer and Schaefer, 2014; Ivanova *et al.*, 2017). Phenotype B has been found to be associated with low HDL-C and high triglycerides and has also been observed in many metabolic diseases (Hoogeveen *et al.*, 2014; Fan *et al.*, 2019). Additionally, apolipoprotein B (ApoB), the primary apolipoprotein of LDL, and LDL particle number may be of greater importance than LDL size (Sniderman *et al.*, 2019). Higher circulating levels of ApoB indicate an increased risk of trapping ApoB within the arterial wall and depositing atherogenic cholesterol resulting in increased risk of a CVD event (Sniderman *et al.*, 2019). Due to this only measuring LDL-C as whole may not indicate an accurate change in metabolic health.

In addition to typical clinical markers, emerging novel biomarkers have also been revealed to be associated with metabolic health. These biomarkers include but are not limited to Fibroblast growth factor 21 (FGF21), adiponectin, C-reactive protein (CRP), leptin and plasminogen activated inhibitor-1 (PAI-1) (Mertens *et al.*, 2006; Soto González *et al.*, 2006; Esteghamati *et al.*, 2011; Ahl *et al.*, 2015; Lakhani *et al.*, 2018). For example, FGF21 has shown to be elevated in individuals with poor metabolic health, whereas adiponectin is reduced (Hung *et al.*, 2008;

Lakhani *et al.*, 2018). While weight loss strategies decrease FGF21 and leptin and increase adiponectin (Ma *et al.*, 2016; Gómez-Ambrosi *et al.*, 2017), research is limited in response to VLC diets in free living populations.

Therefore, the aim of this study is to investigate the impact of an 8-week VLC diet on markers of metabolic health compared with a HC diet (the current UK guidelines) in adults. Although 8 weeks is considered a short intervention, many of the novel markers of metabolic health have yet to be investigated in response to a VLC diet. A study of shorter duration would enhance recruitment and adherence to the VLC diet while also providing enough time to stimulate changes in blood markers.

The objectives are:

1. To determine if a VLC diet improves markers of MetS (HDL-C, triglycerides, IR and inflammation) compared to a HC diet,
2. investigate if a VLC diet improves emerging lipoprotein markers such as sdLDL-C and ApoB compared to a HC diet,
3. investigate if a VLC diet improves novel biomarkers of CVD risk compared a HC diet and
4. determine if a VLC diet improves body composition compared to a HC diet.

Hypothesis: a VLC compared to a HC diet will show greater improvements in the following four key areas:

1. markers of MetS (HDL-C, triglycerides, BP, WC, IR and inflammation
2. lipoprotein metabolism (sdLDL-C and ApoB),
3. novel biomarkers of CVD risk (FGF21, Leptin and adiponectin);
4. body composition (body fat %, VAT (l)).

3.3. Methods

3.3.1. Participants and study design

The study was an 8-week dietary intervention, parallel design, randomised control trial in Caucasian males (*n*=9) and females (*n*=7) aged 19 – 64 years with a BMI of 18.5 – 29.9 kg/m^2. During a fasted finger prick screening test (Cholestech LDX®, Alere Ltd, Stockport, UK) with the addition of blood pressure (BP) and waist measurements, participants cardiometabolic risk was calculated based on a points system modified from the RISCK (Reading, imperial, Surrey, Cambridge, and Kings) trial (Jebb *et al.*, 2010). A score between 4 and 11 indicated a small-elevated cardiometabolic risk (Table 3.1).

Table 3.1: Cardiometabolic risk scoring system.

Cardiometabolic measure	Points
Fasting glucose: > 5.5 mmol/L	3
Fasting triglycerides: > 1.3 mmol/L	1
Fasting HDL-C: < 1.0 mmol/L (males), < 1.3 mmol/L (females)	2
Waist circumference: > 94 cm (males), > 80 cm (females)	1
Waist circumference: > 102 cm (males), > 88 cm (females)	2
Systolic blood pressure: > 130 mmHg	1
Diastolic blood pressure: > 85 mmHg	1

Participants were excluded if they, were a smoker, vegan/vegetarian, take dietary supplements, had any known food allergies or intolerances, consume alcohol above the weekly UK recommendations, suffer from an eating disorder, suffer from current or previous renal impairment, have a history of cardiometabolic diseases, or take lipid, BP or blood glucose-lowering medication. All procedures were approved by the Liverpool John Moores University research ethics committee (REC number: 16/ELS/029) and all participants provided written informed consent. This study is registered with clinical trials (REF: NCT03257085).

3.3.2. Study protocol and Diet

After successful screening, participants were randomly assigned to either the UK Eatwell guide (HC) (*n*=8), (PHE 2016) or a VLC diet (*n*=8) for 8 weeks. Participants were given a personalised meal plan, recipes and a detailed guide to follow each diet by a registered nutritionist (Chapter 9, appendix 1 and 2). Participants in the HC group were required to eat a diet composed of 50% carbohydrates, 15% protein and at most, 35% fat per day. This calculated as consuming at least 333 g and 267 g of carbohydrates, 55 g and 45 g of protein and at most 97 g and 78 g of fat per average male and female energy requirements of 2500 kcal and 2000 kcal per day respectively (Committee on Medical Aspects of Food Policy (COMA), 1991). The diet also was required to be high in fibre and low in free sugars. Conversely, participants in the VLC group were required to eat a diet consisting of no more than 50 g of carbohydrates per day to induce ketosis (Westman *et al.*, 2007), increase the amount of fat consumed while consuming similar amounts of protein to the HC group. Participants were also given information on how to increase fibre intake while also keeping carbohydrates low (Chapter 9, appendix 1). Examples of foods consumed by the VLC participants included unlimited amounts of beef, poultry, fish, eggs, oils and heavy cream; moderate amounts of hard cheeses, low carbohydrate vegetables and salad dressings; and small amounts of nuts, nut butters and seeds. Participants restricted fruit and fruit juices, dairy products (with the exception of heavy cream and hard cheese), breads, grains, pasta, cereal, high carbohydrate vegetables, and desserts. Subjects were instructed to avoid all low carbohydrate breads and cereal products. To maintain healthy micronutrient levels participants in the VLC group were provided and instructed to consume a daily multivitamin (Centrum Women, Centrum, Pfizer Consumer Healthcare Ltd, UK). No specific instructions on caloric intake was given to participants in either group, as they were required to eat *ad libitum*. Participants were also contacted at least on a weekly basis by telephone or email to check if assistance was needed with recipes or meals to promote adherence.

Participants were required to attend LJMU laboratories in the morning having been fasted for 12 hours at 0, 4 and 8 weeks of the diet. On each testing day, anthropometrics, a venous blood sample, BP and body composition were collected. During the week prior to each testing day participant's physical activity was measured for 7 days by wearing an activity watch on their non-dominant arm (Actigraph GT9X, Florida, USA). On the 2nd and final testing day participants were required to complete a fibre intake questionnaire and the VLC group were also to report any adverse events associated with the diet (Chapter 9, Appendix 3).

3.3.3. Blood measurements

Whole blood was collected into tubes with EDTA for plasma and tubes with a silica separator for serum (plus 30 min rest at room temperature) and centrifuged at 3000 g for 15 min and 4°C, and quickly aliquoted into storage tubes and stored at -80 °C until further analysis.

3.3.4. Biochemical markers

The Evidence Investigator ^{TM} Biochip Array technology (Randox Laboratories Ltd, UK) that uses sandwich chemiluminescent immunoassays to simultaneously detect multiple analytes from a single sample (see section 2.3.7.) was used to determine the following plasma cytokines and hormones; ferritin, interleukin-6 (IL-6), insulin, Leptin, PAI-1, resistin, tumour necrosis factor-α (TNFα), adiponectin, CRP and cystatin C. The Daytona, (Randox Laboratories Ltd, UK) an automated random-access clinical chemistry analyser (see section 2.3.6 for further details) was used to detect total cholesterol, triglycerides, LDL-C, sdLDL-C, HDL-C, glucose, non-esterified fatty acids (NEFA) and apolipoproteins A1 and B and the ketone D-3 hydroxybutyrate. FGF21 and apolipoprotein CIII (ApoCIII) was detected by ELISA analysis. The homeostatic model of insulin resistance (HOMA IR) was calculated by (fasting plasma insulin x fasting plasma glucose)/22.5 (Wallace, Levy and Matthews, 2004).

3.3.5. Blood pressure and body composition

Blood pressure was monitored 3 times using a digital sphygmomanometer (Omron Healthcare Europe B.V.) with systolic and diastolic measures being recorded. Body composition was measured via bioelectrical impedance (SECA mBCA 515, Germany) and although participants were encouraged to drink a glass of water when arriving fasted, hydration was not controlled. Waist measurements were 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).

3.3.6. Food diaries

Overall compliance with the dietary recommendations over the course of the intervention was assessed via 4-day food diaries. All were completed on 3 occasions at 0, 4 and 8 weeks. Analysis of food diaries was completed with Dietplan 7 (Forestfield Software Ltd, UK).

3.3.7. Statistics

All normally distributed data are presented as mean ± SD whereas non-normally distributed data are presented as medians ± interquartile range (IQR). All data was explored for distribution using the Shapiro Wilks test. Normally distributed data underwent a 2 x 3 mixed ANOVA with 2 between factors (VLC *vs.* HC) and 3 within factors (Baseline *vs.* interim *vs.* endpoint) to investigate significant differences for main and interaction effect. If repeated measures data had a missing value, mixed effects analysis was used instead of ANOVA. Data with a significant interaction effect, the mean change from baseline and endpoint was calculated and if normally distributed, underwent ANCOVA analysis using 2 different models. Model one consisted of using the baseline measure and VAT as covariates as the starting point may have influenced the results. Model 2 consisted of using the △ change in calories and MVPA as these confounding factors may impact metabolic health. Non-normally distributed data underwent Mann Whitney U tests at each time point to investigate differences between groups, Friedman's test for differences within groups throughout the diets and Dunn's test for post-hoc analysis. Independent t-test was also carried out to investigate differences in baseline measurements. The alpha level for significance was set at $P < 0.05$, while a trend was defined as $P = 0.05$ to < 0.08, and GraphPad Prism (California, USA) and SPSS version 25 (PASW, Chicago, IL) statistical software was used for statistical analysis. R studio 1.1.463 was used to analyse the raw accelerometer data using GGIR (van Hees *et al.*, 2013).

3.4. Results

Out of the 58 people screened, 34 attended for clinical screening, 22 were excluded leaving 12 participants randomly assigned to either diet. Due to difficulties in recruitment and time constraints the screening criteria was relaxed. A further 7 participants were screened and were included in the study. Two participants withdrew before and another after baseline measurements due to time constraints. A further person withdrew from the study after the interim time point due to illness most likely from following the VLC diet, therefore 15 participants completed the intervention but all 16 were included in analysis. Exclusion of this participant had no significant effect on the results except for sdLDL-C which changed from a trend (P < 0.056) to being statistically significantly (P = 0.03) different between groups. Participants consisted of 9 males and 7 females with 8 in the VLC group (M/F: 4/4) and 8 in the HC group (M/F: 5/3). Baseline measurements are shown in Table 3.2. Although there was significant (P < 0.05) differences in body composition (body mass, body mass index (BMI), fat mass, fat free mass (FFM), skeletal muscle & WC) between groups, leptin was the only biochemical marker that was also significantly different at baseline (P < 0.01).

Table 3.2. Baseline differences between the VLC (n=8) and HC (n=8) groups.

	VLC	**HC**	
	Baseline		(P value)
Age	43.8 ± 10.4	44.6 ± 15.27	-
Height (cm)	176 ± 11.6	168 ± 11.02	-
Mass (kg)	89.53 ± 14.85	72.48 ± 14.72	0.04
BMI (kg/m^2)	28.75 ± 2.15	24.56 ± 3.83	0.02
FM (%)$^{\$}$	35.65 ± 7.43	25.5 ± 2.5	0.01
FFM (%)$^{\$}$	64.2 ± 7.4	74.5 ± 2.5	0.01
SkM (%)	32.05 ± 4.92	35.8 ± 4.2	0.07
SBP (mmHg)	130 ± 10	127 ± 12	0.67
DBP (mmHg)	83 ± 9	78 ± 8	0.37
VAT (l)$^{\$}$	3.21 ± 3.43	2.65 ± 1.35	0.33
WC (cm)$^{\$}$	101.2 ± 15.87	93.75 ± 22.34	0.05
TC (mmol/L)	5.98 ± 0.95	5.37 ± 1.18	0.28
TG (mmol/L)$^{\$}$	1.14 ± 1.51	1.24 ± 0.45	0.88
Glucose (mmol/L)$^{\$}$	5.47 ± 0.75	5.72 ± 0.59	0.52
HDL-C (mmol/L)	1.28 ± 0.24	1.36 ± 0.38	0.62
Non-HDL-C (mmol/L)	4.69 ± 1.06	4.01 ± 1.07	0.22
Calculated LDL-C (mmol/L)	4.08 ± 0.87	3.40 ± 1.03	0.17

LDL-C (mmol/L)	3.98 ± 0.87	3.50 ± 1.33	0.44
Adiponectin (ug/ml)$	2.11 ± 1.98	2.69 ± 3.27	0.44
Apo A1 (mg/dL)	158.71 ± 14.27	164.71 ± 30.25	0.64
Apo B1 (mg/dL)	107.25 ± 20.35	94.38 ± 20.79	0.23
Apo CIII (mg/dL)$	17.83 ± 9.81	19.8 ± 7.21	0.31
ApoB1:ApoA1 (mg/dL)	0.70 ± 0.15	0.58 ± 0.15	0.14
CRP (ng/ml)	1.57 ± 0.83	1.25 ± 0.50	0.36
Ferritin (ng/ml)	172.61 ± 168.17	155.38 ± 83.3	0.80
IL-6 (pg/ml)$	1.16 ± 1.14	1.11 ± 1.61	0.57
Insulin (pmol/L)	67.21 ± 27.64	60.26 ± 15.48	0.55
HOMA IR	2.9 ± 1.3	2.5 ± 0.6	0.48
NEFA (mmol/L)	0.79 ± 0.34	0.76 ± 0.25	0.86
PAI-1 (ng/ml)$	7.19 ± 10.04	5.14 ± 8.02	0.72
Resistin (ng/ml)$	2.76 ± 2.41	3.82 ± 2.57	> 0.99
sdLDL-C (mmol/L)	1.00 ± 0.50	0.77 ± 0.36	0.32
sdLDL:LDL-C (mmol/L)	0.26 ± 0.08	0.22 ± 0.09	0.45
TG:HDL (mmol/L)	1.12 ± 0.77	1.19 ± 1.08	0.89
TNFa (pg/ml)	6.41 ± 0.69	6.52 ± 1.81	0.87
D3-Hydroxybutarate	0.07 ± 0.02	0.08 ± 0.05	0.48
Cystatin C (pg/ml)	0.35 ± 0.11	0.41 ± 0.13	0.47
Leptin (ng/ml)$	3.69 ± 2.01	1.31 ± 0.71	0.007
Leptin/adiponectin (ng/µg)$	1.58 ± 2.63	0.37 ± 0.41	0.007
FGF21 (pg/ml)	147.9 ± 114.6	180.7 ± 138.5	0.61

Values are expressed as means ± SD. $ denotes values expressed as medians ± interquartile range (non-parametric data). Significance set at P < 0.05.
Apo, Apolipoprotein; BMI, Body mass index; CRP, C-Reactive protein;; DBP, Diastolic blood pressure; FERR, Ferritin; FGF21, Fibroblast growth factor 21; FM, Fat Mass; FFM, Fat-free mass; HDL-C, High density lipoprotein-Cholesterol; HOMA IR, *homeostatic model of insulin resistance; IL-6, interleukin-6; LDL-C, Low density lipoprotein-cholesterol; NEFA, non-esterified fatty acids; PAI-1, Plasminogen activated inhibitor-1; sdLDL-C, small dense Low density lipoprotein-cholesterol; SkM, Skeletal muscle mass; TC, total cholesterol; TG, Triglycerides; TNFα, Tumour necrosis factor-α; VAT, Visceral adipose tissue.*

3.4.1. Diet

Food diary analysis showed no significant ($P > 0.05$) differences in energy or macronutrient composition at baseline. As intended, the percentage of energy derived from fat and carbohydrate significantly ($P < 0.001$) increased from 34% to 61% and decreased from 42% to 10% respectively in the VLC group. Whereas in the HC group, fat percentage decreased slightly from 36% to 33% and carbohydrate percentage remained unchanged at 41% which was significantly different from the VLC group ($P < 0.001$) (Table 3.3). No significant

differences were observed in energy intake between groups or throughout the intervention ($P > 0.05$). The percentage of total sugar derived from energy also significantly ($P < 0.001$) decreased in the VLC group compared to the HC group. The increase in dietary fat in the VLC group also led to a significant ($P < 0.01$) increase in energy derived from saturated (13% to 24%), monounsaturated (8% to 17%) and polyunsaturated (3% to 9%) fatty acids compared to the HC group. There was also a significant ($P = 0.04$) decrease in fibre intake in the LC group compared to the HC group which remained unchanged. The percentage energy of protein significantly ($P < 0.001$) increased within the VLC group by 7% compared to the HC group which only increased by 2%. Plasma D-3 hydroxybutyrate, a marker of ketosis, significantly ($P = 0.035$) increased within the VLC group and was significantly ($P < 0.05$) different from the HC group at week 4 and 8 of the intervention (Table 3.4).

3.4.2. Body composition

Body composition largely improved in the VLC group but remained unchanged within the HC group over 8 weeks (Table 3.4). Body mass significantly ($P < 0.001$) decreased in the VLC group and led to a significant ($P < 0.001$) decrease in BMI compared to the HC group which remained unchanged throughout. This decrease in body mass was largely attributed to a significant ($P < 0.001$) 3 kg reduction in fat mass in the VLC group. A significant ($P < 0.01$) interaction in FFM was observed between groups, due to a small increase in FFM in the HC group and small decrease in the VLC group. However, FFM % significantly ($P = 0.02$) increased in the VLC group and remained unchanged in the HC group. At each time point FFM % was significantly ($P = 0.02$ higher in the HC group. Furthermore, skeletal muscle mass % did not significantly ($P > 0.05$) change with either diet and was only significantly ($P = 0.02$) different between groups at baseline. In the VLC group, WC showed a tendency ($P = 0.05$) of decreasing, with post-hoc analysis showing a significant ($P = 0.049$) decrease at endpoint vs baseline only. No significant ($P = 0.35$) change was observed in the HC group and no significant ($P > 0.05$) difference was observed between groups at any time point. Similarly, In the VLC group, VAT showed a tendency ($P = 0.05$) of decreasing, with post-hoc analysis showing a significant ($P = 0.049$) decrease at endpoint vs baseline only. No significant ($P = 0.54$) change was observed in the HC group and no significant ($P > 0.05$) difference was observed between groups at any time point.

Table 3.3. Changes in dietary composition with the VLC and HC diets.

	VLC			HC			(*P* value)	
Nutrients	**Week 0**	**Week 4**	**Week 8**	**Week 0**	**Week 4**	**Week 8**	**TIME**	**T X G**
Energy (kCal)	1974 ± 618	1970 ± 387	1728 ± 482	2263 ± 686	2165 ± 587	2304 ± 742	0.79	0.45
CHO (g)	218 ± 74	50 ± 18^{bb}	45 ± 20^{bb}	252 ± 189	249 ± 66	258 ± 113	<0.001	<0.001
CHO (%)	42 ± 9	10 ± 5^{bbb}	10 ± 4^{bb}	41 ± 8	43 ± 8	41 ± 6	<0.001	<0.001
Sugar (g)	70 ± 21	28 ± 11^{bbb}	26 ± 13^{bbb}	85 ± 45	95 ± 46	86 ± 49	<0.01	<0.001
Sugar (%)	15 ± 6	6 ± 2^{bb}	6 ± 2^{bb}	14 ± 4	17 ± 6	14 ± 4	<0.01	<0.001
Fibre (g)	22 ± 4	11 ± 4^{bbb}	16 ± 10	25 ± 12	25 ± 8	27 ± 10	0.03	0.04
PRO (g)	88 ± 37	120 ± 26	106 ± 33	95 ± 20	95 ± 13	106 ± 23	0.09	0.08
PRO (%)	17 ± 3	25 ± 5^{bb}	24 ± 4^{bb}	17 ± 3	18 ± 3	19 ± 3	<0.001	<0.001
Fat (g)	75 ± 25	132 ± 49^{b}	118 ± 39	89 ± 25	82 ± 30	85 ± 31	0.04	<0.01
Fat (%)	34 ± 4	58 ± 13^{bb}	61 ± 6^{bbb}	36 ± 7	33 ± 7	33 ± 7	<0.001	<0.001
SFA (g)	28 ± 9	56 ± 25	45 ± 18	28 ± 9	26 ± 11	28 ± 12	0.03	<0.01
SFA (%)	13 ± 3	24 ± 8^{b}	23 ± 4^{bb}	11 ± 3	10 ± 4	11 ± 3	<0.001	0.001
PUFA (g)	10 ± 4	20 ± 13	21 ± 10	12 ± 5	11 ± 4	10 ± 4	0.07	0.02
PUFA (%)	3 ± 1	7 ± 3^{b}	9 ± 5^{b}	4 ± 2	4 ± 2	4 ± 2	<0.01	<0.001
MUFA (g)	22 ± 13	41 ± 17	40 ± 15	24 ± 13	25 ± 13	21 ±9	0.03	0.02
MUFA (%)	8 ± 4	15 ± 8^{b}	17 ± 7^{bb}	8 ± 4	8 ± 5	7 ± 4	<0.01	<0.01

Values are expressed as means ± SD of n=8 VLC & n=8 HC. ^{b} *P < 0.05,* ^{bb} *P < 0.01,* ^{bbb} *P < 0.001, denotes significantly different to baseline.*

CHO, Carbohydrates; MUFA, monounsaturated fatty acids, PUFA, polyunsaturated fatty acids; PRO, protein; SFA, Saturated fatty acids

3.4.3. Clinical biochemical markers of metabolic health

Both diets exerted benefits in markers of metabolic health (Table 3.4). Systolic (SBP) and diastolic blood pressure (DBP) both significantly improved (P = 0.01) in the VLC group compared to the HC group which remained unchanged. SBP decreased by 7 mmHg and DBP by 8 mmHg in the VLC group over the 8 weeks. Both diets showed similar significant (P < 0.05) improvements in triglycerides, insulin and HOMA-IR. Fasting insulin decreased from 67.21 ± 27.64 pmol/L at baseline to 55.53 ± 12.77 pmol/L at interim and 41.13 ± 17.4 pmol/L at endpoint in the VLC group and 60.26 ± 15.48 pmol/L at baseline to 52.44 ± 8.42 pmol/L at interim and 44.02 ± 11.23 pmol/L at endpoint in the HC group (Figure 3.1). Fasting triglyceride levels decreased similarly in both groups from baseline (VLC; 1.33 ± 0.77, HC; 1.34 ± 0.68 mmol/L) to interim (VLC; 1.07 ± 0.4, HC; 1.15 ± 0.32 mmol/L) and endpoint (VLC; 1.11 ± 0.29, HC; 1.24 ± 0.56 mmol/L) (Figure 3.2). HOMA-IR decreased by 1.1 points and 0.6 points in the VLC and HC groups respectively. Similarly, in both groups total cholesterol significantly (P < 0.05) increased; in the VLC group by 0.37 mmol/L and 0.40 mmol/L in the HC group. LDL-C, glucose and HDL-C did not significantly (P > 0.05) change throughout the intervention; however, HDL-C did show a trend for improvement with the VLC diet (P = 0.09). While apolipoproteins A1 (apo A1), B (Apo B) and C-III (apo CIII) showed no change throughout the diet, apo CIII was significantly (P < 0.05) different between groups at week 4, with higher levels observed in the HC group. sdLDL-C showed a trend (P = 0.056) of decreasing in the VLC group (Baseline: 0.99 ± 0.5; Interim: 0.92 ± 0.34; Endpoint: 0.86 ± 0.3 mmol/L) compared to the HC group (Baseline: 0.77 ± 0.36; Interim: 0.85 ± 0.32; Endpoint: 0.84 ± 0.43 mmol/L) (Figure 3.3). This change in sdLDL-C led to a significant (P = 0.01) decrease in sdLDL/LDL-C ratio in the VLC group compared to the HC group which showed a slight increase. NEFA showed a trend of (P = 0.08) of increasing in both groups after 4 weeks and then decreasing at 8 weeks (Table 3.4).

3.4.4. Physical activity

There was no significant difference at baseline in total physical activity (VLC; 218 ± 44.1 min p/day vs HC; 216.15 ± 49 min p/day) or total moderate to vigorous intensity (MVPA) (VLC; 214 ± 44.5 min p/day vs HC; 205.9 ± 48 min p/day). There was also no significant (P > 0.05) difference in total physical activity over 8 weeks (VLC; 218 ± 44.1 to 270.81 ± 101.5 min p/day vs HC; 216.15 ± 49 to 222.7 ± 70.4 min p/day) between groups or MVPA (VLC; 214 ± 44.5 to 266.5 ± 102.5 min p/day vs HC; 205.9 ± 48 to 209.2 ± 71.7 min p/day).

3.4.5. Novel biochemical markers of metabolic health

FGF21 showed a trend (P = 0.05) in overall change throughout in the VLC diet, an initial decrease after 4 weeks (Median ± IQR: 148.16 ± 203.49 to 99.4 ± 92.43 pg/ml) and increased at 8 weeks (Median ± IQR: 167.38 ± 152.08 pg/ml), whereas no change was observed in the HC group (Median ± IQR: 138 ± 243.56 to 136.4 ± 233.41 to 201.3 ± 406.55 pg/ml, P = 0.94) (Figure 3.4). No significant (P > 0.05) difference was observed between groups at any time point. In contrast, adiponectin showed a trend (P = 0.08) of increasing in the HC group (Median ± IQR: 2.69 ± 3.27 to 3.33 ± 1.91 to 3.19 ± 3.33 µg/ml) but remained unchanged in the VLC group (Median ± IQR: 2.01 ± 2.09 to 2.12 ± 1.51 to 2.12 ± 1.85 µg/ml P = 0.96) (Figure 3.5). No significant (P > 0.05) difference was observed between groups at any time point although, there was a trend (P = 0.07) of adiponectin being higher at week 4 in the HC group. Leptin also significantly (P = 0.001) decreased within the VLC group from being significantly (P < 0.01) different to the HC group at baseline to similar levels (P = 0.40) at 8 weeks (Table 3.4). The changes observed in adiponectin and leptin led to a significant (P < 0.05) decrease in leptin/adiponectin ratio (LAR) in both groups (Table 3). Groups were significantly (P < 0.05) different at baseline and week 4. Ferritin showed a significant (P < 0.05) decrease with both diets (Table 3). No significant (P > 0.05) change in C-reactive protein (CRP) was observed following both diets; however, CRP was significantly (P <0.01) different between groups at 4 weeks only (Table 3). No significant (P > 0.05) change within or between groups was observed in cystatin C, IL-6, TNFα, resistin and PAI-1 (Table 3.4).

Table 3.4. Changes in anthropometric variables and biochemical markers of metabolic health following the VLC and HC diets for 8 weeks.

Measurement	VLC Week 0	VLC Week 4	VLC Week 8	HC Week 0	HC Week 4	HC Week 8	TIME	T X G
Mass (kg)	89.53 ± 14.85	86.51 ± 14.51^{bb}	86.39 ± 14.25	72.48 ± 14.72	72.01 ± 14.88	72.27 ± 14.7	<0.001	<0.001
BMI (kg/m^2)	28.75 ± 2.15	27.76 ± 1.98^{bb}	27.61 ± 1.26^{b}	24.56 ± 3.83	24.40 ± 3.98	24.50 ± 3.98	<0.001	<0.001
FM (kg)	32.08 ± 4.28	29.80 ± 3.6^{bb}	29.10 ± 3.41^{bb}	19.89 ± 7.46	19.48 ± 7.89	19.60 ± 8.22	0.01	<0.001
FFM (kg)	57.39 ± 11.62	56.71 ± 11.87	57.28 ± 11.95	52.59 ± 11.63	52.52 ± 11.74	52.67 ± 11.54	0.008	0.003
SBP (mmHg)	130 ± 10	120 ± 10^{b}	123 ± 9^{b}	127 ± 12	128 ± 12	129 ± 14	0.04	0.01
DBP (mmHg)	83 ± 9	77 ± 9	75 ± 9^{bb}	78 ± 8	79 ± 8	78 ± 9	0.01	0.01
TC (mmol/L)	5.98 ± 0.95	6.22 ±1.15	6.35 ± 0.64	5.37 ± 1.18	5.64 ± 1.07	5.73 ± 1.27	0.047	0.71
Non-HDL-C (mmol/L)	4.69 ± 1.06	4.91 ± 1.36	4.92 ± 0.85	4.01 ± 1.06	4.26 ± 0.88	4.27 ± 1.08	0.11	0.47
Calculated LDL-C (mmol/L)	4.08 ± 0.87	4.42 ± 1.22	4.41 ± 0.77	3.40 ± 1.03	3.73 ± 0.81	3.70 ± 1.00	0.02	0.76
sdLDL:LDL-C (mmol/L)	0.26 ± 0.08	0.21 ± 0.03	0.20 ± 0.03	0.22 ± 0.09	0.23 ± 0.06	0.23 ± 0.08	0.08	0.01
Apo A1 (mg/dL)	158.71 ± 14.27	166.50 ± 23.09	173.00 ± 29.42	164.71 ± 30.25	172.50 ± 29.44	174.00 ± 32.83	0.46	0.88
Apo B (mg/dL)	107.25 ± 20.35	111.38 ± 24.81	111.43 ± 19.93	94.38 ± 20.79	105.00 ± 20.13^{b}	99.00 ± 29.09	0.14	0.66

ApoB:ApoA1 (mg/dL)	0.70 ± 0.15	0.68 ± 0.17	0.66 ± 0.16	0.58 ± 0.15	0.62 ± 0.13	0.58 ± 0.19	0.21	0.76
Glucose (mmol/L)	5.69 ± 0.41	5.78 ± 0.47	5.68 ± 0.47	5.79 ± 0.35	5.60 ± 0.54	5.80 ± 0.53	0.84	0.16
HOMA IR	2.9 ± 1.3	2.4 ± 0.6	1.8 ± 0.8	2.5 ± 0.6	2.2 ± 0.5	1.9 ± 0.6	<0.01	0.54
Cystatin C (ug/ml)	0.36 ± 0.11	0.31 ± 0.06	0.36 ± 0.09	0.41 ± 0.13	0.43 ± 0.17	0.43 ± 0.15	0.62	0.20
Ferritin (ng/ml)	172.61 ± 168.17	162.49 ± 159.66	173.14 ± 162.87	155.38 ± 83.3	128.14 ± 72.66	141.38 ± 89.82	0.04	0.29
NEFA (mmol/L)	0.79 ± 0.34	0.96 ± 0.30	0.80 ± 0.24	0.76 ± 0.25	0.79 ± 0.22	0.62 ± 0.17	0.08	0.59
Non-parametric testing							**VLC (Time)**	**HC (Time)**
FM (%)	34.4 ± 9.0*	32.8 ± 7.5[b]*	30.6 ± 8.0[b]*	25.5 ± 2.5	24.9 ± 2.68	25.05 ± 3.98	0.02	0.53
FFM (%)	65.3 ± 9.0*	67.2 ± 7.5[b]*	66.7 ± 8.0[b]*	74.5 ± 2.5	75.05 ± 2.67	74.95 ± 3.97	0.02	0.53
SKM (%)	33.0 ± 5.6*	33.7 ± 6.2	33.3 ± 7.5	35.8 ± 4.2	36.25 ± 3.88	35.9 ± 4.37	0.26	0.77
WC (cm)	101 ± 15.8	99.1 ± 17.2	98.4 ± 16.9[b]	93.8 ± 22.4	93.1 ± 21.9	92 ± 22.1	0.05	0.35
VAT (l)	4.23 ± 3.09	3.83 ± 3.09[b]	3.19 ± 3.06	2.65 ± 1.98	2.46 ± 1.90	2.46 ± 1.84	0.05	0.54
LDL-C (mmol/L)	3.62 ± 1.26	4.7 ± 2.02	4.27 ± 1.59	3.31 ± 1.91	3.66 ± 0.71	3.65 ± 1.23	0.43	0.39
HDL-C (mmol/L)	1.26 ± 0.39	1.27 ± 0.47	1.28 ± 0.32	1.43 ± 0.72	1.33 ± 0.63	1.47 ± 0.77	0.09	0.24
Apo CIII (mg/dL)	17.83 ± 9.81	15.94 ± 27.02*	13.60 ± 5.04	19.80 ± 6.67	24.64 ± 22.35*	19.40 ± 14.47	0.12	0.19

TG:HDL (mmol/L)	0.76 ± 1.38	1.02 ± 0.68	0.97 ± 0.53	0.82 ± 0.65	0.87 ± 0.41	0.72 ± 0.92	0.19	0.72
CRP (ug/ml)	1.1 ± 1.62	1.69 ± 1.66**	1.24 ± 1.29	1.06 ± 0.79	0.88 ± 0.51	0.98 ± 0.86	0.31	0.24
IL-6 (pg/ml)	1.3 ± 1.37	1.23 ± 1.27	1.54 ± 0.38	1.11 ± 1.61	0.83 ± 0.92	1.04 ± 1.99	0.96	0.79
TNFα (pg/ml)	6.74 ± 1.28	5.83 ± 1.93	5.97 ± 1.74	6.50 ± 3.19	6.23 ± 3.21	6.15 ± 3.45	0.62	0.53
Leptin (ng/ml)	3.98 ± 1.86**	1.98 ± 1.84b**	1.20 ± 2.42bb	1.31 ± 0.71	0.95 ± 0.54	1.07 ± 0.86	<0.01	0.29
Leptin:adiponectin (ng:ug)	1.70 ± 2.63**	0.96 ± 1.4b**	0.70 ± 1.37bb	0.37 ± 0.41	0.23 ± 0.28	0.31 ± 0.7	<0.01	0.03
Resistin (ng/ml)	2.75 ± 1.15	2.76 ± 1.47	2.60 ± 1.51	3.82 ± 2.57	2.93 ± 1.78	2.97 ± 1.70	0.96	0.15
PAI-1 (pg/ml)	7.30 ± 9.61	6.53 ± 4.45	6.32 ± 5.20	5.14 ± 8.02	4.11 ± 5.37	4.47 ± 9.99	0.11	0.79
D-3-Hydroxybutyrate (mmol/L)	0.07 ± 0.01	0.31 ± 0.06b*	0.19 ± 0.27	0.09 ± 0.05	0.13 ± 0.11	0.08 ± 0.03	0.04	0.58

Values are expressed as means ± SD of n=8 VLC & n=8 HC. Non-parametric testing values are expressed as median ± IQR of n=7 VLC & n=8 HC. [b] *P < 0.05,* [bb] *P < 0.01, denotes significantly different to baseline, *P < 0.05 denotes significantly different between groups at that timepoint.*
Apo, Apolipoprotein; BMI, Body mass index; CRP, C-Reactive protein;; DBP, Diastolic blood pressure; FERR, Ferritin; FM, Fat Mass; FFM, Fat-free mass; HDL-C, High density lipoprotein-Cholesterol; HOMA IR, homeostatic model of insulin resistance; IL-6, interleukin-6; LDL-C, Low density lipoprotein-cholesterol; NEFA, non-esterified fatty acids; PAI-1, Plasminogen activated inhibitor-1; sdLDL-C, quantitative insulin sensitivity check index; small dense Low density lipoprotein-cholesterol; SBP, systolic blood pressure; *TC, total cholesterol; TG, Triglycerides; TNFα, Tumour necrosis factor-α; VAT, Visceral adipose tissue.*

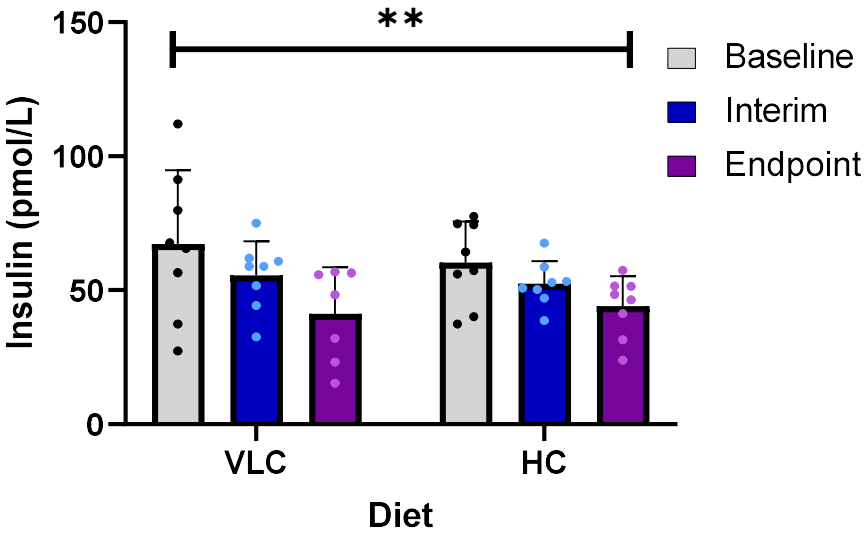


Figure 3.1. Effect of VLC and HC diet on insulin levels at 0, 4 and 8 weeks.

Values are expressed as means ± SD of n=8 VLC & n=8 HC, ** indicates significant ($P < 0.01$) difference effect of time.

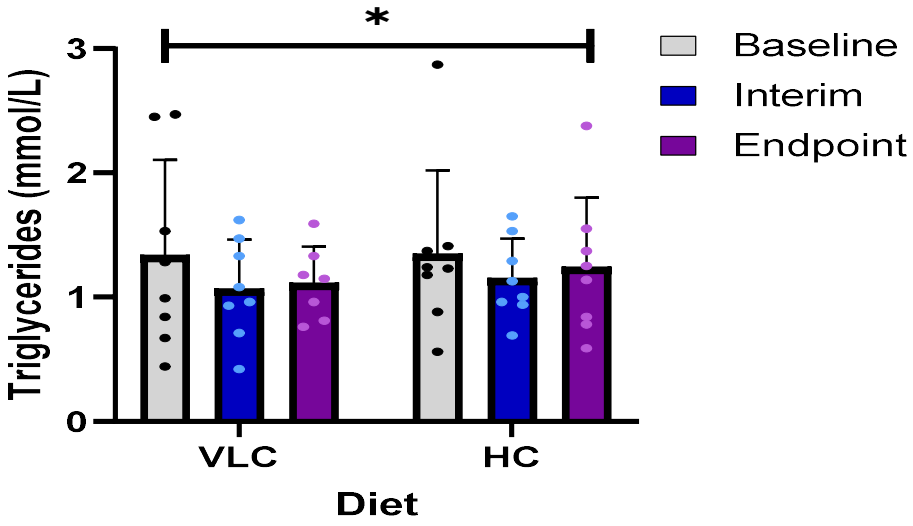


Figure 3.2. Effect of VLC and HC diet on triglyceride levels at 0, 4 and 8 weeks.

Values are expressed as means ± SD of n=8 LC & n=8 HC, * indicates significant ($P < 0.01$) difference effect of time

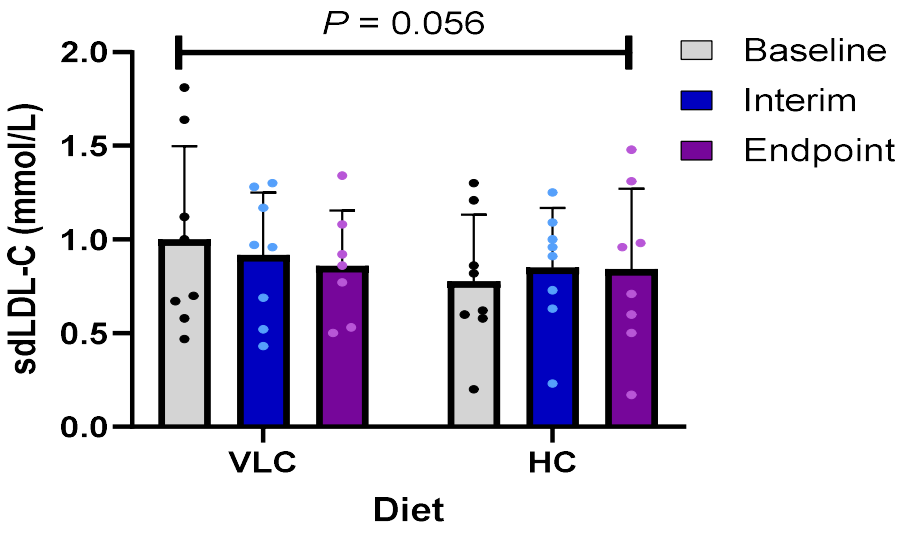


Figure 3.3. Effect of VLC and HC diet on sdLDL-C levels at 0, 4 and 8 weeks.

Values are expressed as means ± SD of n=8 LC & n=8 HC.

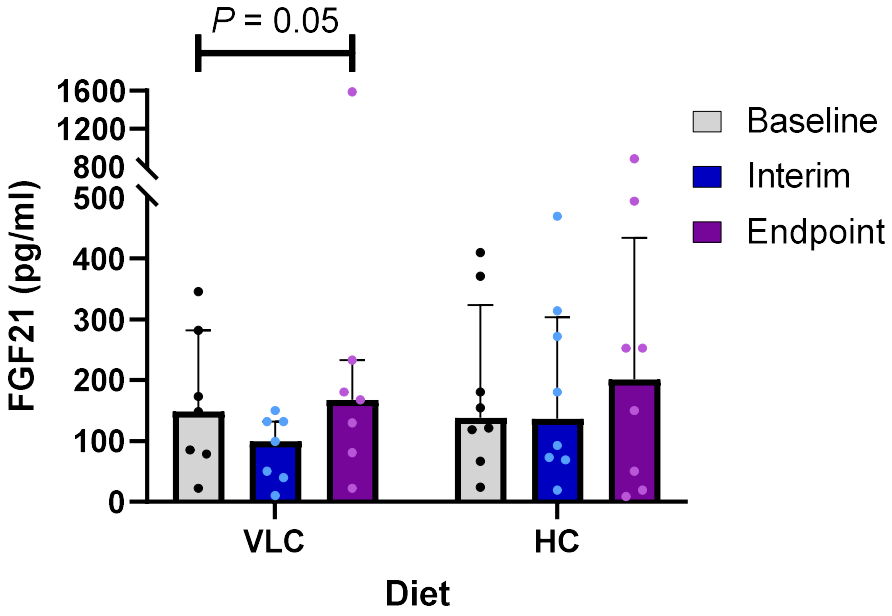


Figure 3.4. Effect of LC and HC diet on FGF21 levels at 0, 4 and 8 weeks.

Values are expressed as medians ± IQR of n=7 LC & n=8 HC.

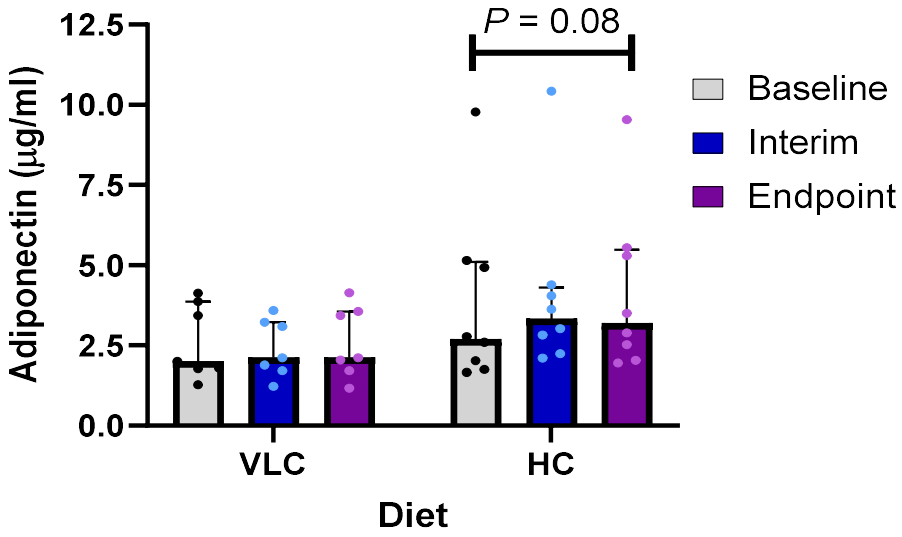


Figure 3.5. Effect of VLC and HC diet on adiponectin levels at 0, 4 and 8 weeks.
Values are expressed as medians ± IQR of n=7 VLC & n=8 HC.

3.4.6. Covariate analysis

Body mass, fat mass, FFM, SBP, DBP, sdLDL-C, sdLDL/LDL-C ratio, VAT and adiponectin showed a significant response to diets in the primary analysis and were able to undergo ANCOVA analysis using two models (Table 3.5 and 3.6). Model 1 appeared to diminish the effect the diet had on sdLDL-C (*P* = 0.08) that was shown in the primary analysis (*P* = 0.06) however, the remaining variables did not change with ANCOVA analysis. Model 2 reduced the effects the diets had on the change in VAT (*P* = 0.05) but enhanced the effect on sdLDL-C from *P* = 0.06 to *P* = 0.03. No change was observed with the remaining variables.

Table 3.5. Model 1.

Variable	**VLC** (Adjusted Δ 8 weeks, CI 95%)	**HC** (Adjusted Δ 8 weeks, CI 95%)	***P* value**
Body mass (kg)	-5.14, -6.64 to -3.64	-0.33, -1.71 to 1.05	0.001
FM (kg)	-3.94, -5.42 to -2.45	-0.01, -1.37 to 1.34	<0.01
FFM (kg)	-1.42, -2.23 to -0.6	-0.08, -0.83 to 0.68	0.03
SBP (mmHg)	-8.85, -14.69 to -3.02	1.95, -3.45 to 7.36	0.02
DBP (mmHg)	-9.18, -12.67 to -5.69	-0.34, -3.58 to 2.9	0.01
sdLDL-C (mmol/L)	-0.17, -0.31 to -0.2	0.02, -0.12 to 0.15	0.08
sdLDL/LDL-C (mmol/L)	-0.06, -0.09 to -0.02	-0.01, -0.04 to 0.03	0.04
Adiponectin (ug/ml)	-0.0, -0.21 to 0.22	0.31, 0.12 to 0.51	0.05

Adjusted change in anthropometric and biochemical markers of metabolic health due to baseline levels of the measure and baseline measure of VAT. *DBP, diastolic blood pressure; fat Mass; FFM, fat-free mass; small dense low-density lipoprotein-cholesterol; SBP, systolic blood pressure.*

Table 3.6. Model 2

Variable	**VLC** (Adjusted Δ 8 weeks, CI 95%)	**HC** (Adjusted Δ 8 weeks, CI 95%)	***P* value**
Body mass (kg)	-5.33, -6.77 to -3.88	0.83, -1.36 to 1.53	<0.001
FM (kg)	-3.74, -4.96 to -2.52	0.46, -1.18 to 1.27	0.001
FFM (kg)	-1.52, -2.38 to -0.71	0.34, -0.78 to 0.85	0.02
SBP (mmHg)	-7.75, -12.76 to -2.74	1.65, -3.35 to 6.66	0.02
DBP (mmHg)	-8.3, -11.62 to -4.99	-1.22, -4.53 to 2.1	0.01
VAT (l)	-0.42, -0.68 to -0.15	-0.19, -0.46 to 0.8	0.23
sdLDL-C (mmol/L)	-0.22, -0.4 to -0.04	0.8, -0.1 to 0.25	0.03
Adiponectin (ug/ml)	-0.03, -0.3 to 0.24	0.32, 0.5 to 0.59	0.08

Adjusted change in anthropometric and biochemical markers of metabolic health due to changes in energy intake and physical activity. *DBP, diastolic blood pressure; fat Mass; FFM, fat-free mass; small dense low-density lipoprotein-cholesterol; SBP, systolic blood pressure.*

3.5. Discussion

The main outcome of this investigation is that in accordance with hypothesis 1, an 8-week *ad libitum* VLC diet showed greater significant ($P < 0.05$) improvements in markers of CMD such as BP, WC and VAT but both diets showed similar significant ($P < 0.05$) improvements in triglycerides, insulin and HOMA IR. In agreement with hypothesis 2, a VLC diet showed favourable improvements in lipoprotein metabolism due to a trend ($P = 0.056$) of a reduction in sdLDL-C leading to a significant ($P = 0.01$) reduction in sdLDL:LDL-C compared to a small increase with the HC diet. In contrast with hypothesis 3, both diets showed similar favourable improvements in novel biomarkers of metabolic health such as ferritin and LAR; however, only the VLC diet showed a significant ($P < 0.001$) reduction in leptin and a trend ($P = 0.05$) of decreasing levels of FGF21 whereas only the HC diet showed a trend ($P = 0.08$) of increasing adiponectin. Somewhat, in agreement with hypothesis 4, a VLC diet showed significant reductions in fat mass ($P < 0.001$) but also a small significant decrease ($P < 0.01$) in FFM compared to the HC diet which remained unchanged.

Poor metabolic health is highly associated with the development of T2D and CVDs (Sperling *et al.*, 2015). Following a poor lifestyle including poor dietary intake can lead to the accumulation of metabolic abnormalities such as MetS and dysregulated lipid and glucose metabolism (Phillips *et al.*, 2013). Although the current UK dietary guidelines suggest a diet high in carbohydrates and restricted fat (< 35% of energy) intake particularly SFA (PHE, 2015; 2016) VLC diets have shown to at least perform as well as HC diets in reducing body fat and improving metabolic health risk factors even with an increase in SFA intake (Mansoor *et al.*, 2016; Tay *et al.*, 2018). Similarly, in the current study, participants who followed the VLC diet significantly increased their SFA intake by approximately 2-fold but still significantly improved markers of metabolic health. It should be noted though that the positive effects of fat loss may have outweighed the negative impact of increased SFA intake. However, in the absence of weight loss a VLC diet with increased SFA intake has been shown to be superior to a HC diet in reducing MetS markers (Hyde *et al.*, 2019).

Body composition significantly ($P < 0.05$) improved in the VLC group resulting in a trend towards a significant ($P = 0.05$) improvement in WC and VAT levels but remained unchanged in the HC group. Fat loss tends to be similar between diets when caloric intake is matched between diets (Tay *et al.*, 2008, 2018; Brinkworth *et al.*, 2009; Hall *et al.*, 2015; Veum *et al.*, 2017; Gardner *et al.*, 2018; Hyde *et al.*, 2019) thus, the increased weight loss observed in the VLC group may be due to negative energy balance. No restriction on energy intake was implemented and although participants were instructed to maintain their current physical

activity, these variables may have influenced weight loss. While no significant differences were observed over time or between groups, the VLC group decreased their calorie intake slightly at 8 weeks (-246 kCal per day) and increased their MVPA (51 min per day) throughout the study whereas the HC groups calorie intake and MVPA remained unchanged. The VLC group also significantly ($P < 0.01$) increased their protein intake which may have augmented energy expenditure due to an increase in thermogenesis (Westerterp-Plantenga, Lemmens and Westerterp, 2012). The increase in protein intake in addition to elevated ketones may result in elevated satiety levels leading to a reduction in calorie intake (Westerterp-Plantenga, Lemmens and Westerterp, 2012). Therefore, these small accumulative effects of reduced energy intake and increased energy expenditure may have affected weight loss and therefore metabolic health. To account for this, ANCOVA analysis was carried out on body composition, BP and biochemical variables with the change in energy intake and MVPA used as covariates. This resulted in the change in VAT not being significantly ($P = 0.23$) different between diets; however, the remaining markers of body composition and BP were significantly lower with the VLC diet compared with the HC diet. Interestingly, the change in sdLDL-C in response to the VLC diet was more effective compared with the HC diet, going from a trend ($P = 0.056$) in primary analysis to a significant ($P = 0.03$) reduction in ANCOVA analysis. Although, the largest decrease in fat mass was at 4 weeks and no change in energy intake was observed at this time. It could also be argued that a VLC diet increases energy expenditure as doubly labelled water studies have shown that compared to a HC diet, a LC diet can cause ~200-300 kcal/d increase in energy expenditure (Ebbeling *et al.*, 2018, 2020). However, others have argued that if energy expenditure increases with a LC diet its most likely around 50-100 kcal/d (Hall *et al.*, 2016, 2019). It is unlikely that energy expenditure increased as large as ~200 kcal/d in the current study as after week 4, it would be estimated that a further energy deficit of ~450 kcal/d would be observed with the VLC diet; however, no further weight loss was observed after 4 weeks. Also, the use of self-reporting food diaries may not be totally accurate as participants are likely to under report foods they don't eat regularly (Natarajan *et al.*, 2010).

Hypertension is a key risk factor in the development of CVDs and primary feature of MetS (Alberti *et al.*, 2009; Yusuf *et al.*, 2020). Similarly to other research (Tay *et al.*, 2008; Westman *et al.*, 2008; Brinkworth *et al.*, 2009; Veum *et al.*, 2017), the VLC diet significantly ($P < 0.05$) improved BP over the 8 week period. This is most likely due to the significant ($P < 0.05$) improvement in body composition in the VLC group rather than the dietary composition as when weight loss is induced with a HC calorie restricted diet similar effects on BP are observed (Tay *et al.*, 2008; Westman *et al.*, 2008; Brinkworth *et al.*, 2009; Veum *et al.*, 2017). Furthermore, in participants with obesity following a matched non-caloric restricted VLC and HC diet, no weight loss occurred in either group nor was there any improvement in blood

pressure (Hyde *et al.*, 2019). Nonetheless the improvement in BP in the VLC group has major clinical significance as a reduction of 10 mmHg in SBP can lead to an estimated decrease of 13% overall reduction in mortality and 20% reduction in CVD events (Ettehad *et al.*, 2016).

A common feature of following a LC diet is in an increase in LDL-C and HDL-C (Mansoor *et al.*, 2016; Gjuladin-Hellon *et al.*, 2019). In the current study, both diets significantly ($P < 0.05$) increased LDL-C and were above recommended healthy levels (>3.0 mmol/L) but had no impact on HDL-C which remained in the healthy range (>1.0 mmol/L) (Mach *et al.*, 2020). sdLDL-C showed a trend ($P = 0.056$) of decreasing with the VLC diet leading to a significant ($P = 0.01$) decrease in sdLDL:LDL-C compared to the HC diet. An increase in sdLDL-C indicates a greater risk of CVD events compared to total LDL alone (Hoogeveen *et al.*, 2014; Ivanova *et al.*, 2017). The current results suggest the HC groups LDL particle size has shifted towards a more atherogenic phenotype (an increase in sdLDL, pattern B) whereas the VLC group to a less atherogenic (a decrease in sdLDL pattern A) (Ivanova *et al.*, 2017). The weight loss achieved by the VLC group may have impacted the results; however, participants with dyslipidaemia following a calorie matched restricted (1500 kCal per day) VLC (n=20) or HC (n=20) diet for 12 weeks reported similar results in which the VLC and HC groups shifted towards a pattern A and B phenotype respectively (Volek *et al.*, 2009). This change in LDL phenotype occurred when both diets similarly reduced fat mass percentage (VLC, -2.4%; HC, -2.2%) (Volek *et al.*, 2009). However, a greater reduction in VAT was achieved in the current study and by Volek *et al.* (2009), which is more associated with CMD compared with subcutaneous adipose tissue and may play a role in improving LDL phenotype (Després *et al.*, 2008; Sperling *et al.*, 2015). As baseline levels of CMD markers may influence the response to each diet, ANCOVA analysis was undertaken to control for such confounders. When using baseline VAT and sdLDL-C as a covariate, the interaction ($P = 0.08$) between diets in sdLDL-C only slightly diminished compared with primary analysis ($P = 0.056$). Additionally, the sdLDL/LDL-C ratio did not change when baseline VAT and sdLDL/LDL-C ratio were used as covariates. While the present study adjusted for confounding variables such as VAT, a study by Hyde et al. (2019) investigated the effect of a VLC diet under isocaloric conditions. In a randomised crossover design, obese participants with MetS (n=16) followed either a VLC (6% CHO), HC (57% CHO) or moderate carbohydrate (MC) (32% CHO) diet for 4 weeks each (16 weeks total and 2 week washout periods between diets) tailored to their caloric requirements to maintain a stable body weight (Hyde *et al.*, 2019). Body composition including WC remained unchanged with each diet; however, only the VLC diet showed favourable improvements in shifting LDL pattern from phenotype B to phenotype A compared to the MC and HC diet, highlighting a lack of effect of fat mass on LDL phenotype (Hyde *et al.*, 2019). Furthermore, in a crossover design, healthy participants (n = 63),

supposedly following a LC diet (although 45% of energy was derived from carbohydrates) and then a HC diet for 4 weeks each and were measured at the end of each diet (Faghihnia *et al.*, 2010). Although there was no washout between diets and no baseline measure of LDL subclasses, all sdLDL-C subclasses (pattern B) were significantly ($P < 0.01$) lower and VLDL subclasses were significantly ($P < 0.01$) higher (pattern A) at the end of the LC diet compared with the HC diet (Faghihnia *et al.*, 2010).

High triglyceride levels (>1.7 mmol/L) are a component of MetS and indicates an increase in CVD risk (Alberti *et al.*, 2009; Mach *et al.*, 2020). Although not a component of MetS, high fasting insulin levels and IR is highly associated with MetS and indicates a risk of developing T2D and CVD (Sperling *et al.*, 2015). Interestingly, in the absence of weight loss or change in dietary composition, the HC diet showed similar improvements in triglyceride and insulin levels and subsequent indicators of IR; HOMA IR. Normally, triglyceride levels do not improve with a HC diet even with weight loss but improve with a LC diet (Tay *et al.*, 2008, 2018; Volek *et al.*, 2009; Hall *et al.*, 2015; Gardner *et al.*, 2018). Furthermore, a VLC diet has shown to also improve triglyceride levels independent of weight loss (Hyde *et al.*, 2019). However, this may be dependent on carbohydrate quality and type as a diet with lower glycaemic index (GI) and load has been reported to significantly lower triglyceride levels in participants with T2D after 12 weeks compared with a high glycaemic load and index diet (Jenkins *et al.*, 2012). In contrast, in a randomised cross-over design, participants with obesity who were provided with food to maintain body weight, did not show improvements in triglyceride levels following a low GI high carbohydrate (58% of energy) diet ($n = 153$) compared with a high GI high carbohydrate diet ($n = 150$) for 5 weeks (Sacks *et al.*, 2014). However, when following a lower carbohydrate (40% of energy) diet, low GI significantly improved triglyceride levels compared with high GI, while both diets showed significantly greater improvements compared with the high carbohydrate diets (Sacks *et al.*, 2014). This may explain the results of the current study as the HC participants carbohydrate intake was ~40%. It may also indicate that low GI foods may only exert beneficial effects on triglyceride levels with carbohydrate intakes < 50% of energy intake. In addition to reducing glycaemic load and index, it is also possible that participants following the HC diet increased their polyphenol intake without changing their macronutrient intake. An increase in polyphenol consumption has previously shown to reduce triglyceride levels in obese but metabolically healthy participants (Annuzzi *et al.*, 2014). The improvement of both diets on fasting insulin levels and indicators of insulin sensitivity and resistance agrees with previous research although, in HC diets that induce weight loss (Tay *et al.*, 2008, 2018; Volek *et al.*, 2009; Hall *et al.*, 2015; Veum *et al.*, 2017; Gardner *et al.*, 2018). Within IR tissue, normal insulin levels are insufficient to reduce blood glucose levels resulting in an increase in insulin secretion by the pancreatic β-cells (Gastaldelli, 2011). HOMA IR has

shown to be a good tool in estimating IR in specific populations based on fasting insulin and glucose levels (Wallace, Levy and Matthews, 2004). In the body, skeletal muscle accounts for the largest uptake of glucose in response to insulin stimulation and poor lifestyle habits can increase circulating fatty acids, inflammatory cytokines and reactive oxygen species which can inhibit this mechanism (Samuel and Shulman, 2016). Therefore, both a healthy VLC and HC diet can reduce inhibitory molecules to improve insulin sensitivity and restore normal insulin levels.

Adiponectin is a hormone that is synthesized in adipose tissue and unlike most adipokines, adiponectin is anti-inflammatory, insulin sensitizing (Cheng *et al.*, 2014) and expressed at lower levels in the metabolically unhealthy (Balsan *et al.*, 2015). Conversely, leptin is another adipokine that is involved in body weight regulation and is expressed at higher levels in individuals with MetS and this increase is most likely due to impaired leptin signalling induced by increased inflammation (Sáinz *et al.*, 2015) . Adiponectin showed a trend (P = 0.08) of increasing in response to the HC diet only. Typically, weight loss has shown to increase adiponectin levels whilst also improving insulin sensitivity (Bruun *et al.*, 2003; Cheng *et al.*, 2014; Ma *et al.*, 2016). Therefore, it is unusual no change was observed in the VLC group. However, Ma et al. (2016) showed a significant change after 2 years of a hypocaloric high fat diet but not after 6 months in participants with obesity; an 8-week diet may be too short to elicit an increase in adiponectin. As adipokines have an important role in the pathophysiological function in metabolism, it has been shown that the LAR is a reliable and useful marker in predicting markers of metabolic health (Frühbeck *et al.*, 2017, 2019). The LAR has shown to be highly correlated with body fat, WC, insulin, HOMA IR, triglycerides, HDL-C levels and T2D (Frühbeck *et al.*, 2017, 2019). However, the LAR was found not to predict the future incidence of coronary heart disease in healthy middle aged men and women indicating it may primarily be an indicator of CMD (Karakas *et al.*, 2010). The present study showed a significant (P < 0.05) improvement in the LAR in both groups thus suggesting an improvement in adipocyte function and metabolic health. This indication was also reinforced by the similar significant improvements (P < 0.05) in insulin and HOMA-IR with both diets. Although adiponectin and leptin are regularly measured in LC diet intervention studies, there is currently a lack of use of the LAR, indicating a novel observation in the current study. However, in T2D patients following either a low fat diet (n = 67), Mediterranean diet supplemented with olive oil (n = 74) or mixed nuts (n = 50) for 1 year all showed similar improvements in LAR with reductions in WC but no change in HOMA IR (Lasa *et al.*, 2014). The lack of change in HOMA IR is most likely due to the T2D patients being used by Lasa *et al.* (2014). In obese participants (n = 10) following a very low caroie diet, LAR signifcantly improved along with HOMA IR, triglycerides and body compostion (Oberhauser *et al.*, 2012). These results, in addition to the data from the

current study, indicate that a diet that improves fasting triglyceride levels will reduce insulin resistance and improve adiocyte function as shown by improvements in HOMA IR and LAR.

FGF21 is also a hormone that regulates metabolic energy homeostasis and is primarily expressed by the liver but is also expressed by muscle and other tissues (Fisher and Maratos-Flier, 2016). FGF21 has been shown to be involved in glucose and lipid regulation and animal studies have shown FGF21 to increase in response to starvation and ketogenesis (Kim and Lee, 2014; Fazeli *et al.*, 2015). Pharmacological administration of FGF21 has shown to improve the metabolism of obese and diabetic animals (Xu *et al.*, 2009; Kharitonenkov and Larsen, 2011). Conversely, in humans with obesity or metabolic disease FGF21 is increased compared to healthy people indicating potential FGF21 resistance and a ketogenic diet does not increase its levels (Domouzoglou *et al.*, 2015; Gómez-Ambrosi *et al.*, 2017; Lakhani *et al.*, 2018). In the current study FGF21 showed a tendency of decreasing ($P = 0.05$) in response to a VLC diet but rebounded by endpoint of diet but no change was observed in the HC. The decrease in FGF21 levels in response to a ketogenic diet have been reported by Christodoulides *et al.*, (2009) in which 7 subjects (5 diabetic) reduced their FGF21 levels (394 ± 193 to 201 ± 134, $P < 0.05$) after 4 months. In the current study, the increase in FGF21 experienced by the participants between 4-8 weeks may be attributed to a lack of adherence. Although the food diaries suggest adherence was maintained, measurement of D-3 hydroxybutyrate, an indicator of ketosis, was highest at 4 weeks (0.31 ± 0.06 mmol/l) and decreased by 8 weeks (0.19 ± 0.27 mmol/l). Yet, these increased ketone levels would only be considered small as they are below the recommended ketosis threshold of 0.5 mM and therefore unlikely to have a significant metabolic effect (Feinman *et al.*, 2015). Christodoulides *et al.*, 2009 reported higher levels of ketones (0.5 ± 0.5 mmol/l) throughout which may have maintained the reduction in FGF21; however, as this study was only a pre-post design with no control or comparative group it is difficult to ascertain what the potential mechanisms of reduced FGF21 levels. Participants with obesity undergoing a calorie restricted HC diet also showed a decrease in FGF21 (Gómez-Ambrosi *et al.*, 2017). The participants reduced body fat, insulin, HOMA IR and triglycerides. Similar results are observed in the current study in the VLC group. The HC also showed significant decreases in insulin, HOMA and triglycerides but not body fat or FGF21. This suggests weight loss alone rather than diet composition contributes to reduced FGF21 levels. Large sample sizes are required when investigating the role of FGF21 in the regulation of CMD due to a larger variation in concentrations between participants.

3.6. Limitations

Although sufficiently powered (94%) to detect significant changes in sdLDL/LDL-C, small sample size (n = 16), may not be fully representative of the population. Furthermore, coupled with the small samples size, females were mixed in age and the stage of their menstrual cycle was not accounted for resulting in a more heterogenous population. However, a smaller sample size allowed for greater management of participants to increase adherence to dietary protocols which was successful as shown by dietary analysis. Although all participants were given sufficient support throughout and food diary analysis showed both groups to be close to recommended dietary intakes. It is possible that adherence may have decreased as the study progressed ketone levels decreased at week 8 compared with week 4, indicating a higher carbohydrate intake. The use of self-reporting food diaries has limitations as participants are likely to under report foods they don't eat regularly (Natarajan *et al.*, 2010).

As directed, the HC group did not change their dietary intake throughout the intervention however, exhibited changes in biochemical markers therefore, the current study does not contain a control group thus, conclusions may be limited to comparative results between interventions only and there is evidence of change over time rather than the interventions (Barnett, van der Pols and Dobson, 2005; Bland and Altman, 2011). Furthermore, although all participants were instructed to maintain normal physical activity levels, the VLC group markedly increased their MVPA by approximately 52 mins per day which may have influenced results. The unintentional observed changes in physical activity in the VLC group and metabolic health markers in the HC group demonstrate a potential Hawthorne effect in the current study which can have implications for the generalisability of the results (Sedgwick and Greenwood, 2015). Bioelectrical impedance is dependent on body water and VLC diets are associated with a decrease in total body water primarily due to a reduction in glycogen stores (Malik and Hu, 2007) thus this may have impacted weight loss and FFM levels. However, if the weight loss observed was primarily due to water loss greater decreases in FFM would be observed. Considering this, the fat loss achieved by the VLC group is a lot higher than what would be expected from dietary analysis. Confounding factors described in the discussion and above such as the small increases in MVPA and limitations with body composition methods and underreporting of food diaries may have hidden the true cause of fat loss rather being attributed to the VLC diet alone.

Participants were randomly assigned to either group, yet unfortunately, this has led to a possible imbalance in baseline measures as the VLC group was significantly ($P < 0.05$) heavier due to increased fat mass. This may explain some of the greater improvements within

the VLC diet as they may have had higher levels of CMD at baseline. Due to this ANCOVA analysis was carried out on significant variables using the baseline measure and baseline levels of visceral adipose tissue (VAT) as covariates. ANCOVA analysis reported only slightly diminished effects of the VLC diet on sdLDL-C (P = 0.08) that was shown in the primary analysis (P = 0.06). In future, stratified randomisation by sex, fat mass and age could be used to consider for covariates that may influence results at baseline (Altman and Bland, 1999). Nonetheless, all participants appeared to be metabolically similar at baseline. This study is also short in duration limiting extrapolation of findings to long term health; however, the objective of this study was to investigate changes in metabolic health and associated mechanisms rather than long-term compliance and these changes were evident.

3.7. Conclusion

In conclusion, an 8-week *ad libitum* VLC diet can improve metabolic health at least as well as the current UK guidelines (HC diet) even with an increase in SFA. Both diets similarly improved triglycerides, insulin, LARand HOMA-IR levels; however, only the VLC diet showed improvements in body composition (fat mass and VAT) which may have led to significantly greater improvements in LDL composition and BP compared to the HC diet. These improvements ultimately reduce the risk of developing metabolic diseases such as T2D and CVD. These results may also lead to a change in future guidelines on how to lead a healthy diet. Additionally, emerging biomarkers of metabolic health such as FGF21 and adiponectin showed differential responses to each diet as FGF21 showed a tendency of decreasing with the VLC diet, but adiponectin showed a tendency of increasing with the HC diet. Yet, higher-powered longitudinal studies are needed to reveal the impact of diet on such insulin sensitising hormones and subsequent metabolic health. Furthermore, the cellular mechanisms in which each diet exerts its potential benefits on metabolic health are yet to be investigated and are to be examined in chapter 4.

Chapter 4: The impact of human serum on insulin signalling in C2C12 skeletal muscle cells: Effect of differing carbohydrate diets.

4.1. Abstract

Introduction: The cellular environment of skeletal muscle is key in regulating insulin signalling and subsequent glucose uptake. Diet has a profound effect on metabolic health, muscle insulin sensitivity and on associated biomarkers (fibroblast growth factor 21; FGF21). The impact of diet on the cellular environment and subsequent *in vitro* insulin signalling mechanisms are yet to be investigated. The objective was to investigate the effect of human serum from participants following a low carbohydrate (LC) vs. high carbohydrate (HC) diet on insulin signalling and cellular stress using skeletal muscle cells as a model. As both diets improved metabolic health, it was hypothesised, both diets would improve the endocrine environment resulting in improved insulin signalling, glucose uptake and reduced cellular stress.

Methods: Human serum was collected from participants, who followed LC or a HC diet, at 0, 4 and 8 weeks. C2C12 myoblasts and myotubes were pre-conditioned in conventional DMEM with horse serum (DM), LC or HC serum for 3 hours before being measured for insulin signalling and glucose uptake. In the absence or presence of 100 nM insulin +/- fibroblast growth factor 21 (FGF21; 1500 pg/ml), Akt^{ser473} phosphorylation (p-Akt^{ser473}) was determined after 20 min. Insulin induced glucose uptake was measured after 30 mins using 2-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino)-2-Deoxyglucose. AMP-activated protein kinase phosphorylation (p-AMPK^{thr172}) was also measured at 0-, 15- and 30-mins post serum incubation in myotubes.

Results: Both diets resulted in significantly ($P < 0.05$) improved markers of metabolic health. In myoblasts, both diets showed a tendency ($P = 0.07$) of increasing p-Akt^{ser473} but had no effect on the insulin stimulated fold-change in p-Akt^{ser473}. FGF21 enhanced insulin stimulation in DM but had no effect on LC and HC serum and insulin stimulated glucose uptake did not significantly change with either diet in myoblasts. In myotubes, both diets showed a significant ($P < 0.01$) decrease in p-Akt^{ser473} but had no effect on the insulin stimulated fold-change. Similar to myoblasts, FGF21 enhanced insulin stimulation in DM but had no effect on LC and HC serum in myotubes. Insulin stimulated glucose uptake also did not significantly change with either diet. In myotubes, both diets resulted in a significant ($P < 0.05$) decrease in p-AMPK^{thr172} and significantly correlated ($R = 0.62$, $P < 0.01$) with insulin induced p-Akt^{ser473}. As AMPK activity can regulate p-Akt, insulin stimulated fold-change in p-Akt^{ser473} and glucose uptake were relativised to p-AMPK^{thr172} in myotubes. Both diets showed a tendency ($P = 0.07$) of increasing p-Akt^{ser473} and significantly ($P < 0.01$) increased glucose uptake in myotubes.

Conclusion: Serum derived from humans following a LC or HC diet can improve the *in vitro* skeletal muscle response to insulin stimulation and energy stress signalling. When relativised to p-AMPK^{thr172}, both the LC and HC diets that improved metabolic health *in vivo* improved insulin stimulated p-Akt^{ser473} and glucose uptake in a similar fashion, *in vitro*.

4.2. Introduction

The C2C12 murine cell line is an established model for studying skeletal muscle *in vitro*, particularly investigating insulin signalling (Turner *et al.*, 2018; Wong, Al-Salami and Dass, 2020). However, cellular models rarely replicate the physiological environment seen *in vivo* in human skeletal muscle. Cell culture methods have been developed over many years to enable questions, not feasible *in vivo* and to reduce experimental costs; however, they potentially under-estimate what actually occurs in muscle adaptation (Cantor *et al.*, 2017). An alternative method of using human derived serum in combination with traditional cell culture protocol may replicate a more physiologically relevant cellular environment (Carson *et al.*, 2018; Cogan *et al.*, 2019) and warrants further investigation.

Under euglycaemic-hyperinsulinaemic conditions skeletal muscle accounts for approximately 80% of total glucose uptake and is mediated by the anabolic hormone insulin thus highlighting its importance in regulating whole-body glucose homeostasis (Defronzo *et al.*, 1981; DeFronzo and Tripathy, 2009).

Insulin resistance (IR) is a precursor in the development of type 2 diabetes (T2D) and is associated with a reduction in skeletal muscle insulin-induced glucose uptake resulting in hyperglycaemia and hyperinsulinaemia (Martin *et al.*, 1992; Petersen and Shulman, 2002). IR is a major contributor to the pathogenesis of the metabolic syndrome (MetS), which is also highly associated with obesity (particularly visceral adipose tissue), dyslipidaemia, hypertension, inflammation and cardiovascular disease risk (CVD). (Després and Lemieux, 2006; Nguyen *et al.*, 2008; Meshkani and Adeli, 2009; Klöting and Blüher, 2014; Sperling *et al.*, 2015; Grundy, 2016). Leading an unhealthy lifestyle, such as sedentary behaviour and poor nutrition resulting in obesity, is a significant contributor in the development of disrupted metabolism (Pattyn *et al.*, 2013; Rodríguez-Monforte *et al.*, 2017; Stein, Ferrari and Scolari, 2019).

A major regulator of insulin induced glucose uptake is the serine/threonine kinase Akt (Whiteman, Cho and Birnbaum, 2002). There are 3 isoforms of Akt, Akt1, 2 & 3, with Akt2 being primarily expressed in metabolic tissues and playing a key role in insulin induced glucose uptake (Garofalo *et al.*, 2003; Gonzalez and McGraw, 2009). Within insulin sensitive tissues, insulin binds to the insulin receptor causing autophosphorylation of tyrosine and trans-activation of the insulin receptor substrate-1 (IRS-1) (Boucher *et al.*, 2014). The activated IRS-1 allows the recruitment of the Type IA phosphatidylinositol 3' kinase (PI3K). PI3K catalyses the formation of PI(4,5)-bisphosphate (PIP_2) to PI(3,4,5)-trisphosphate (PIP_3) thus recruiting 3' phosphoinositide-dependent kinase-1 (PDK-1) (Meshkani and Adeli, 2009; Samuel and

Shulman, 2016). PDK-1 phosphorylates Akt and the atypical protein kinase C (PKC) (Alessi *et al.*, 1997; Boucher *et al.*, 2014). Activated Akt phosphorylates 160-kDa substrate of Akt (AS160) which stimulates glucose transporter 4 (GLUT4) translocation to the plasma membrane allowing glucose uptake (Karlsson *et al.*, 2005; Sakamoto and Holman, 2008). Furthermore, conversion of PIP_2 to PIP_3 by insulin and growth factors results in mammalian target of rapamycin complex 2 (mTORC2) activation which also causes phosphorylation and activation of Akt promoting cell survival, proliferation and glucose uptake (Saxton and Sabatini, 2017; Liu and Sabatini, 2020). AMP-activated protein kinase (AMPK) is another regulator of skeletal muscle glucose uptake that is independent of insulin (Hardie, Ross and Hawley, 2012). During times of cellular stress, such as low energy availability triggered by muscle contractions, activation of AMPK occurs to inhibit anabolic processes and increase catabolic processes to restore cellular ATP concentrations (Hardie, Ross and Hawley, 2012). AMPK, similarly to Akt2, also phosphorylates AS160 to increase glucose uptake (Cartee, 2015). Activation of AMPK can also cause phosphorylation and activation of mTORC2 thereby increasing Akt phosphorylation leading to enhanced insulin stimulated Akt activation (Kazyken *et al.*, 2019). Within IR tissue under basal conditions, AMPK activity is upregulated (Al-bayati, Brown and Walker, 2019), while the insulin dependent PI3K/Akt signalling cascade is diminished. Poor lifestyle habits can increase circulating fatty acids, inflammatory cytokines and reactive oxygen species (ROS) which can inhibit tyrosine activation of IRS-1 by insulin (Shulman, 2000; Meshkani and Adeli, 2009; Tanti and Jager, 2009; Samuel and Shulman, 2016). Due to this, therapeutic interventions targeting an improvement in metabolic health to reduce obesity, hyperlipidaemia and inflammation may improve insulin sensitivity and reduce the risk of developing MetS, T2D and CVD. Additionally, novel hormones may also regulate insulin sensitivity such as fibroblast growth factor 21 (FGF21) (Tezze, Romanello and Sandri, 2019). For example, FGF21 administration improves insulin stimulated glucose uptake in primary human skeletal muscle cells yet, a lack of an effect has been observed on Akt signalling (Mashili *et al.*, 2011; Jeon *et al.*, 2016). Although conversely, FGF21 positively correlates with the MetS and obesity therefore, it may play a role in the transition from insulin sensitive to IR or in the maintenance of metabolic health (Lakhani *et al.*, 2018; Tezze, Romanello and Sandri, 2019).

A typical western diet has been implicated in the development of IR, dyslipidaemia and inflammation leading to MetS (Rodríguez-Monforte *et al.*, 2017; Drake *et al.*, 2018). The current governing body dietary guidelines advocate eating a diet high in carbohydrates (>50%) although low in sugar, and low to moderate in fat (<35%), particularly low in saturated fat (European Commission, no date; Public Health England, 2016; World Health Organization, 2018a; U.S. Department of Health and human services, 2020). However, a diet high in

carbohydrates (HC) may not be optimal for individuals who are IR or live with T2D. Very low carbohydrate diets (LC) (<50 g per/day) have been reported to improve markers of MetS including IR and may be better for IR individuals (Schwingshackl and Hoffmann, 2014; Feinman *et al.*, 2015; Meng *et al.*, 2017). Although both diets may improve markers of MetS, the mechanisms in which they improve insulin sensitivity are not fully understood.

HC and LC diets exert differential metabolic effects on whole body homeostasis (Hall *et al.*, 2016); however, the specific interaction of their microenvironment on insulin signalling in skeletal muscle has yet to be investigated. Therefore, the aim of this study was to investigate the effect of serum from participants who followed an 8-week LC or HC diet and demonstrated improved metabolic health (both diets significantly ($P < 0.05$) improved VAT levels, fasting triglyceride and insulin levels, and HOMA-IR (McCullough *et al.*, 2020) (in study 1, chapter 3)), on insulin signalling and glucose uptake in C2C12 cells. The objectives were to:

1. Determine if insulin signalling changes in response to serum from participants following LC or HC diets,
2. Investigate if potential insulin sensitising FGF21 can mediate an increase in insulin signalling in different serum conditions,
3. Study if serum incubation has an effect on insulin induced glucose uptake and if a change in diet enhances/decreases glucose uptake, and
4. Determine the impact of serum on cellular energy stress using AMPK as a marker.

The objectives were underpinned by standard cell culture conditions (e.g. conventional differentiation medium) to confirm establishment the model.

It is hypothesised that: 1; as both diets improved metabolic health (study 1, chapter 3, (McCullough *et al.*, 2020)), insulin signalling in serum conditioned from LC and HC participants would cause an improvement in insulin signalling. 2; that the conditioned serum would change from a high stress environment to a lower stressful environment in response to diet, 3; both diets would improve glucose uptake at subsequent timepoints, and 4; that FGF21 would enhance insulin signalling.

4.3. Methods

4.3.1. Participants

Serum derived from participants following either a LC (n= 8) or HC diet (n= 8) for 8 weeks was used for this study. Within-group serum was pooled and heat inactivated at 56 C° for 30 mins. For details of methods and results see study 1, chapter 3. Briefly, participants were requested to attend LJMU laboratories for blood sampling in the morning, having been fasted for 12 hours, at 0, 4 and 8 weeks of the diet.

4.3.2. Cell culture

C2C12 cells were purchased from ATCC and passages 8-12 were used. For detailed cell culture see general methods section 2.2. Briefly, cells were grown in T75 flasks containing growth media (GM) (DMEM, 10% FBS, 10% NBCS, 1% PS, 2mM L-G) in a humidified atmosphere of 5% CO_2 at 37 °C. Upon reaching 80% confluence, cells were split into 6 well plates at a density of 4 x10^5 cells/ml and cultured for 48 hrs in a humidified atmosphere of 5% CO_2 at 37 °C until 80% confluency. Cells were incubated in differentiating media (DM) (DMEM, 2% HS, 1% PS, 2mM L-G) to induce differentiation to myotubes. Myotubes formed over 96-144 hours with the media being topped up with 1 ml of DM every 48 hours in a humidified atmosphere of 5% CO_2 at 37 °C.

4.3.3. Insulin stimulation

To investigate insulin signalling in myoblasts, after attainment of 80% confluence in 6 well plates, cells were washed twice with phosphate buffered saline (PBS) and incubated for 3 hours at 37 °C and 5% CO_2 in DM or LCDM (DMEM, 2% HI LC serum, 1% PS, 2mM L-G) or HCDM (DMEM, 2% HI HC serum, 1% PS, 2mM L-G). To investigate insulin signalling in myotubes, fully formed myotubes were washed twice with PBS and incubated for 3 hours in DM, LCDM or HCDM. Cells were spiked with 100 nM insulin and incubated at 37 °C and 5% CO_2 for 20 mins. To investigate impact of FGF21 on insulin signalling, 1.5 ng/ml FGF21 was co-incubated with serum for 3 hours prior to insulin stimulation. This dose was selected as it was the highest physiologically relevant dose observed in participants from study 1 and this incubation time was selected as it showed to elicit a greater response in p-Akt compared to 1hr in myoblast.

Following insulin stimulation, myoblasts were washed twice with ice cold PBS, trypsinised and centrifuged at 800 g at 4 °C for 5 mins. Cells were fixed with 2% paraformaldehyde and

incubated for 30 mins at room temperature. Cells were permeabilised with ice-cold 100% methanol and stored at -20 °C before processing for flow cytometry. Myotubes were lysed and scraped with RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.25% deoxycholic acid, 1% NP-40, 1 mM EDTA.) before processing for western blot. Myotubes could not be subjected to flow cytometric analyses, hence the need to adopt Western blotting. For further details of see section 2.3.5.

4.3.4. Assessment of serum on myotube metabolism

To assess the impact of serum on myotube metabolism, myotubes were washed twice with PBS and incubated for 3 hours in serum free media (1% PS, 2mM L-G) at 37 °C and 5% CO_2. After incubation in serum free media, myotubes were washed twice with PBS and incubated in DM, LCDM and HCDM at 37 °C and 5% CO_2. At 0, 15 and 30 mins, myotubes were lysed and scraped with 1x RIPA buffer before processing for western blot (section 2.3.5).

4.3.5. Glucose uptake

For full details on glucose uptake assay see section 2.3.1. Briefly, in 96 well plates cells were washed twice with PBS and incubated in serum free, low glucose, 1% BSA DMEM for 24 hours, prior to being washed twice with PBS and transferred DM, LCDM and HCDM containing 0% glucose and 100 µM 2-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino)-2-Deoxyglucose, (2NBDG). Myoblasts and myotubes were incubated at 37 °C and 5% CO_2 in the absence or presence of 100 nm of insulin for 30 mins. After incubation cells were placed on ice and washed 3 times with ice cold PBS. Cell fluorescence was detected (λex = 460 to 490 nm, λem =530 to 550 nm) using a microplate reader (Clariostar, BMG LABTECH, Ortenberg, Germany). Protein concentration was determined by BCA™ (see methods section 2.3.3) assay and glucose uptake was relativised to protein content and expressed as fold change of basal glucose uptake.

4.3.6. Flow cytometry

To detect phosphoproteins in myoblasts, flow cytometry (BD Accuri C6, BD Biosciences, Wokingham, UK) was performed. For detailed description see section 2.3.4. Briefly, cells were washed with flow buffer (PBS + 0.5% horse serum) and centrifuged at 600 g at 4 °C for 5 minutes. Cells were resuspended at 1 x 10^6 cells/ml in flow buffer. The anti-human/mouse phospho-AKT (S473; APC; 675/25 nm; 0.5 µg) antibody (Thermo Fisher Scientific inc, Waltham, USA) was added to samples and incubated at RT in the dark for 60 minutes. Cells were washed 2 times by centrifuging at 600 g at 4 °C for 5 minutes and resuspending in flow buffer. Data from 1000 events were recorded by flow cytometry.

4.3.7. SDS-Page and immunoblotting

Total protein and phosphoproteins in myotubes were detected by Western blot, (see section 2.3.5 for further details). Protein concentrations of samples were determined by BCA™ assay (section 2.3.3) and samples were subsequently resuspended in 5x Laemmli buffer at 1 mg/ml. 40 µg of sample was loaded and electrophoresed on 10% SDS-polyacrylamide gels. Semi-dry transfer of proteins to a nitrocellulose membrane using BioRad transfer pack was carried out. Following blocking for 1-hour in 5% non-fat dried milk, membranes were incubated overnight with rabbit anti-phosphorylated or total; Akt^{ser473} and $AMPK^{thr172}$, both at a dilution of 1:1000 (Cell Signalling Technology, London, UK). After overnight incubation, the membrane was washed 3 times in TBS-Tween at 0.1% and incubated for 1 hour in HRP-conjugated goat anti-rabbit antibodies at dilution of 1:1000 (Thermo Fisher Scientific inc, Waltham, USA). Proteins were visualised by enhanced chemiluminescence (Thermo Fisher Scientific inc, Waltham, USA) and quantified by densitometry (ChemiDoc^{TM} MP imaging system, Bio-Rad Laboratories, Inc. CA, USA)

4.3.8. Statistical analysis

GraphPad Prism version 9 (California, USA) statistical software was used for statistical analyses. Data were assessed and confirmed for normal distribution by Shapiro Wilks test. To compare the effects of DM on insulin signalling, data were subjected to a paired t-test to detect significant difference between basal and stimulated conditions. To investigate the effects of DM and FGF21 on insulin signalling, data underwent a 2 x 2 mixed ANOVA with 2 between factors (DM *vs.* DM/FGF) and 2 within factors (0 nM *vs.* 100 nM of insulin). To investigate the effect of DM on insulin induced glucose uptake and on cellular stress, data underwent a one-way ANOVA. To determine the effect of diet on insulin signalling, data underwent a 3-way mixed ANOVA with 2 between factors (LCDM *vs.* HCDM), 3 within (time) factors (baseline *vs.* interim *vs.* endpoint) and 2 within (dose) factors (0 nM *vs.* 100 nM of insulin) at each timepoint. To investigate the effect of diet and FGF21 on insulin signalling, data underwent a 3-way mixed ANOVA with 2 between (group) factors (LCDM *vs.* HCDM), 2 between (condition) factors (DM *vs.* DM/FGF) and 2 within (dose) factors (0 nM *vs.* 100 nM of insulin). To compare the effects of diet on cellular stress at 0 mins, 15 mins and 30 mins, data underwent a 3-way mixed ANOVA with 2 between factors (LCDM *vs.* HCDM), 2 within (time) factors (0 min *vs.* 15 mins, 0 min *vs.* 30 mins and 15 min *vs.* 30 min) and 3 within (timepoints) factors (baseline, interim and endpoint). To investigate the effect of diet on glucose uptake at 0 nM and 100 nM of insulin, data underwent a 3-way mixed ANOVA with 2 between factors (LCDM *vs.* HCDM), 2 within (dose) factors (0 nM *vs.* 100 nM) and 3 within (timepoints) factors (baseline, interim

and endpoint). Bonferroni post hoc pairwise comparisons test was used when significant main effects and interactions were present. Pearson's correlation analysis was done on normally distributed data to investigate if there were any associations between p/t-Akt^{ser473}, p/t-AMPK^{thr172} and glucose uptake. Non-normally distributed data underwent Spearman's correlation. All data are presented as mean ± SEM and significance set as $P \leq 0.05$.

4.4. Results

4.4.1. Both a LC and HC diet for 8 weeks improve markers of metabolic health (Study 1, Chapter 3).

Participants following the LC diet significantly ($P < 0.001$) decreased their carbohydrate intake and significantly ($P < 0.001$) increased their fat intake. The HC group showed no significant change in diet. Both diets similarly improved metabolic health due to significant ($P < 0.05$) improvements in, fasting triglyceride and insulin levels, and HOMA-IR.

4.4.2. Insulin increases Akt^{ser473} phosphorylation in skeletal muscle C2C12 myoblasts in DM.

Prior to determining the effect of diet on serum and subsequent cellular mechanisms, the cellular mechanisms in response to routine cell culture were investigated. Following incubation of myoblasts in DM, 100 nM of insulin stimulation significantly ($P < 0.001$) increased p-Akt^{ser473} by 28%, from 68908 ± 4172 AU to 88349 ± 4599 AU after 20 mins (Figure 4.1).

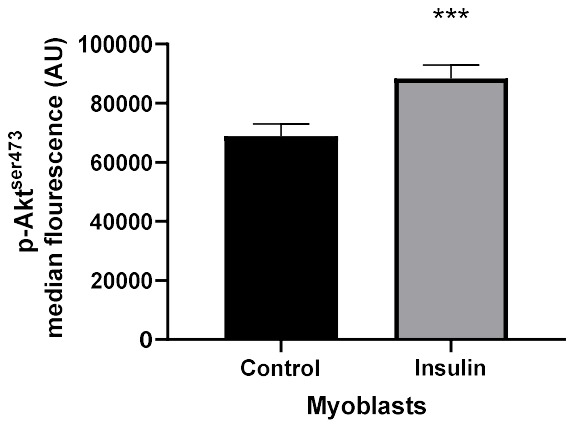


Figure 4.1. The effect of insulin stimulation in myoblasts on p-Akt^{ser473} in DM after 20 min. *** $P \leq 0.001$ indicates significantly different to control (0 nM). All data (n=6 in duplicate replicates in each group) are presented as mean ± SEM.

4.4.3. Co-incubation of skeletal muscle C2C12 myoblasts in DM with FGF21 enhances insulin stimulated Akt^{ser473} phosphorylation.

In the presence of FGF21 alone, there was no significant (P = 0.22) increase in basal p-Akt^{ser473} from 60256 ± 9960 to 73982 ± 9960 AU. Insulin significantly (P < 0.001) increased basal p-Akt^{ser473} to 68486 ± 10587 AU in the absence of FGF21 and to 99302 ± 12776 AU in the presence of FGF21 (Figure 4.2). The higher levels of p-Akt^{ser473} with FGF21 led to a significant (P < 0.01) interaction between groups. Insulin increased p-Akt^{ser473} in DM and DM/FGF by 1.15 ± 0.05 and 1.34 ± 0.02-fold respectively (Table 4.1). The fold change in DM/FGF21 was also significantly (P = 0.04) greater than the fold change in DM alone.

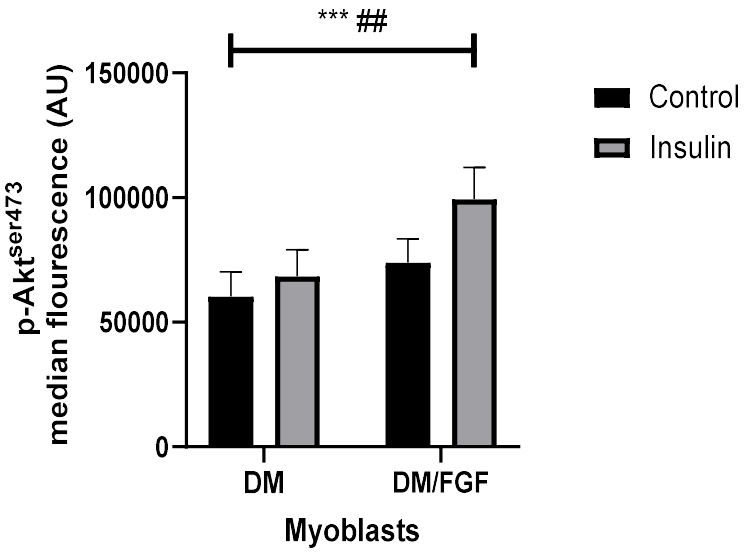


Figure 4.2. The effect of insulin stimulation in myoblasts on p-Akt^{ser473} in DM and DM/FGF after 20 min. *** P < 0.001 indicates significantly different to control (0 nM). ## P < 0.01 indicates significant interaction between groups. All data (n=4 replicates in duplicate in DM and n=3 replicates DM/FGF) are presented as mean ± SEM.

4.4.4. Insulin stimulation of skeletal muscle C2C12 myoblasts in DM shows a tendency of increasing glucose uptake

Having confirmed the cells were responsive to insulin induced Akt phosphorylation, the impact on glucose uptake was investigated. Following incubation in DM, myoblasts were treated with 0 nM and 100 nM of insulin for 30 min (Figure 4.3). Insulin stimulation showed a trend (P = 0.06) of increasing glucose uptake by a fold change of 1.38 ± 0.16.

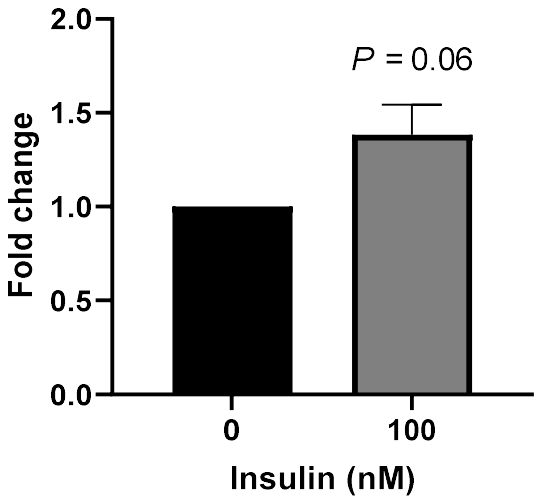


Figure 4.3. The effect of insulin stimulation in myoblasts on relative fold change in glucose uptake in DM after 30 min. All data (*n*=6 replicates in duplicate in DM) are presented as mean ± SEM.

4.4.5. Insulin increases Akt^{ser473} phosphorylation in skeletal muscle C2C12 myoblasts in LCDM and HCDM, but diet has no effect on insulin signalling.

In the presence of LCDM or HCDM, stimulation of myoblasts with 100 nM insulin for 20 min did not significantly (*P* = 0.09) increase p-Akt^{ser473} at baseline (LCDM; 80052 ± 12723 to 100095 ± 11965 AU, HCDM; 84923 ± 11726 to 93993 ± 16360 AU), interim (LCDM; 89788 ± 8007 to 118427 ± 17774 AU, HCDM; 83241 ± 14645 to 108877 ± 10756 AU) or endpoint (LCDM; 86737 ± 20170 to 119451 ± 25588 AU, HCDM; 86275 ± 14392 to 116092 ± 27058 AU) (Figure 4.4). There was a tendency (*P* = 0.07) of p-Akt^{ser473} to increase throughout the diet. Post-hoc analyses showed endpoint p-Akt^{ser473} to be significantly (*P* = 0.02) higher compared to baseline. There was no significant interaction between timepoints, groups and insulin stimulation.

The relative insulin induced p-Akt^{ser473} fold change vs control after 20 mins significantly (*P* < 0.001) increased at baseline (LCDM; 1.29 ± 0.07, HCDM; 1.1 ± 0.08) interim (LCDM; 1.3 ± 0.12, HCDM; 1.41 ± 0.16) and endpoint (LCDM; 1.4 ± 0.12, HCDM; 1.32 ± 0.11) (Table 4.1). No significant interaction between groups at any timepoint was observed. Furthermore, the fold change of p-Akt^{ser473} did not significantly change with time. There was no significant interaction between timepoints, groups and insulin stimulation.

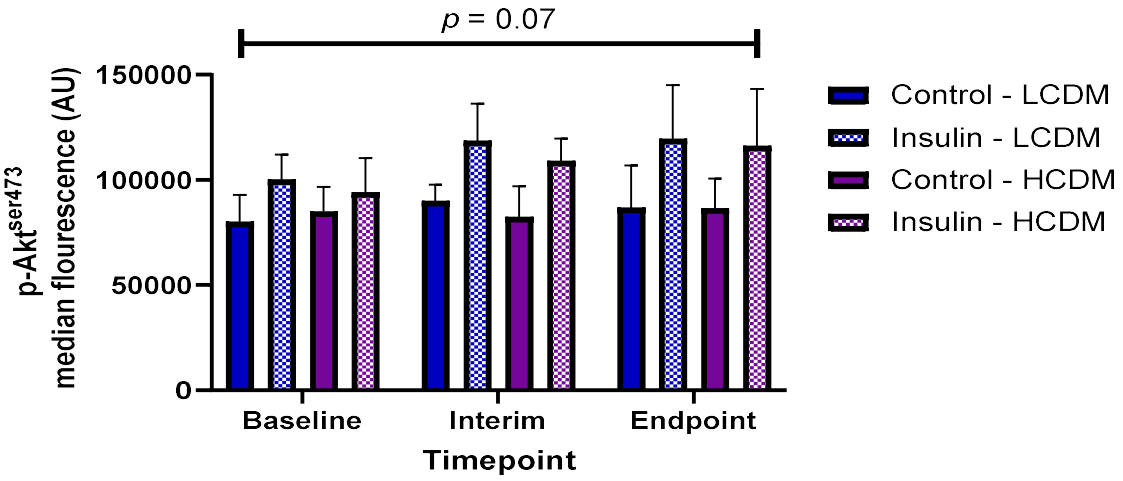


Figure 4.4. The effect of insulin stimulation in myoblasts on absolute p-Akt^{ser473} in LCDM and HCDM after 20 min at each timepoint. All data (n=5 replicates in each group) are presented as mean ± SEM.

4.4.6. Co-incubation of FGF21 reduces Akt^{ser473} phosphorylation but has no effect on insulin stimulated Akt^{ser473} phosphorylation in skeletal muscle C2C12 myoblasts in LCDM and HCDM.

Following co-incubation of FGF21 with baseline LCDM or HCDM, stimulation of myoblasts with 100 nM insulin for 20 min significantly ($P < 0.01$) increased p-Akt^{ser473} in cells incubated with LC/FGF (45370 ± 3839 to 52811 ± 2886 AU) and HC/FGF (48868 ± 4289 to 57482 ± 3448 AU) (Figure 4.5). There was no significant interaction between groups. The p-Akt^{ser473} levels observed in LC/FGF and HC/FGF were significantly ($P < 0.05$) lower than the p-Akt^{ser473} baseline levels of LCDM and HCDM. There was no significant interaction between condition, group or insulin stimulation.

The relative insulin induced p-Akt^{ser473} *vs.* control (0 nM) after 20 mins significantly ($P < 0.001$) increased in LC/FGF by 1.2 ± 0.1-fold and HC/FGF by 1.19 ± 0.07-fold (Table 4.1). The fold changes in LC/FGF and HC/FGF were not significantly different between each other or with baseline levels of LCDM and HCDM alone. There was no significant interaction between condition, groups and insulin stimulation.

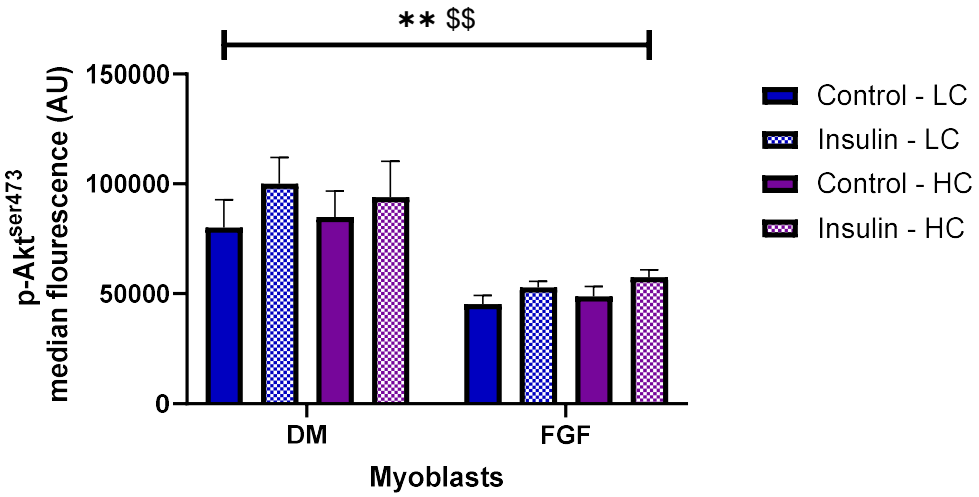


Figure 4.5. The effect of insulin stimulation in myoblasts after 20 min on p-Akt^{ser473} in LC/FGF, HC/FGF and baseline LCDM and HCDM. ** $P \leq 0.01$, indicates significantly different to control (0 nM), $^{\$\$}P \leq 0.01$ significantly different between FGF and DM conditions. All data (n=5 replicates LCDM and HCDM, n=3 replicates in LC/FGF and HC/FGF) are presented as mean ± SEM.

4.4.7. Insulin stimulation of skeletal muscle C2C12 myoblasts in LCDM and HCDM does not increase glucose uptake.

Following incubation in LCDM and HCDM glucose uptake in myoblasts was measured after 30 min in the absence and presence of 100 nM of insulin (Figure 4.6). No significant relative fold change in insulin induced glucose uptake was observed from 0 nM to to 100 nM (BL = LCDM; 1.13 ± 0.16, HCDM; 0.93 ± 0.08, INT = LCDM; 1.23 ± 0.26, HCDM; 1.08 ± 0.17, END = LCDM; 1.15 ± 0.12, HCDM; 1.03 ± 0.15) at any timepoint. No significant interaction was observed between timepoints or groups. This may be due to methodical issues as described previously (see methods 2.3.1).

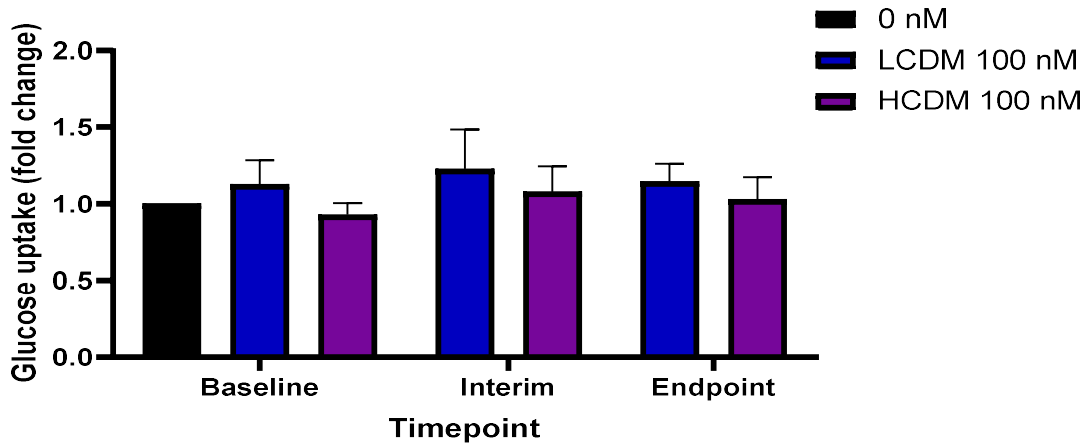


Figure 4.6. The effect of insulin stimulation in myoblasts on glucose uptake in LCDM and HCDM after 30 min. All data (n=3 replicates in duplicate) are presented as mean ± SEM.

4.4.8. Summary of the effects of serum on insulin signalling and glucose uptake in myoblasts (Figure 4.17).

Stimulation of myoblasts with 100 nM insulin for 20 mins showed significant (P < 0.001) increases in p-Akt^{ser473} in DM, LCDM and HCDM. Diet showed a tendency (P = 0.07) to increase p-Akt^{ser473} but had no effect on the relative fold change with insulin stimulation.

Co-incubation of DM and FGF21 significantly (P < 0.05) enhanced p-Akt^{ser473} compared to DM alone and insulin stimulated fold change of in p-Akt^{ser473}. In contrast, co-incubation of baseline LCDM or HCDM with FGF21 significantly (P < 0.01) decreased p-Akt^{ser473} compared to LCDM or HCDM alone, however, had no effect on the insulin induced fold change of p-Akt^{ser473}.

No significant change in glucose uptake was observed with 100 nM of insulin following incubation in LCDM and HCDM.

These results warrant further research in myotubes to investigate if conditioned serum has similar effects in myoblasts.

4.4.9. Insulin stimulation of skeletal muscle C2C12 myotubes in DM show greater increases in Akt^{ser473} phosphorylation compared to myoblasts.

Following incubation of myotubes in DM, 100 nM insulin stimulation significantly (P < 0.001) increased p/t-Akt^{ser473} by 3.1-fold, after 20 mins from 0.61 ± 0.09 p/t-Akt^{ser473} to 1.9 ± 0.29 p/t-Akt^{ser473} (Figure 4.7).

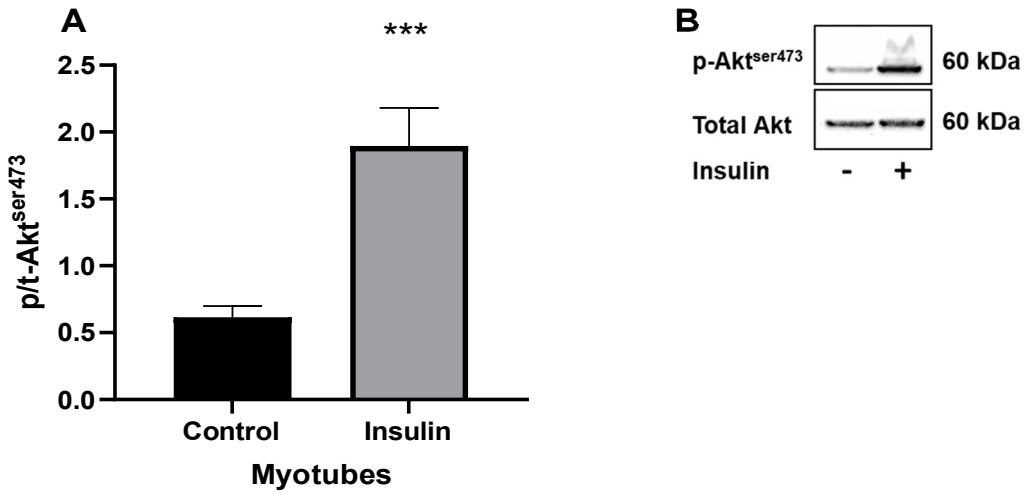


Figure 4.7. The effect of insulin stimulation in myotubes on p/t-Akt^{ser473} in DM after 20 min. A) Quantitative and B) qualitative representation. *** $P \leq 0.001$ indicates significantly different to control (0 nM). All data (n=9 replicates in each group) are presented as mean ± SEM.

4.4.10. Skeletal muscle C2C12 myotubes show a greater increase in insulin stimulated fold change in Akt^{ser473} phosphorylation compared to myoblasts when FGF21 is incubated in DM.

In the presence of FGF21 alone, there was no significant (P = 0.99) change in basal p/t-Akt^{ser473} from 0.61 ± 0.09 to 0.36 ± 0.17 p/t-Akt^{ser473}. Insulin significantly (P < 0.001) increased basal p/t-Akt^{ser473} to 1.89 ± 0.29 in the absence of FGF21 and to 1.58 ± 0.67 p/t-Akt^{ser473} in the presence of FGF21 (Figure 4.8). There was no difference in p/t-Akt^{ser473} in DM vs FGF.

Insulin increased p/t-Akt^{ser473} in DM by a fold change of 3.15 ± 0.31 and in the presence of FGF21 by 6.31 ± 2.19-fold (Table 4.1). The fold change in DM/FGF21 was significantly (P = 0.03) greater than the fold change in DM alone.

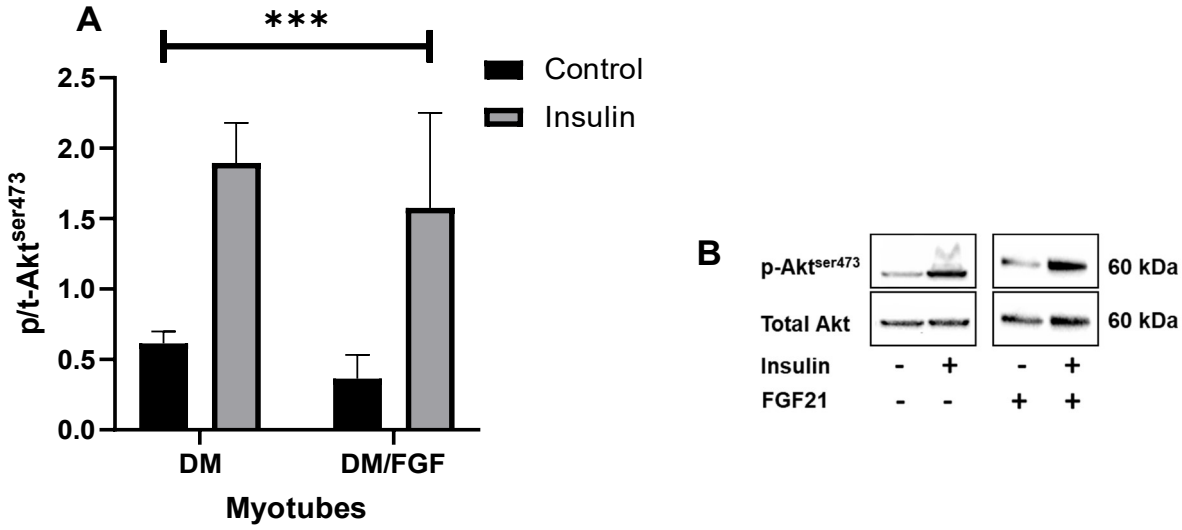


Figure 4.8. The effect of insulin stimulation in myotubes on p/t-Akt^{ser473} in DM and DM/FGF after 20 min. A) Quantitative and B) qualitative representation. *** $P \leq 0.001$ indicates significantly different to control (0 nM). All data (n=3 replicates in triplicate in DM and n=3 replicates DM/FGF) are presented as mean ± SEM.

4.4.11. Insulin stimulation of skeletal muscle C2C12 myotubes in DM increase glucose uptake, similarly to myoblasts.

Following the significant impact of insulin on p/t-Akt phosphorylation in myotubes, the impact of 100 nM of insulin for 30 min on glucose uptake was investigated (Figure 4.9). Glucose uptake significantly (P = 0.04) increased by a fold change of 1.44 ± 0.65 with 100 nM of insulin.

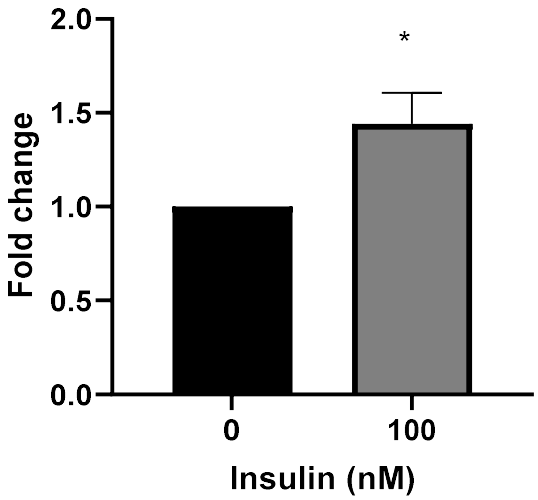


Figure 4.9. The effect of insulin stimulation in myotubes on glucose uptake in DM after 30 min. All data (n=6 replicates in duplicate in DM) are presented as mean ± SEM.* *P* < 0.05 indicates significantly different to 0 nM.

4.4.12. LC and HC diets show a reduction in insulin stimulated Akt^{ser473} phosphorylation in skeletal muscle C2C12 myotubes in contrast to myoblasts.

Having established the impact of control culture conditions on myotube behaviour, the impact of LCDM and HCDM could be further investigated. Under baseline conditions, in the presence of LCDM or HCDM there was no impact on Akt phosphorylation (LCDM; 0.7 ± 0.0 vs. HCDM; 0.85 ± 0.2). Stimulation of myotubes with 100 nM insulin for 20 min significantly (*P* < 0.01) increased p/t-Akt^{ser473} at baseline (LCDM 3.25 fold; 0.7 ± 0.0 to 2.27 ± 0.43 p/t-Akt^{ser473}, HCDM 2.9 fold; 0.85 ± 0.2 to 2.47 ± 0.57 p/t-Akt^{ser473}), interim (LCDM 1.95 fold; 0.65 ± 0.03 to 1.25 ± 0.1 p/t-Akt^{ser473}, HCDM 2.59 fold; 0.74 ± 0.12 to 1.92 ± 0.49 p/t-Akt^{ser473}) and endpoint (LCDM 2.13 fold; 0.64 ± 0.17 to 1.31 ± 0.43 p/t-Akt^{ser473}, HCDM 2.6 fold; 0.43 ± 0.13 to 0.99 ± 0.09 p/t-Akt^{ser473}) (Figure 4.10). There was a significant (*P* = 0.01) decrease of p/t-Akt^{ser473} compared to baseline leading to a significant (*P* = 0.03) interaction between timepoints *vs.* control and insulin stimulation. There was no significant interaction between timepoints, groups and insulin stimulation.

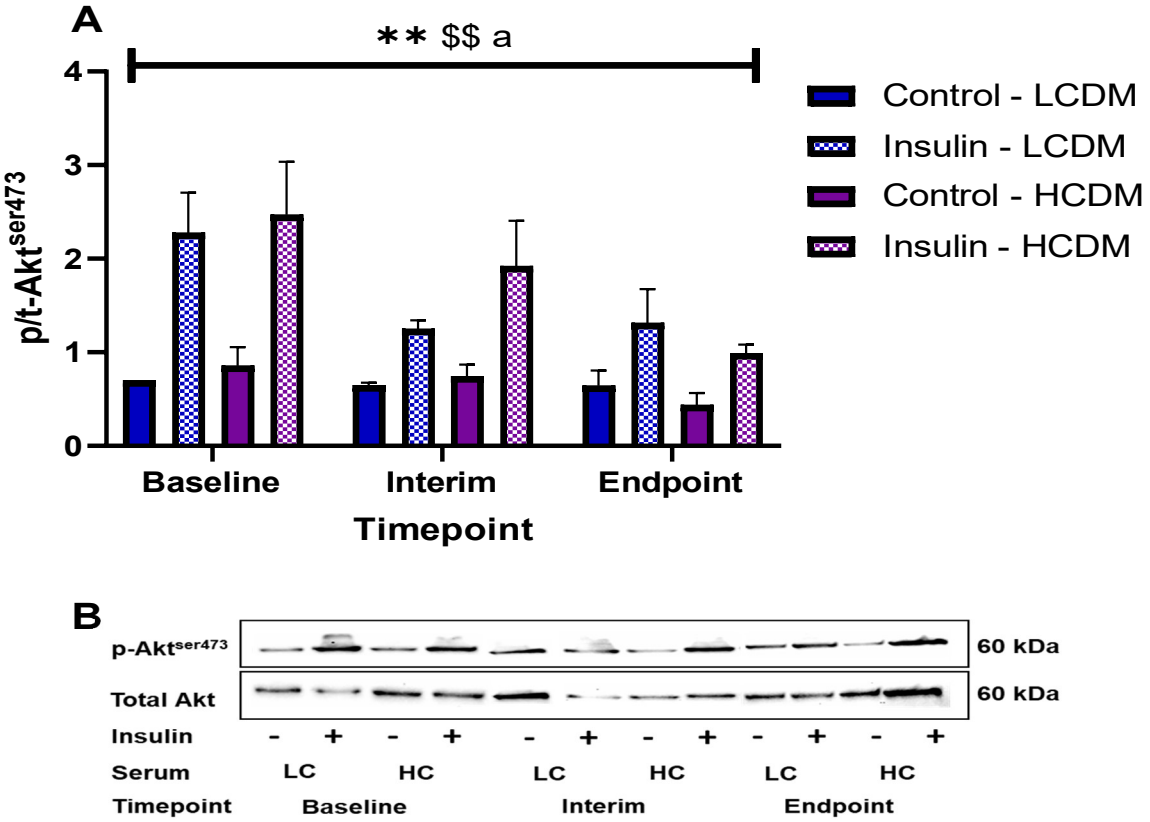


Figure 4.10. The effect of insulin stimulation in myotubes on p/t-Akt^{ser473} in LCDM and HCDM after 20 min at each timepoint. A) Quantitative and B) qualitative representation. ** $P < 0.01$, indicates significantly different to control (0 nM), $^{\$\$}P < 0.01$ indicates significant effect for time, $^{a}P < 0.05$ indicates significant interaction between timepoints *vs.* control and insulin stimulation. All data (n=3 replicates in each group) are presented as mean ± SEM.

4.4.13. FGF21 does not enhance insulin stimulated fold change in Akt^{ser473} phosphorylation in skeletal muscle C2C12 myotubes, similarly to myoblasts, when in LCDM and HCDM.

Under baseline conditions, in the presence of LCDM or HCDM there was no impact on Akt phosphorylation (LCDM; 0.7 ± 0.0 vs. HCDM; 0.85 ± 0.2). In the presence of FGF21, baseline conditions did not change (LCDM; 0.49 ± 0.05 vs. HCDM; 1.25 ± 0.42). Stimulation of myotubes with 100 nM insulin for 20 min significantly ($P < 0.01$) increased p/t-Akt^{ser473} at baseline (LCDM 3.25-fold; 0.7 ± 0.0 to 2.27 ± 0. p/t-Akt^{ser473}, HCDM 2.9-fold; 0.85 ± 0.2 to 2.47 ± 0.57 p/t-Akt^{ser473}). In the presence of FGF21, stimulation with 100 nM of insulin, significantly ($P < 0.01$) increased p/t-Akt^{ser473} (LC/FGF 2.74-fold; 0.49 ± 0.05 to 1.28 ± 0.12 p/t-Akt^{ser473}, HC/FGF 2.8 fold; 1.25 ± 0.42 to 2.51 ± 0.34 p/t-Akt^{ser473}) (Figure 4.11).

There was a significant ($P < 0.05$) interaction between condition *vs.* LC and HC. The p/t-Akt^{ser473} levels observed in LCDM and LCFGF were significantly ($P < 0.05$) lower than the p/t-

Akt^{ser473} baseline levels of HCDM and HC/FGF. There was no significant interaction between condition, group or insulin stimulation.

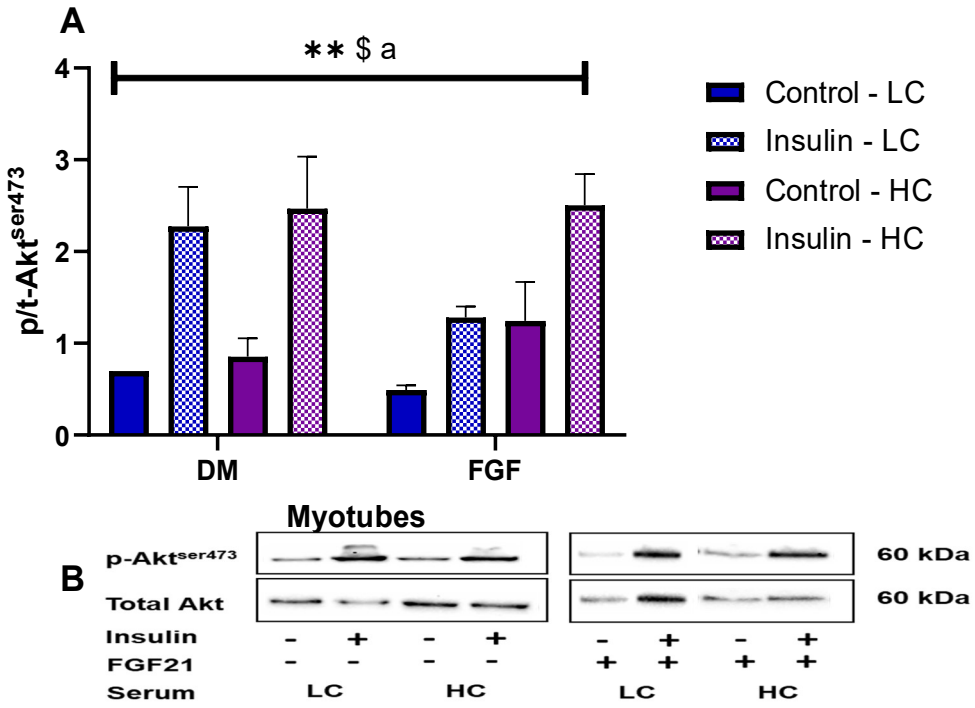


Figure 4.11. The effect of insulin stimulation in myotubes after 20 min p-Akt^{ser473} LC/FGF, HC/FGF and baseline LCDM and HCDM. A) Quantitative and B) qualitative representation. ** $P < 0.01$, indicates significantly different to control (0 nM), $^{\$}$ $P < 0.05$ significantly different between LC and HC groups, ^{a} $P < 0.05$ indicates significant interaction between condition *vs.* LC and HC. All data (n=3 replicates) are presented as mean ± SEM.

4.4.14. Insulin stimulation of skeletal muscle C2C12 myotubes in LCDM and HCDM show no change in glucose uptake, similarly to myoblasts.

Having investigated the impact of LCDM and HCDM on Akt phosphorylation, glucose uptake was measured in response to insulin for 30 min in myotubes (Figure 4.12). No significant relative fold change in insulin induced glucose uptake was observed from 0nM to 100 nM (BL = LCDM; 1.48 ± 0.52, HCDM; 1.03 ± 0.1, INT = LCDM; 1.1 ± 0.24, HCDM; 1.02 ± 0.12, END = LCDM; 1 ± 0.25, HCDM; 1.2 ± 0.12) at any timepoint. No significant interaction was observed between timepoints or groups. This may be due to methodical issues as described previously (see methods 2.3.1).

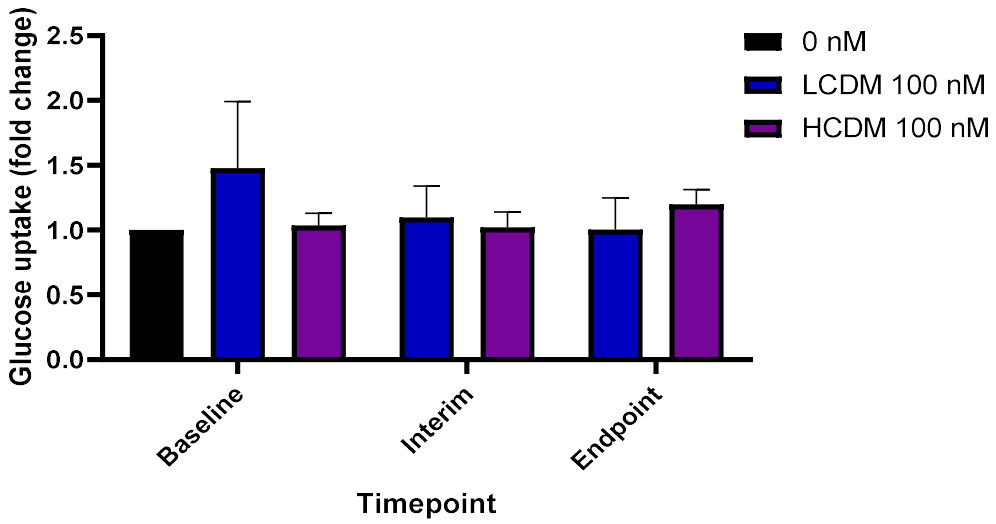


Figure 4.12. The effect of insulin stimulation in myotubes on glucose uptake in LCDM and HCDM after 30 min. All data (n=3 replicates in duplicate in DM) are presented as mean ± SEM.

Table 4.1. Insulin stimulated fold change in Akt^{ser473} phosphorylation in myoblasts and myotubes in each condition.

Condition	Myoblasts	Myotubes
	Fold change p-Akt^{ser473} Vs 0 nM	Fold change p-Akt^{ser473} Vs 0 nM
DM	1.15 ± 0.05	3.15 ± 0.31
DM/FGF	1.34 ± 0.02*	6.31 ± 2.19*
LCDM Baseline	1.29 ± 0.07	3.25 ± 0.62
HCDM Baseline	1.1 ± 0.08	3.33 ± 1.29
LCDM Interim	1.3 ± 0.12	1.95 ± 0.23
HCDM Interim	1.41 ± 0.16	2.59 ± 0.42
LCDM Endpoint	1.4 ± 0.12	2.13 ± 0.31
HCDM Endpoint	1.32 ± 0.11	2.6 ± 0.60
LC/FGF	1.2 ± 0.10	2.74 ± 0.58
HC/FGF	1.19 ± 0.07	2.8 ± 1.32

Data presented as mean ± SEM. * indicates significantly different to DM

4.4.15. DM does not increase AMPK^{thr172} phosphorylation in skeletal muscle C2C12 myotubes.

To investigate the effects of DM on p/t-AMPK^{thr172}, myotubes were incubated with DM for 0, 15 and 30 mins (Figure 4.13). No significant change in p/t-AMPK^{thr172} was observed with time from 0 mins (0.69 ± 0.08 p/t-AMPK^{thr172}) to 15 mins (1.09 ± 0.9 p/t-AMPK^{thr172}) or 30 mins (1.9 ± 0.86 p/t-AMPK^{thr172}).

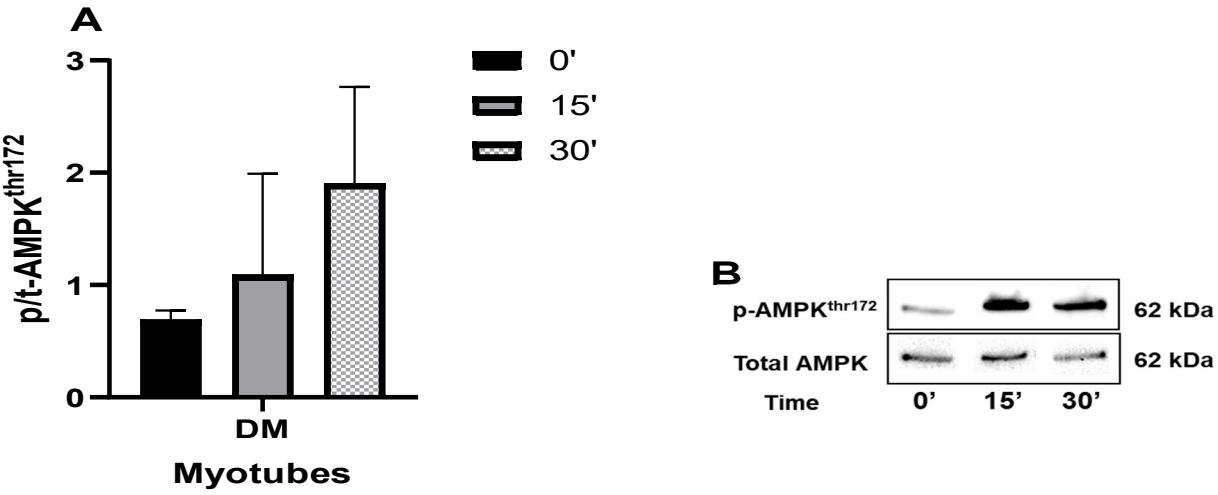


Figure 4.13. The effect of DM serum on p/t-AMPK^{thr172} at 0, 15 and 30 minutes in myotubes. A) Quantitative and B) qualitative representation. All data (n=3 replicates in duplicate in DM) are presented as mean ± SEM.

4.4.16. LCDM and HCDM increase AMPK^{thr172} phosphorylation in skeletal muscle C2C12 myotubes but diet reduces this effect.

To investigate the effects of diet on p/t-AMPK^{thr172}, myotubes were incubated with LCDM and HCDM for 0, 15 and 30 mins (Figure 4.14). LCDM and HCDM relative fold change of p/t-AMPK^{thr172} from 0 min showed a tendency (P = 0.06) of increasing at 15 mins and significantly (P < 0.05) increased from 0 to 30 mins and 15 to 30 mins. LCDM and HCDM relative fold change of p/t-AMPK^{thr172} significantly (P = 0.04) decreased from baseline to endpoint after 15 min (BL = LCDM; 2.25 ± 0.43, HCDM; 2.25 ± 0.74, INT = LCDM; 1.2 ± 0.28, HCDM; 0.9 ± 0.31, END = LCDM; 1.37 ± 0.22, HCDM; 1.97 ± 0.66) and 30 min (BL = LCDM; 6.51 ± 2.16, HCDM; 4.54 ± 0.83, INT = LCDM; 2.16 ± 0.43, HCDM; 2.93 ± 0.64, END = LCDM; 1.33 ± 0.2, HCDM; 1.96 ± 0.44). No significant interaction was observed between groups at 15 or 30 min.

4.4.17. Insulin induced p/t-Akt^{ser473} correlates with p/t-AMPK^{thr172} in myotubes.

To investigate if the differences in p/t-AMPK^{thr172} were associated with differences in insulin induced p/t-Akt^{ser473} Spearman's correlation analysis was undertaken. p/t-AMPK^{thr172} at 30 mins was significantly positively associated (r = 0.62, P < 0.01) with insulin stimulated p/t-Akt^{ser473} at 20 mins (Figure 4.15). No significant (r = 0.05, P = 0.82) association was found between p/t-AMPK^{thr172} after 15 mins and p/t-Akt^{ser473} at 20 mins. No significant association was found with glucose uptake.

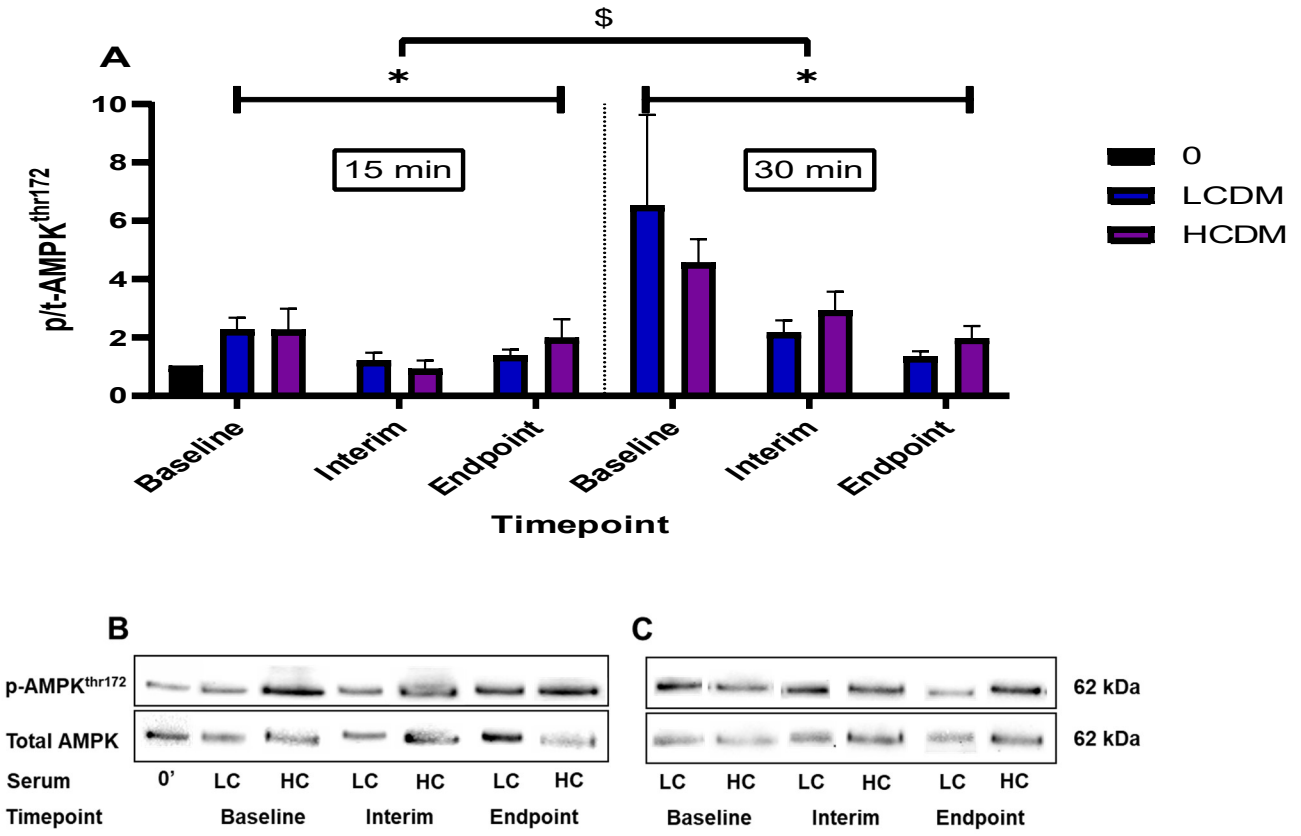


Figure 4.14. The effect of LCDM and HCDM on p/t-AMPK^{thr172} in myotubes after 15 and 30 min. A) Quantitative and B) qualitative representation. * $P \leq 0.05$ indicates significantly different from baseline to endpoint of diet, $^{\$}$ $P \leq 0.05$ indicates significantly different from 15 to 30 mins. All data (n=3 replicates in duplicate in DM) are presented as mean ± SEM

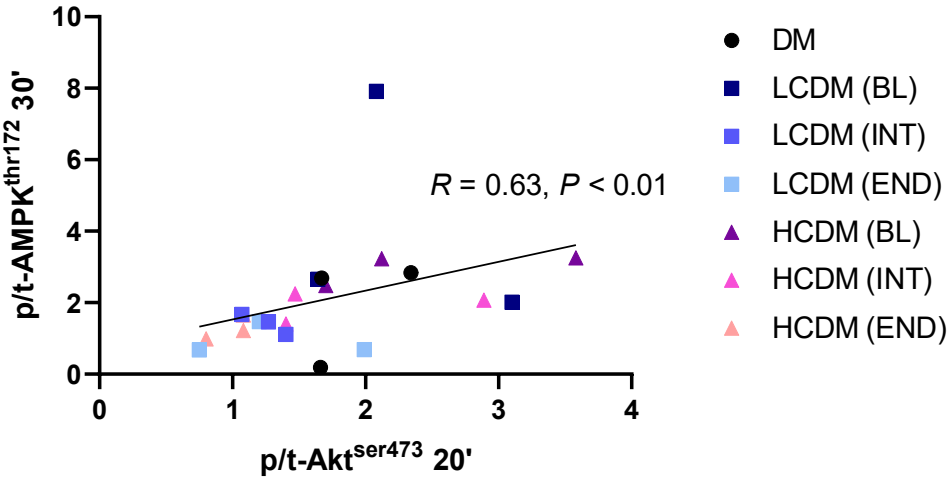


Figure 4.15. Correlation of p/t-AMPK^{thr172} after 30 minutes of serum incubation and insulin induced p/t-Akt^{ser473} after 20 min. Significance set as $P \leq 0.05$.

4.4.18. Diet increases insulin stimulated p/t-Akt^{ser473} and glucose uptake when relativised to p/t-AMPK^{thr172}.

Research shows AMPK activity to influence insulin dynamics. For example, AMPK activation has shown to stimulate insulin induced glucose uptake during IR conditions (Jaiswal *et al.*, 2019) while also activating the Akt pathway via mTORc2 signalling (Kazyken *et al.*, 2019). thus, reduced AMPK activity at each timepoint may have influenced results. Therefore, each timepoint was relativised to p/t-AMPK^{thr172} at 30 mins. Insulin stimulated fold change in p/t-Akt^{ser473}/p/t-AMPK^{thr172} showed a trend (P = 0.07) of increasing from baseline (LCDM; 0.5 ± 0.01, HCDM; 0.73 ± 0.28) to interim (LCDM; 0.9 ± 0.11, HCDM; 0.89 ± 0.14) and endpoint (LCDM; 1.6 ± 0.24, HCDM; 1.33 ± 0.3) in both diets (Figure 4.16A). Glucose uptake when relativised to p-AMPK showed a significant (P < 0.01) increase with 100 nM insulin from baseline (LCDM; 0.23 ± 0.08, HCDM; 0.23 ± 0.02) to interim (LCDM; 0.51 ± 0.11, HCDM; 0.35 ± 0.04) and endpoint (LCDM; 0.76 ± 0.19, HCDM; 0.61 ± 0.06) (Figure 4.16B). No significant difference was observed between diets.

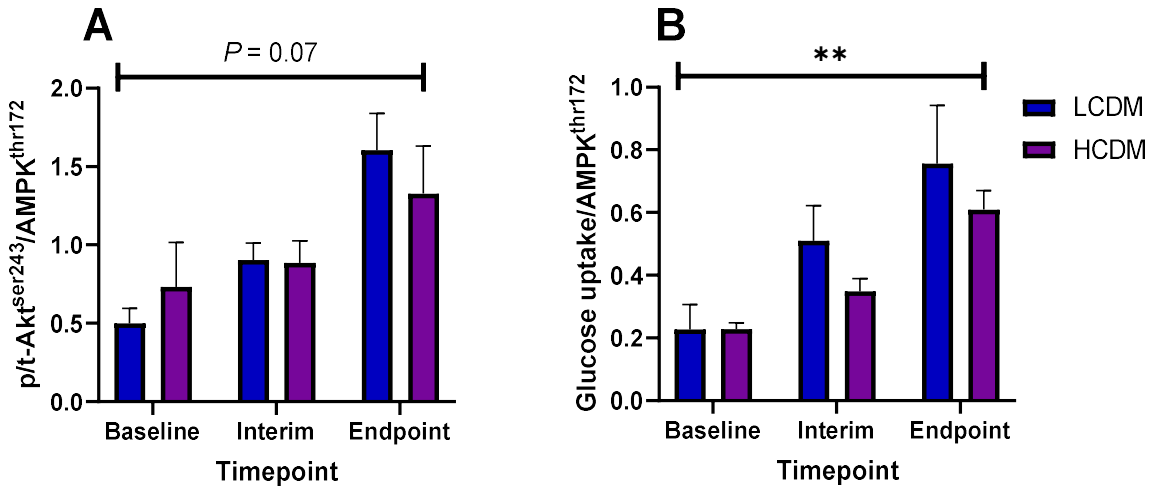


Figure 4.16. The effect of insulin stimulation in myotubes on insulin induced fold change of A) p/t-Akt^{ser473} and B) glucose uptake relativised to p/t-AMPK^{thr172} in LCDM and HCDM. ** P < 0.01 indicates significantly different from baseline to endpoint of diet. All data (n=3) are presented as mean ± SEM.

4.4.19. Summary of the effects of serum on insulin signalling glucose uptake and AMPK in myotubes (Figure 4.17).

Stimulation of myotubes with 100 nM insulin for 20 mins showed significant (P < 0.001) increases in p/t-Akt^{ser473} in DM, LCDM and HCDM. Surprisingly, both diets resulted in a significant (P = 0.01) decrease of p/t-Akt^{ser473} compared to baseline leading to a significant (P < 0.05) interaction between timepoints *vs.* control and insulin stimulation. However, diet had

no effect on the relative fold change with insulin stimulation. In contrast, co-incubation of DM and FGF21 did not significantly ($P > 0.05$) enhance p/t-Akt^{ser473} compared to DM alone but significantly ($P < 0.05$) increased the insulin stimulated fold change of in p/t-Akt^{ser473}. Similarly to myoblasts, co-incubation of baseline LCDM or HCDM with FGF21 did not significantly ($P > 0.05$) increase p/t-Akt^{ser473} compared to LCDM or HCDM alone. The p/t-Akt^{ser473} levels observed in LCDM and LCFGF were significantly ($P < 0.05$) lower than the p/t-Akt^{ser473} baseline levels of HCDM and HC/FGF but no difference was observed in the relative fold change in insulin stimulation.

No significant change in glucose uptake was observed with 100 nM of insulin following incubation in LCDM and HCDM.

Both diets resulted in a significant decrease in AMPK^{thr172} phosphorylation which was significantly positively associated ($R = 0.62$, $P < 0.01$) with insulin stimulated p/t-Akt^{ser473} at 20 mins. Due to the potential effect of reduced AMPK activity on reducing insulin sensitivity, each timepoint was relativised to AMPK^{thr172} phosphorylation. This resulted in both diets showing a trend ($P = 0.07$) at increasing insulin stimulated p/t-Akt^{ser473} and significantly increasing glucose uptake compared to baseline relative to AMPK activity levels.

4.5. Discussion

The main outcome of this investigation is that insulin significantly increased p-Akt^{ser473} in all serum conditions but in contrast to hypothesis 1, although myoblasts significantly (P = 0.02) increased p-Akt^{ser473} by endpoint, the insulin-induced relative fold change did not differ between timepoints. Conversely, myotubes showed a significant (P = 0.01) decrease of p/t-Akt^{ser473} compared to baseline leading to a significant (P = 0.03) interaction between timepoints *vs.* control and insulin stimulation in both diets. However, the relative fold-change was not significantly different between timepoints or groups.

In agreement with hypothesis 2 both diets significantly (P = 0.04) reduced p/t-AMPK^{thr172} compared to baseline, indicating a reduction in cellular stress. There was no significant difference between diets and p/t-AMPK^{thr172} at 30 mins, significantly correlated (R = 0.62, P < 0.01) with insulin induced p/t-Akt^{ser473} at 20 mins. Interestingly, due to the potential role of AMPK on Akt signalling (Kazyken *et al.*, 2019), insulin stimulated p/t-Akt^{ser473} was expressed relative to p/t-AMPK^{thr172} to account for differences in p/t-AMPK^{thr172} between timepoints. This resulted in a tendency (P = 0.07) of insulin stimulated p/t-Akt^{ser473} improving with both diets in myotubes in accordance with hypothesis 1.

Contrary with hypothesis 3, insulin did not have any significant effect on glucose uptake in LCDM or HCDM. This was most likely due to methodical issues as explained in method development. However, when insulin stimulated glucose uptake was relativised to p/t-AMPK^{thr172}, a significant (P < 0.01) improvement in glucose uptake was observed with both diets.

In contrast with hypothesis 4, although FGF21 significantly (P < 0.05) enhanced the relative fold change in p/t-Akt^{ser473} when stimulated with insulin in myoblasts and myotubes conditioned with DM, FGF21 had no significant effect on the insulin induced fold change in p/t-Akt^{ser473} in LCDM and HCDM. In myoblasts, FGF21 significantly (P < 0.01) decreased basal and insulin stimulated p/t-Akt^{ser473} levels similarly in LCDM and HCDM, whereas in myotubes, FGF21 significantly (P < 0.05) decreased basal and stimulated p/t-Akt^{ser473} in LCDM but increased in HCDM.

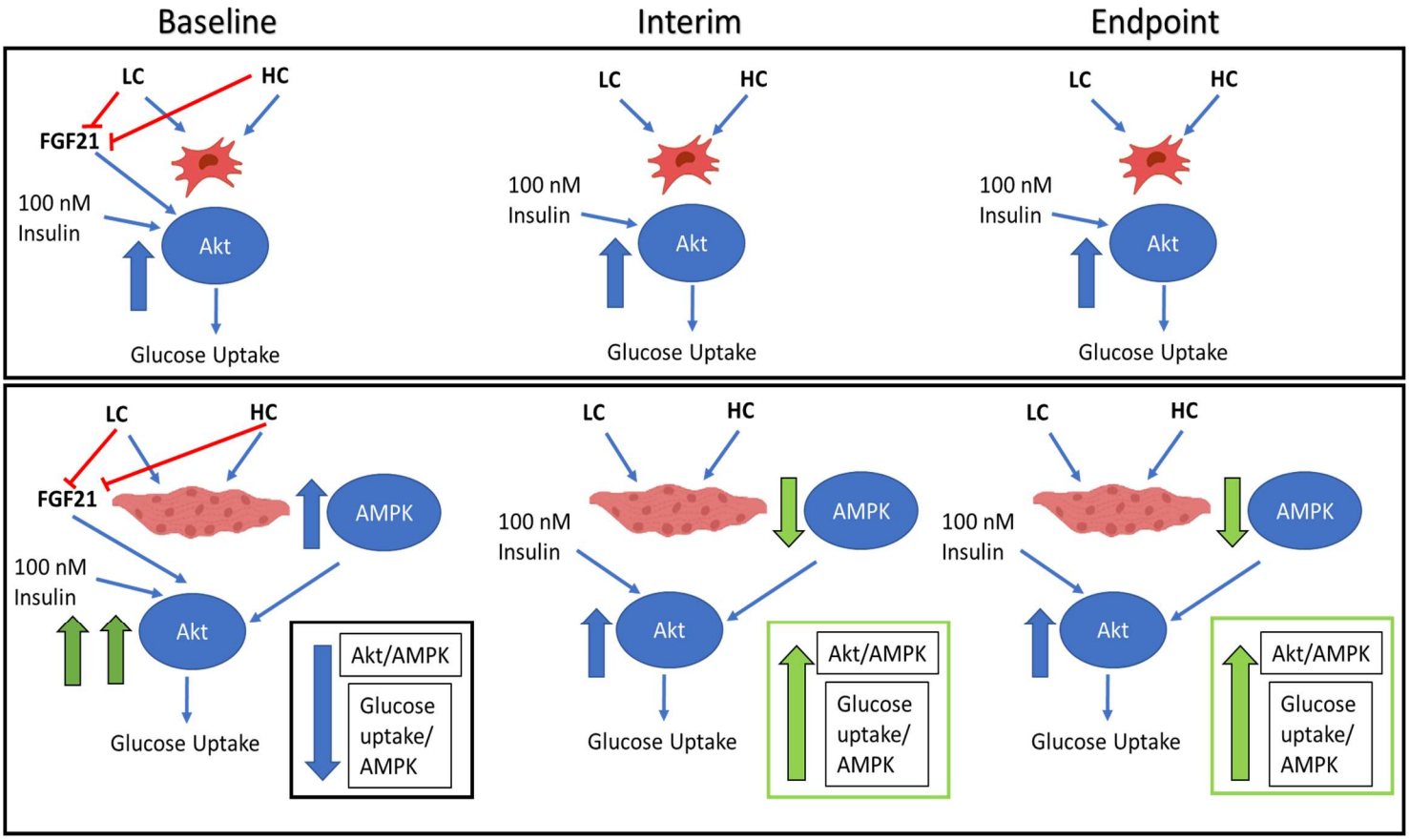


Figure 4.17. Summary of the effects of human derived serum following an 8-week LC or HC diet on insulin signalling, glucose uptake and AMPK phosphorylation in myoblasts (top) and myotubes (bottom).

While reproducible, traditional cellular models typically change only one variable to study insulin signalling and mechanisms of IR (Yuan *et al.*, 2017; Turner *et al.*, 2018). Although it is well established that the *in vivo* cellular environment has an impact on insulin signalling and subsequent IR, *in vitro* cellular models do not mimic the human *in vivo* environment (Samuel and Shulman, 2016; Cantor *et al.*, 2017). IR is the precursor in the development of T2D and is primarily driven by myriad of metabolic disturbances as a result of unhealthy lifestyles (Schellenberg, 2013; Samuel and Shulman, 2016). Therefore, traditional cell culture methods cannot elucidate the impact of the change in metabolic factors (nutrients, hormonal and inflammatory) on insulin signalling in response to interventions, such as a change in diet. The development of cellular models using human serum has allowed investigators to assess post-prandial effects of protein supplementation on GLUT 4 translocation and muscle protein synthesis (Carson *et al.*, 2018; Cogan *et al.*, 2019; Patel *et al.*, 2019). Furthermore, human plasma derived from old participants showed decreases in myoblast migration and myotube diameter compared to young plasma (Kalampouka, van Bekhoven and Elliott, 2018). Typically, mechanisms of human skeletal muscle adaptation are investigated by use of muscle biopsies; however, the use of conditioned serum may allow for such mechanisms to be

investigated less invasively and potentially at reduced costs. C2C12 cells have shown to be a robust model for understanding skeletal muscle mechanisms therefore this model allows for the direct impact of the endocrine environment on peripheral cell behaviour to be assessed without the potential interference from lifestyle or genetic factors observed with human tissue. Due to this, the effect of serum derived from participants who improved metabolic health following a LC or HC diet, on insulin signalling and glucose uptake was investigated *in vitro*.

The primary findings of this study were that both diets showed a tendency ($P = 0.07$) for increasing basal and stimulated p/t-Akt^{ser473} in myoblasts by endpoint; however, in myotubes basal and stimulated p/t-Akt^{ser473} significantly ($P < 0.05$) declined with serum derived following both diets. Although, the relative fold change with insulin stimulation did not change at each timepoint and was similar between diets. Furthermore, acute LCDM and HCDM serum incubation led to a significant ($P < 0.05$) increase in p/t-AMPK^{thr172} after 30 minutes, which was not evident in DM. Both diets showed a significant ($P < 0.05$) decrease in p/t-AMPK^{thr172} at interim and endpoints compared to baseline indicating a decrease in cellular stress. Therefore, baseline serum increased cellular stress perhaps by having higher levels of metabolic factors (fatty acids, ROS and cytokines) that can interfere with glucose uptake thereby reducing energy availability, but diet reduced this effect. Although no significant change was observed in measured cytokines and fatty acids with either diet, fasting triglyceride and insulin levels significantly decreased with both diets which is indicative of an improvement in metabolic health (Gao *et al.*, 2016). These results are in accordance with previous research as cultured myotubes from diabetic patients showed significantly ($P < 0.05$) higher basal p-AMPK^{thr172} compared to healthy controls (Al-bayati, Brown and Walker, 2019).

During times of cellular stress such as low energy availability, allosteric activation of AMPK occurs through phosphorylation of threonine 172 residues by AMP and additional non-canonical mechanisms (Hardie, Ross and Hawley, 2012; Herzig and Shaw, 2018). This increase in AMPK activation results in inhibition of anabolic pathways such as glycogen synthesis and protein synthesis and upregulation of catabolic pathways such as glycolysis and lipolysis (Hardie, Ross and Hawley, 2012; Herzig and Shaw, 2018). Furthermore, activation of AMPK can enhance glucose uptake via insulin-dependent and independent mechanisms. AMPK activation can increase GLUT 4 translocation and subsequent glucose uptake via AS160 independent of insulin induced PI3K/Akt pathway (Cartee, 2015; Kjøbsted *et al.*, 2017). During simulated chronic IR conditions (Akt1 & 2 ablation), mitochondrial dysfunction may occur leading to insulin induced glucose uptake via upregulation of p-AMPK^{thr172} (Jaiswal *et al.*, 2019). Upregulated p-AMPK^{thr172} may also indirectly upregulate glucose uptake as in Hek293 cells, 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) induced activation of p-AMPK^{thr172} increased mTORc2 and downstream signalling such as p-Akt^{ser473} (Kazyken *et*

al., 2019). Co-incubation of AICAR, a compound that activates AMPK, with insulin showed additive effects by further increasing p-Akt^{ser473} compared to insulin alone (Kazyken *et al.*, 2019). Additionally, p/t-AMPK^{thr172} and insulin induced p/t-Akt^{ser473} showed a significant positive (R = 0.62, P < 0.01) correlation, indicating a possible role for AMPK in the observed insulin responses. The increased levels of p/t-AMPK^{thr172} at baseline may explain the higher levels observed of p/t-Akt^{ser473} and glucose uptake at this timepoint and the contradictory results in which diet appeared to reduce insulin sensitivity in myotubes. Therefore, to account for the insulin sensitising effects of higher p/t-AMPK^{thr172} levels, insulin stimulated p/t-Akt^{ser473} and glucose uptake was relativised to p/t-AMPK^{thr172}. This resulted in a trend (P = 0.07) of increasing insulin stimulated fold change in p/t-Akt^{ser473} and significant (P < 0.01) increases of glucose uptake with both diets. Both diets showed similar responses to insulin signalling, glucose uptake and reductions in cellular stress which is in accordance with the results in study 1 (chapter 3) where participants both showed similar significant (P < 0.05) improvements in fasting insulin levels, HOMA IR and markers of metabolic health following either a LC or HC diet (McCullough *et al.*, 2020).

No significant responses of insulin on glucose uptake were observed in myoblasts or myotubes in LCDM or HCDM due to a large variation observed in 2NBDG glucose uptake. This may be due to methodical issues with the use of serum because in DM, glucose uptake showed a tendency of increasing in myoblasts and significantly increased in myotubes after 30 min of insulin stimulation. Similar research using 100 nM of insulin, has also reported significant increases in glucose uptake by 1.2 – 1.4 fold which is similar to the 1.4-fold increase observed in DM conditions the current study (Jeon *et al.*, 2016; Kim, Go and Imm, 2017). However, most observations in LCDM or HCDM conditions showed lower fold changes in glucose uptake (<1.2-fold) indicating a potential interference of the serum. Determination of cellular glucose kinetics typically requires serum free conditions as serum is thought to increase basal activity of the required pathways thereby reducing the potential effect of insulin (Pirkmajer and Chibalin, 2011). In the current study, cells did not differentiate in conditioned media; therefore, to measure glucose uptake, cells were incubated in low serum conditions for 3 hours to allow the serum to effect cell metabolism while also allowing signalling mechanisms to return to basal levels. Yet, the serum may have still interfered with glucose uptake leading to a large variation in insulin induced glucose uptake. Similar results were observed in L6 myotubes, as low serum media showed a similar response in insulin induced Akt^{ser473} phosphorylation compared with serum free however, only serum free myotubes showed increased in glucose uptake (Ching *et al.*, 2010).

Immunoblotting of myoblasts and myotubes have reported similar increases in IRS-1 tyrosine phosphorylation (Lamphere and Lienhard, 1992) and glucose uptake in response to insulin

stimulation (Turner *et al.*, 2018). Furthermore, differentiation of satellite cells into myotubes from T2D patients has been shown to maintain its IR phenotype; therefore, myoblasts may show similar results in insulin signalling and glucose uptake as myotubes (Gaster, 2019). Insulin stimulation significantly ($P < 0.001$) increased the relative fold change in p-Akt^{ser473} in both myotubes and myoblasts although, diet appeared to slightly increase ($P = 0.07$) p-Akt^{ser473} in myoblasts only. Myotubes compared to myoblasts showed a larger increase in the relative fold change in p/t-Akt^{ser473} although, the different methods used may explain this. Flow cytometry allows for single cell analysis unlike Western blot potentially allowing for greater accuracy however, myotubes are large multinucleated cells which cannot be subjected to flow cytometry measurements due to issues of size (Krutzik *et al.*, 2004).

In study 1, participants following a LC diet showed a trend ($P = 0.051$) in changing FGF21 levels as FGF21 decreased after 4 weeks but increased similar to baseline by 8 weeks. The maximum FGF21 concentration observed in participants was 1586 pg/ml and was therefore used for cellular experiments. Baseline levels of FGF21 were also similar between participants in LC (mean ± SD; 162.1 ± 115.9) and HC (mean ± SD; 180.7 ± 138.5) groups. FGF21 expression is upregulated in skeletal muscle in response to insulin induced PI3K/Akt signalling and has shown to enhance insulin induced glucose uptake in myotubes (Izumiya *et al.*, 2008; Mashili *et al.*, 2011; Jeon *et al.*, 2016). Due to its potential insulin sensitizing effects, we sought to investigate if FGF21 administration could enhance myoblast and myotube insulin sensitivity in normal serum conditions and in baseline conditioned sera when participants were estimated to be at worse metabolic health.

In DM, administration of FGF21 during serum incubation significantly ($P < 0.01$) increased basal and insulin stimulated p-Akt^{ser473} compared to insulin alone in myoblasts only. In both myoblasts and myotubes FGF21 significantly ($P < 0.05$) increased the insulin induced relative fold change in p-Akt^{ser473}. These results are in contrast to previous research in which FGF21 enhanced insulin induced glucose uptake but did not activate the PI3K/Akt signalling pathway (Mashili *et al.*, 2011). However, differences in methods such as FGF21 dose (1 µg/ml) and incubation times may explain this. In cardiomyocytes, FGF21 was shown to phosphorylate FGFR1 and PI3K/Akt pathway (Liu *et al.*, 2013). In skeletal muscle, FGF21 administration phosphorylated FGFR1 and increased glucose uptake however, PI3K/Akt signalling was not measured (Jeon *et al.*, 2016).

Conversely, co-incubation of FGF21 in conditioned baseline serum significantly decreased p-Akt^{ser473} in myoblasts similarly in both diets. In myotubes, FGF21 administration significantly ($P < 0.05$) decreased p/t-Akt^{ser473} in LC but increased in HC leading to a significant ($P < 0.05$) interaction between groups. However, FGF21 had no significant effect on the insulin

stimulated fold change in p-Akt^{ser473} in either group in myoblasts or myotubes. Although, FGF21 influences basal and stimulated p-Akt^{ser473} in all groups it had no significant effect on the relative fold change in LCDM and HCDM.

This suggests FGF21 alone is insufficient at improving the cellular environment to enhance insulin signalling. It is possible that the cellular environment in LCDM and HCDM interferes with FGF21 signalling as shown in T2D and obesity conditions. Paradoxically, although FGF21 has been shown to increase insulin sensitivity in a variety of tissues, it is commonly upregulated in conditions of obesity and T2D indicating a state of FGF21 resistance in these populations (Mashili *et al.*, 2011; Domouzoglou *et al.*, 2015; Lakhani *et al.*, 2018). The saturated fatty acid palmitate has been shown to regularly induce IR and reduce glucose uptake in skeletal muscle (Schmitz-Peiffer, Craig and Biden, 1999). Human myotubes exposed to palmitate reduced glucose uptake and increased FGF21 expression while downstream signalling of FGF21 was decreased as shown by reduced phosphorylation of FGFR, FRS2α and ERK 1/2 (Jeon *et al.*, 2016). FGF21 requires it cofactor β-Klotho to exert its potential metabolic effects (Samms *et al.*, 2016; Tezze, Romanello and Sandri, 2019). The reduction in FGF21 signalling may be due to a decrease in β-Klotho expression by palmitate (Jeon *et al.*, 2016). Additionally, FGF21 administration enhanced insulin induced glucose uptake and was able to partly reduce the negative impact of palmitate (Jeon *et al.*, 2016). These results support the FGF21 resistance model seen in humans with obesity or T2D, while also demonstrating its insulin sensitising effects on skeletal muscle. It is likely that the stressful cellular environment seen in baseline sera may cause similar inhibitory effects due to increased levels of fatty acids, ROS and/or cytokines that may not have been measured. We used 1500 pg/ml of FGF21 as it was similar to the highest amount observed in participants from study 1, chapter 3. However, higher amounts of FGF21 (100 - 1000 ng/ml) have been used to increase glucose uptake, therefore increasing FGF21 levels may elicit different results (Mashili *et al.*, 2011; Jeon *et al.*, 2016).

4.6. Limitations

Although serum was analysed for many markers of metabolic health the composition of the serum is largely unknown; therefore, the precise impact of specific metabolites or proteins within the serum on cellular metabolism remain to be determined. Identification of metabolites via metabolomics could lead to specific pathways being attributed to changes in cellular metabolism (Cantor *et al.*, 2017; Yang, Vijayakumar and Kahn, 2018). Furthermore, serum was pooled, and heat inactivated which may have weakened the ability of the *in vivo* environment to influence myocyte metabolism and signalling. However, the study was exploratory in nature and these steps were necessary to improve consistency and reduce costs associated with experiments as outlined in methods section 2.2.5. With these methods as a foundation for a future model, future studies can investigate individual serum responses rather than pooled serum to while heat inactivation may not be necessary when traditional methods are not used as a control.

Serum incubation was relatively short (3 hours) which may only indicate acute effects on cellular metabolism; however, it was not possible for myoblasts to differentiate in serum derived from participants which would have provided a greater indication of the chronic effects. The use of human serum on murine cells may also be a limitation as it may limit the relevance to human skeletal muscle. Furthermore, C2C12 cells may not be the best cell line at studying the effects of skeletal muscle insulin stimulated glucose uptake. C2C12 cells are enriched in genes associated with contraction and muscle development but express lower concentrations of metabolism related genes relative to rat L6 myotubes and primary human myotubes (Abdelmoez *et al.*, 2020). Nonetheless, the use of the C2C12 cell line has shown to be a n established model of skeletal muscle to study metabolic disease and reduces variability associated with genetic factors of human muscle (Wong, Al-Salami and Dass, 2020). A reduction in the genetic factors allows collected data to be attributed to changes in serum conditions. However, when a serum model is established, further research is warranted on human muscle conditioned with human serum.

The impact of serum on the effect insulin-induced glucose uptake was difficult to ascertain as it is recommended to perform the 2NBDG glucose uptake assay in serum free conditions (Yamamoto *et al.*, 2015). As the objective of this study was to investigate the effect of serum on insulin induced glucose uptake, serum was co-incubated with 2-NBDG and insulin which may have impacted results. Most observations in LCDM or HCDM conditions showed lower fold changes in glucose uptake (<1.2-fold) compared with standard cell culture conditions (DM, 1.4-fold) indicating a potential interference of the serum. Furthermore, to provide optimal conditions for the glucose uptake assay, the glucose uptake data was collected in the different

conditions relative to the insulin signalling data therefore, disagreements between experiments may be down to different methodological approaches.

Finally, cells were dosed with 2% serum which would have significantly diluted the biochemical properties of serum limiting its potential effect on cellular metabolism. However, larger increases in serum (50-100%) reduces cell viability (Carson *et al.*, 2018). Serum concentrations of 2% are standard cell culture conditions and ≥2% will compromise myoblast fusion.

Furthermore, previous research in L6-GLUT4myc cells demonstrated that incubation with 5% serum for 1 hour from fasted participants resulted in maximal GLUT4 translocation (Cogan *et al.*, 2019). Therefore, serum concentrations ≥5% are likely to be detrimental to the experiments in the current study as it may not allow for insulin to exert any further effect.

4.7. Conclusion

To summarise, traditional reductionist cell culture models are a powerful tool in understanding biology and have been essential in elucidating a myriad of signalling molecules related to lifestyle and their impact on insulin signalling. However, traditional *in vitro* methods cannot elucidate the unpredictable the change in metabolic factors (nutrients, hormonal and inflammatory) in response to interventions, such as a change in diet. Here we show that sera derived from humans following a LC or HC diet can impact the *in vitro* skeletal muscle response to insulin stimulation and energy stress signalling. Both diets improved markers of metabolic health as seen in study 1, chapter 3. This improvement in metabolic health resulted in an improved cellular environment and subsequently reduced cellular energy stress as observed by reducing acute AMPK^{thr172} phosphorylation. Furthermore, both diets showed similar effects on changing basal and insulin stimulated p-Akt^{ser473}; however, the relative fold change in insulin stimulation did not change with diet. When accounting for the potential compensatory effect of high AMPK^{thr172} phosphorylation at baseline, insulin stimulated fold change in Akt^{ser473} phosphorylation and glucose uptake increased similarly with both diets. Additionally, the potential of FGF21 to enhance insulin signalling in skeletal muscle may be due to the endocrine environment which agrees with the current FGF21 resistance hypothesis in MetS and T2D populations. The effects diet had on serum and subsequent acute impact on energy stress and insulin signalling demonstrate the important influence of the cellular environment. These results also highlight the potential role of using human derived serum to investigate cellular mechanisms relating to human interventions.

Chapter 5: Markers of metabolic health in anabolic androgenic steroid users

5.1. Abstract

Introduction: It is currently estimated that 3.3% of the global population use anabolic androgenic steroids (AAS) and usage is reportedly on the rise, particularly in recreational sports people to improve body image. Individuals who use AAS are at a higher risk of developing cardiovascular diseases (CVD) due to development of impaired cardiac function and lipoprotein disturbances. Use of AAS may also lead to development of MetS and associated markers, therefore the aim of this study is to investigate markers of metabolic health in individuals who use AAS compared with healthy resistance trained controls.

Methods: In a cross-sectional design, healthy resistance trained males aged between 19-45 years old who currently use AAS and have never used AAS were recruited for this study. Participants were excluded if they smoked, suffered from medically diagnosed lipid disorders, currently taking lipid or glucose lowering medication. After providing informed consent, participants were separated by AAS (n = 9) use or no use of AAS (n = 10) and were required to provide a fasted venous blood sample in addition to measurements of anthropometrics, body composition by skin-fold measures, dietary intake by 24 hr dietary recall and food frequency questionnaire and a 7-day training log. A semi-structured interview on drug use was conducted with individuals in the AAS group. Blood samples were processed for plasma and analysed for lipids, hormones and cytokines related to metabolic health. Plasma also underwent density gradient ultracentrifugation for the analysis of low-density lipoprotein (LDL) subclass phenotypes, pattern A and B.

Results: The AAS group showed significantly (P < 0.05) lower levels of body fat % and higher levels of fat free mass. No significant difference was detected in training volume or any measure of dietary intake. The AAS group showed significantly (P < 0.05) lower levels of high-density lipoprotein cholesterol (HDL-C), apolipoprotein (Apo) A1 and plasminogen activator inhibitor 1. The highest users of AAS showed a significantly moderate positive relationship lipoprotein cholesterol including: LDL-Cholesterol (r = 0.65, P = 0.03), ApoB (r = 0.69, P = 0.02), ApoB/A1 (r = 0.64, P = 0.03), leptin (r = 0.59, P = 0.048) and strong significantly positive association with pattern A (r = 0.72, P = 0.02) and pattern B (r = 0.96, P < 0.001).

Conclusion: Use of AAS results in suppression of HDL-C and its primary apolipoprotein ApoA1 leading to increased CVD risk but does not result in MetS. Increased AAS use may lead to increased Apo B and LDL pattern B phenotype. Future studies should investigate changes in metabolic health throughout the long-term cycling of AAS to help elucidate which practices may be the most detrimental to cardiovascular health. Furthermore, the effect of using a myriad of anabolic drugs on cardiometabolic disease and subsequent cellular mechanisms has yet to be investigated and would provide further insight into their relationship.

5.2. Introduction

It is currently estimated that 3.3% of the global population use anabolic androgenic steroids (AAS) and usage has been shown to be on the rise (Dunn, Mckay and Iversen, 2014; Sagoe *et al.*, 2014; McVeigh and Begley, 2017). Recreational sports people are reported as being the highest users with the greatest motivation to use AAS being primarily to improve body image, while competitive bodybuilding and athletic performance are secondary (Parkinson and Evans, 2006; Dunn, Mazanov and Sitharthan, 2009; Sagoe *et al.*, 2014; Begley, McVeigh and Hope, 2017). Particularly when used in combination with resistance exercise, AAS use is reported to increase muscle mass and strength in a dose response fashion (Bhasin *et al.*, 1996, 2001). Although this population are characteristically highly active, the potential benefits acquired from regular exercise may be reduced with chronic AAS use. Individuals who use AAS are at a higher risk of developing cardiovascular diseases (CVD), psychological disorders, hypogonadism and some cancers (Pope *et al.*, 2014; Baggish *et al.*, 2017; Goldman, Pope and Bhasin, 2019). The number one cause of death globally is CVD and with the increasing prevalence of AAS use, it may further exacerbate this global issue (World Health Organization, 2018b).

Metabolic syndrome (MetS) is the constellation of the often interrelated metabolic abnormalities that lead to increased risk of CVD's (Sperling *et al.*, 2015). It is most commonly associated with sedentary/obese populations and is defined by having a combination of some, but not all of high triglycerides, low high-density lipoprotein cholesterol (HDL-C), insulin resistance (IR), inflammation, hypertension and high levels of visceral adipose tissue (VAT) (Sperling *et al.*, 2015). However, AAS use may also lead to development of MetS and other associated metabolic abnormalities. For example, a literature review of AAS use has shown HDL-C to be reduced by 20 ≥ 70% and low-density lipoprotein cholesterol (LDL-C) levels to increase by > 20% (Achar, Rostamian and Narayan, 2010). Additionally, testosterone enanthate (TestE) was reported to show a dose-response suppression of HDL-C, with increasing doses (25 ≥ 600 mg/week) showing increased HDL-C reduction (Bhasin *et al.*, 2001). Early studies reported that after 8-weeks of AAS administration, HDL-C and LDL-C significantly ($P < 0.01$) decreased by 49% and increased by 31% respectively (Lenders *et al.*, 1988). A more recent cross sectional study has reported that this negative impact of AAS on lipoprotein metabolism is still prevalent today with AAS use (Rasmussen *et al.*, 2017). Individuals who use AAS typically use a range of AAS types, doses, cycles and methods of administration which may impact health differently. For instance, in addition to the effect of AAS dose, the type of AAS and route of administration may also have an impact on lipoprotein levels (Thompson *et al.*, 1989). Orally administered stanozolol showed a significant ($P < 0.05$)

increase of 29% and decrease of 33% in LDL-C and HDL-C respectively compared to 200 mg/week of TestE injections which decreased LDL-C by 16% and HDL-C by 9% after 6 weeks (Thompson *et al.*, 1989).

Skeletal muscle is the largest tissue for glucose disposal and increases in muscle mass should improve insulin sensitivity, yet paradoxically, research suggests chronic AAS use may be detrimental to glucose metabolism (Cohen and Hickman, 1987; DeFronzo and Tripathy, 2009). Powerlifting steroid users (mean AAS use 4 years) were shown to have similar fasting glucose levels as non-using powerlifters and sedentary participants; however, they had significantly ($P < 0.05$) higher fasting insulin levels that were similar to those observed in obese participants (Cohen and Hickman, 1987). More recently, an oral glucose tolerance test revealed AAS users (mean AAS use 2 years) to have reduced glucose tolerance compared to healthy controls (Rasmussen *et al.*, 2017). Interestingly, although users of AAS had lower body fat %, compared to healthy controls, they had greater levels of VAT, C-reactive protein (CRP) and reduced leptin levels which are all independently associated with IR and MetS (Soto González *et al.*, 2006; Rasmussen *et al.*, 2017; D'Elia *et al.*, 2019). These results indicate AAS use may lead to MetS as shown by low HDL-C, IR, increased VAT and inflammatory markers. Due to this, additional markers that are associated with MetS such as ferritin, interleukin-6 (IL-6), resistin, tumour necrosis factor-α (TNF-α) and plasminogen activator inhibitor-1 (PAI-1) (Soto González *et al.*, 2006; Esser *et al.*, 2014) may also be dysregulated with AAS use but are yet to be investigated.

Poor dietary intake and low physical activity are modifiable risk factors of MetS and CVD (Strasser, 2013; Rodríguez-Monforte *et al.*, 2017). A typical western diet (high in sugar, fat and calories) has shown to be positively associated with markers of MetS, whereas a Mediterranean diet (high in fruit, vegetables and low in red and processed meats) is inversely associated with MetS (Medina-Remón *et al.*, 2018; Norde *et al.*, 2020). Due to this, assessment of diet and physical activity levels must be considered when assessing markers of MetS between populations.

Therefore, the aim of this study is to investigate markers of metabolic health in individuals who use AAS compared with healthy resistance trained controls.

The objectives are:

1. To determine if markers of MetS (HDL-C, triglycerides, IR and inflammation) are different in users of AAS compared to healthy resistance trained controls.
2. To investigate if additional markers of CVD risk are different in AAS users compared to controls.
3. To investigate if AAS dose is associated with specific health markers.

It is hypothesised that:

1. Individuals who use AAS display features of MetS.
2. AAS participants also display an increase in additional markers of CVD risk compared to healthy controls.
3. That doses of AAS use will also be associated with higher CVD risk.

5.3. Methods

5.3.1. Participants and Design

In a cross-sectional design, healthy caucasian males aged between 19-45 years old were invited to take part in this study in the North-West England area. To be included they must be resistance trained i.e. at least 3 years' experience following a resistance training programme and either never have used AAS (control group) or currently using AAS (experimental, AAS group). Potential participants were excluded if they smoked, suffered from medically diagnosed lipid disorders, currently taking lipid or glucose lowering medication. Anonymity was explicitly guaranteed. Following completion of a screening form and If eligible, participants provided informed consent if they wished to volunteer to take part. The study was approved by Liverpool John Moores Research Ethics Committee (17/SLN/005). Participants were then allocated to either the control group (CON) or AAS group.

Participants were required to attend LJMU laboratories in the morning after fasting for 12 hours. Anthropometrics, a venous blood sample, body composition, dietary intake, 7-day training log and drug use were all recorded.

5.3.2. Anthropometrics

For body mass, participants were instructed to stand on a digital scale (Seca 704, Birmingham, UK), with shoes off and all heavy items removed. For height measurement, participants were asked to stand under a portable stadiometer (Seca 213, Birmingham, UK) and instructed to look forward and breathe in before the measurement was taken.

5.3.3. Body composition

Participant body composition was assessed via Harpenden skinfold callipers (Cranlea, Birmingham, UK) by an ISAK (International Society for the Advancement of Kinanthropometry) qualified practitioner. Skinfold thicknesses were determined utilising standard techniques agreed by ISAK. Briefly, anthropometric landmarks were identified, and skinfold sites marked prior to beginning the measurement. Skinfold thickness was determined at 8 sites (bicep, triceps, subscapular, supraspinale, iliac crest, abdominal, anterior thigh and medial calf). Each skinfold site was measured sequentially and then repeated. Where the duplicate measure deviated from the first measurement by >5% the site was re-measured for a third time and the closest of the measures used. Adipose tissue % was calculated according to the following

formula: % BF = 5.174 + (0.124 × thigh) + (0.147 × abdominal) + (0.196 × triceps) + (0.130 × calf) (Reilly *et al.*, 2009).

5.3.4. Blood collection

Blood was collected from the antecubital vein in 5 ml plasma EDTA or serum vacutainer tubes. After gentle inversions, plasma vacutainers were immediately placed on ice whereas serum vacutainers were allowed to stand at room temperature for 30 minutes and then placed on ice until processing. Vacutainers were centrifuged at 3000 g for 15 min and 4°C, and quickly aliquoted into storage tubes and stored at -80°C until analysis.

5.3.5. Blood analysis

The Evidence Investigator ^{TM} Biochip Array technology (Randox Laboratories Ltd, UK) that uses sandwich chemiluminescent immunoassays to simultaneously detect multiple analytes from a single sample (see section 2.3.7.) was used to determine the following plasma cytokines and hormones; Ferritin, IL-6, insulin, Leptin, PAI-1, Resistin, TNF-α. The Daytona Plus, (Randox Laboratories Ltd, UK) an automated random-access clinical chemistry analyser (see section 2.3.6.) was used to detect total cholesterol, triglycerides, LDL-C, HDL-C, glucose, and Apolipoproteins A1 (ApoA1) and B (ApoB).

5.3.6. Separation of LDL subclasses

Low-density lipoprotein subclasses were separated by density gradient ultracentrifugation using iodixanol (Optiprep^{TM} Sigma Aldrich, Poole UK) (Davies, Graham and Griffin, 2003). Details of separation are described in section 2.3.8. Briefly, lipoproteins were separated using a two-step gradient of a lower layer of plasma at a concentration of 120 g/L in iodixanol and clear Tris-buffered saline solution at a concentration of 90 g/L iodixanol of the upper layer in Beckman Optiseal centrifuge tubes. In a Beckman NVT65 near-vertical rotor, samples were centrifuged at 341000$g_{(av)}$ and 16 °C for 3 h (at speed) in a Beckman Optima XL-100 ultracentrifuge. After centrifugation, 0.5 ml gradient fractions were collected from the top of sample tubes using an Autodensiflow gradient fractionator (Labconco), coupled with a Gilson FC 204 automated fraction collector. Using refractometry, the following formula: $p = na - b$, where a = 3.2984, b = 3.3967, n = refractive index, and p = density; was used to determine each fractions density. Based on the density, LDL subfractions were categorised as phenotype A (1.022-1.028 kg/L) and phenotype B (1.028-1.036 kg/L) (Davies, Graham and Griffin, 2003). Cholesterol was determined in each fraction using the Daytona Plus (Randox Laboratories Ltd, UK).

5.3.7. Diet, training and AAS use

Participants diet was assessed via 24 h dietary food recall and a food frequency questionnaire (EPIC-Norfolk) to assess average food intake in the past year (Mulligan *et al.*, 2014). A 7-day training log was used to estimate participants weekly training volume, intensity and frequency. Participants in the AAS group also took part in a semi-structured interview (Table 5.1.) to assess history of drug use.

5.3.8. Statistical analysis

GraphPad Prism (California, USA) statistical software was used for statistical analysis. Data were assessed and confirmed for normal distribution by Shapiro Wilks test. Normally distributed data underwent an independent t-test whereas non-normally distributed data underwent a Mann-Whitney U test to investigate significant differences between groups. Pearson's correlation analysis was done on normally distributed data to investigate if there were any associations between diet, training, AAS use and health markers. Non-normally distributed data underwent Spearman's correlation. All data are presented as mean ± SD unless non-normally distributed which is presented as median ± interquartile range and significance set as $P < 0.05$ and a trend as $P > 0.05 < 0.1$.

5.4. Results

5.4.1. Participants

A total of 19 participants (control participants, n=10 and AAS, n=9) were included in this cross-sectional study. Both groups were well matched for age, height and training volume but unsurprisingly, markers of body composition were significantly different (Table 5.1.). Fat free mass index (FFM/I) was significantly ($P < 0.01$) higher in the AAS group and body fat % (BF%) also showed a trend ($P = 0.05$) at being lower compared to CON (Figure 5.1). Training volume, as determined by weekly sets per muscle groups, was not significantly different between groups (Table 5.2.). Furthermore, diet was not significantly ($P > 0.05$) different between groups as shown by the 24-hr dietary recall and FFQ (Table 5.3). Dietary variables were relativised to body mass to account for differences in energy requirements. An FFM/I score > 25 kg/m^2 has been reported to be a simple tool to identify AAS users (Kouri *et al.*, 1995). One participant in the CON group had an FFM/I score of 34 kg/m^2 and had very low levels of HDL-C, characteristics of AAS use. Therefore, analysis was repeated without this participant. Removal of this participant did not significantly alter any results except for TC/HDL-C (changed from a trend ($P = 0.05$) to being significantly ($P = 0.03$) higher in the AAS group) and PAI-1 (changed from being significantly ($P = 0.03$) lower in the AAS group to a trend ($P = 0.06$)). Low levels of PAI-1 are an indication of AAS use therefore, it is unlikely this participant is an AAS user as the high levels reported by this individual is at least 10x greater than the highest reported in the AAS group. The AAS participants revealed their drug use in the previous year. All participants used injectable AAS (Figure 5.2) and some used oral AAS in addition to a range of other drugs (Figure 5.2). A variety of doses and cycles were also reported.

Table 5.1. Participant characteristics.

	CON (n=10) Mean ± SD	**CON (n=9) Mean ± SD**	**AAS (n=9) Mean ± SD**	***P* value**
Age (years)	25.75 ± 4.53	25.75 ± 4.53	27.29 ± 7.41	0.64
Height (cm)	177.03 ± 6.73	176.08 ± 6.39	177.66 ± 3.61	-
Weight (kg)	85.10 ± 19.61	79.15 ± 5.91	95.13 ± 10.10**	<0.01
Body Fat (%)	11.07 ± 2.46	10.58 ± 2.03	9.21 ± 0.90	0.09
FFM (kg)	75.37 ± 15.34	70.73 ± 4.73	86.37 ± 9.12**	<0.01
FFM/I (kg/m^2)	23.96 ± 3.82	22.84 ± 1.55	27.37 ± 2.84**	<0.01
Pushing (sets/wk)	46.1 ± 23.63	46.67 ± 24.99	42.88 ± 16.92	0.58
Pull (sets/wk)	34.6 ± 17.34	33.56 ± 18.06	34 ± 15.9	0.69
Legs (sets/wk)	25.7 ± 18.04	21.78 ± 13.9	26.88 ± 8.66	0.33
Total sets (sets/wk)	106.4 ± 40.1	102 ± 39.89	96.13 ± 29.57	0.73

Data are presented as mean ± SD, ** denotes significantly ($P < 0.01$) different from control group. *FFM, Fat free mass; FFM/I, fat free mass index*.

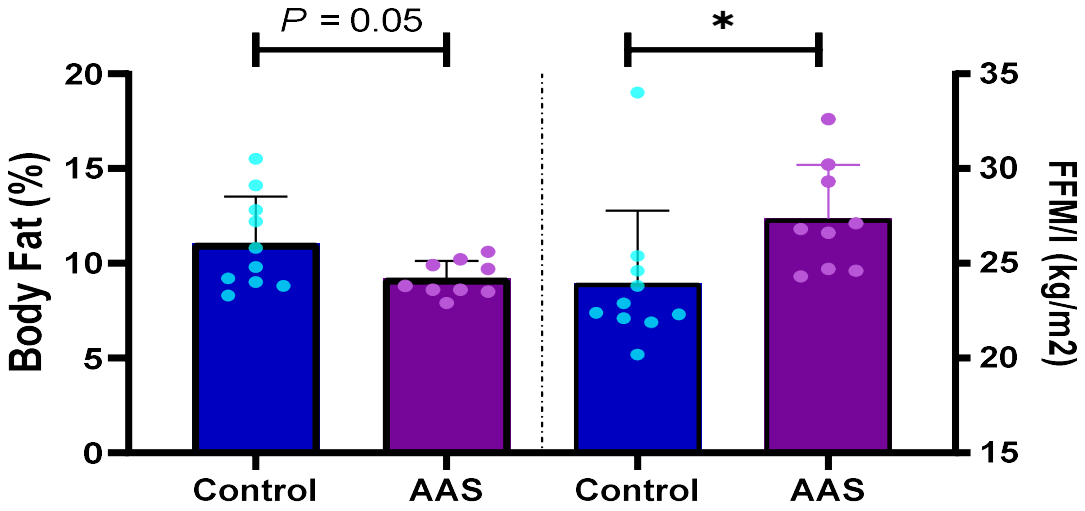
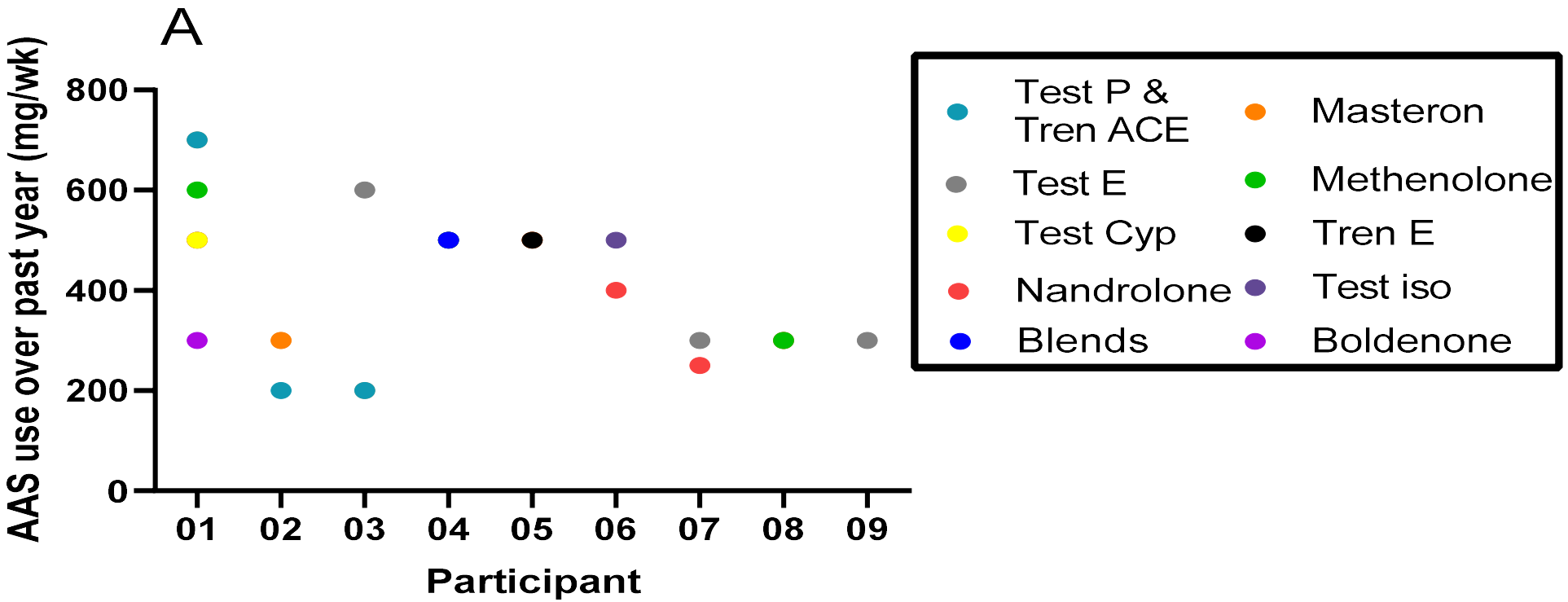


Figure 5.1. Comparison of body fat % and fat free mass index between CON and AAS users. Mean ± SD are shown, * indicates significant ($P < 0.05$) difference between groups.

Table 5.2. Comparison of diet from 24hr dietary recall and FFQ relativised to body mass.

	24 hr dietary recall			Food Frequency questionnaire		
	CON	AAS	*P* value	CON	AAS	*P* value
	Mean ± SD	Mean ± SD		Median ± IQR	Median ± IQR	
Energy (kCal/kg)	35.53 ± 13.48	38.34 ± 12.42	0.66	25.0 ± 20.22	25.32 ± 13.75	0.97
CHO (g/kg)	3.36 ± 1.38	4.78 ± 1.95	0.09	2.7 ± 2.18	2.61 ± 1.37	0.57
Sugar (g/kg)	1.09 ± 0.63	1.26 ± 0.91	0.65	1.18 ± 1.34	1.07 ± 0.53	0.36
Fibre (g/kg)	0.35 ± 0.20	0.31 ± 0.13	0.64	0.19 ± 0.23	0.17 ± 0.27	0.57
PRO (g/kg)	2.37 ± 1.0	2.62 ± 0.78	0.57	1.48 ± 1.56	1.76 ± 1.08	0.97
Fat (g/kg)	1.45 ± 0.8	1.03 ± 0.51	0.22	0.94 ± 0.74	1.13 ± 0.9	0.83
SAT (g/kg)	0.55 ± 0.39	0.35 ± 0.17	0.18	0.38 ± 0.28	0.38 ± 0.36	>0.99
MUFA (g/kg)	0.35 ± 0.27	0.22 ± 0.16	0.24	0.35 ± 0.28	0.46 ± 0.35	0.83
PUFA (g/kg)	0.13 ± 0.08	0.09 ± 0.07	0.21	0.19 ± 0.22	0.18 ± 0.18	0.83

CHO, Carbohydrates; PRO, Protein; SAT, Saturated fatty acids; MUFA, Monounsaturated fatty acids; PUFA, Polyunsaturated fatty acids


A
AAS use over past year (mg/wk)
800
600
400
200
0
01
02
03
04
05
06
07
08
09
Participant
Test P & Tren ACE
Masteron
Test E
Methenolone
Test Cyp
Tren E
Nandrolone
Test iso
Blends
Boldenone

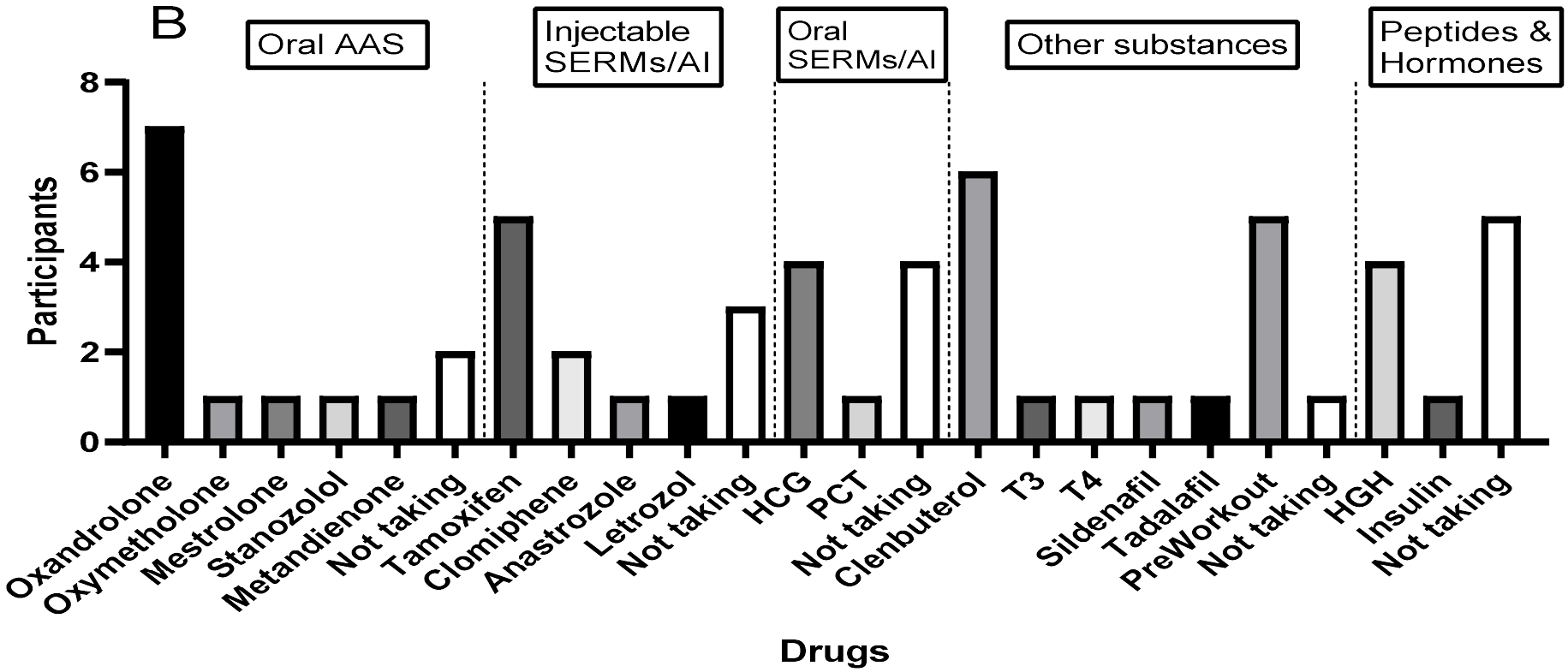


Figure 5.2. Drug use during the past year in the AAS group. A, type and amount of injectable AAS used in past year. B, the number of participants using oral AAS, injectable oestrogen, oral oestrogen, fat loss and other substances and peptides and hormones in the past year. AI, Aromatase inhibitors; HCG, Human chorionic gonadotropin; HGH, human growth hormone; PCT, post-cycle therapy; SERMs, Selective oestrogen modulators; T3, Triiodothyronine; T4, thyroxine; Test Cyp, Testosterone cypionate; Test E, Testosterone enanthate; Test Iso, Testosterone isocaporate; Test P, Testosterone propionate; Tren Ace, Trenbolone acetate; Tren E, Trenbolone Enanthate.

5.4.2. Metabolic health markers

A key feature of MetS, HDL-C was shown to be significantly (*P* = 0.04, Cohens *d*: -1.36) lower in AAS users compared to CON (Figure 5.3A). Other features of MetS, plasma triglycerides, and IR (determined by HOMA IR) were not significantly different between groups (Table 5.3). Additional measures that are associated with metabolic health; ApoA1 (Cohens *d*: -1.42) and PAI-1 (Cohens *d*: -0.70) were significantly (*P* < 0.05) lower in the AAS group (Figure 3B & C). The ratio of ApoB/A1 (*P* = 0.06) and TC/HDL-C (*P* = 0.05) also showed trends of being higher in the AAS group compared with CON, whereas, LDL-C, non-HDL-C, Insulin, ApoB, LDL-C/ApoB, glucose, triglycerides/HDL-C, leptin, ferritin, IL-6 and TNFα were not significantly different (Table 5.3).

Table 5.3. Comparisons of markers of metabolic health in AAS users and controls.

	CON (n=10)	CON (n=9)	AAS (n=9)	*P* Value, *Effect size*
TC (mmol/L)	4.01 ± 0.75	4.07 ± 0.77	4.00 ± 0.66	0.86, *-0.09*
TGs (mmol/L) $	0.79 ± 0.5	0.7 ± 0.49	0.8 ± 0.42	0.80, *0.37*
ApoB (mg/dl)	63.80 ± 14.76	64.33 ± 15.56	68.89 ± 15.43	0.54, *0.29*
LDL-C (mmol/L)	2.57 ± 0.70	2.55 ± 0.74	2.81 ± 0.84	0.50, *0.33*
LDL-C/ApoB	0.72 ± 0.07	0.71 ± 0.07	0.73 ± 0.08	0.61, *0.22*
Glucose (mmol/L)	5.60 ± 0.28	5.60 ± 0.29	5.55 ± 0.77	0.87, *-0.09*
TG/HDL-C$	0.72 ± 0.62	0.62 ± 0.45	0.89 ± 0.58	0.28, *0.80*
HDL-C/Apo1$	0.14 ± 0.03	0.15 ± 0.03	0.13 ± 0.02	0.14, *-0.81*
ApoB/A1	0.46 ± 0.11	0.45 ± 0.11	0.60 ± 0.19	0.05, *1.02*
Non-HDL-C (mmol/L)	2.91 ± 0.71	2.91 ± 0.75	3.12 ± 0.66	0.55, *0.29*
TC/HDL-C	3.75 ± 0.86	3.58 ± 0.73*	4.72 ± 1.17	0.03, *1.20*
Pattern A (mmol/L)	2.4 ± 1.25	2.4 ± 1.33	3.51 ± 1.53	0.12, *0.31*
Pattern B (mmol/L)	1.08 ± 0.73	0.92 ± 0.55	1.03 ± 0.51	0.65, *0.40*
Insulin (pmol/L) $	27.06 ± 28.38	24.33 ± 24.72	34.08 ± 31.47	0.54, *0.32*
HOMA IR$	1.1 ± 1.2	1.0 ± 1.05	1.4 ± 1.45	0.66, *0.38*
Leptin (ng/ml) $	1.59 ± 0.88	1.46 ± 0.63	1.2 ± 1.11#	0.21, *-0.78*
Ferritin (ng/ml)	78.15 ± 46.39	74.26 ± 48.0	99.67 ± 80.44	0.45, *0.40*
IL-6 (pg/ml) $	0.91 ± 0.84	0.88 ± 0.59	1.24 ± 2.1	0.15, *0.09*
Resistin (ng/ml)	2.36 ± 0.82	2.36 ± 0.88	2.76 ± 1.22	0.46, *0.38*
TNFα (pg/ml)	4.22 ± 1.16	4.04 ± 1.11	4.15 ± 1.66	0.88, *-0.70*

Data are presented as mean ± SD unless otherwise stated. $ indicates presented as median ± interquartile range. * *P* < 0.05, denotes significantly different vs AAS group.# 2 participants reported undetectable levels of leptin. *ApoA1, apolipoprotein A; ApoB, apolipoprotein B; HDL-C, high-density lipoprotein cholesterol; HOMA IR, homeostatic model of insulin resistance; IL-6, interleukin 6; LDL-C, low-density lipoprotein cholesterol; TC, total cholesterol; TG, triglycerides; TNFα, Tumour necrosis factor alpha.*

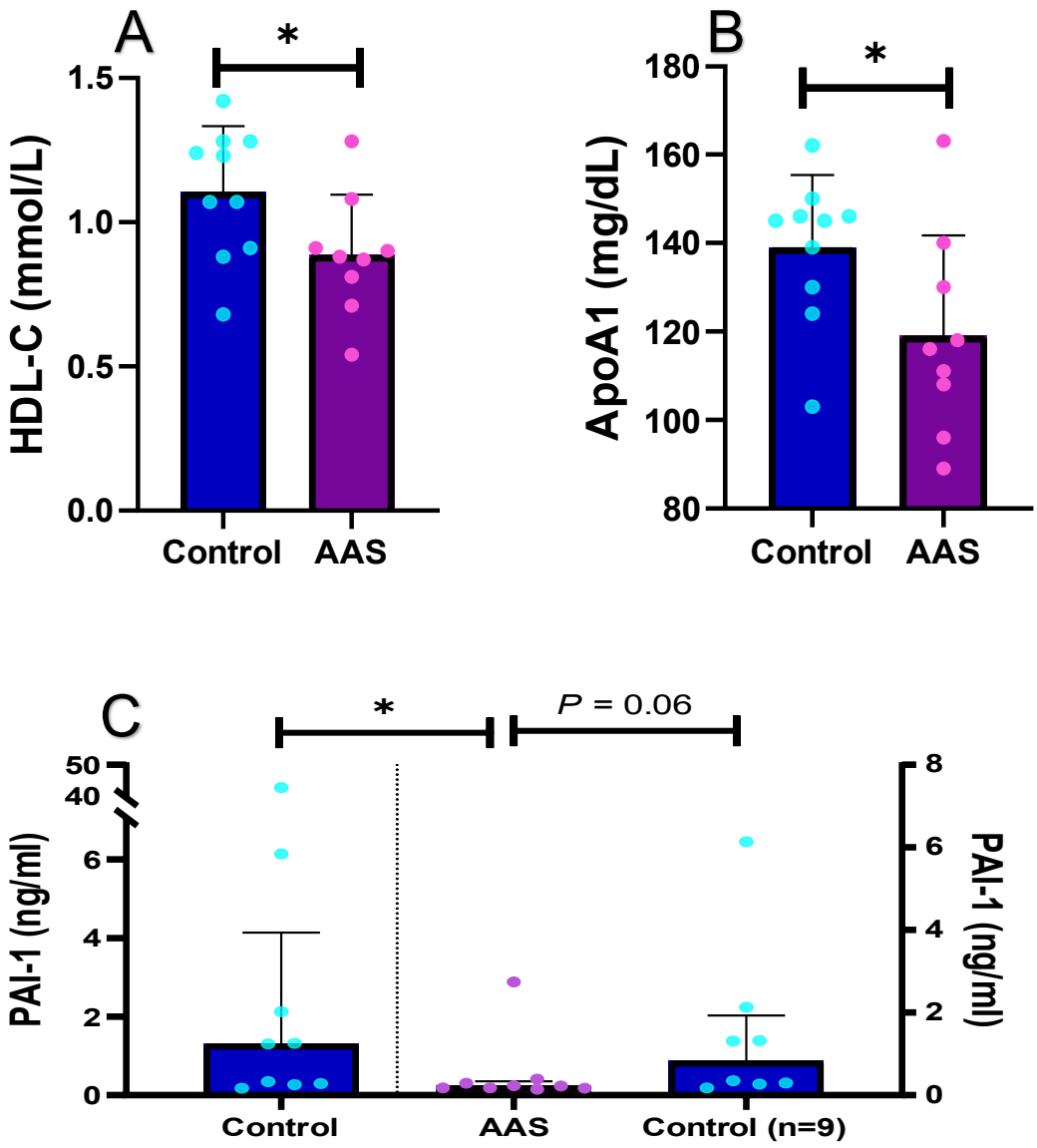


Figure 5.3. Comparison of MetS markers and markers of CVD risk. A, HDL-C (mean ± SD); B, ApoA1 (mean ± SD); C, PAI-1 (median ± Interquartile range) with outlier also removed. * indicates significant (P < 0.05) difference between groups. *ApoA1, apolipoprotein A; HDL-C, high-density lipoprotein cholesterol; PAI-1, plasminogen activator inhibitor 1.*

5.4.3. Association of AAS use with markers of metabolic health and CVD risk.

Interestingly, higher doses of AAS may showed a positive association with LDL-C and composition, indicating an increased CVD risk (Table 5.4). Increasing doses of AAS showed a moderate, significantly positive relationship with total cholesterol (r = 0.60, P = 0.04), LDL-C (r = 0.65, P = 0.03), non-HDL-C (r = 0.67, P = 0.02) ApoB (r = 0.69, P = 0.02), ApoB/A1 (r = 0.64, P = 0.03), leptin (r = 0.59, P = 0.048) and strong significantly positive association with pattern A (r = 0.72, P = 0.02) and pattern B (r = 0.96, P < 0.001) (Figure 5.4).

Table 5.4. Correlation of markers of metabolic syndrome and body composition with AAS use.

Cardiometabolic health measure	**AAS dose** (*n*=9) **P, *r***
Triglycerides	0.34, *0.17*^{S}
Total cholesterol	0.04, *0.60*
HDL-C	0.29, *-0.21*
LDL-C	0.03, *0.65*
Non-HDL-C	0.02, *0.67*
Pattern A	0.02, *0.72*
Pattern B	<0.001, *0.96*
Triglycerides/HDL-C	0.37, *0.13*^{S}
Apolipoprotein A1	0.30, *-0.21*
Apolipoprotein B	0.02, *0.69*
Apolipoprotein B/A1	0.03, *0.64*
Total cholesterol/HDL-C	0.06, *0.50*
HDL-C/Apolipoprotein A1	0.44, *-0.06*
LDL-C/Apolipoprotein B	0.14, *0.41*
Glucose	0.44, *-0.05*
Insulin	0.38, *-0.12*
HOMA IR	0.44, *0.05*
Leptin	0.048, *0.59*
Interleukin-6	0.50, *0.01*^{S}
TNFα	0.21, *-0.30*
Ferritin	0.45, *-0.05*
Resistin	0.37, 0.13
PAI-1	0.22, *0.29*^{S}
Body Fat %	0.48, *0.02*
FFM/I	0.47, *-0.03*

All data analysed by Pearsons correlation unless stated. ^{S} denotes Spearmans correlation. Significance set at $P < 0.05$. *FFM/I, fat free mass index; HDL-C, high-density lipoprotein cholesterol; HOMA IR, homeostatic model of insulin resistance; LDL-C, low-density lipoprotein cholesterol; PAI-1, plasminogen activator inhibitor 1 TNFα, Tumour necrosis factor alpha.*

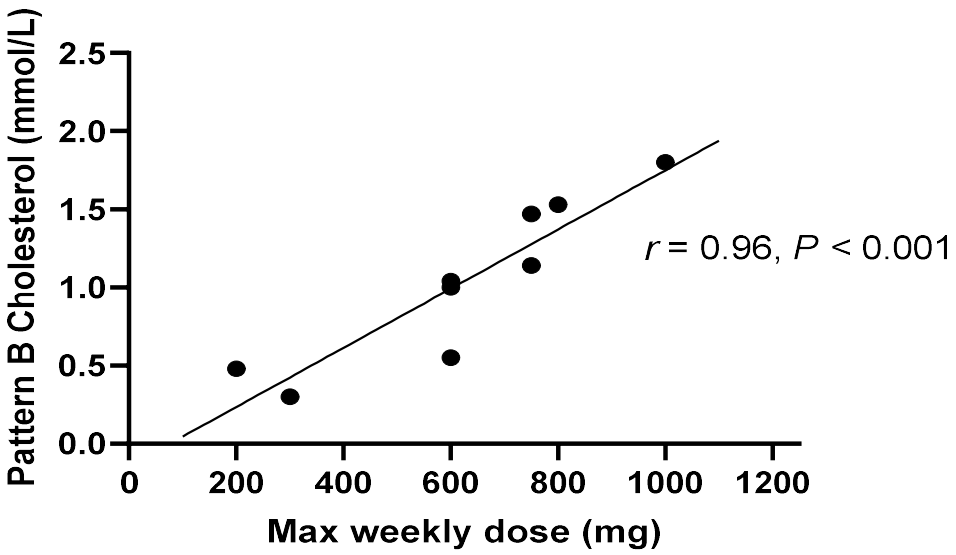


Figure 5.4. Association of maximum weekly AAS dose with pattern B LDL-C.

5.5. Discussion

The main outcome of this study, in somewhat in agreement with hypothesis 1, users of AAS display a significant (P = 0.04) suppression of HDL-C, a key feature of MetS. However, they cannot be considered to have MetS as they did not exhibit hyperglycaemia (>5.5 mmol/L) or increased plasma triglyceride levels (>1.7 mmol/L) (Alberti *et al.*, 2009). Furthermore, associated markers of MetS, IR (HOMA IR) and inflammation (TNF-α, IL-6) were also not significantly (P > 0.05) different between groups. Furthermore, in agreement with hypothesis 2, users of AAS also displayed significant (P = 0.04) decreases in ApoA1 and a trend (P = 0.06) of increasing ApoB/A1 ratio, both indicating an increase in CVD risk. In accordance with hypothesis 3, The highest users of AAS were also observed to be associated with significantly higher levels of with LDL-C (r = 0.65, P = 0.03), ApoB (r = 0.69, P = 0.02), pattern A (r = 0.72, P = 0.02) and pattern B (r = 0.96, P < 0.001).

It is well established that AAS use results in abnormal lipid metabolism (Glazer, 1991; Achar, Rostamian and Narayan, 2010). Use of AAS has shown a reduction in HDL-C of ≥70% and increased LDL-C levels of >20% (Achar, Rostamian and Narayan, 2010). HDL-C is a key feature of MetS, the HDL particle (HDL) transports cholesterol away from cells back to the liver to be excreted, through a process of reverse cholesterol transport, thereby reducing CVD risk (Fuster *et al.*, 2005; Santos-Gallego *et al.*, 2011). HDL of which apolipoprotein A1 (ApoA1) is the major apolipoprotein, also has an anti-inflammatory and antioxidant effect on the vascular system further reducing the potential of CVD (Barter *et al.*, 2004; Rhee, Byrne and Sung, 2017). In the current study, the AAS group showed significantly (P < 0.05) lower levels of HDL-C (-20%) and ApoA1 (-15%). This lower HDL-C level (0.89 ± 0.21 mmol/L) with AAS use is also deemed clinically significant as <1 mmol/L is the clinical cut-off point for identification of MetS and indicates increased CVD risk (Sperling *et al.*, 2015). Similarly, although a greater decrease, a recent cross-sectional study in males reported that current users of AAS (n = 37) showed a significant (P < 0.01) 45% reduction in HDL-C compared to non-AAS using controls (n = 30) (Rasmussen *et al.*, 2017). These results are also in accordance with AAS self-administration and randomised control trials (Lenders *et al.*, 1988; Thompson *et al.*, 1989; Bhasin *et al.*, 2001; Hartgens *et al.*, 2004). Prospective studies on the effects of AAS self-administration on lipoprotein metabolism have reported significant (P < 0.01) decreases in HDL-C and ApoA1 by 53% and 41% respectively after 8 weeks of administration (Lenders *et al.*, 1988; Hartgens *et al.*, 2004). This negative impact on HDL-C and ApoA1 are maintained after 14 weeks of self-administration (Hartgens *et al.*, 2004). A randomised crossover trial with a 6 to 9-week washout between AAS groups in males (n = 11), has shown oral stanozolol (42 mg/wk) use to significantly decrease HDL-C and ApoA1

greater than 200 mg/wk of intramuscular Testosterone Enanthate (Test E) administration by 24% and 32% respectively after 6 weeks (Thompson *et al.*, 1989). Furthermore, a randomised double blind placebo control trial in males (*n* = 16) without previous AAS use, reported no significant effect of 200 mg/wk of nandrolone decanoate on HDL-C or ApoA1 (Hartgens *et al.*, 2004). The lack of effect of nandrolone on HDL-C and ApoA1 is most likely due the dose and length of time of use as Singh *et al.* (2002) reported an inverse dose response (25, 50 125, 300, 600 mg/wk) effect of Test E administration for 20 weeks on HDL-C and ApoA1. However, post-hoc analysis showed that only a dose of 600 mg/wk significantly reduced HDL-C and ApoA1 after 20 weeks (Singh *et al.*, 2002). Although, self-administration prospective studies may be less controlled, they may be more representative of the population as it replicates the methods used by this unique population. Nonetheless, these differences in methods may be why in the current study LDL-C and its major lipoprotein ApoB were not significantly ($P > 0.05$) different to healthy controls. Although not considered a feature of MetS but rather an association, small-dense low-density lipoprotein cholesterol (sdLDL-C), is a stronger predictor of CVD risk compared with larger LDL particles (Hoogeveen *et al.*, 2014). LDL primarily comprises of ApoB and can be separated into 2 distinct phenotypes, pattern A, which contains primarily large buoyant LDL particles and pattern B which contains small dense lipoprotein particles; the latter considered a more atherogenic phenotype (Diffenderfer and Schaefer, 2014). Although LDL-C and ApoB levels were similar between controls and AAS users, higher doses of AAS were observed to have a moderate positive association with LDL-C ($r = 0.65$, $P = 0.03$) and ApoB ($r = 0.69$, $P = 0.02$) and a strong positive association with pattern A ($r = 0.72$, $P = 0.02$) and pattern B ($r = 0.96$, $P < 0.001$).

Previous cross-sectional research has shown LDL-C to be significantly higher in AAS users, up to 37%, compared to healthy controls (Lenders *et al.*, 1988; Rasmussen *et al.*, 2017). Furthermore, pre/post self-administration trials have reported LDL-C to increase by 30% after 8 weeks and its primary apolipoprotein ApoB to increase by 27% (Lenders *et al.*, 1988; Hartgens *et al.*, 2004). Similarly, to its deleterious effects on HDL-C, orally administered stanozolol increased LDL-C and ApoB by 22% and 26% respectively after 6 weeks. Surprisingly in the same study Test E alone had perhaps beneficial reducing effects on LDL-C and ApoB (Thompson *et al.*, 1989). This highlights that AAS administration route may have a significant impact on metabolic health.

The mechanisms by which AAS negatively impact lipid metabolism are not fully understood, but the upregulated activity of hepatic triglyceride lipase (HTGL) has been implicated (Thompson *et al.*, 1989; Glazer, 1991). Phospholipase activity of HTGL catabolises HDL and its removal from the plasma and conversion of idLDL to sdLDL (Glazer, 1991; Santamarina-Fojo *et al.*, 2004). The contrasting effects of orally administered stanozolol on lipoprotein

metabolism compared with injected Test E may be explained by its significant (P < 0.05) increase in HTGL activity (Thompson *et al.*, 1989). Therefore, orally administered AAS which are 17α-alkylated steroids, may have a greater detrimental effect on metabolic health perhaps as it has to circulate via the liver thereby increasing HTGL activity compared to parentally administered AAS, in addition to its increased risk of hepatoxicity (Hartgens and Kuipers, 2004; Solbach *et al.*, 2015; Niedfeldt, 2018). Therefore, in addition to, dose, type and length of AAS use, the route of AAS administration also has a profound impact on lipoprotein metabolism which can lead to a large variability in AAS self-administration observational studies.

Although this cross-section of AAS users display suppressed HDL-C, they are not more likely to display MetS as there was no significant (P < 0.05) difference in triglycerides, HOMA IR and inflammatory markers compared to healthy controls. Furthermore, only HDL-C was below the recommended clinical levels for MetS identification (Alberti *et al.*, 2009). Previous cross-sectional research in male bodybuilders revealed that current and former AAS users had impaired glucose tolerance compared to healthy controls (Rasmussen *et al.*, 2017). AAS users were also reported to have significantly (P < 0.01) reduced leptin levels however, in contrast, high leptin levels are associated with IR and MetS (Esteghamati *et al.*, 2011; Rasmussen *et al.*, 2017; D'Elia *et al.*, 2019). Leptin is pleiotropic hormone produced by adipocytes which regulates body weight and energy homeostasis (Sadaf Farooqi and O'Rahilly, 2009). However, leptin resistance typically occurs in MetS and obese populations thereby reducing its ability to help regulate energy homeostasis (Sáinz *et al.*, 2015). Although no significant difference in leptin levels were observed in the current study between AAS and CON groups, the leptin levels of the AAS group (*median* ± *IQR*: 1.2 ± 1.1 ng/ml) are similar to what was reported by Rasmussen *et al*. (2017) (*median* ± *IQR*: 1.2 ± 1.7 ng/ml). Furthermore, 2 participants from the AAS group reported undetectable leptin concentrations. AAS use may induce leptin deficiency which is also associated with obesity and IR (German *et al.*, 2010). In some cases, AAS use may induce leptin deficiency perhaps due to reducing body fat to extremely low levels, resulting in impaired glucose tolerance. Consequently, treatment with leptin may improve glucose sensitivity and metabolic health (Yaspelkis *et al.*, 2001; German *et al.*, 2010). Yet, further research is required on the effects of AAS use on leptin concentrations and potential mechanisms of action.

Fibrinolysis involves the breakdown of fibrin to control blood vessel patency and is largely regulated by PAI-1 (Alessi and Juhan-Vague, 2006). PAI-1 is prothrombotic and increasing levels are associated with MetS and CVD risk (Mertens *et al.*, 2006). Interestingly, AAS use has shown to decrease circulating levels of PAI-1 (Chang *et al.*, 2018). A previous cross-sectional study showed a trend (P = 0.07) of lower PAI-1 levels in AAS users compared to

healthy controls (Ferenchick *et al.*, 1995). Furthermore, oral oxandrolone treatment decreased PAI-1 levels in healthy males after 3 days and remained suppressed during treatment (Kahn *et al.*, 2006). This decrease in PAI-1 increases fibrinolytic activity thus reducing vascular thrombosis; however, AAS use simultaneously increases pro-coagulatory factors resulting in a balanced haemostatic system (Kahn *et al.*, 2006). The low levels of PAI-1 maybe more of a biomarker of AAS use due to a protective regulatory mechanism rather than a reduction in CVD risk.

Interestingly, both groups were observed to be very well matched in terms of training volume and dietary intake indicating that differences in results are largely due to AAS use. Training variables such as exercise selection, rep ranges and load were highly variable making it difficult to compare training volume accurately. Using weekly sets in a plane of motion was used to provide an indication of training volume as there is a dose response relationship of weekly sets with increasing muscle mass (Schoenfeld, Ogborn and Krieger, 2017). No differences in dietary intake were also observed between groups yet, both methods reported divergent results. Relative carbohydrate and protein intake was estimated to be higher and fat intake was estimated to be lower in the AAS group when determined by the 24-hr dietary recall. However, when measured by the food frequency questionnaire relative carbohydrate and fat intake was slightly lower and higher respectively in the AAS group. Use of a 24hr dietary recall and food frequency questionnaire allowed for differences in current dietary intake and average yearly dietary intake to be assessed. Use of both measures are recommended to account for limitations of both methods (Shim, Oh and Kim, 2014). This is particularly needed in the current population as they typically cycle between phases of “bulking” and “cutting” (Lenzi *et al.*, 2019).

5.6. Limitations

As with all cross-sectional designs, causation cannot be determined; therefore, only associations between AAS use and metabolic health can be inferred. Furthermore, this study may have included type II errors as a sample size of n = 9 AAS users (70% power) is small and limits the relevance to a larger population. However, access to this population is limited due to the illicit nature of acquiring AAS and requires a long-term relationship with AAS using communities to build trust. Moreover, the NAT and AAS groups were very well matched as shown by their dietary and training records which would increases the reliability of attributing the difference in metabolic health to AAS use. Due to the unregulated use of AAS, individuals use a variety of doses, types, frequencies of AAS and AAS-related polypharmacy. This increases the variability of results in the association between AAS use and metabolic health in comparison with similar research. Several biochemical analyses were unable to be measured due to resource or logistic limitations such as testosterone and VAT levels. As testosterone levels were not measured it was difficult to determine the effect of AAS use on circulating levels of testosterone which may have impacted results. However, AAS use was recorded which demonstrated that all users were taking supraphysiological doses of AAS which would have resulted in increased circulating levels. Furthermore, although all participants arrived fasted for 12 hours, the time between blood sample collection and last AAS administration and/or exercise session was not controlled for prior to this which may have triggered acute effects in addition to the chronic effects on blood metabolic health markers.

5.7. Conclusion

In conclusion, use of AAS results in suppression of HDL-C and its primary apolipoprotein ApoA1 leading to increased CVD risk. This cross-section of AAS users did not display MetS as they did not show significantly different levels of plasma triglycerides, inflammatory markers or IR. Although no significant difference in LDL-C levels were observed between groups, higher doses of AAS use showed a moderate positive association with LDL-C and its major apolipoprotein, ApoB and a strong positive association with LDL phenotypes pattern A and B. Methods of AAS use are highly variable between individuals due to different doses, administration techniques and cycles being used which may lead to contrasting results between studies. However, these prospective self-administration studies may be more indicative of the health implications of AAS use as they replicate the exact practices of this unique population. Future studies should investigate changes in metabolic health throughout the long-term cycling of AAS to help elucidate which practices may be the most detrimental to cardiovascular health. Furthermore, the effects of using a myriad of anabolic pharmaceuticals are yet to be investigated on mechanisms of skeletal muscle health and are to be examined in chapter 6.

Chapter 6: The impact of human serum on insulin and anabolic signalling in C2C12 skeletal muscle cells: Effect of anabolic-androgenic steroids.

6.1. Abstract

Introduction: Anabolic-androgenic steroids (AAS) increase skeletal muscle hypertrophy by upregulating muscle protein synthesis (MPS), satellite cell activation and possibly by decreasing catabolic pathways. However, AAS use is associated with dyslipidaemia and possibly impaired insulin sensitivity, contributing to increased risk of cardiovascular disease (CVD). Use of serum from AAS-using or non-using participants, in cell culture, may represent a physiological model to investigate insulin sensitivity. The objective of this study was to investigate the effect of serum from AAS using and natural (NAT) resistance trained males on C2C12 skeletal muscle metabolism. It was hypothesised that chronic AAS serum stimulation would impair insulin signalling and glucose uptake, increase myoblast fusion and myotube anabolic signalling.

Methods: Human serum was collected from AAS-using (n = 9) or non-using (n = 10) resistance trained males and pooled within their groups. C2C12 myoblasts and myotubes were pre-conditioned in conventional horse serum (DM), NAT or AAS serum for 3 or 120 hours, respectively before being measured for insulin signalling and glucose uptake. Akt^{ser473} phosphorylation (p-Akt^{ser473}) was determined after 20 and 30 mins. Insulin induced glucose uptake was measured after 30 mins using 2-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino)-2-Deoxyglucose. Creatine kinase activity was measured at 0, 48, 96 and 120 hours after initiation of differentiation in DM, NAT and AAS serum. Signalling (Akt^{ser473}, $mTOR^{ser2448}$ (myoblast only), $ERK1/2^{T202/Y204}$ and $P38^{t180/Y182}$), responses of myoblasts and myotubes were determined at 0, 15, 30, 60 mins post serum stimulation. Puromycin incorporation (SUnSET method) was measured to determine MPS at 1, 6 and 24 hr and puromycin removal was measured to determine protein breakdown (MPB) at 30, 48 and 72 hr post serum stimulation.

Results: C2C12 myoblasts and myotubes significantly (P < 0.01) increased p-Akt^{ser473} in response to 100 nM of insulin in DM, NAT and AAS conditions. No differences were observed between groups. Insulin significantly (P = 0.04) increased glucose uptake in myotubes and myoblasts (trend, P = 0.08) comparably between conditions. CK activity significantly (P < 0.01) increased comparably between conditions yet protein abundance was significantly (P = 0.04) higher in AAS serum *vs.* DM after 96 hr. In myoblasts, all conditions significantly (P < 0.05) increased Akt^{ser473}, $mTOR^{ser2448}$, $ERK1/2^{T202/Y204}$ and $P38^{t180/Y182}$ phosphorylation. ERK and P38 phosphorylation were significantly (P < 0.05) higher in NAT and AAS serum compared to DM. In myotubes, all conditions similarly significantly (P < 0.05) increased p-Akt^{ser473} and p-$ERK^{T202/Y204}$ at 30 mins. MPS and the rate of MPB was similar between groups at all timepoints.

Conclusion: Serum derived from AAS users does not impair insulin signalling or glucose uptake compared to non-users. However, muscle fusion and anabolic signalling was similar between AAS and NAT serum indicating a lack of hypertrophic response with AAS use.

6.2. Introduction

Testosterone and its anabolic-androgenic steroid (AAS) derivatives are known to increase skeletal muscle hypertrophy by upregulating muscle protein synthesis (MPS) and accretion, satellite cell activation and possibly by decreasing catabolic pathways (Cheung and Grossmann, 2018). When AAS are used in combination with resistance exercise, skeletal muscle hypertrophy is enhanced most likely due to a synergistic increase in anabolic signalling (Bhasin *et al.*, 1996; Zeng, Zhao and Liao, 2017). However, AAS users are at a higher risk of developing cardiovascular disease (CVD) and range of other disorders including: psychological disorders, neuroendocrine disorders and sex-specific disorders (aromatisation and hypogonadism in males and virilisation in females) (Pope et al., 2014; Baggish et al., 2017; Bjørnebekk et al., 2017; Westlye et al., 2017; Goldman et al., 2019). Nevertheless, the direct impact of AAS use on health is difficult to determine as other substances such as insulin-like growth factor-I (IGF-I) and growth hormone; drugs to prevent AAS-related adverse effects, other image enhancing drugs (clenbuterol, diuretics and thyroid hormones) and psychoactive drugs are also reportedly used to complement their range of AAS types, doses and cycles (Sagoe et al., 2015; Begley et al., 2017).

AAS exert their effects via genomic and non-genomic pathways. Genomic actions of AAS occur when androgens bind to the androgen receptor located in the cytoplasm which results in their translocation to the nucleus (Davey and Grossmann, 2016). At the nucleus the androgen/receptor complex moderates gene transcription by binding to the androgen response element of the DNA (Bennett *et al.*, 2010; Parr *et al.*, 2018). This in turn, upregulates expression of genes related to protein accretion and anabolism such as insulin growth factor-I (IGF-I), nutrient sensing, storage and transporting (Lipin, GLUT3 and System A Transporter 2) and satellite cell differentiation (myogenin), while also increasing satellite cell number (Sheffield-Moore, 2000; Lee, 2002; Sinha-Hikim *et al.*, 2003; Haren *et al.*, 2011). Androgen response element binding may also downregulate genes involved in muscle atrophy such as inhibitory-Kappa kinase alpha (Haren *et al.*, 2011; Rossetti, Steiner and Gordon, 2017). Non-genomic actions of AAS are characterised by the speed in which they exert their effects (within minutes) thus indicating activities independent of transcription (Deng *et al.*, 2017; Parr *et al.*, 2018). AAS have been reported to exert non-genomic effects via membrane-located receptors; membrane-located AR, endothelial growth factor receptor and sex hormone binding globulin receptor (Parr *et al.*, 2018). Binding of these receptors by AAS leads to an increase in intracellular calcium and activation of several second messenger signalling cascades including; mitogen-activated protein kinases (MAPK), extracellular regulated kinases 1/2 (ERK 1/2), protein kinase A (PKA), calmodulin and phosphatidylinositol-3-phosphate kinase

(PI3K)/Akt/mammalian target of rapamycin complex 1 (mTORc1) pathways (Hamdi and Mutungi, 2010; Antinozzi *et al.*, 2017; Parr *et al.*, 2018). Activation of PI3K/Akt by testosterone, triggers mTORc1, a key regulator of protein turnover via activation of the eukaryotic initiation factor 4E-binding protein 1 (4E-BP1) and p70S6 kinase S6K1 (S6K1) (Basualto-Alarcón *et al.*, 2013; Liu and Sabatini, 2020). These signalling cascades upregulate transcription, satellite cell proliferation, MPS and reduce apoptosis ultimately resulting in skeletal muscle hypertrophy (Estrada *et al.*, 2003; Hamdi and Mutungi, 2010; Antinozzi *et al.*, 2017; Parr *et al.*, 2018).

The use of AAS reportedly results in polycythaemia, reduced left ventricular and diastolic function and accelerated atherosclerosis compared to non-use (Stergiopoulos *et al.*, 2008; Baggish *et al.*, 2017). AAS use may affect blood pressure (BP) and metabolism which ultimately increases CVD risk in addition to altered cardiac function (Achar, Rostamian and Narayan, 2010). Furthermore, AAS use can increase low-density lipoprotein cholesterol (LDL-C) and decrease high-density lipoprotein cholesterol (HDL-C) increasing the risk of developing atherosclerosis (Achar, Rostamian and Narayan, 2010). AAS use may also result in lower insulin sensitivity and higher levels of visceral adipose tissue (VAT) compared to matched controls thereby sharing similar metabolic characteristics of obese/sedentary populations such as Metabolic syndrome (MetS) and increasing risk of CVD (Rasmussen *et al.*, 2017). For example, an oral glucose tolerance test (OGTT) between healthy controls, steroid using bodybuilders and former steroid using bodybuilders (mean discontinuation of 2.5 years) revealed that current and former AAS users had impaired glucose tolerance compared to healthy controls (Rasmussen *et al.*, 2017). Interestingly, although current users of AAS had lower % body fat compared to healthy controls and former users, they had significantly greater levels of VAT and reduced adiponectin and leptin levels which are all independent predictors of insulin resistance (IR), type 2 Diabetes (T2D) and MetS (Hung *et al.*, 2008; Preis *et al.*, 2010; Frühbeck *et al.*, 2017; Rasmussen *et al.*, 2017; D'Elia *et al.*, 2019). Additionally, powerlifting steroid users had higher fasting insulin levels compared with non-using powerlifters and were similar to those observed in obese participants (Cohen and Hickman, 1987). An OGTT also revealed the powerlifting steroid users to have a 2-fold increase in post-glucose glycaemia compared to non-users, which again was a similar increase to the obese group. Post-glucose insulinaemia in the powerlifting steroid users was also higher compared to all groups and at least 2-fold higher compared to obese participants (Cohen and Hickman, 1987). In contrast, doses (>300 mg/wk) of testosterone for 20 weeks or nandrolone (300 mg/wk) for 6 weeks had no significant effect on insulin sensitivity or glucose tolerance in males (Hobbs, Jones and Plymate, 1996; Singh *et al.*, 2002). These results indicate that AAS polysubstance use may be with the cause of impaired glucose metabolism rather than use of

an individual AAS.

As skeletal muscle is the largest tissue for glucose disposal (Defronzo *et al.*, 1981), increases in muscle mass should improve insulin sensitivity; paradoxically, the results above indicate that chronic AAS polysubstance use may cause tissue IR. This may be due to an imbalance of regulatory adipokines and cytokines from increased VAT levels and circulating lipids leading to a decreased/delayed stimulus of the PI3K/Akt signalling cascade in response to glucose ingestion, as also observed in T2D individuals (Samuel and Shulman, 2016). Furthermore, nutrient overload is reported to increase IR via mTORc1 dependent pathway. Chronic activation of S6K1 mediated by mTORc1, inflicts serine phosphorylation of insulin receptor substrate 1 (IRS1) leading to reduced insulin sensitivity (Um, D'Alessio and Thomas, 2006; Yoon and Choi, 2016). It is possible that chronic AAS use, leading to hyperactivation of mTORc1/S6K1 signalling may cause IR. Oestradiol has also shown to be a key regulator of insulin sensitivity (Gupte, Pownall and Hamilton, 2015) and has been shown to be significantly ($P < 0.01$) elevated with AAS use compared to healthy males (Rasmussen *et al.*, 2017). Deficient levels of circulating oestradiol are associated with IR in males and females, and oestradiol treatment is reported to restore insulin sensitivity (Gupte, Pownall and Hamilton, 2015; Galmés-Pascual *et al.*, 2020). However, in contrast, conversion of testosterone to oestradiol due to an increase in aromatase activity results in a decrease in the testosterone to oestradiol ratio which has been implicated in the development of MetS in older males (Maggio *et al.*, 2010; Carrageta *et al.*, 2019). Furthermore, in a large population of post-menopausal women (N = 3117), increased total oestradiol concentrations were associated with future T2D incidence and was only slightly attenuated ($P = 0.07$) when adjusted for metabolic risk factors (BMI, glucose and insulin) (Muka *et al.*, 2017). Potential mechanisms are yet to be elucidated yet, oestradiol is reported to bind to insulin and the insulin receptor further highlighting its potential role in inducing IR (Root-Bernstein, Podufaly and Dillon, 2014).

The C2C12 murine cell line is an established model for studying skeletal muscle *in vitro* however, cellular models, using highly defined culture conditions, rarely replicate the physiological environment seen *in vivo*. Furthermore, there is a lack of research on the impact of AAS polysubstance use on skeletal muscle health, which is more typical of AAS users. Use of serum derived from individuals who use AAS may replicate a more physiologically relevant cellular environment to help elucidate the impact of AAS on skeletal muscle insulin signalling and metabolism.

Therefore, the primary objective of this study was to investigate the effect of serum derived from resistance trained males who do not use and who do use AAS on skeletal muscle

metabolism including; insulin stimulated signalling and glucose uptake, myoblast differentiation and protein synthesis. The underpinning objectives were:

1. To determine if insulin signalling is different between serum conditions (DM vs NAT vs AAS),
2. Investigate if serum conditions differentially effect insulin induced glucose uptake,
3. Determine the impact of serum conditions on myoblast differentiation to myotubes and potential signalling mechanisms, and
4. Investigate the effect of serum on protein synthesis and degradation using the surface sensing of translation (SUnSET) method (Schmidt *et al.*, 2009).

It was hypothesised that:

1. Chronic AAS serum incubation would impair insulin signalling vs NAT and DM in myotubes but acute incubation in myoblasts would not,
2. Chronic incubation of AAS serum would impair insulin induced glucose uptake vs NAT and DM in myotubes but acute incubation in myoblasts would not,
3. Myoblast differentiation would be enhanced in conditioned serum vs DM, with the AAS group showing enhance anabolic signalling and fusion rates and,
4. That myotube protein synthesis would be enhanced in conditioned serum vs DM, with the AAS group showing greater rates of protein synthesis and reduced rates of protein degradation.

6.3. Methods

6.3.1. Participants

Following ethical approval and informed consent, resistance trained males who do not use AAS (NAT) and who do use AAS (AAS) were recruited for this study, to obtain serum samples. Participants were requested to attend LJMU laboratories in the morning, having been fasted for 12 hours. Within-group serum was pooled and heat inactivated at 56 C° for 30 mins. For details of methods, participants and results relating to the participants see study 3, chapter 5 (section 5.3).

6.3.2. Cell culture

C2C12 murine skeletal muscle cells were purchased from ATCC and passages 8-12 were used. For detailed cell culture see general methods section 2.2. Briefly, cells were grown in T75 flasks containing growth media (GM) (DMEM, 10% FBS, 10% NBCS, 1% PS, 2mM L-G) in a humidified atmosphere of 5% CO_2 at 37 °C. Upon reaching 80% confluence, cells were split into 6 well plates at a density of 4 x10^5 cells/ml and cultured for 48 hrs in a humidified atmosphere of 5% CO_2 at 37 °C until 80% confluency. To induce differentiation to myotubes, cells were washed and incubated in DMEM (1% PS, 2mM L-G) supplemented with 2 % HS (DM) or 2 % serum derived from NAT and AAS groups depending on experiment. Myotubes were allowed to form over 96-144 hours with the media being topped up with 1 ml of media every 48 hours in a humidified atmosphere of 5% CO_2 at 37 °C.

6.3.3. Insulin stimulation

To investigate insulin signalling in myoblasts, after attainment of 80% confluence in 6 well plates, cells were washed twice with PBS and incubated for 3 hours at 37 °C and 5% CO_2 in DM, NAT (DMEM, 2% HI NAT serum, 1% PS, 2mM L-G) or AAS (DMEM, 2% HI AAS serum, 1% PS, 2mM L-G). To investigate insulin signalling in myotubes, myoblasts were differentiated to myotubes in DMEM (1% PS, 2mM L-G) supplemented with 2% HS (DM) 2% NAT or 2% AAS. Myotubes were washed twice with phosphate buffered saline (PBS) and incubated for 3 hours in 2% DM, NAT or AAS. Cells were spiked with 100 nM insulin and incubated at 37 °C and 5% CO_2 for 20 and 30 mins.

Following insulin stimulation, myoblasts were washed twice with ice cold PBS, trypsinised and centrifuged at 800 g at 4 °C for 5 mins. Cells were fixed with 2% paraformaldehyde and incubated for 30 mins at room temperature. Cells were permeabilised with ice-cold 100% methanol and stored at -20 °C before processing for flow cytometry (BD Accuri C6, BD

Biosciences, Wokingham, UK) (2.3.4). Myotubes were lysed and scraped with RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.25% deoxycholic acid, 1% NP-40, 1 mM EDTA) before processing for Western blot (section 2.3.5.). Myotubes could not be subjected to FLOW cytometric analyses, hence the need to adopt Western blotting.

6.3.4. Glucose uptake

Glucose uptake was measured using 2-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino)-2-Deoxyglucose, (2-NBDG), for full details read methods section 2.3.1. Briefly, in 96 well plates myoblasts and myotubes were washed twice with PBS and incubated in serum free, low glucose, 1% BSA DMEM for 24 hours. Following 24 hours, myoblasts were washed twice with PBS and transferred to 2% HS or NAT or AAS DMEM (0% glucose, 100μM 2-NBDG, 1% PS, 2mM L-G), as were myotubes, but in serum free DMEM (0% glucose, 100μM 2-NBDG, 1% PS, 2mM L-G). Myoblasts and myotubes were incubated at 37 °C and 5% CO_2 with 0 or 100 nm of insulin for 30 mins. After incubation cells were placed on ice and washed 3 times with ice cold PBS. Cell fluorescence was detected (λex = 460 to 490 nm, λem =530 to 550 nm) using a microplate reader (Clariostar, BMG LABTECH, Ortenberg, Germany). Protein concentration was determined by BCA™ (see methods section 2.3.3) assay and glucose uptake was relativised to protein content and expressed as fold change of basal glucose uptake.

6.3.5. Creatine kinase

Creatine kinase (CK) activity was measured as a marker of myoblast differentiation to myotubes and relativized to total protein (Sharples, Al-Shanti and Stewart, 2010; Sharples *et al.*, 2011; Dugdale *et al.*, 2018). For detailed description please read methods section 2.8. Briefly, cells were incubated in DMEM (1% PS, 2mM L-G) supplemented with 2 % HS (DM) or serum derived from NAT and AAS groups. Cells were washed twice with PBS and lysed with TMT at 0, 48, 96 and 120 hr. In duplicate, 10 µl of cell lysates were analysed using 96-well UV plates. Using a multichannel pipette 200 µl of CK reagent was added to samples. Over 20 minutes, the change in absorbance was measured continuously using an ELISA plate reader (Biotek, USA) at a wavelength of 340 nm. CK activity was then relativised to protein concentration obtained from BCA™ assay (section 2.3.3).

6.3.6. Measurement of protein synthesis and breakdown.

The SUnSET method was used to measure global protein synthesis (Schmidt *et al.*, 2009). The SUnSET method involves pulsing cells (10 µl/ml) with the antibiotic puromycin which is a structural analogue of aminoacyl-tRNA and therefore can be incorporated into a translating

poly-peptide chain. Incorporation of puromycin into developing peptide chains results in termination of peptide elongation and the rate at which puromycin labelled polypeptides are produced serves as a measure of protein synthesis rates. The labelled polypeptides can then be measured with monoclonal antibodies using immunodetection methods. An adaptation of the SUnSET method was used to allow for determination of MPB measuring the rate of puromycin removal from cells in addition to MPS (Crossland *et al.*, 2017). Myotubes were incubated in DM, NAT or AAS media containing 1 μM of puromycin for 1, 6 and 24 hrs. To determine removal of puromycin from cells, after 24 hrs cells were washed twice with PBS and incubated in DM, NAT or AAS media without puromycin for a further 6, 24 and 48 hrs. Myotubes were washed twice with ice cold PBS, lysed and scraped with RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.25% deoxycholic acid, 1% NP-40, 1 mM EDTA) before processing for Western blot.

6.3.7. Flow cytometry

To detect phosphoproteins in myoblasts, flow cytometry was performed. For detailed description please read methods section 2.9. Briefly, cells were washed with flow buffer (PBS + 0.5% horse serum) and centrifuged at 600 g at 4 °C for 5 minutes. Cells were resuspended at 1 x 10^6 cells/ml in flow buffer. The anti-human/mouse phospho-AKT (S473; PE; 585/40nm; 0.06 μg), anti-human/mouse phospho-P38 (Thr180, Tyr182; PE; 585/40nm; 0.06 μg), anti-human/mouse phospho-mTOR (Thr180, Tyr182; PerCP; 670/LP nm; 0.125 μg) and anti-human/mouse phospho-ERK/12 (Thr202, Tyr204; AlexaFlour; 488 nm; 0.03 μg) antibodies (Thermo Fisher Scientific inc, Waltham, USA) were added to samples and incubated at RT in the dark for 60 minutes. Cells were washed twice by centrifuging at 600 g at 4 °C for 5 minutes and resuspending in flow buffer. Data from 1000 events were recorded by flow cytometry.

6.3.8. SDS-Page and immunoblotting

Total protein and phosphoproteins in myotubes were detected by Western blot, (see section 2.3.5 for further details). Protein concentrations of samples were determined by BCA™ assay (section 2.3.3) and samples were subsequently resuspended in 5x Laemmli buffer at 1 mg/ml. 30 μg of sample was loaded and electrophoresed on 10% SDS-polyacrylamide gels. Semi-dry transfer of proteins to a nitrocellulose membrane using BioRad transfer pack was carried out. Following blocking for 1-hour in 5% non-fat dried milk, membranes were incubated overnight with rabbit anti-phosphorylated or total; Akt^{ser473} and $P38MAPK^{t180/Y182}$ and total $ERK1/2^{T202/Y204}$ at a dilution of 1:1000, and p-$ERK1/2^{T202/Y204}$ at a dilution of 1:2000 (Cell Signalling Technology, London, UK) similarly, anti-puromycin was incubated at a dilution of 1:20000 clone (12D10 mouse monoclonal; Merck Millipore Limited, Carrigtwohill, Co. Cork,

Ireland). After overnight incubation, membrane was washed 3 times in TBS-Tween at 0.1% and incubated for 1 hour in HRP-conjugated goat anti-rabbit antibodies at dilution of 1:1000 (Thermo Fisher Scientific inc, Waltham, USA) with the exception of puromycin which was incubated with goat anti-mouse IgG2a-specific at a dilution of 1:10000 (LI-COR Biosciences UK Ltd, Cambridge, UK). Proteins were visualised by enhanced chemiluminescence (Thermo Fisher Scientific inc, Waltham, USA) and quantified by densitometry (ChemiDoc^{TM} MP imaging system, Bio-Rad Laboratories, Inc. CA, USA).

6.3.9. Statistical analysis

GraphPad Prism (California, USA) statistical software was used for statistical analyses. Data were assessed and confirmed for normal distribution by Shapiro Wilks test. To determine the effect of serum condition on each experiment, data underwent a 2-way mixed ANOVA with 3 between factors (DM *vs.* NAT *vs.* AAS) and 2-4 within (time) factors depending on experiment. Bonferroni post hoc pairwise comparisons test was used when significant main effects and interactions were present. All data are presented as mean ± SEM and significance set as $P \leq 0.05$.

6.4. Results

6.4.1. High-density lipoprotein cholesterol and apolipoprotein A1 are significantly reduced in individuals using AAS (study 3, section 5.4.1.)

Participants who use AAS showed significantly (P = 0.04) lower levels of HDL-C and apolipoprotein A1 compared with healthy controls (Study 3, section 5.4.1.). Furthermore, The highest users of AAS were also observed to be associated with significantly higher levels of with LDL-C (P < 0.01, r = 0.76), apolipoprotein B (P < 0.01, r = 0.77), pattern A (P < 0.01, r = 0.79) and pattern B (P < 0.001, r = 0.94). These results indicate an increased risk of developing CVD in individuals who use AAS.

6.4.2. NAT and AAS serum elicit similar responses in Akt^{ser473}, $mTOR^{ser2448}$, $ERK1/2^{T202/Y204}$ and $P38MAPK^{t180/Y182}$ phosphorylation but show differential responses in $ERK1/2^{T202/Y204}$ and $P38MAPK^{t180/Y182}$ compared with DM during fusion.

To elucidate the impact of serum on potential acute mechanisms of fusion, cells were incubated in each condition for 0, 15, 30 and 60 mins. Upon serum incubation, p-Akt^{ser473} significantly (P < 0.001) increased from 0 min (All groups; 11249 ± 965) to 15 min (DM; 13139 ± 331, NAT; 13230 ± 686, AAS; 13476 ± 1165) to 30 min (DM; 12226 ± 579, NAT; 14536 ± 995, AAS; 14180 ± 676) to 60 min (DM; 16432 ± 784, NAT; 17889 ± 1143, AAS; 17499 ± 434) (Figure 6.1). Post-hoc analyses showed p-Akt^{ser473} to be significantly (P < 0.01) higher at 15, 30 and 60 min compared to 0 min. No difference was observed between 15 and 30 min, but 60 min was significantly higher compared to 15 and 30 mins. No significant interaction between groups was observed.

$mTOR^{ser2448}$ phosphorylation (p-$mTOR^{ser2448}$) significantly (P < 0.01) increased from 0 min (All groups; 60320 ± 2445) to 15 min (DM; 67315 ± 2461, NAT; 59845 ± 3981, AAS; 57240 ± 1345) to 30 min (DM; 65311 ± 4655, NAT; 69130 ± 5297, AAS; 65251 ± 5643) and decreased at 60 min (DM; 51606 ± 6304, NAT; 54745 ± 4959, AAS; 60619 ± 4707) (Figure 6.1). Post-hoc analyses showed p-$mTOR^{ser2448}$ to be significantly (P < 0.05) lower at 60 min compared to

30 min. No difference was observed between any other time. No significant interaction between groups was observed.

ERK1/2$^{T202/Y204}$ phosphorylation (p-ERK1/2$^{T202/Y204}$) significantly ($P < 0.001$) increased from 0 min (All groups; 15342 ± 1952) to 15 min (DM; 15490 ± 228, NAT; 17243 ± 778, AAS; 17113 ± 1535) to 30 min (DM; 16353 ± 492, NAT; 21729 ± 1073, AAS; 20922 ± 1532) to 60 min (DM; 20368 ± 958, NAT; 33241 ± 4526, AAS; 30582 ± 1678) (Figure 6.1). Post-hoc analyses showed p-ERK1/2$^{T202/Y204}$ to be significantly ($P < 0.05$) higher at 15, 30 and 60 min compared to 0 min. p-ERK1/2$^{T202/Y204}$ was also significantly higher at 30 min vs 15 min and 60 min compared to all previous timepoints. There was also a significant ($P < 0.01$) interaction between groups with post-hoc analyses showing AAS to be significantly higher compared to DM at 60 mins.

P38MAPK$^{t180/Y182}$ phosphorylation (p-P38MAPK$^{t180/Y182}$) significantly ($P < 0.001$) increased from 0 min (All groups 5005 ± 283) to 15 min (DM; 5096 ± 151, NAT; 5121 ± 242, AAS; 5166 ± 274) to 30 min (DM; 5382 ± 359, NAT; 5053 ± 55, AAS; 5357 ± 124) to 60 min (DM; 12427 ± 142, NAT; 14462 ± 430, AAS; 14202 ± 677) (Figure 6.1). Post-hoc analyses showed p-P38MAPK$^{t180/Y182}$ to be significantly ($P < 0.001$) higher at 60 min compared to 0, 15 and 30 min. There was also a significant ($P < 0.01$) interaction between groups however, post-hoc analyses showed no differences between groups at any time point.

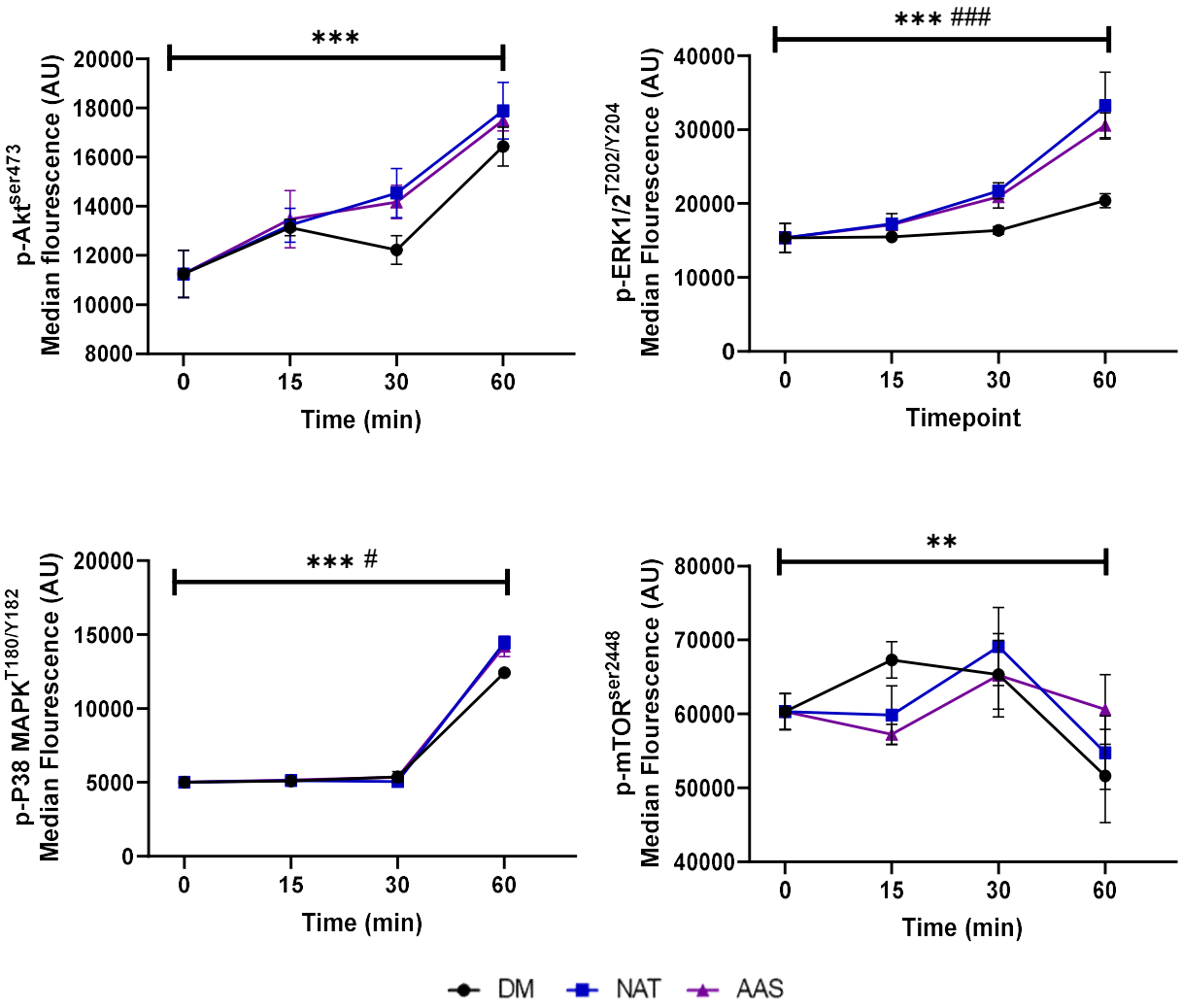


Figure 6.1. The effect of serum on p-Akt^{ser473}, p-mTOR^{ser2448}, p-ERK1/2$^{T202/Y204}$ and p-P38MAPK$^{t180/Y182}$ at 0, 15, 30 and 60 min. All data (n=3 replicates in duplicate in each group) are presented as mean ± SEM. ** ($P < 0.01$) *** ($P < 0.001$) denotes significantly different from 0 min. $^{\#}$ ($P < 0.05$) $^{\#\#\#}$ ($P < 0.001$) denotes significant interaction between groups.

6.3.1. Creatine kinase activity significantly increases during fusion of myoblasts to myotubes in DM, NAT and AAS serum.

Upon attainment of 80% confluence, myoblasts were switched to DM media containing 2% HS or human derived NAT or AAS serum to induce differentiation to myotubes. CK activity, a marker of fusion, significantly ($P < 0.001$) increased in the from 0 hr (All groups; 58.29 ± 7.4) to 48 hr (DM; 179.49 ± 77.69, NAT; 328.24 ± 105.5, AAS; 284.37 ± 96.47) to 96 hr (DM; 255.31 ± 69.54, NAT; 358.52 ± 143.52, AAS; 364.46 ± 94.83) to 120 hr (DM; 350.44 ± 113.97, NAT; 348.07 ± 110.52, AAS; 456.95 ± 59.61) (Figure 6.2C). Post-hoc analyses showed CK activity to be significantly ($P < 0.01$) higher at 48, 96 and 120 hr compared to 0 hr. CK activity was not significantly different between 48, 96 and 120 hr. There was no significant interaction between

groups at any time. Protein concentration was significantly ($P < 0.01$) different between groups with post hoc analysis showing the AAS group to be significantly ($P = 0.04$) higher than DM at 96 and 120 hr (Figure 6.2A). However, CK activity relativised to protein concentration (Figure 3C) did not reveal different results to non-relativised CK activity (Figure 6.2B).

6.4.3. Summary of the effects of serum conditions on myoblast signalling and fusion.

Compared with DM, myoblasts conditioned in AAS or NAT serum showed similar acute increases in p-Akt and p-mTOR but showed significantly higher p-ERK and p-P38 levels, all implicated in differentiation. CK activity, a marker of differentiation of myoblasts to myotubes significantly increased comparably in all conditions from 0 to 120 hours. Additionally, protein abundance also significantly increased in all groups with greater concentrations in NAT and AAS conditions, with AAS serum being significantly higher compared with DM after 96 hours.

6.4.4. Insulin increases Akt^{ser473} phosphorylation in skeletal muscle C2C12 myoblasts in DM, NAT and AAS serum.

Basal p-Akt^{ser473} was not significantly different between conditions prior to insulin stimulation. Stimulation of myoblasts with 100 nM of insulin for 20 and 30 min significantly ($P < 0.001$) increased p-Akt^{ser473} in the presence of DM (7020 ± 356 to 9007 ± 1122 to 8621 ± 427 AU), NAT (7349 ± 245 to 9195 ± 529 to 10845 ± 1516 AU) and AAS (7398 ± 277 to 10573 ± 2045 to 9139 ± 698 AU) (Figure 6.3). Post-hoc analyses showed p-Akt^{ser473} to be significantly ($P < 0.01$) higher at 20 mins in AAS and at 30 mins in NAT. There was no significant interaction between groups.

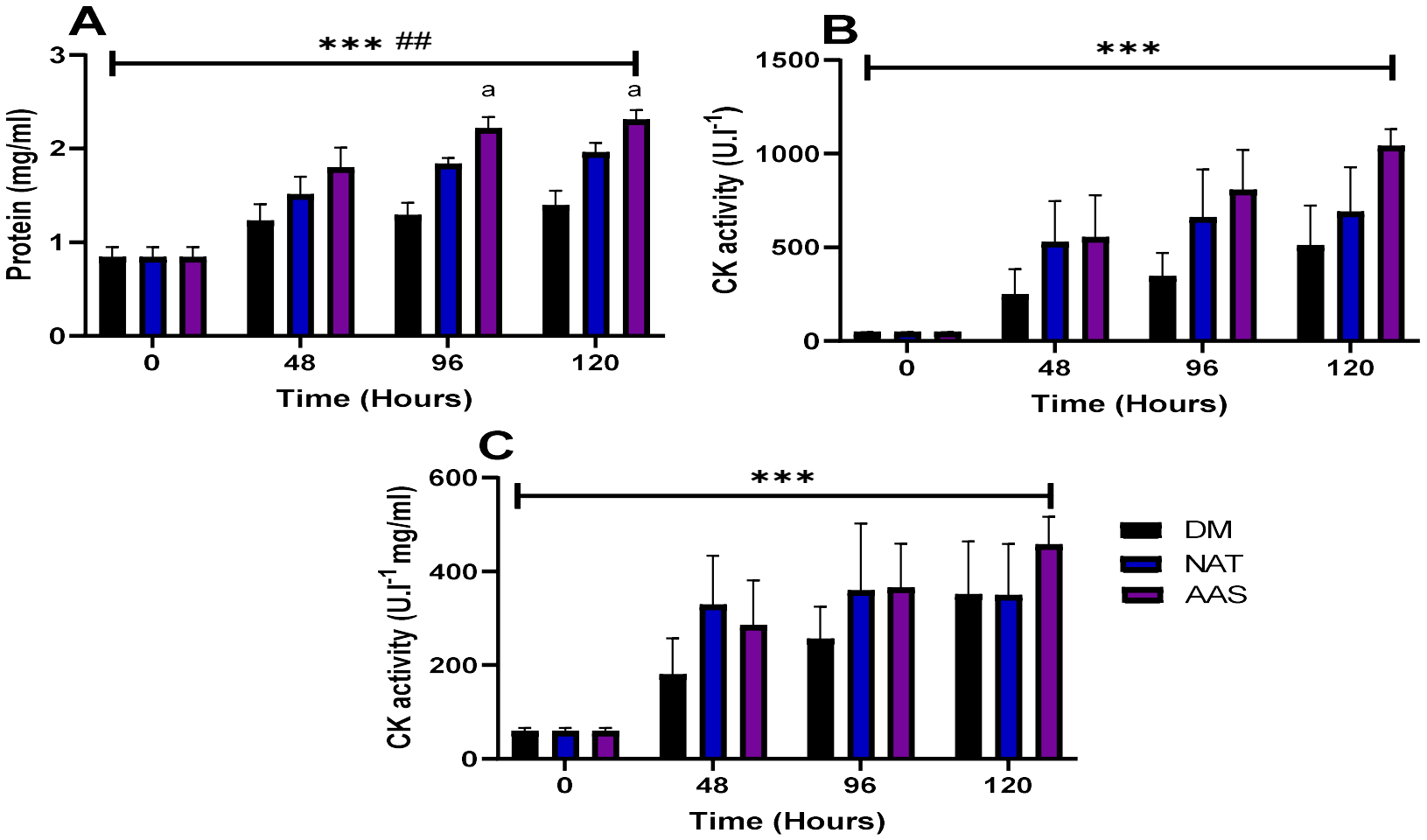


Figure 6.2. The effect of serum on protein abundance and CK activity during fusion of myoblasts to myotubes at 0, 48, 96 and 120 hours. A) Protein abundance, B) CK activity, C) CK activity relativised to protein abundance. All data (n=3 replicates in duplicate in each group) are presented as mean ± SEM.*** denotes significantly ($P < 0.001$) different from 0 hours. ## denotes significant ($P < 0.01$) interaction between groups. a denotes significantly ($P < 0.05$) different *vs* DM.

The relative insulin induced p-Akt^{ser473} fold change vs control significantly (P < 0.001) increased after 20 mins (DM; 1.23 ± 0.06, NAT; 1.20 ± 0.02, AAS; 1.36 ± 0.18) and 30 mins (DM; 1.29 ± 0.06, NAT; 1.53 ± 0.17, AAS; 1.28 ± 0.04) (Table 6.1). No significant interaction between groups at any timepoint was observed.

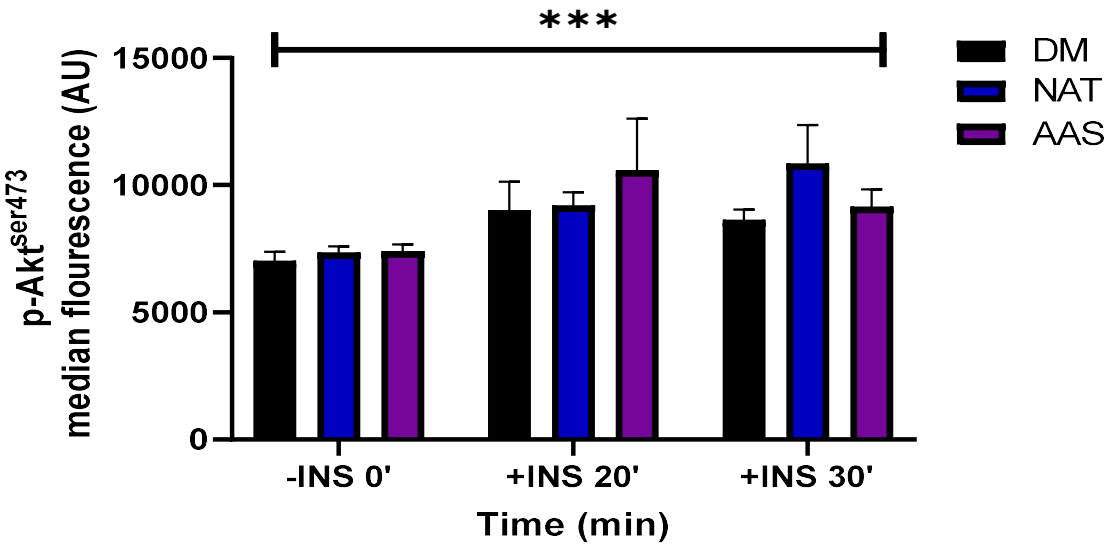


Figure 6.3. The effect of insulin stimulation in myoblasts on p-Akt^{ser473} in DM, NAT and AAS after 20 and 30 min. All data (n=3 replicates in each group) are presented as mean ± SEM.*** denotes significantly (P < 0.001) different from 0 min.

6.4.5. Glucose uptake in response to insulin is comparable in skeletal muscle C2C12 myoblasts in DM, NAT and AAS serum.

Following incubation in DM, NAT and AAS myoblasts were treated in the absence or presence of insulin (100 nM) for 30 min (Figure 6.4). In response to insulin, a trend (P = 0.08) in increasing the fold change in glucose uptake was observed at 30 minutes (DM; 1.22 ± 0.07, NAT; 1.08 ± 0.2, AAS; 1.51 ± 0.33). No significant difference was observed in glucose uptake in the absence or presence of insulin between groups.

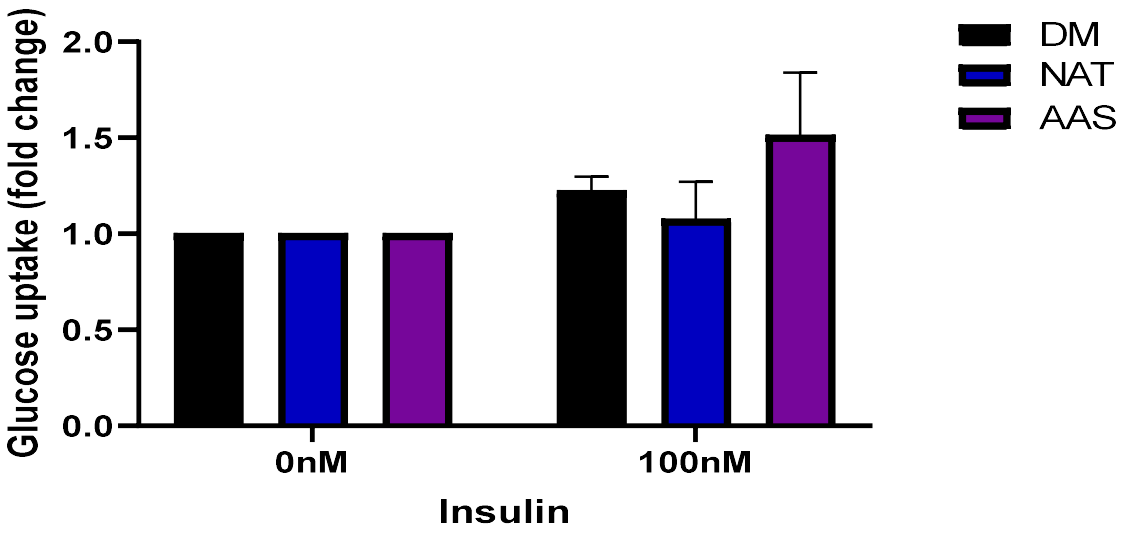


Figure 6.4. The effect of insulin stimulation in myoblasts on glucose uptake in DM, NAT and AAS after 30 min. All data (n=3 replicates in triplicate in DM) are presented as mean ± SEM.

6.4.6. Summary of the effects of insulin on myoblasts conditioned in NAT and AAS serum.

Myoblasts conditioned in NAT and AAS serum showed comparable increases in p-Akt and glucose uptake in response to insulin. The effects of serum on cellular glucose metabolism may only be considered acute due to the short incubation time (<3 hours). As myoblasts showed successful fusion in each condition, the impact of chronic (4-5 days) serum conditions on cellular metabolism in myotubes was warranted.

6.4.7. Insulin increases Akt^{ser473} phosphorylation in skeletal muscle C2C12 myotubes in DM, NAT and AAS serum.

Following the results in myoblasts, stimulation of myotubes with 100 nM insulin for 20 and 30 min was investigated. Serum conditions showed a trend (P = 0.05) of p/t-Akt^{ser473} being different between groups, with post hoc analysis reporting that AAS serum showed a trend (P = 0.06) of overall p/t-Akt^{ser473} being lower compared with DM. Insulin stimulation significantly (P < 0.01) increased p/t-Akt^{ser473} in the presence of DM (0.38 ± 0.11 to 1.6 ± 0.18 to 2.74 ± 0.66 AU), NAT (0.22 ± 0.03 to 1.66 ± 0.79 to 1.83 ± 0.43 AU) and AAS (0.13 ± 0.03 to 0.93 ± 0.40 to 0.93 ± 0.07 AU) (Figure 6.5). Post-hoc analysis showed p/t-Akt^{ser473} to be significantly (P < 0.05) higher at 20 mins and 30 mins compared to baseline however, no difference was observed from 20 to 30 mins. Although there was a trend (P = 0.06) of overall p-Akt^{ser473} being lower in AAS conditions compared with DM, there was no significant interaction between groups in response to insulin stimulation.

The relative insulin induced p/t-Akt^{ser473} fold change vs control significantly (P < 0.01) increased after 20 mins (DM; 4.35 ± 0.47, NAT; 7.70 ± 3.67, AAS; 7.27 ± 3.1) and 30 mins (DM; 7.28 ± 1.74, NAT; 8.53 ± 1.99, AAS; 7.24 ± 0.55) (Table 6.1). No significant interaction between groups at any timepoint was observed.

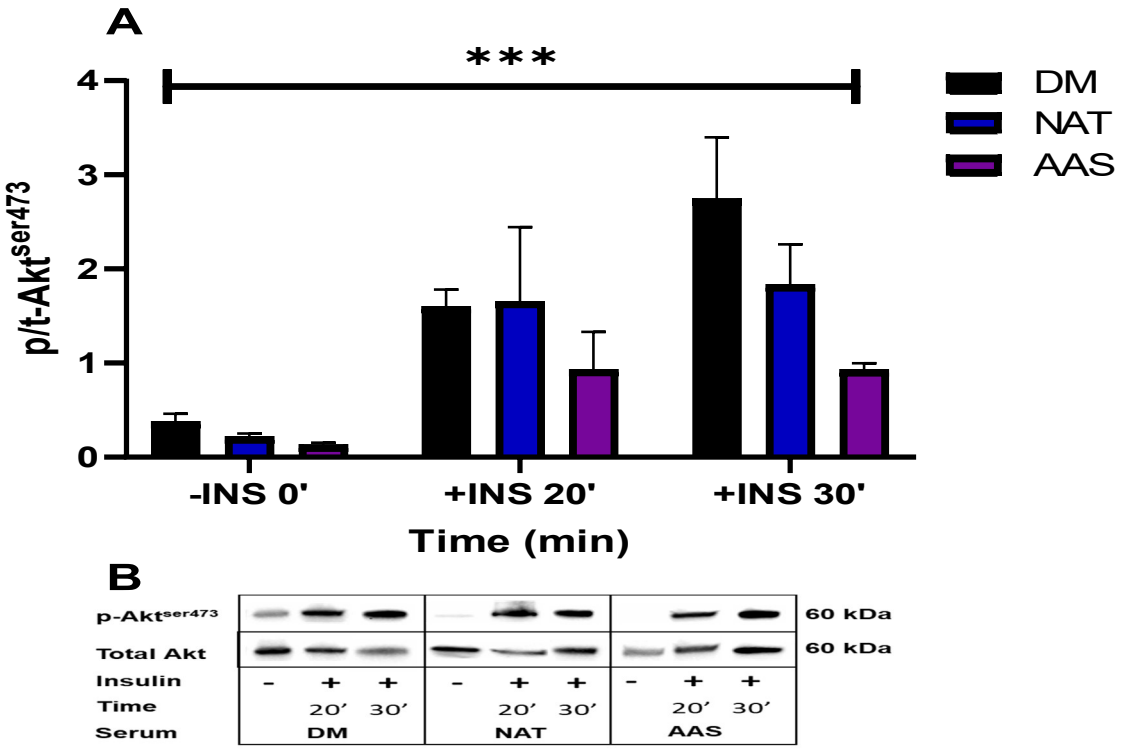


Figure 6.5. The effect of insulin stimulation in myotubes on p/t-Akt^{ser473} in DM, NAT and AAS after 20 and 30 min. A) Quantitative representation, B) qualitative representation. All data (n=3 replicates in each group) are presented as mean ± SEM.*** denotes significantly (P < 0.001) different from 0 min.

6.4.8. Insulin does not increase glucose uptake in skeletal muscle C2C12 myotubes in DM, NAT and AAS serum.

Following incubation in DM, NAT or AAS myotubes were treated with 100 nM of insulin for 30 min (Figure 6.6). Insulin significantly (P = 0.04) increased glucose uptake comparably between conditions (DM; 1.32 ± 0.16, NAT; 1.21 ± 0.29, AAS; 1.48 ± 0.35). Similar to myoblasts, no significant difference was observed in glucose uptake in the absence or presence of insulin between groups.

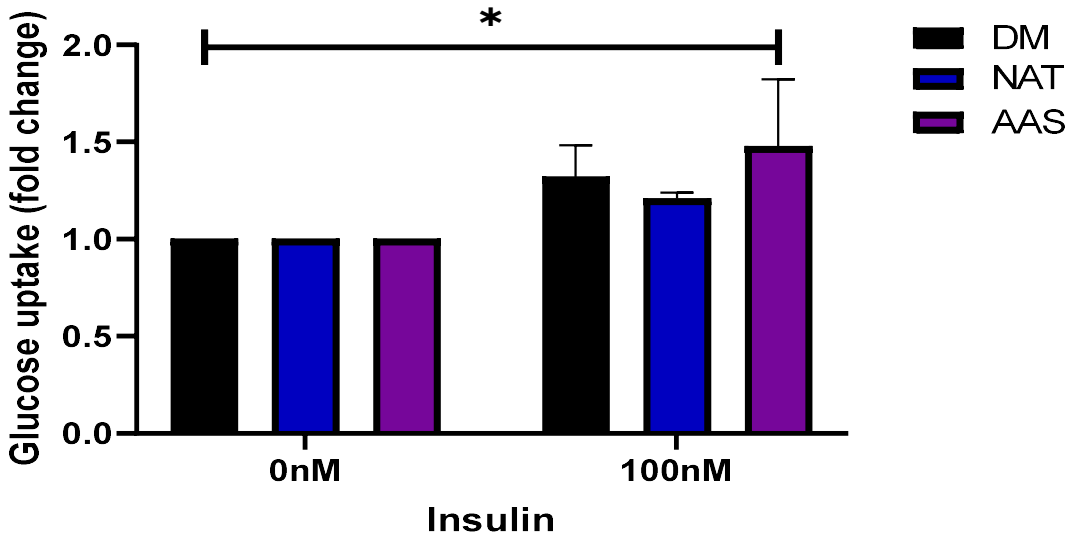


Figure 6.6. The effect of insulin stimulation in myotubes on glucose uptake in DM, NAT and AAS after 30 min. All data (n=3 replicates in triplicate in DM) are presented as mean ± SEM.

6.4.9. Summary of the effect of chronic NAT and AAS serum conditions on glucose metabolism in myotubes.

In myotubes conditioned in DM, NAT or AAS serum for 4-5 days, the effect of insulin on p-Akt and glucose uptake was similar between conditions. However, and in contrast to myoblast observations, AAS serum reported a trend ($P = 0.06$) of expressing lower p-Akt in the absence and presence of insulin compared with DM. This may have potential wider implications on cellular signalling such skeletal muscle anabolism as no difference was observed between groups in glucose metabolism.

Table 6.1. Insulin stimulated fold change in Akt^{ser473} phosphorylation in myoblasts and myotubes in each condition at 20 and 30 minutes.

Condition	Myoblasts Fold change Vs 0 nM at 20 mins	Myoblasts Fold change Vs 0 nM at 30 mins	Myotubes Fold change Vs 0 nM at 20 mins	Myotubes Fold change Vs 0 nM at 30 mins
DM	1.23 ± 0.06	1.29 ± 0.06	4.35 ± 0.47	7.28 ± 1.74
NAT	1.20 ± 0.02	1.53 ± 0.17	7.70 ± 3.67	8.53 ± 1.99
AAS	1.36 ± 0.18	1.28 ± 0.04	7.27 ± 3.1	7.24 ± 0.55

Data presented as mean ± SEM.

6.4.10. DM, NAT and AAS serum elicit similar responses in Akt^{ser473}, ERK1/2$^{T202/Y204}$ and P38MAPK$^{t180/Y182}$ phosphorylation in myotubes.

To elucidate the impact of serum on myotube signalling, cells were incubated in each condition for 0, 15, 30 and 60 mins (Figure 6.7). All groups showed similar p/t-Akt^{ser473} at 15 min (DM; 0.76 ± 0.25, NAT; 1.74 ± 0.36, AAS; 1.03 ± 0.37), 30 min (DM; 0.93 ± 0.12, NAT; 1.31 ± 0.45, AAS; 0.86 ± 0.16) and 60 min (DM; 0.51 ± 0.3, NAT; 0.98 ± 0.34, AAS; 0.67 ± 0.34).

Similarly, all groups showed comparable p/t-ERK1/2$^{T202/Y204}$ at 15 min (DM; 3.74 ± 3.66, NAT; 0.08 ± 0.05, AAS; 0.03 ± 0.03), 30 min (DM; 0.62 ± 0.44, NAT; 1.26 ± 0.19, AAS; 0.45 ± 0.19) and 60 min (DM; 0.47 ± 0.18, NAT; 0.36 ± 0.17, AAS; 0.19 ± 0.04).

All serum conditions also showed similar p/t-P38MAPK$^{t180/Y182}$ at 15 min (DM; 0.85 ± 0.47, NAT; 0.18 ± 0.09, AAS; 0.004 ± 2.56), 30 min (DM; 0.21 ± 0.03, NAT; 0.58 ± 0.23, AAS; 0.09 ± 0.04) and 60 min (DM; 0.10 ± 0.04, NAT; 0.08 ± 0.04, AAS; 0.76 ± 0.71).

All serum conditions significantly (P = 0.02) increased p/t-ERK1/2$^{T202/Y204}$ and showed a trend (P = 0.07) of significantly increasing p/t-Akt^{ser473} from baseline. Post-hoc analyses showed p/t-Akt^{ser473} at 30 min to be significantly (P = 0.046) higher compared to 0 min and p/t-ERK1/2$^{T202/Y204}$ to be significantly (P = 0.03) higher at 30 min compared to 15 min. No significant changes in p/t-P38MAPK$^{t180/Y182}$ were observed in any group.

6.4.11. DM, NAT and AAS serum elicit similar responses in puromycin incorporation, de-corporation and associated signalling.

To investigate the impact of serum on rates of MPS and MPB, myotubes were incubated with DM, NAT or AAS containing 1 µg/ml of puromycin (Figure 6.8). All groups showed similar rates of MPS at 1 hr (DM; $1.19 \times 10^8 \pm 1.87 \times 10^7$, NAT; $1.12 \times 10^8 \pm 1.46 \times 10^7$, AAS; $1.32 \times 10^8 \pm 9.72 \times 10^6$), 6 hr (DM; $1.24 \times 10^8 \pm 5.49 \times 10^6$, NAT; $1.43 \times 10^8 \pm 1.07 \times 10^7$, AAS; $1.37 \times 10^8 \pm 2.17 \times 10^7$) and 24 hr (DM; $9.27 \times 10^7 \pm 1.52 \times 10^6$, NAT; $1.02 \times 10^8 \pm 6.21 \times 10^6$, AAS; $9.11 \times 10^7 \pm 1.92 \times 10^7$). MPS significantly ($P < 0.05$) decreased with time as post-hoc analysis showed puromycin incorporation to be significant lower at 24 h compared to 1 hr and 6 hr. All groups showed similar non-significant responses in p/t-Akt^{ser473} at 1 hr (DM; 0.65 ± 0.28, NAT; 0.98 ± 0.33, AAS; 0.53 ± 0.28), 6 hr (DM; 1.47 ± 0.62, NAT; 1.26 ± 0.39, AAS; 0.83 ± 0.39) and 24 hr (DM; 0.31 ± 0.13, NAT; 0.62 ± 0.23, AAS; 0.62 ± 0.25) and no change was observed between timepoints.

The fold change of puromycin de-corporation from 24 hr was similar between groups at 30 hr (DM; 0.74 ± 0.17, NAT; 0.61 ± 0.06, AAS; 0.72 ± 0.13), 48 hr (DM; 0.81 ± 0.04, NAT; 0.64 ± 0.05, AAS; 0.73 ± 0.12) and 72 hr (DM; 0.58 ± 0.10, NAT; 0.67 ± 0.1, AAS; 0.73 ± 0.14) and significantly ($P < 0.001$) increased with time.

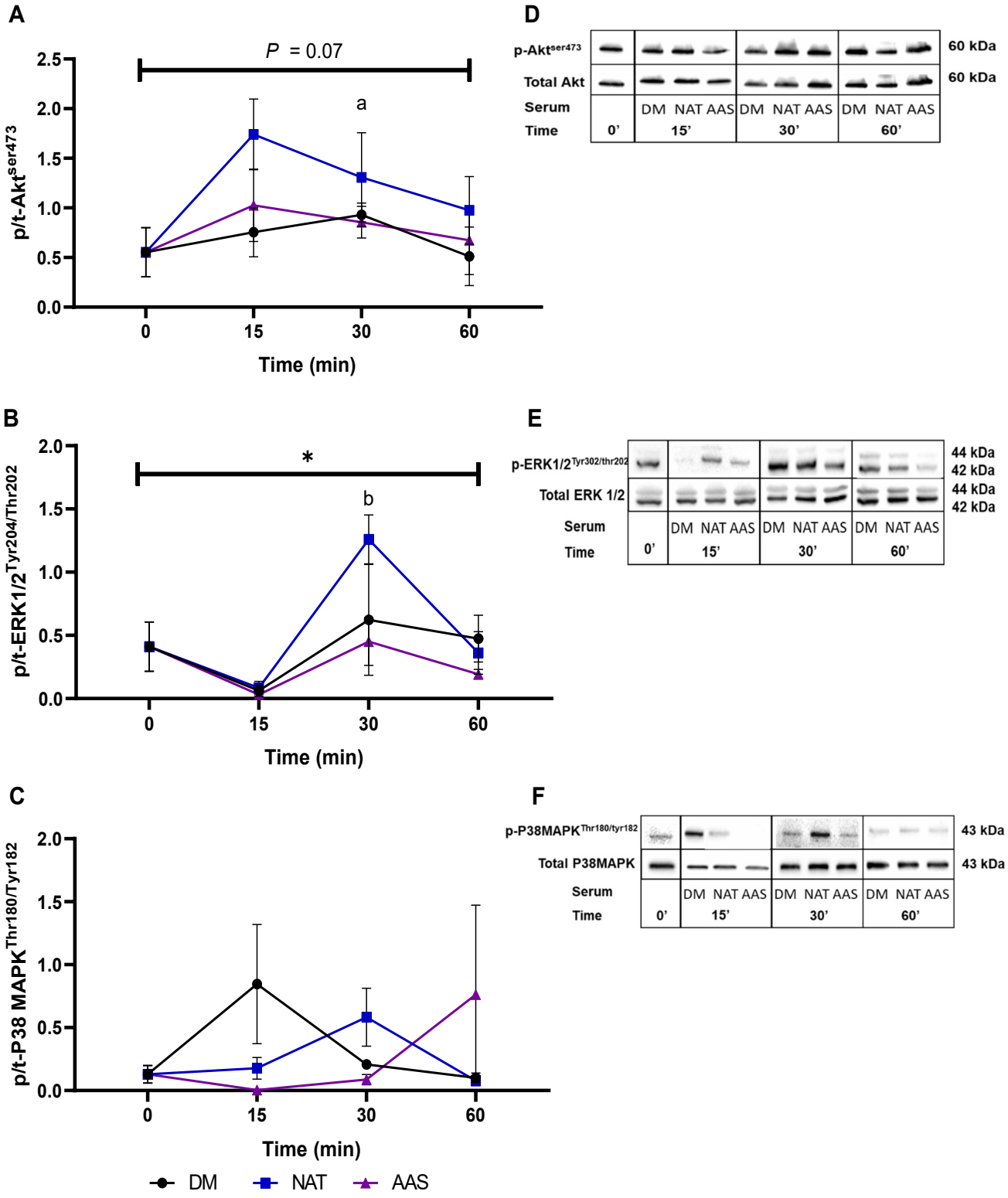


Figure 6.7. The effect of serum on p-Akt^{ser473}, p-ERK1/2$^{T202/Y204}$ and p-P38MAPK$^{t180/Y182}$ at 0, 15, 30 and 60 min. A-C) Quantitative representation, D-F) qualitative representation. All data (n=3 replicates in duplicate in each group) are presented as mean ± SEM. * ($P < 0.05$) denotes significant effect for time, [a] ($P < 0.05$) denotes significantly different to 0 min, [b] ($P < 0.05$) denotes significantly different to 15 min.

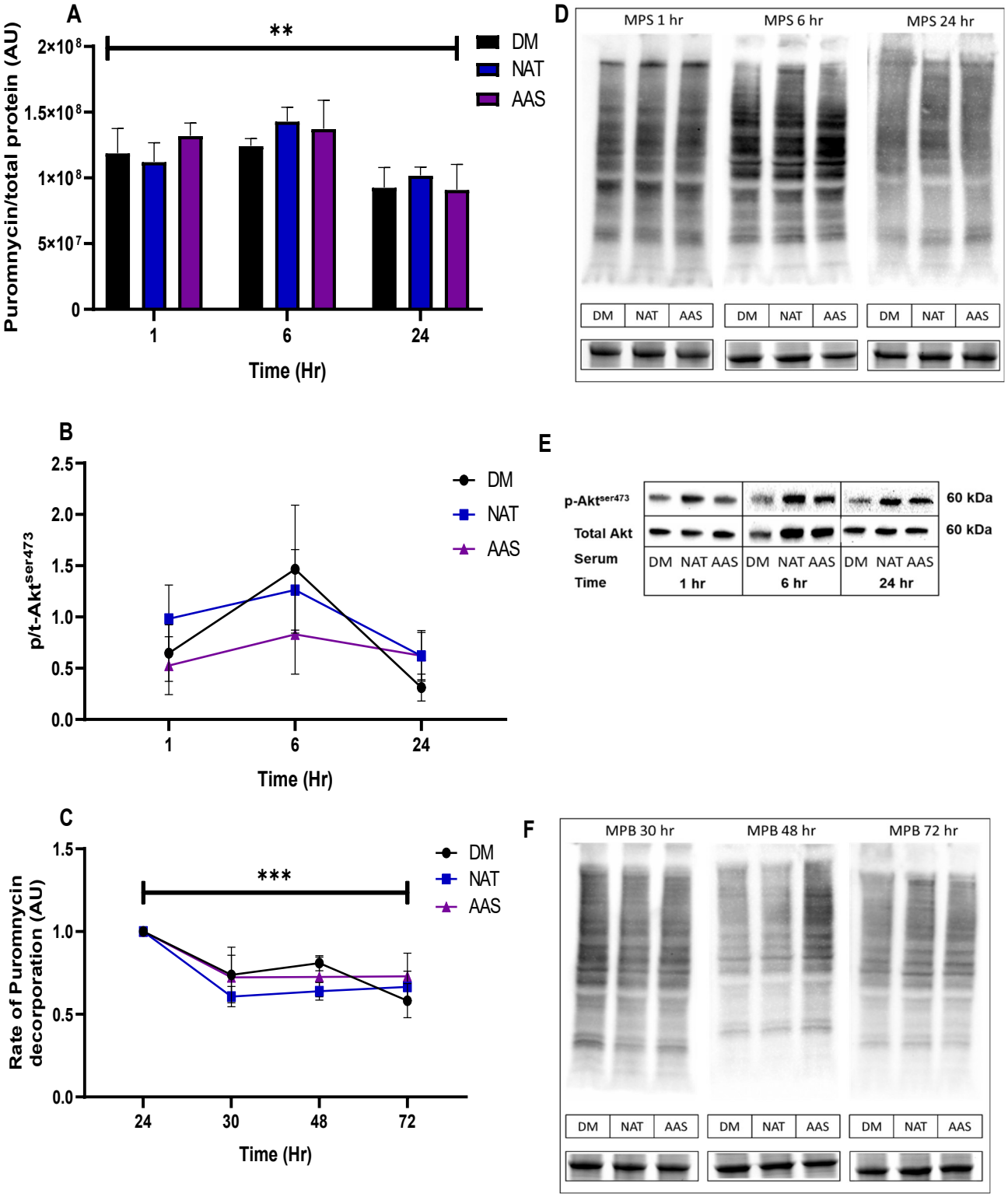


Figure 6.8. The effect of serum on MPS, rate of puromycin de-corporation and p-Akt^{ser473}. A-C) Quantitative representation, D-F) qualitative representation with stain-free protein expression in box below. All data (n=3 replicates in duplicate in each group) are presented as mean ± SEM. ** ($P < 0.01$), *** ($P < 0.001$) denotes significant effect for time.

6.4.12. Summary of the effects of chronic AAS and NAT serum on anabolic signalling, MPS and MPB in myotubes.

Upon incubation with conditioned serum, comparable significant ($P < 0.05$) increases in p-Akt and p-ERK were observed after 30 mins between groups however, NAT serum elicited a greater fold change (Akt, 2.4-fold; ERK, 3.1-fold) from baseline compared with AAS serum ((Akt, 1.6-fold; ERK, 1.1-fold). Although differential spikes were observed in p-P38 by serum conditions, no significant increases or differences was reported. Increased Akt and ERK activity is implicated in many cellular responses including MPS and inhibition of MPB therefore, these markers of cellular metabolism were measured to assess the potential effect of increased p-Akt and p-ERK. Myotubes rapidly incorporated puromycin after 1 hour of serum incubation indicating MPS and was maintained for 6 hours but was reduced after 24 hours. Upon removal of puromycin from media, the rate of puromycin decorporation, an indicator of MPB, was also similar between groups. These data indicate that AAS serum shows similar anabolic effects on skeletal muscle compared with NAT serum.

6.5. Discussion

The main outcome of this investigation was that insulin significantly increased p-Akt^{ser473} in all serum conditions but in contrast to hypothesis 1, there was no significant difference observed between serum conditions in p-Akt^{ser473} or the insulin induced fold change in myoblasts or myotubes. However, in myotubes, AAS serum showed a trend (P = 0.06) of overall lower p/t-Akt^{ser473} compared with DM conditions.

Contrary with hypothesis 2, insulin showed a trend (P = 0.08) of increasing glucose uptake in myoblasts and significantly (P = 0.04) increased glucose uptake in myotubes comparably between all serum conditions.

In contrast with hypothesis 3, myoblast differentiation was not enhanced in conditioned serum as CK activity levels were not significantly different at any timepoint however, p-ERK1/2$^{T202/Y204}$ and p-P38MAPK$^{t180/Y182}$ were enhanced in conditioned serum compared to DM.

Finally, in contrast with hypothesis 4, although anabolic signalling was upregulated MPS and MPB were not significantly different between groups.

Overall, myoblasts exposed to AAS or NAT serum showed elevated acute signalling of differentiation (p-ERK and p-P38) compared with DM however, this did not lead to significantly higher levels of differentiation measured by CK activity over 120 hours. Although the acute signalling of Akt/mTOR pathway was not significantly different between conditions, protein abundance was elevated in NAT and AAS serum, with AAS serum being significantly higher compared with DM after 96 hours. Furthermore, each condition showed comparable p-Akt and glucose uptake in the absence and presence of insulin. Nonetheless, the formation of myotubes in each serum condition allowed for the chronic impact of serum on cellular metabolism to be investigated which may have shown different results.

Myotubes conditioned in AAS serum for 4-5 days reported a trend (P = 0.06) of expressing lower p-Akt in the absence and presence of insulin compared with DM however, the effect of insulin on p-Akt and glucose uptake was similar between conditions. Due to this, other potential mechanisms of skeletal muscle metabolism were explored. The acute impact of serum on anabolic signalling was measured which showed all serum conditions to significantly increase p-Akt and p-ERK which may indicate an increase in anabolism. In addition to this, the effect of serum on MPS and MPB was explored which showed all conditions to have comparable rates of MPS (1-24 hr) and MPB (6-48 hr). These results highlight that although AAS serum does not impair skeletal muscle glucose metabolism, the lack of increased skeletal muscle anabolism at the cellular level indicates that AAS use for hypertrophy is not warranted.

Testosterone and its derivatives AAS are used clinically to increase muscle mass and function and improve metabolic health in ageing populations that are testosterone deficient (Jones *et al.*, 2011; Storer *et al.*, 2017). Controversially, due to AAS hypertrophic potential, supraphysiological dosages are used in combination with resistance exercise to improve perceived body image and muscular performance while putting metabolic health in jeopardy (McVeigh and Begley, 2017; Goldman, Pope and Bhasin, 2019). Therefore, maintenance of testosterone levels within normal healthy ranges is one important factor in the multifactorial regulation of metabolic health and risk of CVD. Furthermore, AAS users commonly use a variety of AAS types, doses and cycles in addition to other substances to increase anabolism or reduce undesirable side effects which was also observed in participants from study 3, chapter 5 (section 5.4.1.) (Sagoe *et al.*, 2015; Begley, McVeigh and Hope, 2017). The chemical interactions of AAS-related polysubstance use may also elicit additional adverse health outcomes (Evans-Brown, Kimergård and McVeigh, 2009).

It is well established that AAS and associated polypharmacy result in an adverse lipoprotein profile (increased LDL-C and reduced HDL-C) (Achar, Rostamian and Narayan, 2010). Somewhat in agreement, in study 3, chapter 5, HDL-C and Apo A1 was significantly ($P < 0.05$) lower while the highest users of AAS were associated with higher levels of LDL-C ($r = 0.65$, $P = 0.03$), ApoB ($r = 0.69$, $P = 0.02$), LDL pattern A ($r = 0.72$, $P = 0.02$) and pattern B ($r = 0.96$, $P < 0.001$). As IR is commonly associated with impaired lipid metabolism and in populations of testosterone deficiency (Muraleedharan and Jones, 2010; Sperling *et al.*, 2015), supraphysiological levels of testosterone induced by AAS use may also cause IR. Furthermore, isolated HDL from humans enhances C2C12 cellular respiration and glucose metabolism in a dose-dependent manner users (Lehti *et al.*, 2013), indicating that deficient HDL concentrations observed in the AAS group may contribute to impaired glucose metabolism. In the current study, myoblasts and myotubes showed similar increases in p-Akt^{473} and glucose uptake in response to insulin in all groups, indicating a lack of IR with acute AAS exposure. However, myoblasts were allowed to differentiate to myotubes in each serum condition for 4-5 days thereby increasing exposure compared to myoblasts. This should have provided enough time for the myotubes to develop a phenotype relevant to the physiological environment prior to insulin stimulation. Myotubes conditioned in AAS serum showed a trend of expressing lower overall p/t-Akt during insulin experimentation compared with DM ($P = 0.06$). Though, no significant interaction was observed in p/t-Akt or the insulin stimulated fold change between conditions. This also did not affect the response of insulin on glucose uptake, therefore, may have wider implications on cellular signalling such skeletal muscle anabolism.

In rodents, by contrast, supraphysiological levels of testosterone and nandrolone have been reported to significantly ($P < 0.05$) diminish the response of insulin-induced glucose uptake

(Holmang and Bjorntorp, 1992; Frankenfeld *et al.*, 2014). Rodents also showed impairments in gluconeogenesis, most likely due to the high fasting insulin levels (Frankenfeld *et al.*, 2014). However in resistance trained males, increasing doses of testosterone (25-600 mg/wk) for 20 weeks had no effect on insulin sensitivity (Singh *et al.*, 2002). Additionally, in a double-blind crossover design, 300 mg/wk of Test enanthate or nandrolone administration for 6 weeks did not affect glucose tolerance or fasting insulin levels in healthy males (Hobbs, Jones and Plymate, 1996). Yet, cross-sections of AAS polypharmacy-using participants (AAS use range, 2-7 years) have reported reduced insulin sensitivity compared to non-using controls, as indicated by OGTT results (Cohen and Hickman, 1987; Rasmussen *et al.*, 2017). The differences in results may be due to AAS polypharmacy having a greater negative effect on glucose metabolism compared with individual AAS use or the length of time in which participants had been using AAS. Participants in the studies of Cohen et al. (1987) and Rasmussen et al. (2017) had a mean AAS use of ~2.5 to 4 years whereas, whereas those in the studies of Singh et al. (2002) and Hobbs et al. (1996) had never used AAS prior to commencing their trials. The results herein, in conjunction with previous research may indicate that short term (< 20 weeks) AAS use or AAS polypharmacy may not have a deleterious impact on glucose metabolism but longer AAS/polypharmacy use might. Long term-controlled studies are required to investigate if AAS polypharmacy has a negative impact on glucose metabolism.

Importantly, testosterone (100 nM) has been shown to have insulin-like effects on human fetal skeletal myoblasts and myotubes following acute administration (30 mins), it has been shown to increase p-Akt and induce glucose transporter 4 (GLUT4) translocation (Antinozzi *et al.*, 2017). The effects of testosterone on increased p-Akt compared to basal levels were also reported to be maintained for at least 12 hours in myoblasts (Antinozzi *et al.*, 2017). It is therefore unsurprising that in myoblasts, AAS serum did not impair insulin signalling or glucose uptake as cells were incubated in serum for only 3 hours which may still elicit an anabolic response. It is perhaps a little surprising though that AAS serum did not upregulate basal p-Akt levels in myoblasts however, it is likely that the testosterone concentration the cells are exposed to are much lower in the current study compared with the reported dose used by Antinozzi et al. (2017) as the AAS serum is diluted in media (2%).

Increasing levels of testosterone have been shown to increase satellite cell proliferation and differentiation and is one mechanism of testosterone-induced muscle hypertrophy (Sinha-Hikim *et al.*, 2003). CK activity, a marker of myoblast differentiation (Sharples, Al-Shanti and Stewart, 2010; Sharples *et al.*, 2011; Dugdale *et al.*, 2018) significantly ($P < 0.01$) increased in all serum conditions. Although no significant difference was observed between conditions at any timepoint, this investigation has highlighted that C2C12 cells can successfully form myotubes in serum derived from humans in at least the same rate as conventional culture

conditions. This should allow cells to acclimatise to a metabolic environment and develop a phenotype that mimics *in vivo.* This model can perhaps be applied to many areas investigating acute and chronic metabolic adaptations seen *in vivo*. Although no significant difference in CK activity was detected between groups, the diversity of muscle function and its adaptability, warranted investigation of the impact of each condition on relevant signalling pathways and subsequent adaptation. The PI3K/Akt and MAPK (ERK and P38) signalling pathways have been implicated in initiating differentiation (Wu *et al.*, 2000; Foulstone *et al.*, 2004; Knight and Kothary, 2011). In agreement with this, upon initiating differentiation in each condition, Akt^{ser473}, $mTOR^{ser2448}$, $ERK1/2^{T202/Y204}$ and $P38MAPK^{t180/Y182}$ phosphorylation significantly increased. Interestingly, only $ERK1/2^{T202/Y204}$ and $P38MAPK^{t180/Y182}$ phosphorylation were significantly higher in NAT and AAS conditions compared with DM. In low serum conditions, P38MAPK is stimulated, resulting in activation of myogenic transcriptional regulators such as myoblast determination 1 (MyoD) and myocyte enhancer factor 2 proteins resulting differentiation (Wu *et al.*, 2000; Keren, Tamir and Bengal, 2006). The resulting effects of increased ERK are difficult to determine as ERK inhibition via the PD98059 inhibitor has shown to increase differentiation possibly through P38MAPK (Al-Shanti and Stewart, 2008). Increased ERK activation via growth factors enhances cell proliferation and prevents cell cycle exit during G_1, preventing premature differentiation (Knight and Kothary, 2011). Therefore, the data herein may indicate that the metabolic environment created by chronic AAS use does not enhance differentiation compared with regular resistance training.

Acute testosterone administration has shown to increase anabolism via a variety of second messenger signals which increase protein synthesis and reduce catabolism resulting in muscle hypertrophy (Basualto-Alarcón *et al.*, 2013; Parr *et al.*, 2018). In contrast to myoblasts observations, neither serum condition increased P38 signalling in myotubes, however, all conditions significantly ($P < 0.05$) increased p-ERK and p-Akt after 30 mins. An increase in Akt and ERK activity is commonly observed in response to serum administration due to growth factors resulting in increased anabolism (mTOR pathway) and reduced catabolism (Forkhead box O pathway) (Frost and Lang, 2007; Lavoie, Gagnon and Therrien, 2020). Both ERK and Akt activation have been reported to increase skeletal muscle growth via parallel phosphorylation of tuberous sclerosis complex 2 resulting mTORc1 activation and downstream signalling (Winter, Jefferson and Kimball, 2011). Although all groups increased p-Akt and p-ERK, surprisingly, the NAT group showed the largest increases (Akt, 2.4-fold; ERK, 3.1-fold) compared to AAS (Akt, 1.6-fold; ERK, 1.1-fold) and DM (Akt, 1.7-fold; ERK, 1.5-fold) at 30 minutes. Nonetheless, it is difficult to determine what exactly is causing the increased Akt and ERK activity in all groups however, it could be expected that the AAS group would show the largest increase in Akt and ERK activity due to the known effect testosterone

has on these anabolic pathways (Basualto-Alarcón *et al.*, 2013; Parr *et al.*, 2018). In response to 100 nM of testosterone, p-ERK and p-Akt have been shown to significantly increase and peak at 5 and 15 min then decrease at 15 and 30 min respectively in myotubes (Basualto-Alarcón *et al.*, 2013). Therefore, it is possible that peak ERK activity in the AAS group has been overlooked in this study.

Moreover, exercise and AAS use are known to increase circulating growth factors which may activate the Akt pathway. For example; regular resistance exercise is reported to increase circulating IGF-I levels and may be implicated in the upregulation of p-Akt observed with NAT serum (Jiang *et al.*, 2020). Testosterone has also shown to increase IGF-I expression (Sculthorpe *et al.*, 2012) and doses of 300 and 600 mg/wk for 20 weeks increased circulating IGF-I levels in males (Bhasin *et al.*, 2001). In contrast, AAS-related polypharmacy has reported serum IGF-I levels to significantly decrease over a period of 24 months in males (n = 20) (Bonetti *et al.*, 2008). However, testosterone levels did not increase during the study by Bonetti et al. (2008) suggesting that the participants did not take sufficient levels of AAS to be deemed supraphysiological. Although unclear, the low doses of exogenous AAS may still have been enough to suppresses the hypothalamic-pituitary-testicular axis resulting in reduced endogenous testosterone production and hypogonadism which is associated with a decline in IGF-1 levels (Sheffield-Moore, 2000; Bonetti *et al.*, 2008; Kanayama *et al.*, 2015).

The metabolic health blood markers measured in chapter 5 are unlikely to directly explain the results *in vitro* as many are not likely to trigger skeletal muscle signalling pathways unlike the diverse range of growth factors present in blood. Furthermore, many of the blood markers measured were similar between groups except for HDL-C, Apo A1 and plasminogen activator inhibitor 1 (PAI-1). Moreover, the acute effects of AAS use cannot be ruled out as some participants are likely to have administered AAS within 48 hrs. As mentioned above HDL-C has shown to have a dose dependent effect on C2C12 glucose metabolism (Lehti *et al.*, 2013) indicating that AAS users may have impaired glucose metabolism due to HDL-C deficiency. However, as no difference was observed in insulin stimulation, glucose metabolism appeared not to be impaired by AAS use although acute effects of AAS use may have negated this effect. The plasminogen system controls the extracellular matrix structure and PAI-1 plays a key role in its regulation via activation of urokinase-type plasminogen activator (Rahman and Krause, 2020). Due to this, PAI-1 is capable of affecting many tissues and PAI-1 deficiency has been shown to enhance skeletal muscle regeneration through increased urokinase-type plasminogen activator activation (Koh *et al.*, 2005; Rahman and Krause, 2020). AAS use is associated with lower levels of PAI-1 (Ferenchick *et al.*, 1995; Chang *et al.*, 2018) and was also observed in chapter 5 therefore, coupled with the potential anabolic effects of AAS use, the lower levels of PAI-1 may contribute to the enhanced differentiation of the AAS group.

Although this does not explain the enhanced effects of the NAT group. It is also likely that metabolites not measured in chapter 5 may also have contributed to the *in vitro* data. For example, testosterone administration stimulates net protein synthesis in fasted conditions, not by amino acid transport to muscle tissue but by increased reutilisation of amino acids from protein breakdown (Ferrando *et al.*, 1998). Therefore, it is likely that AAS administration reduces blood amino acids relative to non-using controls. It may be assumed that the NAT group has a higher concentration of amino acids relative to AAS users which may have enhanced skeletal muscle differentiation and anabolic signalling (Zhang *et al.*, 2018). Further research is warranted on the profiling of serum with high-throughput techniques such as metabolomics to collate many of the possible bioactive compounds that may act on skeletal muscle.

Given the limited impact of AAS vs NAT on any parameters studied thus far, the potential impact of increased ERK and Akt activity by each serum condition on MPS and MPB in myotubes were measured using the modified SUnSET method (Crossland *et al.*, 2017). Puromycin was rapidly incorporated into myotubes when incubated with each serum condition but was reduced at 24 hours, with no differences observed between groups. Similarly, upon removal of puromycin from media, the rate of puromycin decorporation, an indicator of MPB, was similar between groups.

Interestingly, serum derived from humans in the fasted and fed state (amino acid ingestion) also did not show any difference in MPS using a similar method in C2C12 myotubes (Carson *et al.*, 2018). However, mTOR and downstream signalling significantly increased compared to the fasted serum. The level of anabolic stimulus in the serum conditions in this study and in that by Carson et al. (2018) may be at too low a level to observe any difference between conditions using the puromycin method but large enough to increase anabolic signalling. Methods with higher levels of sensitivity such as stable isotope tracer (Griggs *et al.*, 1989; Sheffield-Moore *et al.*, 1999; Shankaran *et al.*, 2016) and dynamic profiling of protein turnover techniques could be used to detect individual and global syntheis rates (Stansfield *et al.*, 2020). However, protein levels were signifcantly higher after 120 hrs with AAS serum during the differentiaition experiment, indicating that MPS did increase with AAS serum and that measuring MPS over 24 hours may be too short to detect diffferences between groups.

Although no negative impact on skeletal muscle glucose metabolism was observed with acute and short-term exposure of AAS serum compared with NAT, the well documented effects on lipoprotein metabolism highlight increased poor cardiometabolic health and CVD risk in this population (Achar, Rostamian and Narayan, 2010). Furthermore, although skeletal muscle is the largest disposal of insulin stimlated glucose uptake (Thiebaud *et al.*, 1982), other insulin

sensitive cells such as hepatocytes and adipocytes could be effected and also play a key role in the pathogenesis of IR leading to T2D (Samuel and Shulman, 2016). The data herein suggest that at the muscle cellular level, anabolic signalling is comparable between both NAT and AAS serum. It could therefore be inferred that the common lifestyle associated with both these groups (diet and resistance training) create a similar anabolic metabolic environment and AAS use does not enhance this. An anabolic cellular enviornment may be be beneficial in maintaining muscle mass and consequently, cardiometabolic health reducing CVD risk (Gordon *et al.*, 2009; Tyrovolas *et al.*, 2020). AAS are used to treat testosterone deficiency and can improve cardiometabolic health and muscle mass in ageing populations (Muraleedharan and Jones, 2010; Jones *et al.*, 2011; Storer *et al.*, 2017). However, due to the associated risks with AAS over-use such as CVD and prostate cancer (Baggish *et al.*, 2017; Goldman, Pope and Bhasin, 2019), where there is no clinical need, a resistance exercise focused lifestyle is likely a much safer option at improving cardiometabolic health by increasing muscle hypertrophy (Ihalainen *et al.*, 2019).

6.6. Limitations

Much of the limitations have been described in chapter 4 (section 4.6) and chapter 5 (section 5.6). Briefly, numerous markers of metabolic health were measured however, there is still many variables within the serum that can be triggering acute and chronic effects on skeletal muscle. Metabolomic analysis could lead to specific pathways being attributed to changes in cellular metabolism (Cantor *et al.*, 2017; Yang, Vijayakumar and Kahn, 2018). Furthermore, proteomic analysis of skeletal muscle could identify potential mechanisms in which chronic serum incubation can impact cellular metabolism (Stansfield *et al.*, 2020).

The use of human serum on murine cells may also be a limitation as it may limit the relevance to human skeletal muscle; however, the C2C12 cell line has shown to be an established model of skeletal muscle and reduces variability in findings (Wong, Al-Salami and Dass, 2020) and the current study may lay the foundations for future research using human serum with human muscle.

Glucose uptake and insulin signalling data were conducted in different conditions therefore data must be considered independent from one another. Different conditions were necessary to provide the optimal environment for the glucose uptake assay. Serum was collected in fasted conditions however, the time between blood sample collection and last AAS administration and/or exercise session was not controlled for thus, subsequent acute effects on serum environment may have impacted results.

Finally, cells were dosed with 2% serum which would have significantly diluted the biochemical properties of serum however, ≥2% would compromise myoblast fusion and cell viability.

6.7. Conclusion

Although muscle mass is typically higher in AAS users, paradoxically, AAS use also increases CVD risk (study 3, chapter 5). Long term AAS use may also increase IR (Cohen and Hickman, 1987; Rasmussen *et al.*, 2017) however, short term testosterone (< 20 weeks) use may not be detrimental (Hobbs, Jones and Plymate, 1996; Singh *et al.*, 2002). The data in the current study show that insulin dynamics are not impaired with AAS use as highlighted by similar p-Akt^{ser473} and glucose uptake in DM, NAT and AAS serum conditions in myoblasts and myotubes. Although CK activity, a marker of differentiation was not significantly different between conditions, AAS and NAT serum showed significant increases in ERK1/2$^{T202/Y204}$ and P38MAPK$^{t180/Y182}$ phosphorylation compared to DM in myoblasts. An increase in MAPK signalling, particularly P38, mediates an upregulation in differentiation via activation of myogenic regulatory transcription factors. Although myotube protein abundance was significantly higher in AAS serum compared with DM after 120 hours, no difference in MPS was observed over 24 hours using the SUnSET method between conditions. However, myotubes showed an increase in p-Akt and p-ERK in response to acute serum stimulation indicating an upregulation in anabolic signalling. No difference was observed between serum conditions in MPS or anabolic signalling which may indicate that at the cellular level AAS use does not elicit a greater hypertrophic response compared with resistance training alone. Due to the associated risks of AAS use, and in combination with the muscle specific data presented here, chronic resistance training provides a safer alternative to AAS prescription in healthy populations to maintain muscle mass and positive metabolic health. Future studies using cultured myoblasts and myotubes from NAT vs. AAS users (given potential epigenetic memory of muscle cells) would represent a good model to investigate the impact of long-term exercise vs exercise +AAS use on skeletal muscle adaptation.

Chapter 7: Synthesis

7.1 Key findings

The overall aim of this thesis was to improve guidance for cardiometabolic disease (CMD) in relation to dietary advice and anabolic-androgenic steroid use by investigating markers of CMD in response to a very low carbohydrate (VLC) diet (objective 1) and among users of anabolic-androgenic steroids (AAS) (objective 3) in addition to investigating the subsequent impact of the metabolic environment on mechanisms of skeletal muscle insulin signalling and metabolism using serum derived from each cohort (objective 2 + 4).

Each study in this thesis aimed to achieve this by completing the following objectives:

1. To explore if an 8-week *ad libitum* VLC diet on key markers of CMD in comparison with a high carbohydrate (HC) diet (current UK guidelines).
2. To investigate if skeletal muscle metabolism and insulin signalling improves in response to serum from participants following VLC or HC diets.
3. To determine if key markers of CMD are different among users of AAS in comparison with healthy controls.
4. To investigate the impact of serum from participants who use and don't use AAS on skeletal muscle metabolism and insulin signalling.

7.1.1 Objective 1

To investigate if a VLC diet improved markers of CMD compared with a HC diet in study 1, consented participants with a slightly elevated metabolic risk followed either a VLC diet (n = 8, <50 g of carbohydrates per day) or a HC diet (n = 8, current UK guidelines) for 8 weeks. Study 1 challenged the hypotheses that a VLC diet would show greater improvements in markers of CMD, which included, metabolic syndrome (MetS) markers (high-density lipoprotein cholesterol (HDL-C), triglycerides, blood pressure (BP), waist circumference (WC), insulin resistance (IR), inflammation, lipoprotein metabolism (small-dense lipoprotein cholesterol (sdLDL-C) and apolipoprotein B (Apo B), novel biomarkers of CMD (fibroblast growth factor 21 (FGF21), Leptin and adiponectin) and body composition (body fat %, visceral adipose tissue (VAT) (l)). In partial acceptance of the hypotheses, an 8-week *ad libitum* VLC diet showed significant (P < 0.05) improvements in markers of CMD such as BP, fat mass, WC, VAT and sdLDL:low-density lipoprotein cholesterol ratio vs. HC but both diets showed similar significant (P < 0.05) improvements in triglycerides, insulin and Homeostatic Model of IR (HOMA IR). Furthermore, the VLC diet induced a trend (P = 0.05) of decreasing FGF21 whereas the HC diet showed a trend (P = 0.08) of increasing adiponectin while both diets also significantly (P < 0.05) improved the leptin:adiponectin ratio (LAR). These data indicate that both a VLC and HC diet can improve cardiometabolic health; however, the VLC diet showed

greater improvements in LDL composition and BP. Both diets are likely to have improved the metabolic environment resulting in improved markers of insulin resistance; therefore, the impact of the serum derived from participants was used to investigate mechanisms of insulin signalling in study 2. Given the reduction in FGF21 in the VLC diet, which is implicated in insulin sensitivity, its impact was also addressed in study 2.

7.1.2 Objective 2

Using serum derived from participants in study 1 and the C2C12 skeletal muscle cell line, research was undertaken to determine if the improvement in cardiometabolic health due to a VLC and HC diets resulted in improved skeletal muscle insulin signalling and metabolism. Study 2 intended to address the hypotheses that serum derived from participants who improved cardiometabolic health following a VLC and HC diet would cause an improvement in insulin signalling, glucose uptake, reduce cellular stress and FGF21 would enhance insulin signalling. In partial acceptance of theses hypotheses, both diets significantly (P = 0.02) increased Akt phosphorylation (p-Akt) in myoblasts at interim and endpoint compared with baseline; myotubes showed a significant (P = 0.01) decrease of p-Akt compared to baseline leading to a significant (P < 0.05) interaction between timepoints vs. control and insulin stimulation in both diets. However, the relative fold-change was not significantly different between timepoints or groups in myoblasts or myotubes. Both diets reduced cellular stress as shown by a decrease in AMP-activated protein kinase phosphorylation (p-AMPK). Additionally, glucose uptake did not significantly change with serum or insulin dosing. Moreover, in standard cell culture conditions FGF21 significantly (P < 0.05) enhanced the insulin induced fold change in p-Akt in myoblasts and myotubes. However, FGF21 had no impact on the effect of insulin on p-Akt in serum derived from participants at baseline. The data produced by study 2 indicate that the improvement in metabolic health exhibited by participants in study 1 resulted in reduced cellular energy stress as observed by reducing acute p-AMPK. As both diets showed decreases in p-Akt in myotubes, it is likely that the high levels of p-AMPK at baseline resulted in increased p-Akt. When p-Akt is relativised to p-AMPK, both diets show a tendency of increasing p-Akt. The lack of effect of FGF21 on insulin signalling in serum derived from participants at baseline may be due to the endocrine environment. For example, the fatty acid palmitate (which is associated with IR) has shown to reduce FGF21 signalling (Jeon *et al.*, 2016), which agrees with the current FGF21 resistance hypothesis in populations with MetS.

7.1.3 Objective 3

To determine if markers of CMD are upregulated among users of AAS, a cohort of resistance-trained males who use (n = 9) or don't use (n = 10, NAT) AAS provided anthropometric data

and blood samples. Study 3 challenged the hypotheses that individuals who use AAS display CMD as measured by MetS markers (HDL-C, triglycerides), IR and inflammation, lipoprotein metabolism (LDL phenotype pattern, and apolipoprotein A1 (Apo A1) and Apo B) and that higher quantities of AAS use would also be associated with higher CVD risk. In accordance with these hypotheses, users of AAS display a significant (P = 0.04) suppression of HDL-C and apolipoprotein A1; however, they showed comparable levels of triglycerides, insulin, glucose and inflammation (tumour necrosis factor- α, IL-6) to the healthy control group. Additionally, AAS use was associated with impaired lipoprotein metabolism with significantly higher levels of with LDL-C (r = 0.65, P = 0.03), ApoB (r = 0.69, P = 0.02), pattern A (r = 0.72, P = 0.02) and pattern B (r = 0.96, P < 0.001). associated with higher AAS use. These data indicate that users of AAS are at increased risk of developing CVD due to suppression of HDL-C and Apo A1 and higher levels of AAS use may further increase CVD risk due to its association with LDL-C and Apo B. Previous *in vitro* studies on the effect of AAS on insulin-induced skeletal muscle signalling and metabolism typically include the use of testosterone alone. However, users of AAS use a variety of pharmaceuticals to enhance growth and minimise unwanted effects, yet the effects of AAS-related polypharmacy are yet to be investigated. Serum derived from participants in this study may represent a relevant physiological model to investigate the effects of AAS-related polypharmacy on the systemic metabolic environment and subsequent skeletal muscle metabolism and insulin signalling.

7.1.4 Objective 4

To investigate the impact of AAS use on the metabolic environment and subsequent skeletal muscle metabolism and insulin signalling, serum derived from participants in study 3 and the C_2C_{12} skeletal muscle cell line was used. Study 4 intended to address the hypotheses that serum derived from AAS users would impair insulin signalling, glucose uptake but increase myoblast differentiation and myotube protein synthesis compared with serum from resistance trained males who don't use AAS (NAT) and standard cell culture conditions (DM). In contrast with these hypotheses, insulin significantly increased p-Akt and glucose uptake comparably in all serum conditions in myoblasts and myotubes. Furthermore, AAS serum did not enhance myoblast differentiation measured by creatine kinase (CK) activity or myotube protein synthesis; however, AAS and NAT conditions significantly upregulated extracellular regulated kinases 1/2 (ERK1/2) and P38 mitogen activated protein kinase (P38MAPK) compared with DM. These data suggest that an acute administration of AAS to myoblasts does not create a metabolic environment that impairs insulin signalling and glucose uptake. As both AAS and NAT serum showed similar increases in myoblast differentiation and upregulated signalling compared with DM, regular resistance training may create an anabolic metabolic environment which may be beneficial to health; although, no difference in protein synthesis was observed

in myotubes over 24 hours between conditions, protein abundance was significantly ($P < 0.05$) higher with AAS serum compared with DM.

7.2 General findings

Metabolic health is largely influenced by poor lifestyle, which can lead to the development and progression of metabolic abnormalities resulting in disease (Martín-Timón, 2014; Sperling *et al.*, 2015). The investigation of mechanisms of disease development and progression is of great clinical importance subsequently that such diseases can be prevented or treated. Research has shown that IR a key feature of CMD, is largely caused by lipotoxicity, inflammation, oxidative stress, hyperglycaemia and mitochondrial dysfunction (Boucher *et al.*, 2014; Samuel and Shulman, 2016). Cellular models have been key in elucidating such mechanisms and have greatly developed the understanding of biology and disease. However, cell culture has been developed to optimise *in vitro* conditions rather than mimic the *in vivo* environment (Yao and Asayama, 2017). Furthermore, cellular investigations of IR typically change one variable at supraphysiological doses thus distancing it from being physiologically relevant to disease states *in vivo* (Yuan *et al.*, 2017; Turner *et al.*, 2018). In IR conditions, a myriad of metabolic disturbances (nutrients, hormonal and inflammatory) are implicated *in vivo.* Therefore, to improve the understanding of how the *in vivo* metabolic environment impacts mechanisms of insulin signalling associated with cardiometabolic health, a more physiologically relevant model is required. Serum derived from humans may increase the physiological relevance of cell culture conditions to investigate systemic influences on *in vitro* insulin signalling and cellular metabolism.

Diet has a profound effect on energy metabolism and the metabolic environment and poor nutrition can manifest in CMD. In line with previous research, study 1 has demonstrated that both a VLC and HC diet can improve markers of CMD although a VLC diet may show favourable improvements in lipoprotein metabolism, as shown in the study and by others (Volek *et al.*, 2009; Gardner *et al.*, 2018; Gjuladin-Hellon *et al.*, 2019; Hyde *et al.*, 2019). Both diets improved markers of IR (LAR and HOMA IR) potentially due to a decrease in inhibitory factors within the metabolic environment (Boucher *et al.*, 2014). For example, in addition to significant changes in LAR, the *ad libitum* VLC diet reported novel favourable changes in FGF21 which is associated with metabolic disease and insulin sensitivity (Mashili *et al.*, 2011; Jeon *et al.*, 2016). As the changes in metabolism associated with each diet would be impossible to replicate *in vitro,* the serum derived from participants following either diet may be able to replicate the *in vivo* systemic environment *in vitro*. This was demonstrated in study 2 where cells incubated with serum from participants following either a VLC or HC diet had an impact on insulin signalling and cellular metabolism. The results demonstrated that serum prior to beginning the dietary intervention increased cellular energy stress as shown by an increase in p-AMPK which may have resulted in a compensatory increase in p-Akt with insulin

stimulation to increase glucose uptake. In accordance with the dietary intervention, over time, cellular stress decreased resulting in a reduction in this compensatory increase in p-Akt. These results provide novel mechanisms on how diet influences the metabolic environment and subsequent insulin signalling.

Testosterone can also greatly influence metabolic health as highlighted by AAS users (Traish and Kypreos, 2011; Goldman, Pope and Bhasin, 2019). AAS use results in supraphysiological levels of circulating testosterone resulting in increased skeletal muscle hypertrophy; however, this can result in dyslipidaemia and possibly additional markers of CMD such as IR and abdominal obesity (Achar, Rostamian and Narayan, 2010; Rasmussen *et al.*, 2017). Study 3 has shown that among users of AAS HDL-C and Apo A1 synthesis is suppressed compared with healthy matched controls, which is in accordance with previous findings (Achar, Rostamian and Narayan, 2010; Rasmussen *et al.*, 2017). Furthermore, triglyceride, insulin, glucose and inflammation (TNF-α, IL-6) levels were similar to the control group indicating that AAS use is associated with impaired localised cardiometabolic health rather than exhibiting CMD. AAS use did not appear to impair glucose metabolism *in vivo* and study 4 used serum derived from AAS and NAT participants to investigate if the mechanisms on insulin signalling and cellular metabolism *in vitro*. In accordance with study 3, serum from AAS users did not impair insulin signalling and glucose uptake compared with NAT serum or standard cell culture conditions. Interestingly, although myoblast differentiation was similar between conditions, AAS and NAT serum induced significantly higher ERK1/2 and P38MAPK activity compared with DM indicating an increase in differentiation potential. These results indicate that AAS use is not associated with impaired insulin signalling and glucose metabolism.

7.3 Future directions

Cardiometabolic health and its regulation is multifaceted, with lifestyle playing a major role. Diet particularly has a great influence on metabolic health and subsequent risk of developing metabolic diseases (Rodríguez-Monforte *et al.*, 2017; Medina-Remón *et al.*, 2018). Although relatively short in length but in accordance with previous research (Volek *et al.*, 2009; Bueno *et al.*, 2013; Mansoor *et al.*, 2016; Hyde *et al.*, 2019), data derived from this thesis has further indicated that a VLC diet with increased saturated fatty acids (SFA) intake can improve cardiometabolic health perhaps to a greater extent than HC diet due to the superior improvements in sdLDL-C. Both diets similarly improved markers of IR (HOMA IR and LAR) which was mirrored *in vitro* in study 2. Both diets showed similar reductions in p-AMPK indicating a reduction in cellular stress and reducing compensatory increases in p-Akt with insulin stimulation. These data highlight that, provided the diet reduces circulating inhibitory molecules, markers of cardiometabolic health will improve particularly insulin sensitivity. This leads to potential questions regarding the effect of diet on circulating metabolites prior to their effect on cardiometabolic parameters:

1. What is the metabolite profile of diets differing in carbohydrate and fat intake associated with improvements in cardiometabolic health?
2. If both a HC and LC diet reduce inhibitory molecules of insulin signalling, what dietary pattern is associated with an increase in these inhibitory molecules resulting in IR?

Therefore, future studies should focus on the effects of different diets on molecules that are associated with insulin signalling and resistance such as circulating branched chain amino acids (BCAA), lipids, ketones, bile acids and nucleotides (Yang, Vijayakumar and Kahn, 2018). Furthermore, the impact of diet on metabolomic signatures in a variety of populations (highly trained, sedentary, obese, AAS-users etc.) should be investigated as this could provide evidence for greater personalised nutrition to maintain or improve cardiometabolic health. This would reduce reliance on blanket recommendations which are not optimal for all populations. For example for every 1 standard deviation increase in baseline metabolites (consisting primarily of BCAA) there was a 2-fold increase in T2D risk (Papandreou *et al.*, 2019). Furthermore, metabolic profiling may predict responders and non-responders to interventions as baseline glycine and N-phenylacetylglycine concentrations were able to predict improved responses in inflammation and oxidative stress in response to Korean blackberry supplementation in participants with obesity (Kim *et al.*, 2017)

Research on the effects of diet on the metabolomic profiles in a variety of populations is growing and is showing promising signs of describing mechanisms of health and diet (Rangel-Huerta, Pastor-Villaescusa and Gil, 2019). Circulating BCAA have been identified as a signature of CMD and have even been able to distinguish between metabolically healthy and unhealthy obese phenotype (Newgard, 2017; Telle-Hansen *et al.*, 2020). Furthermore, in obese participants, fatty acids, BCAA and carboxylic acids were shown to be upregulated compared with lean participants and an 8-week VLC diet resulted in fat loss and a metabolic shift in such metabolites closer to the lean phenotype (Gu *et al.*, 2013). Future research can build on these findings to improve the understanding on how diet impacts the metabolome and subsequent cellular signalling.

Data from this thesis also highlighted that AAS use is associated with low HDL-C and Apo A1 indicating impaired cardiometabolic health; however, markers of IR (HOMA IR and leptin) and *in vitro* insulin signalling were not impaired. Limited research has reported AAS use to be associated with impaired glucose metabolism as shown by oral glucose tolerance tests (OGTT) (Cohen and Hickman, 1987; Rasmussen *et al.*, 2017). Further research is warranted in this population as insulin resistance is a key feature of CMD (Sperling *et al.*, 2015). Controlled studies with testosterone have shown no negative effect on insulin sensitivity with increasing doses (Singh *et al.*, 2002). However, AAS users do not use testosterone in isolation therefore limiting the generalisability of the data. In self-administrating AAS users who had never used AAS previously, HOMA IR was shown to improve after 12 months of AAS use yet, circulating testosterone levels did not increase from baseline (Bonetti *et al.*, 2008). Due to this, further research is warranted on the metabolic effects of AAS-related polypharmacy including markers of IR. Longitudinal assessment of self-administrating AAS users for at least 24 weeks would allow for measurement of markers of metabolic health during on and off cycles to determine causal and perhaps lasting effects of AAS-related polypharmacy.

Although more invasive, the direct impact of dietary interventions on skeletal muscle is yet to be investigated. Satellite cells isolated from participants with T2D maintain their insulin resistant phenotype in culture (Gaster, 2019). In a similar design to study 1 and 2, future studies could investigate the impact of diet on insulin signalling using satellite cells isolated from participants. The impact of serum derived from the same participants could also be used to investigate fusion and mechanisms of insulin signalling. Similar methods could be employed when investigating the effects of AAS use on cardiometabolic health.

In relation to cell culture methods, future research should consider using conditioned serum to study the acute and chronic effects of serum from populations of interest to increase the physiological relevance of media or to investigate the impact of the cellular environment on

skeletal muscle phenotype expression. For example, acute stimulation of cells with serum can result in different metabolic or signalling pathways being regulated due to the new environment. While chronic serum incubation consists of cells differentiating in the new environment which can lead to a change in genotype expression and subsequent phenotype expression. Both acute and chronic serum conditions may resulting in altered metabolic responses to stimuli such as insulin relevant to *in vivo* conditions. In addition to studying mechanisms of insulin signalling observed in this thesis and others (Cogan *et al.*, 2019), serum can be used to investigate mechanisms of ageing (Kalampouka, van Bekhoven and Elliott, 2018). Plasma derived from older (>57 years old) participants showed impaired myoblast migration and myotube regeneration compared with plasma derived from young (<35 years old) participants (Kalampouka, van Bekhoven and Elliott, 2018). These data propose ideas such as the following:

1. Can young serum improve fusion and muscle regeneration in a model of ageing skeletal muscle such as population doublings (Sharples *et al.*, 2011)?
2. Can serum from healthy or endurance athletes improve cellular metabolism in cells isolated from T2D?
3. What impact does serum from different populations have on mechanisms of *in vitro* muscle contractions?
4. Dose serum from different populations alter *in vitro* specific protein turnover using dynamic proteome profiling (Stansfield *et al.*, 2020)

The potential use of conditioned serum in addition to metabolomic profiling represent exciting research areas to investigate phenotypes of interest and their effect on cellular mechanisms.

7.4 Thesis Conclusions

Cardiovascular diseases and type 2 diabetes dominate the top 10 causes of deaths globally. Commonly associated with these diseases are the often-interrelated constellation of metabolic abnormalities also referred as cardiometabolic disease (CMD). Insulin resistance (IR) is a key feature of CMD and involves the inadequate response of insulin to increase cellular glucose uptake in organs and peripheral tissues. Lifestyle plays an integral role in the regulation, prevention and treatment of CMD. Understanding how different lifestyle factors effect cardiometabolic health and mechanisms of insulin signalling are of great clinical importance. Therefore, this thesis investigated markers of cardiometabolic health in response to diets differing in carbohydrate intake and among users of anabolic-androgenic steroids (AAS). In adults at an elevated cardiometabolic risk, an *ad libitum* very low carbohydrate (VLC) and high carbohydrate (HC) diet for 8-weeks induced similar improvements in fasting triglyceride and insulin levels and markers of IR (HOMA IR and leptin:adiponectin ratio). However, the VLC diet showed greater improvements in small-dense low-density lipoprotein cholesterol and body composition (fat mass and VAT). Furthermore, fibroblast growth factor 21 (FGF21) a marker of CMD and potential insulin sensitizer decreased with the VLC diet. Users of AAS were shown to be somewhat metabolically alike with sedentary/obese populations as high-density lipoprotein cholesterol and ApoA1 were shown to be reduced compared with healthy matched controls indicating an elevated impaired cardiometabolic health. Additionally, higher levels of AAS user were positively associated with low-density lipoprotein cholesterol and ApoB. However, markers of IR (HOMA IR and leptin) were not impaired with AAS use.

To elucidate potential mechanisms of insulin signalling and cellular metabolism associated with cardiometabolic health, serum derived from participants was used with the C_2C_{12} skeletal muscle cell line. In accordance with the improvements of diet on markers of IR *in vivo*, both a VLC and HC diet showed comparable reductions in AMPK phosphorylation (p-AMPK) indicating a reduction in cellular energy stress. The increased levels of p-AMPK at baseline (perhaps due to impaired metabolic health) increased cellular insulin sensitivity as shown by increased Akt phosphorylation (p-Akt). When accounting for the potential compensatory effect of high p-AMPK at baseline, both diets increased insulin induced p-Akt. In standard cell culture conditions FGF21 enhanced insulin stimulated fold change in p-Akt however, FGF21 had no impact on the effect of insulin on p-Akt in serum derived from participants at baseline perhaps due to the inhibitory endocrine environment. These results indicate that both a HC and VLC diet improve the metabolic environment leading to increased insulin sensitivity. Serum derived from AAS users did not impair insulin signalling or glucose uptake compared with healthy controls, which is in accordance with *in vivo* findings. Creatine kinase (CK) activity, a marker of differentiation, was not significantly different between serum conditions however, AAS and

NAT serum significantly increased extracellular regulated kinases 1/2 (ERK1/2) and P38 mitogen activated protein kinase (P38MAPK) compared with standard cell culture conditions (DM) which are implicated in myoblast differentiation and fusion. Furthermore, myotube protein synthesis was similar between serum conditions although protein abundance was significantly elevated in the AAS group. As NAT serum showed a similar anabolic metabolic environment to AAS serum, resistance training may improve the endocrine evironment resulting in maintenance of muscle mass in populations of anabolic resistance. This thesis highlights that lifestyle is integral to cardiometabolic health regulation and modulates skeletal muscle insulin signalling via the cellular environment.

The current challenge is to determine what effects diet and AAS have on the cellular enviornment that are associated with changes in cardiometabolic health and regulation of insulin signalling. A greater understanding of these translational mechanisms could help develop optimal treatments and guidelines for the highly prevalent rates of cardiometabolic disease.

Chapter 8: Appendices

8.1: Appendix 1: Very low carbohydrate diet guide and example meal plan

Low-carb Group

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Contents

Introduction

Hello and welcome to the CALIBER study! Nice to have you on board.

This booklet has been designed to be your companion over the next eight weeks whilst you are part of our cohort and to support you to consume a healthy diet whether you are cooking at home, buying ready-made meals or eating out. There are certain rules that can be applied to all of these situations.

We will also give you information on the purpose of our study, what to expect during your visits to our laboratories and once the study has finished and we have analysed the results. This means that not only will you help an important research cause but you can also find out how you and your body did over the course of these eight weeks and how your body composition, blood profile, food cravings and cognition 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?

Maybe you have followed the news over these past couple of years and noticed that there is a lot of controversy and discussion about what makes a diet healthy. The debate has been particularly heated around the issue of carbohydrates. Whilst many public health officials have argued that the vast majority of the population does not follow the dietary UK guidelines (which can be classed as high-carbohydrate, moderate-fat) and that this is the root cause of the UK's problem with ill-health, obesity and diseases such as heart disease and type 2 diabetes, others claim that these guidelines have caused these problems to begin with. The latter group advocates to reduce the amounts of carbohydrates we consume as a nation and for the guidelines to be re-written.

A third group suggests that it is far more complicated than this and that how we react to carbohydrates is actually far more personal and not one-size-fits-all, but some people might be better off on low-carb diets whilst others fare better on high-carb diets in terms of cardiometabolic health.

This is where our study comes in, CALIBER – Carbohydrates, lipids and biomarkers of traditional and emerging cardiometabolic risk factors. We want to compare the effects of consuming either a high-carb or a low-carb diet on these risk factors. The reason why we have asked you to join us is because you showed some slightly elevated risk markers for these illnesses, albeit still at a stage where these should be easy to control and improve through a healthy diet, be this in form of low-carb, high-fat or high-carb, moderate-fat.

The discussion surrounding carbohydrates does not simply stop at their potential impact on risk markers that we find in our blood. There is also some discussion whether carbohydrates or fats lead to an increase in waist circumference and the development of fat deposits in our bodies, whether carbohydrates or fats are the root cause of food cravings that we might experience and whether one or the other somehow helps us to move about more or makes us more sluggish.

As scientists and nutritionists we are naturally curious to find out what might be going on. So thank you for joining us!

What to expect during you visits to our lab

There will be three appointments where we will ask you to come to IM Marsh campus (L17 6BD) for more thorough assessments, one right at the start when you will commence to eat according to the guidelines given to you, one after about 4 weeks and the final visit 8 weeks after your first one.

Each visit is expected to last between 60 and 90 minutes and will entail

A venous blood sample

We take about 8 teaspoons of blood. – Please note that you will have to have fasted for at least 12 hours prior to your appointment as otherwise your blood sugar and your triglycerides might be far higher than normal – painting a wrong picture of how the eating plan is working for you. Just as you had to do before coming in for your initial finger prick appointment you will also have to restrain from drinking alcohol or undertaking 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 and type 2 diabetes might have changed over the course of eight weeks.

Assessing your body composition

This will be done in two 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 ensure that this can be facilitated.**

Secondly, we will ask you to step onto sophisticated 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.

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.

Going through a couple of brief questionnaires with you and conducting one final interview

During your second and your final visit we will go through a check list to see which types of fibre-containing 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 past 4 weeks whilst you were eating according to the low-carb rules.

We will also ask you to bring the container containing your multivitamin and mineral supplement with you containing any remaining pills. During your second lab visit we will provide you will a further supply of supplements for the final four weeks of the study.

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 the diet allocated to you.

Prior to your lab appointments – recording of physical activity

On three occasions (just before your first, second and final visit to our lab) we will ask you to wear a physical activity monitor that looks like a digital watch and has to be worn on the wrist (just like a 'Fit bit') of your non-dominant arm. That means if you are right-handed you will have to wear this on your left wrist.

We will give you this device at least 8 days before your visits to our labs and will ask you to start wearing it for 7 days and at least 10 hours per day commencing on the morning after it has been handed to you and finishing the night before your lab appointment. During this time you will have to complete a wear-time diary on a daily basis, in which you will briefly record the times you are putting the monitor on in the morning, the times you are taking it off at night and any time during the time when you need to remove and put it back on, for example when you are taking a shower or when you are going swimming.

You will need to bring the monitor and the wear time diary with you on the morning of your lab appointment where the research team can collect it from you.

Prior to your lab appointments – food diaries and questionnaires

When you are given your physical activity devices we will also give you a template of a four-day food diary, which you will need to complete for four days before your lab appointment and bring with you on the morning.

We will also give you a number of brief questionnaires, which will assess your cognition and your food cravings over the previous four-week period. These will not take long to complete. Please also bring these questionnaires with you to your lab appointment and pass them on to our research team.

Your involvement in the study – Flow chart low-carb group

Physical activity monitor collection//delivery	First lab visit	Physical activity monitor collection/ /delivery	Second lab visit	Physical activity monitor collection/ /delivery	Third lab visit

All lab visits will take place at LJMU IM Marsh campus in Mossley Hill (L17 6BD).

Your contacts

Your main contact is Tanja Harrison who is also a registered associate nutritionist with the Association for Nutrition.

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If you have any queries or concerns throughout the study, please do not hesitate to contact Tanja.

Other researchers on the team that you will meet on a regular basis

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This study was approved by LJMU's Research Ethics Committee on 16 December 2016 (Ref. 16/ELS/029). 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 complaint, please contact researchethics@ljmu.ac.uk and your communication will be re-directed to an independent person as appropriate.

Further information throughout the study

You can also find all the materials in a private Facebook Group, which you can request to join should you wish to do so at https://www.facebook.com/groups/lowcarbCALIBER/

We will also post regular updates here. However, should you not wish to join all the links and information will be provided to you via email or in hard copy.

Following a low-carb diet – What does that actually mean?

This guideline has been designed to help you follow a low-carbohydrate, high-fat diet over the next eight weeks whilst you are part of our cohort.

The diet that has been assigned to you means that you will eat only small amounts of carbohydrates per day and a higher proportion of fat. Whilst you are taking part in study we ask you to consume a minimum of 30 grams and a maximum of 50 grams of carbohydrates per day.

On a usually consumed Western diet the human body uses the glucose derived from carbohydrates for nearly all its energy needs. However, when carbohydrate intake is restricted the body learns to adapt very quickly and uses dietary and body fat instead. This happens through a process called "ketosis", those fatty acids consumed with our foods and those stored within our body fat cells are broken down in order to produce glycerol and ketone bodies. On very low-carb diets these products will provide the energy required by the human body for normal functioning.

In terms of your diet this means that you will instead eat more fat which will come from a variety of sources.

Carbohydrates come from a range of foods, including potatoes, rice, pasta, bread, fruit and vegetables and to a lesser extend dairy products (in the form of milk sugar, called lactose). This means that there will be restrictions as to which and how much of these foods you will be able to consume.

Potential beneficial effects of a low-carb diet

There is a reason why a growing number of nutrition and health professionals are in favour of a low-carb, high-fat diet.

- You should feel less hungry, especially between meals
- This means that you might eat less than you usually do, leading to a lower calorie intake and consequential potential weight loss
- Your body composition might change meaning less fat mass
- Your insulin levels should decrease as your body needs to produce less insulin to maintain blood sugar levels after a meal
- You will have better blood sugar control, which means that your body needs to produce less insulin to ferry the glucose in your blood into your muscles and you fat depots
- Your cravings for sugary and fatty foods should reduce which means that you will probably eat less of these foods.
- You might feel less bloated

Potential adverse effects of a low-carb diet

However, as we have already told you when you signed up – remember that disclaimer? – some people might have a bit of a harder time adjusting to the new diet. Especially during the first few days on a very low-carb diet you might experience

- Headaches - Drink plenty of fluids.
- Lethargy, weakness - If this is the case try and take it easy for a few days.
- Constipation - This is due to the potentially lower fibre intake. You can address this by increasing your fibre intake and drinking plenty of fluids. Please see the list of fibre-rich permitted foods on page 15.
- Muscle cramps -This is due to your kidneys excreting more sodium which can also impact on your potassium balance. Sodium and potassium are minerals which are used by your body as electrolytes, meaning they regulate the fluid balance in our bodies and stimulate our muscles and nerves. You can deal with this by continuing to take your daily multivitamin and mineral supplement provided by us and by drinking cups of bouillon or home-made bone broth (see recipe). You can also add half a teaspoon of salt to one litre of water and drink this throughout the day. In addition, consume plenty of magnesium and potassium-rich foods from the list provided.
- Brain fog – As your brain has used carbs so far to do all its hard work the initial switch from one fuel (carbs) to another (fats) might take a few days. If you can, try and take it easy for a few days and drink plenty of fluids.
- Nausea, anxiety and palpitations - This might also be due to your electrolyte deficiency (Magnesium and potassium).
- Poor sleep quality – After a while, however, a lot of people report a better quality of their sleep
- Bad breath – A sugar-free mint might help. Bad breath is a sign of your body switching from using carbs to fats (ketones) as fuel source. It will pass!
- Consumption of potentially fewer vitamins and minerals – This is why we are giving you your daily multi-vitamin and mineral supplement.

For these reasons we are actually advising you to start the diet on a less busy day of your week.

How to take your multivitamin supplement

During your first lab visit when you give your first venous blood sample we will also provide you with a multivitamin and mineral supplement, which you will need to take on a daily basis. The container includes 30 pills, which should be sufficient until your second lab appointment where you will receive the supplements for the following 4 weeks.

Please bring the pill container with you to each lab appointment, as we will need to count the number of pills left in the container. We are doing this as the number of pills taken will be taken into account when we analyse the data. It will also help us to explain why some side effects might have occurred – for example if you forgot very often to take your supplement.

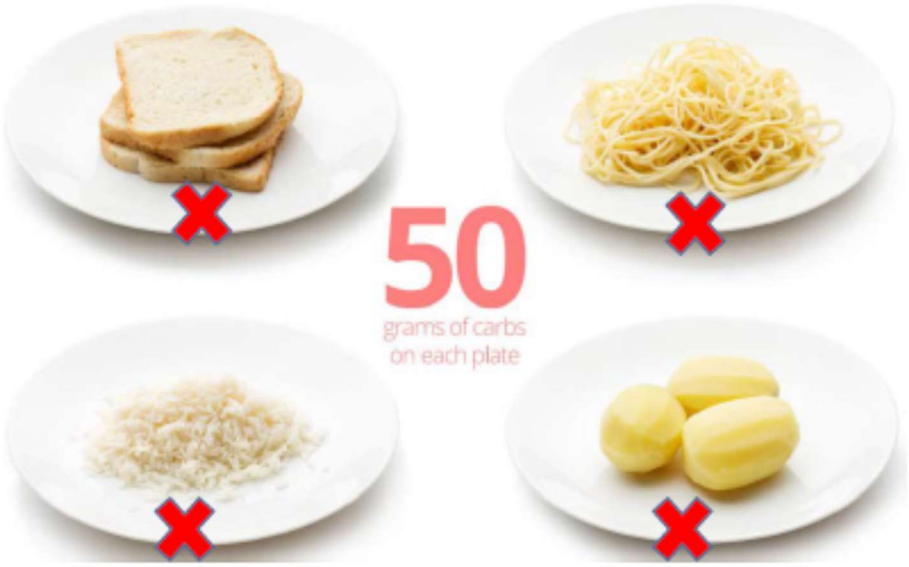
Please do remember to take it on a daily basis. Preferably at mealtimes as some of the vitamins can only be absorbed by our bodies when consumed at the same time as fat. Having said this, the other vitamins will also be more readily available to your body when other nutrients have to be digested with them.

Portion sizes for carbohydrates

So, what do 50 grams of carbs look like?

As there are foods that are naturally high in carbs and those that are naturally low in carbs it can be confusing to decide how much you could eat of these foods.

Below you can see examples of what 50 grams of carbs might look like and how easy it would be to exceed your daily allowance.



(Source: The Dietdoctor, 2017)

(Source: The Dietdoctor, 2017)

Please remember that the foods shown above are on the plates in isolation. Add to these the rest of the foods that you will consume throughout the day and all those carbs soon add up! The foods on the first picture will therefore be found on the list of food items that you will not be allowed to consume over the next eight weeks (see below).

What is fibre and why are we supposed to eat it?

Carbohydrates in our diet come from different sources, with some of them being more readily (if at all) absorbed by our bodies and used for energy to keep us going during the day. When nutritionists and other health professionals talk about carbohydrates and a certain type of carbohydrate diet (in this case "low carbohydrate diet") we mean carbohydrates that are actually available to our bodies. Only these types of carbohydrates are actually being counted when making recommendations for carbohydrate intake. The other type of carbohydrates, which are generally not available, are classed as dietary fibre (see below). There are also different subcategories of these, with some supplying our bodies with small amounts of energy, but these can be disregarded in the context of the foods that we consume every single day as part of a healthy balanced diet.

In the UK it is recommended that everyone in the UK above the age of 14 should aim to consume at least 30 grams of fibre per day. At the moment the majority of the population is not meeting these recommendations. Research has shown that sufficient fibre intake can help prevent heart disease, some cancers and diabetes. Fibre can also aid to improve your digestion and make you feel fuller for longer meaning you eat less and less often (serial snackers beware). Fibre can be found in a number of fruits, vegetables and nuts and seeds, i.e. plant-based foods.

Foods that contain 6 or more grams of fibre per 100g are classed as high-fibre foods, whereas those containing at least 3 grams of fibre per 100g are considered to be 'fibre-rich'.

Please ensure that if you think that your diet has been lacking in fibre so far to increase the amounts that you are consuming gradually over a couple of weeks and to make sure that you drink plenty of fluids. Otherwise you might have to deal with bloating and constipation as side effects.

Carbohydrate and fibre content of fruit and vegetables permitted on a low-carb diet

Food name	Amount available (digestible) carbohydrates in g (per 100g)	Amount fibre in g (per 100g)	Amount available (digestible) carbohydrates in g (per 80g portion)	Amount fibre in g (per 80g portion)
Asparagus, boiled	1.4	1.4	1.12	1.12
Aubergines, fried	2.8	2.3	2.24	1.84
Avocado, average	1.9	3.4	1.52	2.72
Beans, green, boiled	4.0	2.5	3.2	2.0
Beans, soy/edamame	5.1	6.1	4.08	4.88
Broccoli, boiled	2.8	2.3	2.24	1.84
Broccoli, Purple sprouting, boiled	1.3	2.3	1.04	1.84
Brussel sprouts, boiled	3.5	3.1	2.8	2.48
Cabbage, green cooked	2.3	2.6	1.84	2.08
Cabbage, red cooked	2.3	2.0	1.84	1.6
Cabbage, white cooked	3.2	1.4	2.56	1.12
Cabbage, spring greens, cooked	1.6	2.6	1.28	2.08
Carrots, young, boiled	2.3	1.84	4.4	3.52
Cauliflower, cooked	3.5	1.6	2.8	1.28
Celery, raw	0.9	1.1	0.72	0.88
Coconut, desiccated	6.4	13.7	5.12	10.96
Coconut, fresh	3.7	7.3	2.96	5.84
Courgette, boiled	2.0	1.2	1.6	0.96
Cucumber, raw	1.2	0.7	0.96	0.56
Fennel, Florence, boiled	1.5	2.3	1.2	1.84
Kale, curly, boiled	1.0	2.8	0.8	2.24
Kohlrabi, boiled	3.1	1.9	2.48	1.52

Food name	Amount available (digestible) carbohydrates in g (per 100g)	Amount fibre in g (per 100g)	Amount available (digestible) carbohydrates in g (per 80g portion)	Amount fibre in g (per 80g portion)
Leeks, boiled	2.6	1.7	2.08	1.36
Lettuce, cos	1.19	2.1	0.95	1.68
Lettuce, iceberg	1.77	1.2	1.42	0.96
Lettuce, romaine	1.19	2.1	0.95	1.68
Mushrooms, oyster, raw	3.79	2.3	3.03	1.84
Mushrooms, Portobello, grilled	2.24	2.2	1.79	1.76
Mushrooms, white, boiled	0.1	2.1	0	1.68
Olives, green in brine, drained	0.0	2.9	0	2.32
Onions, fried (based on 2 table spoons, chopped)	11.2	1.5	3.36	0.45
Pepper, green, boiled	2.6	1.8	2.08	1.44
Pepper, red, boiled	3.4	0.8	2.72	0.64
Pepper, yellow, boiled	5.3	0.8	4.24	0.64
Pumpkin, boiled	1.9	1.1	1.52	0.88
Radishes, raw	1.9	0.9	1.52	0.72
Raspberries, red	4.6	2.5	3.68	2.0
Rocket	0.0	1.3	0.0	1.04
Spinach, baby, raw	0.2	1.2	0.16	0.96
Spinach, frozen, boiled	0.5	2.1	0.4	1.68
Spring onion, raw	3.0	1.5	2.4	1.2
Strawberries	6.1	1.0	4.88	0.8
Tomato, raw	3.0	1.0	2.4	0.8
Turnip, boiled	2.0	1.9	1.6	1.52
Watercress	0.4	1.5	0.32	1.2

Carbohydrate and fibre content of nuts and seeds

A small handful (or about 30g) of nuts or seeds counts as a portion. Below some guidelines of how much fibre you would get from different types of nuts and seeds.

Food name	Amount available (digestible) carbohydrates in g (per 100g)	Amount fibre in g (per 100g)	Amount available (digestible) carbohydrates in g (per 30g portion)	Amount fibre in g (per 30g portion)
Almonds, raw	2.5	2.7	0.75	0.81
Brazil nuts	3.1	4.3	0.93	1.29
Chia seeds	6	38	1.8	11.4
Flaxseeds (Linseeds)	2	27	0.6	8.1
Hazelnuts	6.0	6.5	1.8	1.95
Pecan nuts	5.8	4.7	1.74	1.41
Pumpkin seeds	5.71	6.0	1.71	1.8
Walnuts	3.3	3.5	0.99	1.05

Permitted foods on a low-carb diet

Dietary fat – the star of the show? Following a high-fat diet

Cutting down on carbs to the extent that is necessary on a low-carb diet means that your energy needs to come from somewhere. Earlier on we already mentioned that fat can also be broken down into products (ketones) that supply our bodies with energy after a period of adaptation.

Fat is also important role in our diet as it helps to absorb some vitamins that would otherwise simply go right through us. It also plays an important role in building the membranes of our cells. The key lies in the quality of the fats consumed with priority given to unsaturated fats, such as olive oil and other vegetable oils, avocados, nuts and oily fish. One type of unsaturated fat, which is essential to human health, are omega-3 fatty acids. These can be found both in animal and plant sources. Oily fish (see below) is an important source of omega-3 fatty acids as these are of the highest quality. However, it is possible for the body to convert the omega-3 fatty acids found in plants foods, such as nuts and seeds and their products, into the same end products found in oily fish. Coconut oil is also a fat recommended on a low-carb, high-fat diet and it is thought to be very healthy, despite a high proportion of saturated fats in it. Some people love to use it for everything, including frying their eggs. However, a small word of caution when it comes to taste as this is very distinct in coconut oil. For some individuals the egg-frying

method might be a bit too much for this reason. In other recipes, however, such as curries, soups and other dishes with lots of other ingredients, the taste of coconut oil complements and enhances the dish instead of tasting too overpowering.

Saturated fats have received a lot of bad press over the past few decades and whilst it is important to prioritise the fatty acids introduced above (mono- and polyunsaturated) a low-carb, high-fat diet doesn't place the same restrictions on saturated fats that you might have been used to so far. A lot of foods that contain a fairly high amount of saturated fats, such as meat, butter and cheese are actually encouraged to be consumed. You therefore don't need to worry about removing the visible fat from beef and pork steaks and other meats or about removing the skin from chicken. This will be one way of ensuring that you will achieve the amount of fat you will need to consume to replace the carbohydrates that you will omit from your diet. In addition, the marbling on cuts of meat will enhance the flavour whilst cooking it and also ensure that the meat stays moist during the cooking process.

One thing to avoid on a low-carb, high-fat diet are margarines as these are not a natural fat but have been manufactured and hardened from vegetable oils – a highly processed food. You should also not use vegetable oils that are high in omega-6 (as opposed to omega-3) fatty acids, such as sunflower oil, corn oil, soybean oil and cotton seed oil. Vegetable oils that should be consumed instead are coconut oil, olive oil and rapeseed oil

Dairy

Dairy products are both a source of fats and also proteins. Consuming a low-carb, high-fat diet means that you should not shy away from using full-fat versions of the product. Think butter, cream and jersey milk or at least full-fat milk. Stay away from low-fat yoghurts and also avoid fruit yoghurts as these contain too much sugar. Dairy is a good source of calcium and should therefore be included in our diet on a daily basis. However, you will also have to careful with the amount of milk that you are consuming as the milk sugar also contains glucose which will be absorbed by our bodies. A bit of milk (full-fat) in your tea and coffee is fine. However, a milkshake or hot chocolate should only be an occasional treat and then you still have to be mindful of the other ingredients.

Dairy products good to consume on a low-carb diet include full-fat versions of cheese, cream, cream cheese, crème fraiche, sour cream, quark and yoghurt.

Proteins to help you build and maintain that temple which is your body

Proteins are important in our diet as they have the vital functions of growth, maintenance and repair. They are also vital to help our immune system function properly. Proteins are composed of compounds called *amino acids* of which there are 20 that play a role in the human body. 8 of these amino acids are classed as essential because unlike the other 12 our bodies cannot produce these themselves, which means that they need to be obtained from our diets. This happens by consuming protein foods.

Whilst protein malnutrition can have disastrous consequences in reality the vast majority of people in the UK consume more than they actually need to.

The average UK male aged 15 to 64 should consume about 55 grams per day, the average UK female in this age group 45 grams per day. In order to achieve these recommendations we should eat two to three portions of protein each day. There are different types of protein sources permitted on a low-carb diet, including:

Animal-based proteins

Animal-based proteins contain all of the essential amino acids. This is because the animals have done the work for us eating a variety of food sources with different amino acid profiles and combing these in their muscles. The same is true for eggs where the egg yolk and the egg white have the role of sustaining the developing chick.

Meat

Meat is permitted on a low-carb diet as it contains virtually no carbohydrates. However, you should not eat more than 70g of processed meat per day, which includes sausages, bacon, cured meats (for example salami, chorizo) and reformed meats products (for example sliced packaged ham). Even on a low-carb diet consuming these foods in excess is not healthy as these not only tend to contain a number of additives but are also high in salt. Unfortunately, depending on the type of processed meat, the manufacturing process and the reputability of the manufacturer this might also mean that you might be consuming a low-quality product consisting of offcuts that will only be saleable if disguised in this form.

You should also be careful with deli products such as pates and hams (breaded is a no-no!) as these might contain hidden sugars, so it is best to check first before eating or even buying them.

Fish and seafood (Shellfish)

You should eat at least two portions of fish per week, one white, one oily. One portion is 140 grams which is about the size of a cheque book. The reason we recommend oily fish because these are an excellent source of omega-3 essential fatty acids, which we have mentioned earlier on. Fish also supply us with a number of vitamins and minerals. Remember that two portions per week is the minimum.

Please remember that you will not be able to eat battered or breaded fish whilst on low-carb!

White fish

Basra	Cod,	Coley	Dab	Flounder	Gurnard
Hake	Haddock	Plaice	Pollock	Red mullet	Tilapia

Some white fish should be eaten no more than once a week due to potentially high levels of pollutants contained in their flesh. These are seabream, seabass, halibut and turbot.

Oily fish

Anchovies	Carp	Herring	Kippers	Mackerel	Pilchard

Salmon	Sardines	Sprats	Trout	Tuna (fresh or frozen)	Whitebait

Swordfish should not be eaten more than once a week due to potentially high levels of pollutants. It should be avoided by children, pregnant women and those wanting to become pregnant.

Shellfish

Cockles	Crab	Langoustines	Mussels (and clams and winkles)
Oysters	Prawns	Scallops	Squid

It is important to note that processed, canned fish like tuna do no longer count as your 'oily' portion as the manufacturing process has taken all the omega-3 fatty acids out of them. However, the good news is – that tuna salad for your lunch still counts towards your portion of white fish.

One thing to bear in mind is choosing where possible sustainable sources of fish as recommended by the Marine Stewardship Council (MSC) in their *Good Fish Guide*. The MSC also run an accreditation scheme, so look out for certified products carrying the following logo:

Further information on the MSC and a regularly updated list of sustainable types of fish can be found at www.msc.org.

Eggs

Eggs are real powerhouses of nutrition and contain all the essential amino acids that humans need in their diet. One medium egg provides about 6.4grams of protein with the egg yolk containing higher concentrations of amino acids. However, as the egg white is larger than the egg yolk the majority of protein supplied when eating an egg will actually come from the egg white.

For animal welfare reasons we would recommend that you avoid buying the eggs from caged hens. However, the choice is absolutely yours.

A word on dairy products

Dairy products, such as milk, cheese, cream, sour cream and yoghurt, are also good sources of protein. However, as they also contain a fair amount of fat, we have included them in the fat section of this guide.

Plant-based proteins

Nuts and seeds are a great source of proteins and are also great sources of unsaturated fats, included important omega-3s.

Other plant-based protein sources include tofu, bean curd, mycoprotein (such as Quorn™) and soya-based meat replacement products, such as supermarkets' own brands. Please remember that the latter two are processed foods so it is always worth checking the label to ensure that there are not too many additives and flavour enhancers, salt and sugar found in

these products. If this is the case, you should either eat these products sparingly to ensure that you do not end up eating lots of hidden sugars by accident!

Beans and pulses, including lentils, although good sources of protein, are generally not recommended on a low-carb diet with the exception of green beans and soya beans as these don't contain as many carbs but are good sources of fibre.

Portion sizes for protein

Non-dairy

Eggs	2 medium-sized
Fish	140g (size of a cheque book) or 3 fish fingers
Meat, cooked	80g (size of a deck of cards or 3 slices)
Meat-replacement products	120g (for example two sausages or about the size of a snooker ball)
Nut butters, such as peanut butter	2 tablespoons (about the size of a golf ball)
Nuts and seeds	40g (1 small handful)

Vegetables

Vegetables growing above ground, for example artichokes, asparagus, aubergines, avocado, bok choi, broccoli, Brussel sprouts, cabbage, cauliflower, courgettes, cucumber, kale, lettuce, mushrooms (careful with shiitake mushrooms however, as they contain a lot more carbohydrates that other varieties!), olives, onions, peppers, spinach and other leafy greens, and tomatoes (Technically a fruit – we know ;-))

You have to be very careful with eating sweetcorn, corn on the cob and peas as these are higher in carbohydrates than other vegetables growing above ground and should only be eaten in moderation.

Fruit

As fruits contain a lot of carbs they should be eaten in moderation, only about 1 piece of fruit per day. Bananas and grapes are the worst offenders in a low-carb diet context. Berries can be eaten a bit more regularly but you should also be careful with these.

There are actually several reasons why eating vegetables and (some) fruits is good for you. Firstly, they contain a considerable amount of carbs in the form of fibre – the good stuff. Secondly, the different colours express the presence of different types of nutrients that are really good for us and are thought to prevent heart disease, diabetes and cancers. These are namely anti-oxidants and polyphenols. The different colours of the fruit and vegetables available for us mean that these contain different types of these nutrients – all-round protection.

If you feel slightly adverse to anything green and orange on your plate (one too many roast dinners with boiled-to-death cabbage) there are ways of incorporating these to help you feel less annoyed by their presence and to gradually get used to them. You could for example add some finely chopped carrots and vegetables to a tomato sauce with your fish or chicken.

Alternatively, you could make a mushroom pâté to create a delicious snack eaten with vegetable sticks.

Magnesium-rich foods

Magnesium plays an important role in our diet and obtaining it from a carefully planned low-carb diet is possible. However, there are some foods that will make it more likely that you will meet your daily requirements. These are artichokes, bone broth (we will give you a recipe for this), fish (highest are fresh tuna and halibut), nuts, spinach and other leafy greens.

Potassium-rich foods

Like magnesium, potassium is also important in a healthy, balanced diet and there are some foods that should be preferred to others in order to achieve your recommended intake. These are avocados, bone broth (again recipe to follow), cooked greens, cooked mushrooms, fish, meat and tomatoes.

Food no-no's on a low-carb diet

Sugar in any of its natural forms – This includes table sugar, brown sugar, demerara etc; honey, syrups in any form, molasses. Be aware of hidden sugars in food products and ready-meals. Later on in this guide we will show you how to read labels and identify these silent assassins that might jeopardise all your efforts.

If you can't do without sweetness for example in your tea or coffee there are some alternatives (in form of natural low-carb sweeteners and sugar alcohols) on the market that you can use sparingly. These are

- Stevia – a plant extract which is 200 to 300 times sweeter than sugar and has been used as a sweetener for many years in Asia and South America. Stevia is sold in UK supermarkets as Stevia sweetener and can also be found under the brand name "Truvia". However, be careful not to pick up a stevia-sugar blend by accident!
- Erythritol – a so-called sugar alcohol, which has about 70% of the sweetness of sugar but does not get broken down by our bodies (unlike sugar). Erythritol can be ordered online.

However, if you feel or experience that the sweet taste of these sweeteners induces cravings for sweet foods and carbs in general (which can happen in some cases) we would advise you to take the plunge and have your tea and coffee unsweetened.

As you will have probably expected chocolate is on the list of foods not to eat over the next 8 weeks. If you are really desperate you can have that odd piece of small (and we mean small!) piece of 70% or above dark chocolate, which contains about 3.5 grams of carbs per square. If you feel that you have to eat more than this small piece once you have started it is best to not eat it at all!

So what should you eat in a day if you are following a low-carb, high-fat diet

Following a low-carb, high-fat diet means that you can eat lots of permitted vegetables (preferably green as they are lower in carbs – but do incorporate some colour as well) and as many sources of high-quality fats as you like – avocado, butter, nuts, seeds and anything from the recommended dairy range. Fat has more calories per gram than carbs or proteins and will help you to feel full and satisfied quicker. Don't forget that if you have eaten a lot of carbs prior to embarking on this diet this might mean that it will take you a few days to adjust to your new style of eating and for your body to send the right signals that you are feeling full. Don't worry about this, after a few days chances are that you will eat less than you used to as you feel fuller sooner.

Fat will be the nutrient that you can eat as much as want of, however, this means only eating until you feel full and satisfied. If you go past the point of feeling full you will still overeat and undo all the goodness that you have done for your body. Listening to your body and knowing when to stop is the key!

One important thing to bear into consideration is to become more mindful when you are eating and to eat slowly, rather than devouring your food within 10 minutes because you feel that there is no time to eat. Eating in a rush and quite frankly 'shoving it in' means that by the time your brain has had the chance to signal your stomach that you have had enough food to satisfy all your physiological needs, thank you very much, you will have most likely overdone it already. You will be surprised to find that when you take it down a notch with the speed eating that you might want to eat far less than anticipated because before you know it you will feel full.

If your day really is that manic and lunch is a 10-minute affair or even takes place behind the wheel of your car (we live in busy, demanding times, we know, and sometimes your boss, children, the situation might not appreciate the eat slowly mantra) the good thing about a high-fat diet is that you can rest assured that once you feel full you will do so for longer. So if your lunch break is hectic eat slightly less at first. The feeling of fullness will come and if this is something that worries you, ensure that you have a snack handy that might fill that hole after all if you get the afternoon slump or your tummy starts rumbling again.

Do not eat more than 2 – 3 portions of protein from the non-dairy list per day. Remember, this is a high-fat diet, not a high-protein diet.

As you should not consume more than 50 grams of carbohydrates per day make sure that you do not eat any items on the 'no-no foods' list. Eating the recommended items from the vegetables and fruit list will help you get there, as will incorporating dairy products, nuts and seeds. This is not a no-carb but a low-carb diet so some carbs are necessary for you to consume and as we do not tend to eat every part of the animal in the UK in the same way as other cultures might do, which addresses their requirements for vitamins and minerals - think seal's eyeball as good sources of these (yum!) - it is important that you incorporate veggies and some fruit in your diet. We will give you a daily multi-vitamin and mineral supplement to

avoid any potential shortfalls, but this can only take you so far. The majority of your nutrients should come from your diet!

Suitable snacks

Overall, after a period of adjustment, we would expect for you not having to snack so much, if at all, as a low-carb, high-fat diet should keep you feel fuller for longer. However, do not despair there are some good snack options out there for you. It just won't be that piece of cake or a packet of crisps.

- Avocados – full of high-quality fats, creamy and delicious
- Canned mackerel in tomato sauce – just be careful with your breath with this one. And read the label to see if any sugars are lurking in that tomato sauce!
- Eggs (hardboiled) – ultimate, easy snack. Put a bit of salt on if you want!
- Slices of ham or salami and cheese rolled up and maybe some cheese spread on – think charcuterie in your favourite tapas restaurant. However, do remember to not eat more than 70 grams of these types of processed meats per day!
- Home-made low-carb crackers – hardly any carbs but lots of fibre. We will give you the recipe
- Nuts – again, pure natural goodness. But if you want some variation, we have some great and easy recipes on how you can flavour these.
- Olives – easy to get hold of with the added benefit of containing high-quality fats
- A piece of cheese – great source of calcium
- Pork scratchings – these can be a good alternative to that packet of crisps
- Some vegetables and a suitable dip – cucumber, celery, peppers, carrots. Again we have some great recipes for dips.

Some staples and alternatives to favourites on a very low-carb diet

- Almond flower – just because you are going low-carb does not mean that you cannot do some baking or have pancakes
- Bone broth – full of magnesium and potassium and other minerals to do your body good
- Bread – we will give you recipes for keto-bread if you can't be without.
- Coconut flower – Another low-carb alternative in cooking and baking
- Psyllium – To make keto crackers, porridge and increase fibre intake
- Pasta – Have spiralised vegetables, such as courgette instead. You can either make these yourself by investing in a spiralizer or they are now readily available from a number of supermarkets and discounters. They have all caught on to this trend.
- Porridge – A breakfast favourite of yours? Although oats are not allowed on low-carb there are some tasty alternatives, and we have the recipes to give you.
- Potatoes – Love mash? How about some caulimash instead? Or other vegetable mashes.
- Rice – A must when you are having curry? Or love risotto? Have cauliflower rice instead. You've guessed it – we do have some recipes for you.

Hydration

This is an important one that often gets overlooked. You should drink 6-8 glasses (200ml or medium-sized) per day to ensure staying hydrated. This will also help your body to cope with a potential increase in dietary fibre following a healthy diet (see above). However, you should stick to water, unsweetened black or herbal teas and black coffee or coffee with small amounts of full fat milk or cream. (We also have a recipe for *bullet proof* coffee.) Be careful with flavoured milks as these also tend to contain a lot of sugar which will catapult you right out of the max. 50g of carbs per day zone! If you think that plain water is too boring there are some ways (and recipes) to make this more interesting, such as adding slices of citrus fruit and/or mint or cucumber for example. If you are struggling with the taste of tea and coffee without sugar there are some alternatives on the market that you can use sparingly. These can be found in the section about sugar on page 21.

You should also not drink any fruit juices as the natural sugar contained in fruits has been freed through the process of liquidisation. This means it is more easily digested and can raise your blood sugar a lot quicker. The fruit sugar contained in fruit (fructose) can actually not be metabolised by our bodies and if not turned into energy will be converted into body fat. In addition, this free sugar can contribute to dental decay.

Alcohol

Small glasses of dry wine are permitted and these contain about 2 grams of carb per glass. So enjoy your glass of wine, but do not overdo it! Spirits such as whisky, brandy, vodka, sugar-free cocktails are also permitted. But be careful not to mix these with soda as this will make your drink too sugary. Beer (think liquid bread!), cider and mixer drinks (even that G&T) with sugary sodas or alcopops are not allowed as these contain far too many carbohydrates, which will be broken down fairly quickly and end up in your blood stream and cells. Bye-bye low-carb diet! Check the labels of diet versions of diet sodas to see whether they contain 0 sugars/carbs and these might be an alternative. However, do bear in mind what we said about artificial sweeteners and their potential to still induce sugar cravings. Furthermore, there is currently debate on whether some artificial sweeteners still elevate your insulin levels. We therefore strongly advise you to proceed with caution here at least whilst you are part of our cohort.

Learning to read labels and nutritional information

This is an important one when food shopping and eating out in places that provide you with information on the nutritional content of their dishes, for example fast food restaurants and pubs. If you are unsure check out the label for the carbohydrate content of the food. Avoid anything with more than 5gram per 100 grams of carbs or more than 5 % of carbs.

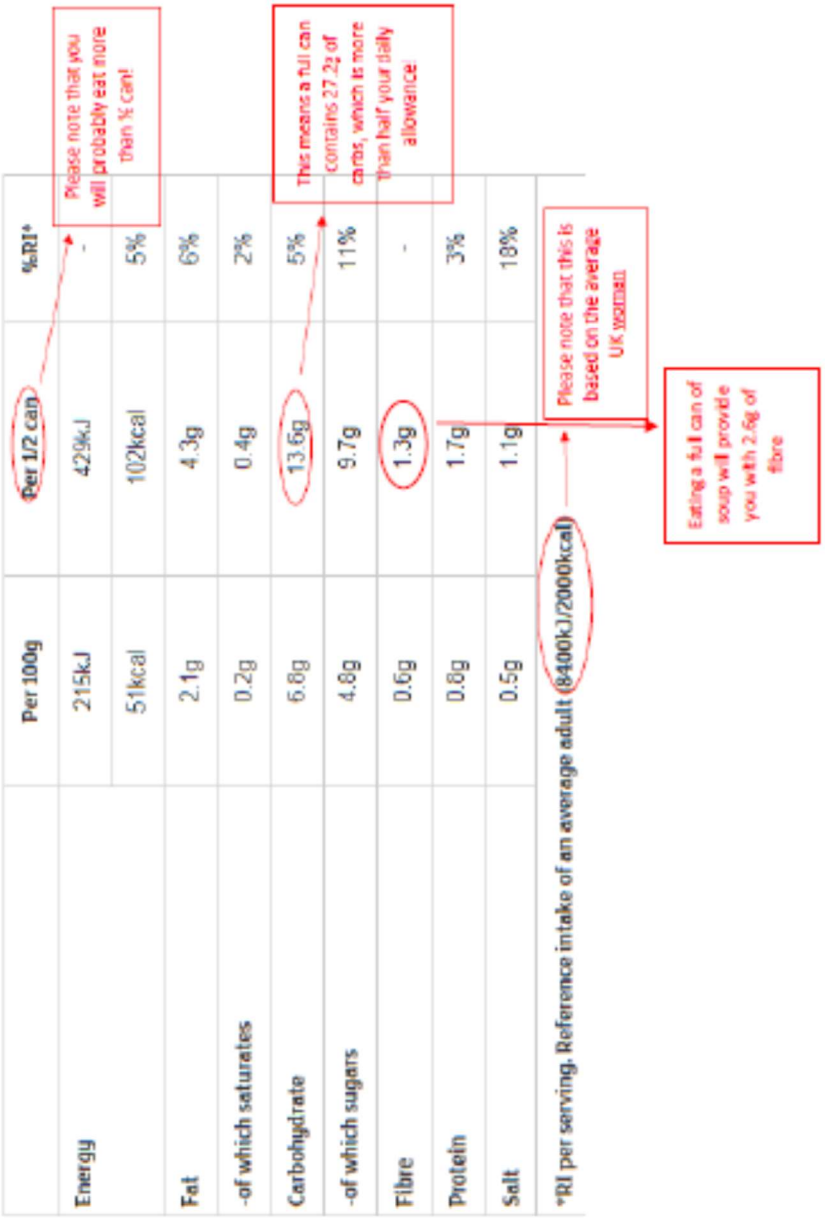
Label reading

In the UK food labels can be found at the back and depending on the manufacturer or retailer also at the front of the packaging.

Labels at the back of the packaging

Below is the back of pack nutrition label for Heinz Tomato soup as an example.

Ea



	Per 100g	Per 1/2 can	%RI*
Energy	215kJ	429kJ	-
	51kcal	102kcal	5%
Fat	2.1g	4.3g	6%
-of which saturates	0.2g	0.4g	2%
Carbohydrate	6.8g	13.6g	5%
-of which sugars	4.8g	9.7g	11%
Fibre	0.6g	1.3g	-
Protein	0.8g	1.7g	3%
Salt	0.5g	1.1g	18%

*RI per serving. Reference intake of an average adult (8400kJ/2000kcal)

There are a few things to be aware of:

The manufacturer's portion size guide might actually not be a realistic reflection of what you might actually eat yourself and it can be very easy to underestimate the amount of carbohydrates you are consuming.

Another thing to take into consideration is the quality of carbohydrates you are eating. The label will not tell what source the total sugars contained in foods derive from and can be a combination of natural sugars found in fruits (fructose) and milk (lactose) where applicable and added sugars.

This is where looking at the ingredients list (also a the back of pack) might necessary as sugar comes under many different names and there might be some surprises in store for you.

As a general rule, the closer an added sugar is towards the top of the ingredients list the more of it will be contained in the product. There might also be different types of added sugar in the same product.

All of these that you might spot in the ingredients list are actually added sugars

> agave sugar, brown sugar, cane sugar, dextrose, fructose, fruit juice concentrate, glucose, golden syrup, HFCS/high fructose corn syrup, honey, hydrolysed starch, invert sugar, isoglucose, levulose, maltose, modified starch molasses, sucrose, syrups (sucrose, glucose, malt, corn, maple), treacle,

Once a product crosses specific thresholds for specific nutrients it will either be classed as 'medium' or 'high' in sugar or other nutrients (see below).

	Low	Medium	High
Total sugars	5g per 100g product (or 5%)	More than 5g but less than 22.5 per 100g product (between 5% and 22.5%)	More than 22.5g per 100g product (or 22.5%)
Salt	0.3g of salt or less per 100g (or 0.1g sodium)	More than 0.3g of salt (0.1g sodium) but less than 1.5g of salt (0.6g sodium) per 100g product	More than 1.5g of salt per 100g (or 0.6g sodium)

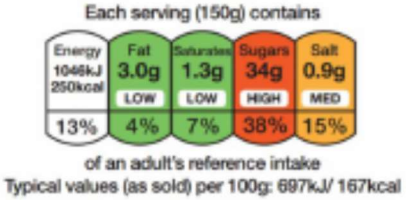
If the portion size means that more than 25g of sugar would be consumed with one portion the product is automatically classed as 'high sugar'.

For drinks containing total sugar the rule is that 2.5g per 100ml is classed as 'low', between 2.5 and 11.25g per 100ml is classed as 'medium' and above 11.25g per 100ml is 'high'. If the

portion size of a drink means that more 13.5g of sugar would be consumed the product is automatically classed as 'high sugar'.

Front of pack labels

Some retailers and manufacturers display a food label at the front of the pack highlighting whether some of the crucial nutrients are contained in the foods to low, medium or high levels. This is called the 'traffic light system'. The main nutrient that you will need to look out for is the 'sugars' category. You do not need to concern yourself with the 'fat' and 'saturates' categories on a low-carb diet as the permitted amounts are different from the standards applied to the traffic light labels. These are based on the UK dietary guidelines.



Interpreting nutritional information provided by fast food restaurant and pub chains

A number of high street fast food outlets and pub chains publish nutritional information on typical serving sizes of their dishes that you can access online, download or on request at the restaurant.

Being aware of the nutritional content of some of these dishes is helpful if you tend to eat out more often as it can be difficult in this case to stick to a low-carb diet and you might unknowingly jeopardise all your efforts.

It is also great to make you aware of how much individual components will add up. Watch out for hidden carbs, including starches and sugars in sauces, salad dressings and coatings.

Some example nutrition guides are attached to this guide to give you an idea what to look out for. However, these are by no means meant as endorsements of particular eateries.

Tips for Eating Out on a low-carb, high-fat diet

Eating out whilst on a low-carb diet can be quite straight forward depending on the type of cuisine that you choose and very often it is easy to make your meal low-carb friendly. We have also provided you with example nutritional information from some of the pub and restaurant chains in the UK to give you examples what to look out for. In general, all chains should have this information readily available for interested diners in the restaurants and on their websites. If you would like us to try and find the nutritional information for a particular place, please do let us know.

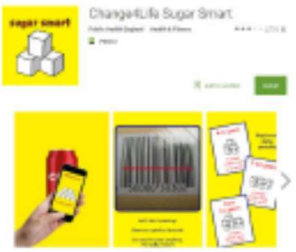
- Many restaurants and pubs now will allow you to swap your potatoes and chips with a side salad.
- If you fancy a burger just have it without the bun or if you feel comfortable doing so ask for it to be wrapped in a large lettuce leaf instead (so you can still pick it up)

- Instead of breaded/battered fish or chicken have the steamed or roasted versions instead.
- If you get a Mexican or a Subway takeaway ask for the salad version of the dish rather than the wrap/burrito.
- Be careful with sauces and gravies as these might contain flour (and carbs!). In order to control how much (and if) you want to eat any of this ask for it to be served on the side rather than on your plate.
- If you fear that you might feel a bit hungry still after you have eliminated the starchy foods from your restaurant plate, ask for (extra) butter or olive oil to make up for this. Some people following a low-carb take a small bottle of olive oil with them when eating out just in case.
- If crave a third course see if there is a cheese platter on the menu (without the crackers!) instead of opting for pudding.
- It might be difficult to eat in Indian or Chinese buffet restaurants or takeaways whilst you are participating in the CALIBER study. Other research undertaken by our nutrition team has shown these dishes to be very high in added sugars. You definitely need to avoid the sweet and sour chicken! However, Indian creamy curries and kebabs might be a good option.
- Go easy on the condiments as ketchup, cocktail and BBQ sauces can contain a lot of added sugar.
- If you know that you have been invited to a dinner party and you don't want to offend, be careful of you carb intake earlier during the day. This way you can at least try and contain some of the damage.
- Otherwise, if your host is understanding give them a fair warning, which should be much appreciated. Some low-carbers excuse their avoidance of starchy foods with stomach issues. If you think that you might not be able to eat enough at the dinner party, have a snack at home before you leave.
- Pizza is a harder one! It should be avoided during your eight weeks on our study. If you really crave pizza, you can use your own using an alternative base. Please see the recipe that we have provided for this.

Appendix - Helpful App to help you to stick to a low-carb diet

Change4Life – Sugar smart App

Change4Life is a public health initiative run by Public Health England. This app lets you scan the barcodes of about 87,000 food products available from UK major manufacturers and retailers. It focuses on the amount of free sugar in these foods and uses a traffic light system to let you know whether these would be high in the nutrients. A good way to find out about any unexpected sugars in products that you might want to buy.



The app can be downloaded for free from the Appstore and GooglePlay.

Appendix - Examples nutritional information fast food, pub and restaurant chains

ASK

Italian

Recipe / Dish	Menu selections	Per 100g** Energy KJ	Kcal	Fat (g)	Saturates (g)	Carbs (g)	sugars (g)	Protein (g)	Salt (g)	Per Serving** Energy KJ	Kcal	Fat (g)	Saturates (g)	Carbs (g)
Italian Olives	Breads / Nibbles	673	163	15	2.0	3.0	0.5	1.0	3.0	841	204	19	2.5	4.8
Rosemary & Sea Salt Bread	Breads / Nibbles	1049	251	5.7	0.8	41	2.8	7	1.1	3069	696	11	1.5	91
Garlic Bread Speciale	Breads / Nibbles	1134	270	11	5.4	33	2.4	11	1.1	3069	729	29	17	89
Garlic Bread	Breads / Nibbles	1144	274	7.8	1.9	38	2.6	8.8	1.1	2455	588	17	4.1	82
Garlic Bread with Mozzarella	Breads / Nibbles	1353	276	11	4.8	29	2.2	11	1.2	3251	784	31	13	83
Cheese Fondata	Breads / Nibbles	1121	284	11	4.2	33	1.2	6.7	1.2	3269	771	33	12	96
Antipasti - The Warm One	Starters & Shares	934	223	13	3.3	17	1.5	8.8	0.8	5948	1422	83	21	106
Antipasti - The Cheese One	Starters & Shares	1140	274	17	7.9	22	4.7	9.9	0.8	5757	1388	85	40	111
Antipasti - The Meat One	Starters & Shares	1123	268	16	3.4	22	1.0	10	1.7	4778	1140	70	15	92
Antipasti - The Mixed One	Starters & Shares	1151	284	16	7.2	21	4.5	9.5	1.0	6258	1535	98	38	111
Mussel Marinara	Starters & Shares	448	106	6.4	0.6	7.9	1.6	3.9	0.6	1564	371	22	2.1	28
Bruschetta	Starters & Shares	660	165	6.5	1.6	16	2.0	4.5	1.0	1343	321	17	3.2	34
Calamari (1 Serving)	Starters & Shares	842	201	14	1.5	11	0.9	7.3	1.0	1507	360	25	2.8	19
Mushrooms Al Forno	Starters & Shares	1291	330	27	5.3	10	1.3	6.3	0.6	2274	540	43	9.6	20
Meatballs	Starters & Shares	515	195	9.7	3.5	16	2.1	12	1.4	1223	292	15	5.3	24
Insalata Caprese	Starters & Shares	776	187	15	7.9	1.6	1.6	11	0.5	1622	391	32	16	3.4
Croquettes	Starters & Shares	888	213	14	3.9	14	3.4	6.8	0.8	1760	423	28	5.7	29
Butterfly King Prawns	Starters & Shares	923	221	11	3.4	14	1.7	9.3	1.0	1737	416	21	6.3	27
Melanzane al Forno	Starters & Shares	521	151	12	3.8	5	2.5	6.8	0.6	1259	301	24	7.3	10
Tagliatelle Carbonara	Fresh Pasta	467	232	15	8.1	15	0.9	9.8	0.9	4535	1079	69	39	70
Aragosta e Gamberoni	Fresh Pasta	628	149	4.9	1.8	17	2.0	8.5	1.0	2756	652	21	8.1	75
Beef Tortellini	Fresh Pasta	513	123	5.5	2.8	13	2.0	6.4	0.9	1919	397	18	8.9	39
Funghi Misti	Fresh Pasta	596	142	7.2	4.1	16	3.4	4.7	0.4	2568	618	33	18	70
Rigatoni con Zucchine e Pesto - without chicken	Pasta - Classic	1182	280	20	3.4	27	2.8	7.1	1.0	3876	899	63	22	80
Fettuccine Bolognese	Pasta - Classic	804	190	5.8	3.2	25	3.1	8.6	0.9	2926	692	21	9.8	87
Spaghetti al Pomodoro	Pasta - Classic	502	120	3.8	1.6	20	3.7	4.2	0.5	2826	672	24	7.8	98
Linguine Carbonara	Pasta - Classic	1209	287	16	9.0	18	3.0	9.5	0.7	4954	1055	58	32	101
Linguine con Frutti di Mare	Pasta - Classic	1084	257	4.8	0.9	38	3.8	7.2	1.3	3977	708	33	2.4	89
Ravioli Manfredi	Pasta - Classic	733	174	7.9	3.0	18	3.6	7.9	0.8	2646	611	28	11	68
Rigatoni di Manzo Piccante	Al Forno	720	171	9.1	3.0	15	2.1	9.2	0.8	2979	945	45	17	85
Penne al Forno della Casa	Al Forno	634	151	7.2	3.7	14	3.8	7.2	0.4	2535	617	43	21	76
Lasagne	Al Forno	650	156	10	4.0	11	2.6	6.4	0.4	3896	742	47	25	51
Melanzane al Forno - Without Side Salad	Al Forno	674	151	11	3.6	5	2.5	5.9	0.6	3547	605	48	24	28
Risotto Pescatore	Risotto	980	181	8.8	3.1	20	1.3	8.9	0.9	3468	812	28	8.8	128
Risotto con Pollo e Funghi	Risotto	578	139	4.0	2.0	19	1.2	8.7	1.1	3428	818	24	12	114
Risotto Rosso	Risotto	568	139	5.0	2.5	20	1.7	8.5	1.0	3333	797	29	14	116
Pork Belly Porchetta	Meat & Fish	813	196	15	5.5	5.8	2.4	10	0.4	4873	1143	87	32	35
Pollo Prosciutto	Meat & Fish	474	112	4.5	1.9	7.0	1.0	12	0.8	2378	562	22	9.5	35
Sea Bass al Forno	Meat & Fish	491	120	9.8	2.4	5.7	0.8	8.4	0.8	2335	568	29	13	31
Prima Pizza Salumi Misti	Pizza & Calzone	858	205	8.9	4	21	2.3	11	1.2	4255	1011	44	20	103

Brewers Fayre

Product/ Dish Description	Nutrition Information Per Portion							
	kJ	kcal	Fat (g)	Saturates (g)	Carbohydrate (g)	Sugars (g)	Protein (g)	Salt (g)
DAYTIME VALUE & SNACKS MENU								
STARTERS								
TOMATO SOUP	960	234	7.7	3.3	33.0	11.8	5.7	1.8
CRISPY POTATO DIPPERS	2045	488	26.6	12.1	34.0	2.7	23.6	1.7
DUDDLE & SQUEAK	1458	349	24.4	10.9	19.5	2.4	10.4	1.4
GARLIC & HERB BREADED MUSHROOMS	1627	389	17.3	1.9	53.0	6.7	8.7	1.5
MAINS								
SMOTHERED CHICKEN	3991	953	45.3	15.1	79.0	11.9	55.4	4.5
LASAGNE	2653	634	26.1	12.1	64.3	17.0	27.9	3.1
GRILLED GAMMON STEAK WITH EGGS	3841	917	37.8	12.5	62.5	5.6	60.1	4.5
GRILLED GAMMON STEAK WITH PINEAPPLE	3655	873	31.3	10.9	81.2	26.4	67.8	4.2
GRILLED GAMMON STEAK WITH ONE OF EACH	3748	895	34.6	11.6	71.9	16.0	74.0	4.3
FISH & CHIPS WITH PEAS	5227	1248	73.2	12.3	107.4	6.9	37.5	1.7
FISH & CHIPS WITH MUSHY PEAS	5386	1286	73.3	12.4	113.9	5.6	39.8	2.6
BATTERED GIANT HADDOCK & CHIPS WITH PEAS	4501	1075	50.3	16.2	86.3	7.7	47.4	1.7
BATTERED GIANT HADDOCK & CHIPS WITH MUSHY PEAS	4660	1113	58.4	16.3	92.8	6.4	49.7	2.6
MEXICAN BEEF CHILLI	2999	716	18.5	5.4	87.1	5.7	38.7	2.3
THREE CHEESE CRUSTLESS QUICHE	2390	571	36.4	18.0	42.2	10.6	19.7	1.2
THE SOUTH WESTERN BURGER	4105	980	47.6	11.0	106.5	12.4	29.6	2.4
BEEF, CHEESE & MUSHROOM BURGER	4846	1157	64.5	23.0	93.3	12.9	48.7	2.5
GRILLED CHICKEN & BACON SALAD	1816	434	19.0	5.6	12.4	11.2	50.9	4.7
CHICKEN TIKKA CURRY	3614	863	24.0	9.2	113.1	25.6	45.3	3.7
BREADED WHOLETAIL SCAMPI WITH PEAS	3885	928	44.7	8.3	107.0	7.0	24.2	3.9
BREADED WHOLETAIL SCAMPI WITH MUSHY PEAS	4044	966	44.8	8.4	113.6	5.7	26.5	4.8
SMOKY PAPRIKA CHICKEN	1802	430	14.7	4.1	31.9	13.1	41.4	2.2
SAUSAGE, EGG & CHIPS	4170	996	57.1	18.9	74.2	7.4	44.0	2.5
SWEET POTATO & FETA LASAGNE	3106	742	39.1	15.8	69.9	17.8	24.2	3.0
HOT 'N' SPICY VEGGIE NACHO BURGER	5160	1233	61.8	19.9	142.3	17.9	23.0	3.0
HAM & CHEESE SANDWICH WITH WHITE BREAD	2613	624	28.3	12.6	55.8	9.0	35.2	2.5

Five Guys

Typical values per portion

	Energy, kJ	Energy, kcal	Total fat, g	Of which Saturated, g	Carbohydrates, g	of which Sugars, g	Fibre, g	Protein, g	Salt, g	Average portion Size (g)
MEAT										
Bacon	2872	686	64.0	22	0.2	0.0	0.1	31.9	0.97	113
Beef Burger Patty	745.4	179.0	13	5.1	0.1	0.1	0.8	18	0.12	81.5
Hot Dog	974.2	235.0	20	8.5	0.5	0.7	0.4	13	1.48	88
BUN										
Burger bun	958.4	227.7	7.20	2.46	38	5.82	1.34	5.78	0.47	72.8
Hot dog bun	1067.8	253.2	7.90	2.86	40	6.98	1.42	6.26	0.56	82.3
FRIES										
Little FRIES - COOKED IN PEANUT OIL	2980.2	716.0	44	5.81	72	0.78	7.66	11	1.14	284.2
Regular FRIES - COOKED IN PEANUT OIL	4823.3	1158.8	72	8.41	118	1.28	12	18	1.85	427.8
Large FRIES - COOKED IN PEANUT OIL	7178.8	1724.8	107	14	178	1.81	18	28	2.75	638.4
Cajun Seasoning	51.8	12.2	0.20	0.03	1.77	0.85	0.86	0.40	0.70	4.25
TOPPINGS										
BBQ Sauce	88.3	20.4	0.08	0.02	4.11	3.85	0.38	0.23	0.25	15
Cheese (1 Slice)	380.9	71.9	5.81	3.72	0.58	0.58	0.00	3.78	0.72	18.4
Green Peppers	8.3	1.9	0.02	0.01	0.43	0.22	0.16	0.08	0.00	8.25
Grilled Mushrooms	51.2	12.1	0.13	0.03	1.87	0.03	0.76	1.14	0.01	22.8
Hot Sauce	8.3	1.9	0.05	0.01	0.14	0.02	0.12	0.19	0.05	2.75
HP Brown Sauce	82.3	12.5	0.01	0.01	3.47	2.83	n/a	0.11	0.18	12.3
Jalapeno Peppers	4.4	1.0	0.03	0.01	0.58	0.33	0.22	0.07	0.02	8
Tomato Ketchup	72.9	17.1	0.02	0.00	3.89	3.82	n/a	0.20	0.30	10.8
Lettuce	8.8	2.9	0.02	0.00	0.27	0.00	0.18	0.14	0.00	15
Mayonnaise	461.2	107.3	12	0.88	0.48	0.48	n/a	0.13	0.24	18.2
Mustard	18.4	4.7	0.24	0.05	0.27	0.05	0.17	0.27	0.17	6.3
Onions	31.9	7.0	0.02	0.00	1.58	0.74	0.30	0.18	0.00	17.5
Grilled Onions	45.2	10.8	0.11	0.03	2.47	1.48	0.00	0.27	0.00	28.8
Pickles	9.0	1.9	0.00	0.00	0.27	0.24	0.36	0.21	0.57	22.8
Relish	104.5	24.6	0.06	0.03	5.82	4.84	0.19	0.06	0.22	18
Tomatoes	24.8	5.8	0.04	0.00	1.27	1.21	0.40	0.84	0.00	40.3

Watch out when you are adding extras to your burger

Harvester

STARTERS AND SHARERS	Energy (KJ)	Energy (Kcal)	Fat (g)	Saturated Fat (g)	Carbohydrate (g)	Sugars (g)	Protein (g)	Salt (g)
Potato Skins with Bacon & Cheese	1,934	460	26.2	13.5	32.2	2.1	22.8	2.15
BBQ Chicken Wings	1,593	379	18.4	4.8	20.3	18.6	32.8	1.98
Crispy Coated Chicken Bites	1,683	401	17.9	3.2	36.9	18.3	22.9	2.21
Cheese Popits with Salsa V	1,759	419	25.9	10.1	33.1	4.4	12.2	1.46
Spicy Crackerjack King Prawns	1,359	324	13.3	3.1	40.0	13.6	9.3	2.02
Breaded Mushrooms V	2,363	563	39.2	6.0	43.6	2.2	8.1	1.88
Chicken & Chorizo skewers NEW	1,990	474	36.5	12.4	6.7	5.5	28.3	1.81
Ultimate Nachos V	3,633	865	48.7	15.8	82.0	4.8	20.3	3.03
Why not add Three Bean Chilli V	464	110	3.0	0.4	15.1	6.8	4.2	0.78
Or add BBQ Pulled Pork	1,298	309	10.1	3.3	40.4	38.7	13.6	1.43
Creamy Tomato & Basil Soup V NEW	1,268	302	5.7	2.7	53.5	4.9	8.0	2.26
Sticky Duck Wings	1,839	438	19.1	5.1	35.5	32.5	29.2	2.69
Fish Basket NEW	2,994	713	45.2	7.5	54.5	7.5	21.7	4.00
Cheesy Garlic Bread Board V NEW	5,795	1,380	92.6	31.8	108.4	15.3	34.0	5.15
CHICKEN	**Energy (KJ)**	**Energy (Kcal)**	**Fat (g)**	**Saturated Fat (g)**	**Carbohydrate (g)**	**Sugars (g)**	**Protein (g)**	**Salt (g)**
Harvester's Famous 1/2 Rotisserie Chicken	1,885	449	22.9	5.0	2.4	3.5	57.1	1.58
Whole Rotisserie Chicken NEW	3,525	839	41.2	10.0	2.4	4.0	113.5	3.07
Chargrilled Chicken Breast	1,126	268	9.1	1.0	2.5	3.1	43.0	1.48
Triple Chicken	2,783	658	27.7	3.7	13.6	3.6	87.3	2.94
Chicken Skewer	1,879	447	25.9	6.0	2.5	3.3	49.7	0.92
Salsa Chicken & Pepper Stack	1,588	378	14.0	1.2	11.4	11.4	49.0	1.90
HARVESTER RECOMMENDS BBQ Brushed & Basted	4,487	1,068	43.8	9.3	93.9	47.4	63.0	3.67
HARVESTER RECOMMENDS Piri Piri Brushed & Basted	3,952	941	48.0	9.6	63.8	8.0	62.1	6.22
Garlic & Parsley Brushed & Basted	6,259	1,490	40.3	12.9	59.9	73.1	69.8	3.1
Farmer Rikki's Hot Chilli Sauce Brushed & Basted	4,117	980	52.0	9.9	62.7	5.8	63.1	2.99

CALIBER Menu 1 (DK18) – Low-carb, high-fat – Week 1

	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Breakfast	Simply eggs and avocado Coffee (no sugar) with a little full-fat milk	Greek yoghurt, mixed berries and chia seeds Coffee (no sugar) with a little full-fat milk	Judy's fabulous oatmeal Coffee (no sugar) with a little full-fat milk	Greek yoghurt, mixed nuts and chia seeds Coffee (no sugar) with a little full-fat milk	Judy's fabulous oatmeal Coffee (no sugar) with a little full-fat milk	Grilled parmesan tomatoes Coffee (no sugar) with a little full-fat milk	1 sausage, mushrooms, tomatoes, avocado Coffee (no sugar) with a little full-fat milk
Lunch	Cheese and herb muffins Cherry tomatoes, snack cucumbers	Bacon-wrapped haloumi Cheese and herb muffins	(Left-over) Courgetti Bolognese	Greek salad with walnuts and avocado	lChef's salad bowl	Left-over butter chicken curry with cauli-rice	Roast lamb with summer vegetables and mint pesto
Dinner	Rosemary salted sirloin with lemon and avocado butter, fried mushrooms and steamed broccoli	Courgetti Bolognese with parmesan cheese	Fish stew with dill and prawns, cauli-rice and steamed broccoli	Stir-fried pork with watercress Cherry tomato salad with balsamic vinegar and olive oil	Creamy butter chicken curry with cauli-rice	Meatloaf with courgette and garlic gratin and heavenly sauce	Cauli-pizza with ham, salami and olives
Snacks	Devilled nuts, 1 bag of Pork scratchings, 2 handfuls of strawberries	2 x mini baby bel 1 handful of walnuts	Vegetable sticks (red peppers, carrot sticks, cucumber) with mushroom pate	Salami, cheese and cucumber rolls 1 handful of almonds 2 handful of raspberries	Avocado, 1 handful of olives	1/2 tub of olives with cheese, nutty crackers with (left-over) mushroom pate	2x mini baby bel, vegetable sticks, 1 handful of walnuts
Drinks	Bullet-proof coffee, Coffee (no sugar) with a little full-fat milk or cream; Tea (no sugar) with a little full-fat milk or unsweetened herbal teas; Water (unflavoured or flavoured with citrus fruit, mint)						

8.2: Appendix 2: High carbohydrate diet guide and example meal plan

Dietary Guidelines Group (High-carb)

CALIBER study Merseyside

Liverpool John Moores University

School of Sport Studies, Leisure and Nutrition

Tanja Harrison BSc (Hons) ANutr AFHEA

SEDA

Contents

Quick guide

For the next eight weeks you have been allocated to the high-carb group of the CALIBER study, which means that you will be asked to follow the UK dietary recommendations.

We will undertake a variety of assessments with you when you come to see us in our laboratory at IM Marsh campus on three occasions, including blood samples, body composition, dietary intake and impact on this diet on food cravings and cognition. We will also ask you to wear a wrist-worn physical activity monitor on three occasions.

These guidance notes will give you further information on which types of food to eat, what makes a portion size for carbohydrates, fats, proteins and fruits and vegetables 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.

Introduction

Hello and welcome to the CALIBER study! Nice to have you on board.

This booklet has been designed to be your companion over the next eight weeks whilst you are part of our cohort and to support you to consume a healthy diet whether you are cooking at home, buying ready-made meals or eating out. There are certain rules that can be applied to all of these situations.

We will also give you information on the purpose of our study, what to expect during your visits to our laboratories and once the study has finished and we have analysed the results. This means that not only will you help an important research cause but you can also find out how you and your body did over the course of these eight weeks and how your body composition, blood profile, food cravings and cognition 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?

Maybe you have followed the news over these past couple of years and noticed that there is a lot of controversy and discussion about what makes a diet healthy. The debate has been particularly heated around the issue of carbohydrates. Whilst many public health officials have argued that the vast majority of the population does not follow the dietary UK guidelines (which can be classed as high-carbohydrate, moderate-fat) and that this is the root cause of the UK's problem with ill-health, obesity and diseases such as heart disease and type 2 diabetes, others claim that these guidelines have caused these problems to begin with. The latter group advocates to reduce the amounts of carbohydrates we consume as a nation and for the guidelines to be re-written.

A third group suggests that it is far more complicated than this and that how we react to carbohydrates is actually far more personal and not one-size-fits-all, but some people might be better off on low-carb diets whilst others fare better on high-carb diets in terms of cardiometabolic health.

This is where our study comes in, CALIBER – Carbohydrates, lipids and biomarkers of traditional and emerging cardiometabolic risk factors. We want to compare the effects of consuming either a high-carb or a low-carb diet on these risk factors. The reason why we have asked you to join us is because you showed some slightly elevated risk markers for these illnesses, albeit still at a stage where these should be easy to control and improve through a healthy diet, be this in form of low-carb, high-fat or high-carb, moderate-fat.

The discussion surrounding carbohydrates does not simply stop at their potential impact on risk markers that we find in our blood. There is also some discussion whether carbohydrates or fats lead to an increase in waist circumference and the development of fat deposits in our bodies, whether carbohydrates or fats are the root cause of food cravings that we might experience and whether one or the other somehow helps us to move about more or makes us more sluggish.

As scientists and nutritionists we are naturally curious to find out what might be going on. So thank you for joining us!

What to expect during you visits to our lab

There will be three appointments where we will ask you to come to IM Marsh campus (L17 6BD) for more thorough assessments, one right at the start when you will commence to eat according to the guidelines given to you, one after about 4 weeks and the final visit 8 weeks after your first one.

Each visit is expected to last between 60 and 90 minutes and will entail

A venous blood sample

We take about 8 teaspoons of blood. – Please note that you will have to have fasted for at least 12 hours prior to your appointment as otherwise your blood sugar and your triglycerides might be far higher than normal – painting a wrong picture of how the eating plan is working for you. Just as you had to do before coming in for your initial finger prick appointment you will also have to restrain from drinking alcohol or undertaking 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 and type 2 diabetes might have changed over the course of eight weeks.

Assessing your body composition

This will be done in two 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 ensure that this can be facilitated.

Secondly, we will ask you to step onto sophisticated 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.

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.

Going through a brief questionnaire with you and conducting one final interview

During your second and final visit we will go through a checklist to see which types of fibre-containing foods you have consumed over the previous four weeks.

During your final visit we will also ask you to stay with us for a little longer to conduct a brief interview with you asking you about your experiences with the diet allocated to you.

Prior to your lab appointments – recording of physical activity

On three occasions (just before your first, second and final visit to our lab) we will ask you to wear a physical activity monitor that looks like a digital watch and has to be worn on the wrist (just like a 'Fir bit') of your non-dominant arm. That means if you are right-handed you will have to wear this on your left wrist.

We will give you this device nine days before your visits to our labs and will ask you to start wearing this device for 7 days and at least 10 hours per day commencing on the morning after it has been handed to you and finishing the night before your lab appointment. During this time you will have to complete a wear-time diary on a daily basis, in which you will briefly record the times you are putting the monitor on in the morning, the times you are taking it off at night and any time during the time when you need to remove and put it back on, for example when you are taking a shower or when you are going swimming.

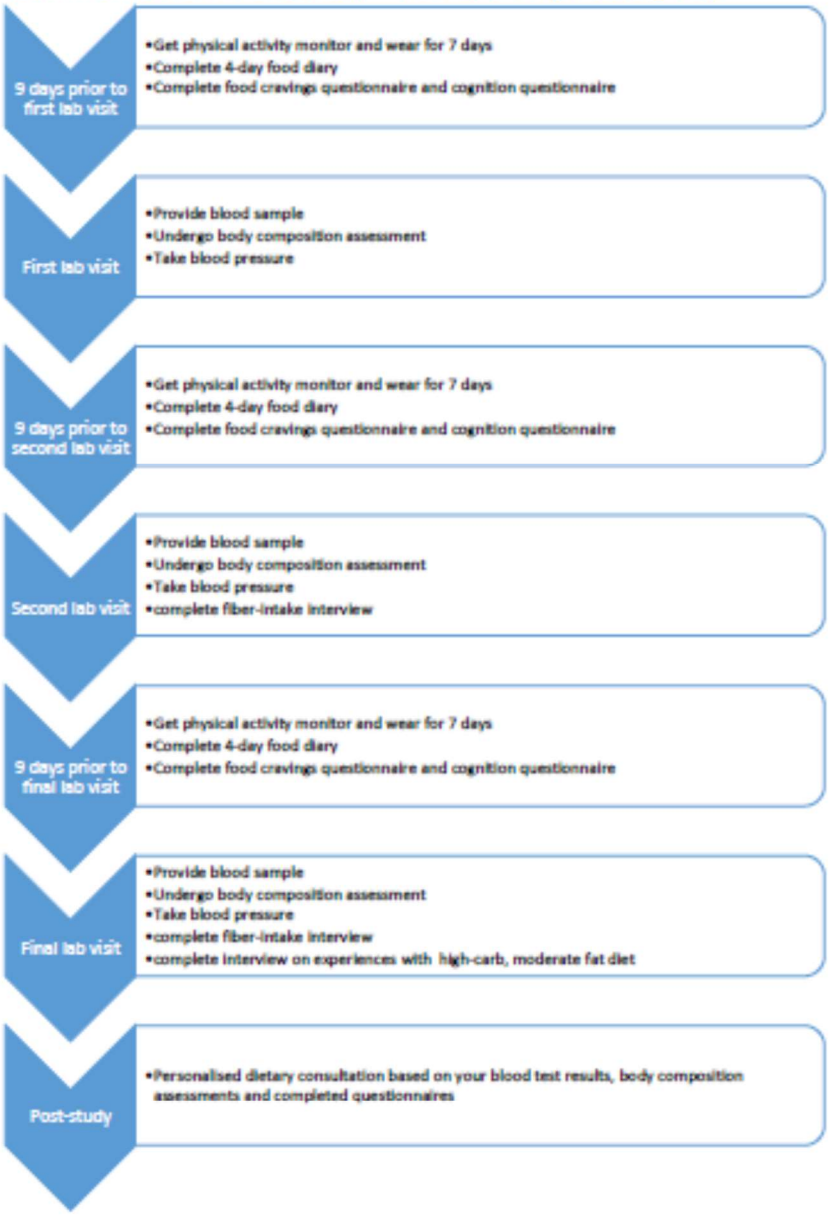
You will need to bring the monitor and the wear time diary with you on the morning of your lab appointment where the research team can collect it from you.

Prior to your lab appointments – food diaries and questionnaires

When you are given your physical activity devices we will also give you a template of a four-day food diary, which you will need to complete for four days before your lab appointment and bring with you on the morning.

We will also give you a number of brief questionnaires, which will assess your cognition and your food cravings over the previous four-week period. These will not take long to complete. Please also bring these questionnaires with you to your lab appointment and pass them on to our research team.

Your involvement in the study – Flow chart high-carb group



Following a high-carb diet – What does that actually mean?

First of all what a good time you have picked to join our study and follow a healthy, balanced diet. With summer just around the corner there is an abundance of foods that will make your eating delicious, interesting and affordable.

This guideline has been designed to help you follow the UK dietary recommendations over the next eight weeks whilst you are part of our cohort.

These recommendations are based around **starchy foods**, which means that at least **50%** of your diet should consist of carbohydrates. For the average man in the UK this means at least 333 grams per day, for the average woman in the UK at least 267 grams per day. (Women tend to be smaller than men and therefore require less energy and less food.) Due to the amount of carbohydrates recommended for consumption the UK recommended standard diet is classed as a high-carb diet.

These carbohydrates will come from a variety of sources, including potatoes, rice (brown is better!), pasta (wholemeal), bread (whole meal), fruit and vegetables and to a lesser extend dairy products (in the form of milk sugar, called lactose).

Portion sizes for carbohydrates

So, what do at least 50% carbs look like?

Over the course of the day we should eat between 6 to 8 portions of starchy foods. If you are not a very active person (i.e. sit mainly at a desk in an office and only also do not move that much in your spare time) you should aim for 6 portions. If you are more active in your job, either your leisure time or both you can go for the 8 portions.

Try to consume about 2 portions at each main meal of the day.

Breakfast cereal - e.g. bran flakes, cornflakes, rice crispies, porridge oats	3 tablespoons (about 20g)
Shredded wheat	1 biscuit
Weetabix	1 biscuit
Muesli	2 tablespoons
Bread	1 slice (medium thick)
Bagel	Half
Bread roll	Half
Crackers	3
Crispbreads	4
Crumpet	1
Muffin	Half
Naan read, plain	1
Pitta bread	Half

Potatoes	2 small boiled (egg-sized)
Jacket potatoes	Half (size of a computer mouse)
Rice, cooked	2 heaped tablespoons (80g)
Noodles, boiled	3 heaped tablespoons (80g)
Pasta, boiled	3 heaped tablespoons (80g)
Malt loaf	1 small slice
Muesli bar	1

Here are some examples on how to achieve between 6 and 8 portions of starchy food per day:

	Day 1	Portions	Day 2	Portions	Day 3	Portions
Breakfast	1 bowl of cereals (60g = 9 tablespoons)	3	2 crumpets	2	2 slices of toast	2
Lunch	Sandwich with two slices of bread	2	1 filled pitta bread	2	Pasta salad (240g = 9 tablespoons of cooked pasta)	3
Dinner	Noodle stir-fry (160g = 6 heaped tablespoons of cooked noodles)	2	Rice with your meal (240g = 6 heaped tablespoons)	3	4 small potatoes as side dish	2
Total		7		7		7

Beware – Not all carbohydrates are equal!

This might actually be one of the issues with a high-carb diet, as with nearly everything in life, there is good and not so good, and the key is to eat more of the good stuff and hold back on the other stuff.

Many of the carbohydrates we consume in the Western world are highly processed – and the give-away is in the colour. Think white - so-called starchy foods – bread (flour), pasta, rice. Whilst it is okay to eat these occasionally, as adults we should focus on also incorporating wholemeal varieties into our daily eating. Maybe you are not used to eating these types and need some convincing? You could ease yourself into eating these by mixing for example white rice with whole-grain varieties or mixing different types of wholegrains. There are even some commercially available blends that can get you started.

The main reason for holding back on these white, processed varieties of starches is firstly that they are digested more easily by our bodies and reach our bloodstreams in the form of glucose a lot quicker. (These are also known as high-glycaemic index foods which you might have heard of before.) Secondly, the white colour is achieved by polishing the original grain and therefore removing a lot of vitamins and minerals that are a natural part of the food

matrix. In case you were wondering this is exactly the reason why wheat flour in the UK is fortified with a range of vitamins and minerals – the goodness that got taken out to begin with needs to be added again somehow.

When we are taking about wholegrain foods we do not only mean flour-based products. Other alternatives are naked barley, buckwheat, oats, quinoa, rye, teff and wild rice.

What is fibre and why are we supposed to eat it?

Carbohydrates in our diet come from different sources, with some of them being more readily (if at all) absorbed by our bodies and used for energy to keep us going during the day. When nutritionists and other professionals talk about carbohydrates and a certain type of carbohydrate diet (in this case "high carbohydrate diet") we mean carbohydrates that are actually available to our bodies. Only these types of carbohydrates are actually being counted when making recommendations for carbohydrate intake. The other type of carbohydrates, which are generally not available, are classed as dietary fibre (see below). There are also different subcategories of these, with some supplying our bodies with small amounts of energy, but these can be disregarded in the context of the foods that we consume every single day as part of a healthy balanced diet.

The UK dietary recommendations state that everyone in the UK above the age of 14 should aim to consume at least 30 grams of fibre per day. At the moment the majority of the population is not meeting these recommendations. Research has shown that sufficient fibre intake can help prevent heart disease, some cancers and diabetes. Fibre can also aid to improve your digestion and make you feel fuller for longer meaning you eat less and less often (serial snackers beware). Fibre can be found in a number of starchy foods and fruit and vegetables, i.e. plant-based foods. This is also where wholegrain varieties of foods come into their own. They contain more fibre than their polished cousins.

Foods that contain 6 or more grams of fibre per 100g are classed as high-fibre foods, whereas those containing at least 3 grams of fibre per 100g are considered to be 'fibre-rich'.

Please note that if you think that your diet has been lacking in fibre so far to increase the amounts that you are consuming gradually over a couple of weeks and to ensure that you drink plenty of fluids. Otherwise you might have to deal with bloating and constipation as side effects.

Foods containing fibre

Wholegrains (see list above), wholegrain cereals, bran cereals, wholegrain bread, brown rice, potatoes and oats are good sources of fibre. In addition, there is a wide variety of fruit and vegetables, and nuts and seeds that can be consumed to maintain or increase your fibre intake.

Fruit and vegetables (1 portion = 80g)

The table below shows the amount of fibre contained in one portion of 80g fruit or vegetable to give you an idea which foods might be good to prioritise in order to obtain sufficient fibre in your diet.

Food name	Amount fibre in g (per 100g)	Amount fibre in g (per 80g portion)
Apples, eating, raw	1.2	0.96
Asparagus, boiled	1.4	1.12
Aubergines, fried	2.3	1.84
Avocado, average	3.4	2.72
Banana	1.5	1.3
Beans, baked in tomato sauce (tinned)	3.8	3.04
Beans, broad, boiled	5.4	4.32
Beans, butter, boiled	5.2	4.16
Beans, green, boiled	2.5	2.0
Beans, red kidney	6.2	4.96
Beans, soy/edamame	6.1	4.88
Beetroot, boiled	1.9	1.52
Blackberries	3.1	2.48
Broccoli, boiled	2.3	1.84
Broccoli, Purple sprouting, boiled	2.3	1.84
Brussel sprouts, boiled	3.1	2.48
Butternut squash	1.4	1.12
Cabbage, green cooked	2.6	2.08
Cabbage, red cooked	2.0	1.6
Cabbage, white cooked	1.4	1.12
Cabbage, spring greens, cooked	2.6	2.08
Carrots, young, boiled	2.3	1.84
Cauliflower, cooked	1.6	1.28
Celery, raw	1.1	0.88
Chickpeas, canned, re-heated	4.1	3.28
coconut, desiccated	13.7	10.96
Coconut, fresh	7.3	5.84
Courgette, boiled	1.2	0.96
Cucumber, raw	0.7	0.56
Fennel, Florence, boiled	2.3	1.84
Kale, curly, boiled	2.8	2.24
Kohlrabi, boiled	1.9	1.52
Leeks, boiled	1.7	1.36
Lentils, green or brown	3.8	3.04
Lentils, red	1.9	1.52
Lettuce, cos	2.1	1.68

Lettuce, iceberg	1.2	0.96
Lettuce, romaine	2.1	1.68
Mushrooms, oyster, raw	2.3	1.84
Mushrooms, Portobello, grilled	2.2	1.76
Mushrooms, shitake	2.1	1.68
Mushrooms, white, boiled	2.1	1.68
Olives, green in brine, drained	2.9	2.32
Onions, fried (based on 2 table spoons, chopped)	1.5	0.45
Parsnip, boiled	4.7	3.76
Peanut butter, smooth	5.4	1.62
Peas, green, frozen, boiled	4.0	3.2
Pepper, green, boiled	1.8	1.44
Pepper, red, boiled	0.8	0.64
Pepper, yellow, boiled	0.8	0.64
Potatoes, boiled	1.0	0.8
Pumpkin, boiled	1.1	0.88
Radishes, raw	0.9	0.72
Raspberries, red	2.5	2.0
Rocket	1.3	1.04
Spinach, baby, raw	1.2	0.96
Spinach, frozen, boiled	2.1	1.68
Spring onion, raw	1.5	1.2
Strawberries	1.0	0.8
Sweetcorn kernels, canned in water, drained	2.5	2.0
Sweet potato, boiled	2.3	1.84
Tomato, raw	1.0	0.8
Turnip, boiled	1.9	1.52
Watercress	1.5	1.2

Nuts and seeds (1 portion = 30g)

A small handful (or about 30g) of nuts or seeds counts as a portion. Below some guidelines of how much fibre you would get from different types of nuts and seeds.

Food name	Amount fibre in g (per 100g)	Amount fibre in g (per 30g portion)
Almonds, raw	2.7	0.81
Brazil nuts	4.3	1.29

Chia seeds	38	11.4
Flaxseeds (Linseeds)	27	8.1
Hazelnuts	6.5	1.95
Peanuts	6.2	1.86
Peanut butter, smooth	5.4	1.62
Pecan nuts	4.7	1.41
Pistachios	6.1	1.83
Pumpkin seeds	6.0	1.8
Sunflower seeds	6.0	1.8
Walnuts	3.5	1.05

Sugar is a carbohydrate – right?

Yes, it is indeed! However, remember when we spoke about that some carbs are better (or worse) than others. Sugar in the form of free sugar should be kept to a minimum and again only be eaten occasionally as a treat. This means less than 31 grams per day for men (about 7 teaspoons) and less than 25 grams per day for women (about 6 teaspoons).

The trouble is that free sugar, which is the stuff that most people mean when they talk about sugar, does not simply come in the form of table sugar, honey or syrups where you control yourself how you add. It is actually hidden in many foods that we buy. Unsurprisingly the usual suspects are sweet-tasting foods such as chocolate, confectionary, cakes and sweetened and unsweetened fruit juices but the list of culprits also includes condiments (such as ketchup), crisps, bread, ready meals and canned goods, mayonnaise, the list goes on.

The best way to avoid free sugars is to do the majority of your cooking yourself and to become an avid food label-reader (and you don't even need a degree to crack this!)

Eat a rainbow

You will have surely heard of the 5-a-day campaign, 5 portions of fruit and veg that is. There are actually several reasons why these are good for you. Firstly, they contain a considerable amount of carbs in the form of fibre – the good stuff. Secondly, the different colours express the presence of different types of nutrients that although not specifically named in the UK dietary guidelines (as there is actually thousands of them) that are really good for us and are thought to prevent heart disease, diabetes and cancers. These are namely anti-oxidants and polyphenols. The different colours of the fruit and vegetables available for us mean that these contain different types of these nutrients – all-round protection.

In general, it is recommendable to consume slightly more vegetables than fruit as these tend to contain more fibre and less fructose (fruit sugars).

If you feel slightly adverse to anything green and orange on your plate (one too many roast dinners with boiled-to-death cabbage) there are ways of incorporating these to help you feel less annoyed by their presence and to gradually get used to them. You could for example add

some finely chopped carrots and vegetables to a tomato sauce with your pasts. Alternatively, you could make a hummus with also contains vegetables such as beans, beetroot or peas to create a delicious snack.

What exactly is a portion of fruit and veg?

A portion is defined as 80g of any fruit or vegetable and looks as follows:

Fruit

Small fruit

Apricots	3
Blackberries	9 – 10 (One handful)
Blackcurrants	4 heaped tablespoons
Blueberries	4 heaped tablespoons (Two handfuls)
Cherries	14
Damsons	5-6
Figs, fresh	2
Fruit salad (fresh)	3 heaped tablespoons
Grapes	14 (One handful)
Kiwi	2
Kumquats	6-8
Lychee	6
Passion fruit	5 - 6
Plums	2
Raspberries	20 (One handful)
Rhubarb, cooked	2 heaped tablespoons
Satsumas	2
Strawberries	7
Tangerines	2

Medium fruit

Apple	One
Avocado	Half
Banana	One
Nectarine	One
Pear	One
Sharon fruit	One

Large fruit

Grapefruit	Half
Mango	Two slices (5cm/2 inches)
Melon	One slice (5cm/2 inches)
Papaya	One slice (5cm/2 inches)
Pineapple	One large slice

Dried fruit

30 grams of dried fruit count as one portion. Be careful with eating too much of these as the sugar in them is concentrated and can lead to tooth decay. It is better to incorporate these with other foods rather than eating on their own.

Apple rings	4
Apricots	3
Banana chips	1 handful
Cherries	1 heaped tablespoon
Cranberries	1 heaped tablespoon
Dates	3
Figs	2
Mango	1 heaped tablespoon
Mixed fruit	1 tablespoon
Peach	2 halves
Pear	2 halves
Pineapple	1 heaped tablespoon or 2 rings
Prunes	3
Raisins, currants or sultanas	1 heaped tablespoon

Fruit or vegetable juices

Note that only 150ml of any of vegetable or fruit juices or smoothies count towards one of your 5-a-day. This is because of the amount of free sugars (remember the 5% for sugar rule above!) contained in these juices. Try to choose unsweetened versions and limit consumption to meal times to prevent tooth decay.

Tinned/canned/jarred fruit

Some of these also contain a lot of sugar if syrup rather than water is used to contain the fruit. Check the label to be aware how much added sugar you might be consuming in this case!

Apples	2 heaped tablespoons
Apricots	6 halves
Cherries	11 (Three heaped tablespoons)
Fruit salad	3 heaped tablespoons
Grapefruit segments	3 heaped tablespoons or 8 segments
Lychee	6
Mandarin oranges	3 heaped tablespoons
Peaches	2 halves or 7 slices
Pear	2 halves or 7 slices
Pineapple	2 rings or 7 chunks
Prunes	6
Raspberries	20 (One handful)
Rhubarb, cooked	5 chunks
Strawberries	9

Vegetables

Fresh, frozen or cooked vegetables

Artichoke hearts	2
Asparagus	5 spears
Aubergine	1/3
Beetroot	3 whole baby beetroot or 7 slices
Beans, French or runner	4 heaped tablespoons
Broccoli	2 spears or 8 florets
Brussel sprouts	6-8
Butternut squash	3 heaped tablespoons
Cabbage, cooked	4 heaped tablespoons
Cabbage, shredded	3 heaped tablespoons
Carrot, sliced	3 heaped tablespoons
Carrot, shredded	3 heaped tablespoons
Cauliflower	8 florets
Chinese leaves or Pak Choi, shredded	4 heaped tablespoons
Corn in the cob	1
Leek, medium-sized (white portion only)	1
Mange-tout	1 handful (about 22)
Marrow, diced	3 heaped tablespoons
Mixed frozen vegetables	3 heaped tablespoons
Mushrooms, sliced	3-4 heaped tablespoons
Mushrooms, button	14
Okra, medium	9
Onion	1
Parsnip, medium	1
Peas	3 heaped tablespoons
Pepper	half
Pumpkin	3 heaped tablespoons
Spinach, cooked	4 heaped tablespoons
Spring greens, cooked	4 heaped tablespoons
Spring onions	8
Swede	3 heaped tablespoons
Sweetcorn, baby	6-8
Sweet potato, medium	1
Tomato puree (Yes, technically a fruit)	1 heaped tablespoon
Turnip, diced	3 heaped tablespoons

Salad vegetables

Celery stick	3
Cucumber	5 cm/2-inch piece
Lettuce or mixed leaves	1 cereal/dessert bowl
Radishes	10
Rocket	1 cereal/dessert bowl

Spinach, fresh	1 cereal/dessert bowl
Tomato, cherry	7
Tomato, medium	1
Watercress, fresh	1 cereal/dessert bowl

Tinned/canned/dried

Try and choose brands that are low in added salt and sugar (read the label).

Asparagus	7 spears
Beetroot	3 whole baby or 7 slices
Carrots, canned	3 heaped tablespoons
Mushrooms, dried	2 tablespoons
Peas	3 heaped tablespoons
Sun-dried tomatoes	4
Sweetcorn	3 heaped tablespoons
Plum tomatoes	2

Pulses (beans and lentils)

No matter how many portions you eat over the course of the day due to their high protein content only one portion of beans or lentils (pulses) will be counted as your 5-a-day.

Pulses include cooked baked beans, borlotti, black eye, broad, butter, cannellini, kidney, pinto, soy beans, chickpeas, green, yellow, red or black lentils. One portion is 3 heaped tablespoons.

Potatoes

These do not count as one of your 5-a-day as they are already part of the starchy portion of the Eatwell Guide, the UK dietary guidelines.

Dietary fat – the evil nutrient? Following a moderate-fat diet

No, not quite! The UK dietary guidelines actually de facto recommend a moderate-fat diet of less than 35%, which is about 97 grams per day for the average UK man and 78 grams per day for the average UK woman aged 11-64. One tablespoon of oil equals 15 grams, this means that the UK average man should consume no more than 6 ½ tablespoons of fat per day, the average UK woman no more than about 5 tablespoons. It is important to note that the majority of fat we consume will not be visible to the eye but incorporated into foods and dishes that we buy and eat.

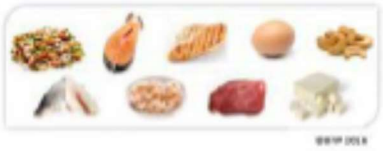
Although it is recommended to stick to low-fat products overall, fat does play an important role in our diet. It helps to absorb some vitamins that would otherwise simply go right through us. It also plays an important role in building the membranes of our cells. The key lies in the

quality of the fats consumed with priority given to unsaturated fats, such as olive oil and other vegetable oils, avocados, nuts and oily fish. One type of unsaturated fat which is essential to human health are omega-3 fatty acids. These can be found both in animal and plant sources. Oily fish (see below) is an important source of omega-3 fatty acids as these are of the highest quality. However, it is possible for the body to convert the omega-3 fatty acids found in plants foods, such as nuts and seeds and their products, into the same end products found in oily fish.

Men should consume no more than 30g (about 2 tablespoons) and women no more than 20g (about 1 ½ tablespoons) of saturated fats, which can be found in chocolate, pastries, cakes and biscuits, dairy products (such as full-fat milk, cream, butter) and fatty cuts of meat and sausages. This means to go easy on the butter and use unsaturated fat spreads instead, use lower-fat milk (green top or red top for example) and only have that hot chocolate with lots of whipped cream on top only as the occasional treat. The same goes for cakes, biscuits, crisps and other snack foods. This means being careful with the snacks that you choose over the course of the day should you get a bit peckish.

When looking for products that are lower in fat it is important to know that there is a difference between 'low-fat' and 'reduced fat' products. The first category means that the food contains 3g or less fat per 100g whereas a reduced fat food contains 25% less fat than the standard product. This might mean that even the reformulated product is still fairly high in fat.

Proteins to help you build and maintain that temple which is your body



Proteins are important in our diet as they have the vital functions of growth, maintenance and repair. They are also vital to help our immune system function properly. Proteins are composed of compounds called *amino acids* of which there are 20 that play a role in the human body. 8 of these amino acids are classed as essential because unlike the other 12 our bodies cannot produce these themselves, which means that they need to be obtained from our diets. This happens by consuming protein foods.

Whilst protein malnutrition can have disastrous consequences in reality the vast majority of people in the UK consume more than they actually need to.

The average UK male aged 15 to 64 should consume about 55 grams per day, the average UK female in this age group 45 grams per day. In order to achieve these recommendations we should eat two to three portions of protein each day and another two to three portions of dairy. There are different types of protein sources, including:

Animal-based proteins

Animal-based proteins contain all of the essential amino acids. This is because the animals have done the work for us eating a variety of food sources with different amino acid profiles

and combing these in their muscles. The same is true for eggs where the egg yolk and the egg white have the role of sustaining the developing chick.

Meat

Aim for lean cuts of meat and lean meat mince as meat can be high in saturated fatty acids. For the same reason it is advisable to cut off the visible fat from beef and pork steaks and to remove the skin from chicken. The way you cook your meat also plays an important role in your diet. Frying meats can add to your fat intake, so it is better to grill your meat instead, to use non-stick frying pans or to use spray bottles for oil, which will help you to avoid unnecessary fat to your pan. Other lower-fat methods include boiling and steaming.

You should not eat more than 70g of processed meat per day, which includes sausages, bacon, cured meats (for example salami, chorizo) and reformed meats products (for example sliced packaged ham).

Fish and seafood (Shellfish)

You should eat at least two portions of fish per week, one white, one oily. One portion is 140 grams which is about the size of a cheque book. The reason we recommend oily fish because these are an excellent source of omega-3 essential fatty acids, which we have mentioned earlier on. Fish also supply us with a number of vitamins and minerals.

White fish

Basra	Cod,	Coley	Dab	Flounder	Gurnard
Hake	Haddock	Plaice	Pollock	Red mullet	Tilapia

Some white fish should be eaten no more than once a week due to potentially high levels of pollutants contained in their flesh. These are seabeam, seabass, halibut and turbot.

Oily fish

Anchovies	Carp	Herring	Kippers	Mackerel	Pilchard
Salmon	Sardines	Sprats	Trout	Tuna (fresh or frozen)	Whitebait

Swordfish should not be eaten more than once a week due to potentially high levels of pollutants. It should be avoided by children, pregnant women and those wanting to become pregnant.

Shellfish

Cockles	Crab	Langoustines	Mussels (and clams and winkles)
Oysters	Prawns	Scallops	Squid

It is important to note that processed, canned fish like tuna do no longer count as your 'oily' portion as the manufacturing process has taken all the omega-3 fatty acids out of them.

However, the good news is – that tuna mayo sandwich for your lunch still counts towards your portion of white fish.

One thing to bear in mind is choosing where possible sustainable sources of fish as recommended by the Marine Stewardship Council (MSC) in their *Good Fish Guide*. The MSC also run an accreditation scheme, so look out for certified products carrying the following logo:

Further information on the MSC and a regularly updated list of sustainable types of fish can be found at www.msc.org.

Eggs

Eggs are real powerhouses of nutrition and contain all the essential amino acids that humans need in their diet. One medium egg provides about 6.4grams of protein with the egg yolk containing higher concentrations of amino acids. However, as the egg white is larger than the egg yolk the majority of protein supplied when eating an egg will actually come from the egg white.

For animal welfare reasons we would recommend that you avoid buying the eggs from caged hens. However, the choice is absolutely yours.

Dairy

Dairy products are both a source of protein and fats. Aim therefore for low-fat versions of dairy where possible. Dairy is also a good source of calcium and should therefore be included in our diet on a daily basis. It is recommended that we consume 2 to 3 portions of dairy (or plant-based, calcium-fortified alternatives) per day in addition to the other protein sources.

Dairy products include milk, cream, yoghurt, cheese, cream cheese, sour cream and quark. If you dislike dairy products, it is recommended to use calcium-fortified plant milks instead, such as soya, rice, oat and almond milks.

Plant-based proteins

These include beans, peas, lentils and chickpeas, which are high in fibre, vitamins and minerals and low in fat.

Nuts and seeds are also a great source of proteins but can contain a lot of fat. However, these are great sources of unsaturated fats, included important omega-3s.

Other plant-based protein sources include tofu, bean curd, mycoprotein (such as Quorn™) and soya-based meat replacement products, such as supermarkets' own brands. Please remember that the latter two are processed foods so it is always worth checking the label to ensure that there are not too many additives and flavour enhancers, salt and sugar found in these products. If this is the case, you should either eat these products sparingly or ensure that you count these towards your salt and sugar consumption for the day.

The proteins found in plants do normally not contain all of the essential amino acids (a more widely consumed exception are soya beans). This means that a combination of plant foods needs to be consumed to achieve a complete profile. Examples of this are beans on toast (i.e.

beans and flour), rice and beans, rice and lentils, crostini/melba toast with broad bean spread. Combing a cereal with a pulse in general will achieve a complete amino acid profile.

Portion sizes for protein

Meat, cooked	80g (size of a deck of cards or 3 slices)
Fish	140g (size of a cheque book) or 3 fish fingers
Eggs	2 medium-sized
Milk	200ml (medium glass)
Plant-based milks, such as soya, rice, almond, oat – fortified with calcium	250ml (large glass)
Yoghurt	125g (small container)
Hard cheese	40g (matchbox-size piece) or 40g (2 slices)
Cottage cheese	200g (large pot)
Cream cheese, light	80g (about 2 small matchboxes)
Fromage frais	150g (small container)
Sour cream	2 tablespoons
Baked beans	200g (small tin)
Pulses, cooked	4-5 tablespoons
Nuts and seeds	40g (1 small handful)
Nut butters, such as peanut butter	2 tablespoons (about the size of a golf ball)
Meat-replacement products	120g (for example two sausages or about the size of a snooker ball)

Treats or foods full of bliss – AKA Foods high in fats, salts and sugars

These are foods that are in general of no nutritional value to our bodies and in consumed in excess can do more harm than good, for example chocolate, cakes, biscuits, crisps, soft drinks, ice-cream, processed pork pies and sausage rolls. Remember the rules not to consume more than 7 teaspoons of sugar and 20 to 30 grams of saturated fats per day? The foods in the *treats* category might jeopardise this goal. If you find yourself eating these things to reward yourself, because you are having a bad day or simply because they are there – talk to us so that we can try and help you to find ways of limiting their consumption.

One of these ways might include to really sit down with a cup of coffee and that doughnut or slice of cake and take your time enjoying it rather than eating mindlessly. Taking your time and appreciating this type of food will also allow your digestive system enough time to connect with your brain and consequently to signal your stomach that you are full and tell your mind that you are happy.

Hydration

This is an important one that often gets overlooked. You should drink 6-8 glasses (200ml or medium-sized) per day to ensure staying hydrated. This will also help your body to cope with a potential increase in dietary fibre following a healthy diet (see above). The UK dietary

recommendations advise that you best stick to water, unsweetened teas and coffee and lower-fat milk.

Limit intake of fruit juices and smoothies (only 150ml per day count as one of your five-a-day) as the natural sugar contained in fruits has been freed through the process of liquidisation. This means it is more easily digested and can raise your blood sugar a lot quicker. The fruit sugar contained in fruit (fructose) can actually not be metabolised by our bodies and if not turned into energy will be converted into body fat. In addition, this free sugar can contribute to dental decay.

Eating whole fruit is not so much of a problem as the sugar is contained within a matrix plus whole fruit has the added benefit of fibre (see above).

That can of coca cola that you might enjoy at lunchtime should become an occasional treat only (see above). Soft drinks contain up to nearly 16 grams (or nearly four teaspoons) of sugar per 100ml, which means you will go over the recommended daily intake very quickly.

Learning to read labels and nutritional information

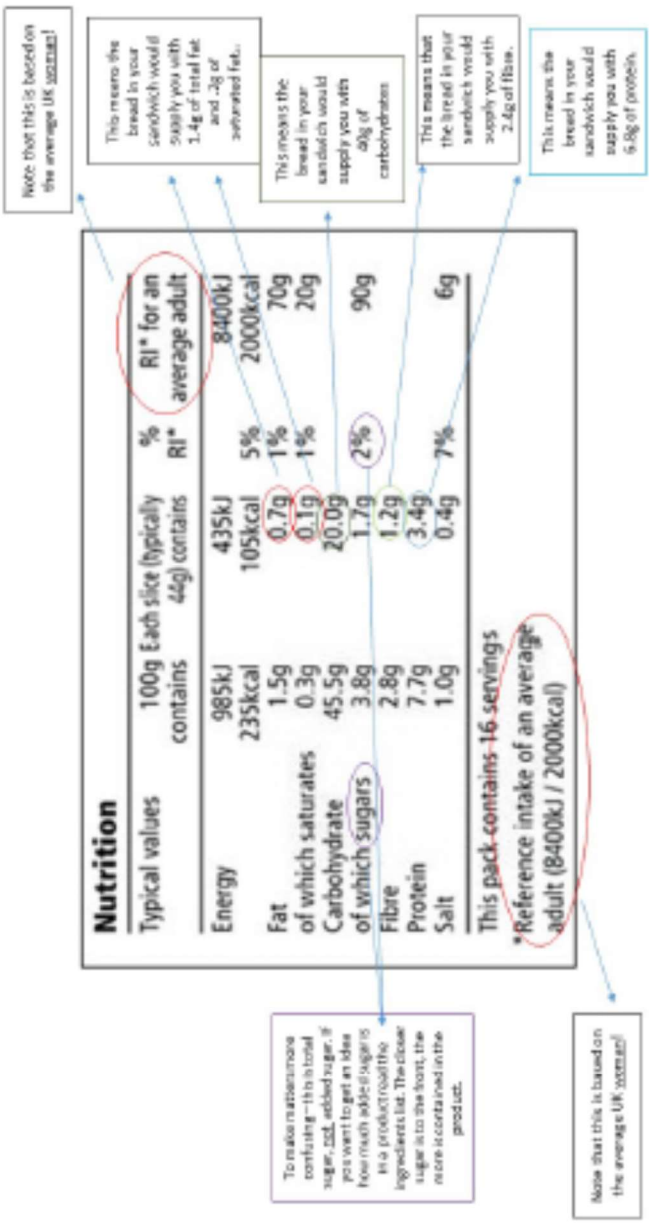
This is an important one when food shopping and eating out in places that provide you with information on the nutritional content of their dishes, for example fast food restaurants and pubs.

Label reading

In the UK food labels can be found at the back and depending on the manufacturer also at the front of the packaging.

Labels at the back of the packaging

Below is the back of pack nutrition label for a loaf of white bread as an example.



There are a few things to be aware of:

The manufacturer's portion size guide might actually not be a realistic reflection of what you might actually eat yourself and it can be very easy to underestimate the amount of sugars and fats that you are consuming.

Whilst it is fairly easy to work out how many carbohydrates you might consume over the course of a day using food label, calculating how much free sugars (remember, the 5% rule!) is an entirely different matter. The label will not tell you these things and total sugars will be a combination of the natural sugars contained in foods and can also include the sugars found in fruits (fructose) and milk (lactose) where applicable.

This is where looking at the ingredients list (also a the back of pack) might necessary as sugar comes under many different names and there might be some surprises in store for you.

As a general rule, the closer an added sugar is towards the top of the ingredients list the more of it will be contained in the product. There might also be different types of added sugar in the same product.

All of these that you might spot in the ingredients list are actually added sugars

> agave sugar, brown sugar, cane sugar, dextrose, fructose, fruit juice concentrate, glucose, HFCS/high fructose corn syrup, honey, hydrolysed starch, invert sugar, isoglucose, levulose, maltose, modified starch molasses, sucrose, syrups (sucrose, glucose, malt, corn, maple), treacle,

Once a product crosses specific thresholds for specific nutrients it will either be classed as 'medium' or 'high' in sugar or other nutrients (see below).

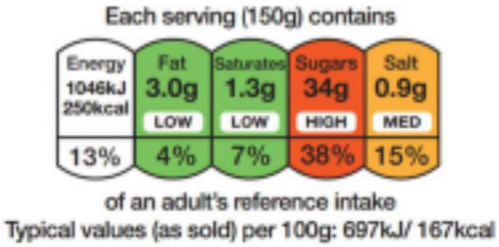
	Low	Medium	High
Total sugars	5g per 100g product (or 5%)	More than 5g but less than 22.5 per 100g product (between 5% and 22.5%)	More than 22.5g per 100g product (or 22.5%)
Total fats	3g per 100g product (or 3%)	More than 3g but less than 17.5 per 100g product (between 3% and 17.5%)	More than 17.5g per 100g product (or 17.5%)
Saturated fats	1.5g per 100g product (or 1.5%)	More than 1.5g but less than 5g per 100g product (between 1.5% and 5%)	More than 5g per 100g product (or 5%)
Salt	0.3g of salt or less per 100g (or 0.1g sodium)	More than 0.3g of salt (0.1g sodium) but less than 1.5g of salt (0.6g sodium) per 100g product	More than 1.5g of salt per 100g (or 0.6g sodium)

If the portion size means that more than 25g of sugar would be consumed with one portion the product is automatically classed as 'high sugar'.

For drinks containing total sugar the rule is that 2.5g per 100ml is classed as 'low', between 2.5 and 11.25g per 100ml is classed as 'medium' and above 11.25g per 100ml is 'high'. If the portion size of a drink means that more 13.5g of sugar would be consumed the product is automatically classed as 'high sugar'.

Front of pack labels

Some retailers and manufacturers display a food label at the front of the pack highlighting whether some of the crucial nutrients are contained in the foods to low, medium or high levels. This is called the 'traffic light system'.



typical serving sizes of their dishes that you can access online, download or on request at the restaurant.

Being aware of the nutritional content of some of these dishes is helpful if you tend to eat out more often as it can be difficult in this case to stick to a healthy, balanced diet and you might unknowingly jeopardise this balance.

It is also great to make you aware of how much individual components will add up. A lot of menus will inform you about the extra calories that adding chips or a jacket potato will add to your dish. However, they fail to explain where exactly these calories are coming from. A good example is choosing a chicken burger thinking that the saturated fat content of these won't be too bad but adding extra cheese and bacon might add more saturated fats than you think. The same goes for building that perfect sandwich. Even more care has to be taken with hidden fats and added sugars in salad dressings.

However, if you do not eat out very often and are overall doing fine following the guidelines, then by all means make this your occasional treat!

Some example nutrition guides are attached to this guide to give you an idea what to look out for. However, these are by no means meant as endorsements of particular eateries.

Your appointment schedule

Physical activity monitor collection//delivery	First lab visit	Physical activity monitor collection/ /delivery	Second lab visit	Physical activity monitor collection/ /delivery	Third lab visit

All lab visits will take place at LJMU IM Marsh campus in Mossley Hill (L17 6BD).

Association for Nutrition.

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If you have any queries or concerns throughout the study, please do not hesitate to contact Tanja.

Other researchers on the team that you will meet on a regular basis

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This study was approved by LJMU's Research Ethics Committee on 16 December 2016 (Ref. 16/ELS/029). 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 complaint, please contact researchethics@ljmu.ac.uk and your communication will be re-directed to an independent person as appropriate.

Further information throughout the study

You can also find all the materials in a private Facebook Group, which you can request to join should you wish to do so at https://www.facebook.com/groups/CALIBERhighcarb/

We will also post regular updates here. However, should you not wish to join all the links and information will be provided to you via email or in hard copy.

Appendix - Helpful App to help you following the official UK dietary recommendations

Change4Life Be Foodsmart App

Change4Life is a public health initiative run by **Public Health England**. This app lets you scan the barcodes of about **130,000** food products available from **UK** major manufacturers and retailers. It focuses on the amount of sugar, salt and saturated fats in these foods and uses a traffic light system to let you know whether these would be high in the nutrients.

The app can be downloaded for free from the Appstore and GooglePlay.

Change4Life – Sugar smart App

This app lets you scan the barcodes of about 87,000 food products available from UK major manufacturers and retailers. It focuses on the amount of free sugar in these foods and uses a traffic light system to let you know whether these would be high in the nutrients.

The app can be downloaded for free from the Appstore and GooglePlay.

Appendix - Examples nutritional information fast food, pub and restaurant chains

Brewers Fayre

Product/ Dish Description	Nutrition Information Per Portion							
	kJ	kcal	Fat (g)	Saturates (g)	Carbohydrate (g)	Sugars (g)	Protein (g)	Salt (g)
DAYTIME VALUE & SNACKS MENU								
STARTERS								
TOMATO SOUP	550	334	7.7	3.3	33.0	11.8	5.7	1.8
CRISPY POTATO DIPPERS	2045	488	29.6	12.1	34.0	2.7	23.6	1.7
BUBBLE & SOUDAK	1459	348	24.4	10.9	19.5	0.4	10.4	1.4
GARLIC & HERB BREADED MUSHROOMS	1627	389	17.3	1.9	53.0	0.7	8.7	1.5
MAINS								
SMOTHERED CHICKEN	3991	953	45.3	15.1	79.0	11.8	55.4	4.5
LASAGNE	2653	634	28.1	12.1	64.3	17.8	27.9	3.1
GRILLED GAMMON STEAK WITH EGGS	3841	917	37.8	12.5	62.5	5.6	80.1	4.5
GRILLED GAMMON STEAK WITH PINEAPPLE	3655	873	31.3	10.8	81.2	26.4	67.8	4.3
GRILLED GAMMON STEAK WITH ONE OF EACH	3748	895	34.6	11.6	71.9	16.0	74.0	4.3
FISH & CHIPS WITH PEAS	5227	1248	73.2	12.3	107.4	6.9	37.5	1.7
FISH & CHIPS WITH MUSHY PEAS	5386	1286	73.3	12.4	113.9	5.6	39.8	2.6
BATTERED GIANT HADDOCK & CHIPS WITH PEAS	4501	1075	59.3	10.2	85.3	7.7	47.4	1.7
BATTERED GIANT HADDOCK & CHIPS WITH MUSHY PEAS	4660	1113	59.4	10.3	92.8	6.4	49.7	2.6
MEXICAN BEEF CHILLI	2999	716	18.6	5.4	97.1	5.7	38.7	2.2
THREE CHEESE CRUSTLESS QUICHE	2390	571	36.4	18.0	42.2	10.8	19.7	1.2
THE SOUTH WESTERN BURGER	4135	988	47.8	11.0	105.5	12.4	29.6	2.4
BEEF, CHEESE & MUSHROOM BURGER	4846	1157	64.5	23.0	93.3	12.3	48.7	2.5
GRILLED CHICKEN & BACON SALAD	1016	434	19.0	5.6	12.4	11.3	50.9	4.7
CHICKEN TIKKA CURRY	3514	843	24.0	6.3	113.1	28.8	45.3	3.7
BREADED WHOLETAIL SCAMPI WITH PEAS	3895	928	44.7	8.3	107.0	7.0	54.3	3.9
BREADED WHOLETAIL SCAMPI WITH MUSHY PEAS	4048	966	44.8	9.4	113.6	5.7	56.6	4.8
SMOKY PAPRIKA CHICKEN	1932	466	14.7	4.1	31.9	13.1	41.4	2.3
SAUSAGE, EGG & CHIPS	4170	996	57.1	18.9	74.2	7.4	44.0	2.5
SWEET POTATO & FETA LASAGNE	3105	742	39.1	15.8	69.9	17.8	54.2	3.0
HOT 'N' SPICY VEGGIE NACHO BURGER	5160	1233	61.8	19.8	142.3	17.8	33.0	3.0
HAM & CHEESE SANDWICH WITH WHITE BREAD	2513	604	28.3	12.8	55.8	3.0	35.2	3.5

Harvester

STARTERS AND SHARERS	Energy (kJ)	Energy (Kcal)	Fat (g)	Saturated Fat (g)	Carbohydrate (g)	Sugars (g)	Protein (g)	Salt (g)
Potato Skins with Bacon & Cheese	1,934	460	26.2	13.5	32.2	2.1	22.8	2.15
BBQ Chicken Wings	1,595	379	18.4	4.8	20.3	18.8	32.8	1.98
Crispy Coated Chicken Bites	1,685	401	17.9	5.2	36.9	18.3	22.9	2.21
Cheese Popits with Salsa V	1,769	419	25.9	10.1	33.1	4.4	12.2	1.46
Spicy Cracker Jack King Prawns	1,359	324	13.2	2.1	40.0	13.6	9.3	2.02
Breaded Mushrooms V	2,365	563	39.2	6.0	45.6	2.2	8.1	1.86
Chicken & Chorizo skewers NEW	1,990	474	26.5	12.4	6.7	5.5	26.3	1.91
Ultimate Nachos V	3,633	865	48.7	15.8	82.8	4.8	20.3	3.05
Why not add Three Bean Chilli V	464	110	3.0	0.4	15.1	6.8	4.2	0.78
Or add BBQ Pulled Pork	1,268	304	10.1	3.3	40.4	38.7	13.6	1.43
Creamy Tomato & Basil Soup V NEW	1,268	302	5.7	2.7	53.5	4.9	8.8	2.26
Sticky Duck Wings	1,829	438	19.1	5.1	26.5	32.5	29.2	2.69
Fish Basket NEW	2,884	710	45.2	7.5	54.5	7.5	21.7	4.00
Cheesy Garlic Bread Board V NEW	5,755	1,380	92.6	31.8	108.4	15.3	34.0	3.15
CHICKEN	**Energy (kJ)**	**Energy (Kcal)**	**Fat (g)**	**Saturated Fat (g)**	**Carbohydrate (g)**	**Sugars (g)**	**Protein (g)**	**Salt (g)**
Harvester's Famous ½ Rotisserie Chicken	1,885	449	22.9	5.0	2.4	3.5	57.1	1.38
Whole Rotisserie Chicken NEW	3,525	839	41.2	10.9	2.4	4.0	113.5	3.37
Chargrilled Chicken Breast	1,106	268	9.1	1.0	2.5	2.1	43.0	1.48
Triple Chicken	2,763	658	27.7	5.7	13.6	3.6	87.3	2.94
Chicken Skewer	1,879	447	23.9	6.0	2.5	3.3	49.7	0.92
Salsa Chicken & Pepper Stack	1,500	378	14.0	1.2	11.4	11.4	49.0	1.90
HARVESTER RECOMMENDS BBQ Brushed & Basted	4,487	1,068	43.8	9.5	103.9	47.4	63.0	3.87
HARVESTER RECOMMENDS Piri Piri Brushed & Basted	3,952	941	48.0	9.6	65.8	8.0	62.1	6.22
Garlic & Parsley Brushed & Basted	4,259	1,680	40.2	12.9	58.6	72.1	69.8	3.1
Farmer Ritch's Hot Chilli Sauce Brushed & Basted	4,177	980	52.0	9.9	62.7	5.8	63.1	2.99

Pizza Hut

	Pizza Weight (g)	Number of Slices per Pizza	Energy per Slice (kcal)	Protein per Slice (g)	Carbohydrate per Slice (g)	Sugar per Slice (g)	Fat per Slice (g)	Saturated Fat per Slice (g)	Salt per Slice (g)	Energy per 100g (kcal)	Protein per 100g (g)	Carbohydrate per 100g (g)	Sugar per 100g (g)	Fat per 100g (g)	Saturated Fat per 100g (g)	Salt per 100g (g)
Triple Pepperoni																
Individual Pan (9")	459	6	228	9.2	20.5	1.4	12.0	4.0	1.08	298	12.0	26.8	1.8	15.7	5.3	1.42
Sharing Pan (13")	954	8	353	11.2	32.6	2.1	18.1	6.1	1.70	296	12.0	27.3	1.8	15.2	5.1	1.42
Individual Thin (11")	445	6	168	7.5	18.7	0.9	8.9	3.5	0.71	252	12.7	25.2	1.2	12.0	4.7	0.95
Sharing Thin (14")	768	8	258	9.7	21.3	1.4	14.0	6.1	1.58	269	12.7	22.2	1.5	14.6	6.4	1.64
Individual Stuffed Crust (11")	561.5	6	259	11.2	19.9	0.9	13.8	6.5	1.48	277	14.1	21.2	0.9	14.7	6.9	1.58
Sharing Stuffed Crust (14")	762	8	279	14.9	25.4	1.4	14.3	4.5	1.34	293	12.6	26.6	1.5	15.0	4.7	1.41
Cheesy Bites (14")	762	8	279	14.9	25.5	1.4	14.5	4.6	1.34	293	12.6	26.8	1.5	15.2	4.8	1.41
Gluten Free (9" Square)	456	6	209	4.8	20.8	1.3	10.3	3.7	1.19	275	8.9	27.4	1.7	13.6	4.8	1.57
Meat Feast																
Individual Pan (9")	475	6	203	9.6	20.8	1.5	8.8	3.0	0.91	256	12.2	26.2	1.9	11.2	3.7	1.15
Sharing Pan (13")	1001	8	327	15.8	33.0	2.4	14.3	4.8	1.49	261	12.7	26.4	1.9	11.4	3.8	1.19
Individual Thin (11")	474	6	178	10.0	20.1	1.0	6.1	2.5	1.02	226	12.6	25.4	1.3	7.8	3.2	1.29
Sharing Thin (14")	829	8	242	14.4	22.8	1.5	9.9	4.0	1.43	234	13.9	22.0	1.5	9.6	3.9	1.38
Individual Stuffed Crust (11")	578	6	233	13.5	20.2	1.1	10.6	5.4	1.28	242	14.0	21.0	1.1	11.0	5.6	1.33
Sharing Stuffed Crust (14")	1107	8	336	19.6	30.2	1.6	14.7	7.0	1.91	243	14.1	21.9	1.1	10.6	5.1	1.38
Cheesy Bites (14")	1107	8	336	19.6	30.2	1.6	14.7	7.0	1.91	243	14.1	21.9	1.1	10.6	5.1	1.38
Gluten Free (9" Square)	474	6	196	7.5	21.2	1.4	8.4	3.1	1.05	249	9.5	26.8	1.8	10.6	3.9	1.33

Subway

SUBWAY® Nutrition Information

LOW FAT SUBS per 6-inch	Energy (kJ)	Energy (kcal)	Fat (g)	of which Saturates (g)	Sugars (g)	Salt (g)
Chicken Breast	1078	256	3.7	1.5	7.1	1.2
Chicken Teriyaki	1047	302	3.8	1.5	10.0	1.7
Chicken Tikka	1287	303	3.7	1.3	7.0	1.3
Ham‡	1213	290	4.4	1.0	7.0	1.0
Turkey Breast‡	1188	277	3.2	1.2	7.0	1.4
Turkey Breast‡ & Ham‡	1228	293	3.8	1.4	7.5	1.6
VEGGIE DELITE®	824	221	2.2	1.0	6.7	0.8

SUBS per 6-inch	Energy (kJ)	Energy (kcal)	Fat (g)	of which Saturates (g)	Sugars (g)	Salt (g)
Big Beef Melt‡	1686	403	19.3	8.1	7.9	1.8
Chicken Bacon Ranch Melt	2100	505	19.7	8.3	7.3	2.4
Chicken Pizziola (includes American Style Processed Cheese)	1963	440	14.4	6.7	9.8	2.3
Italian B.M.T.®	1725	412	17.7	7.3	7.3	2.4
Meatball Marinara	1668	400	16.2	8.8	13.5	1.9
Spicy Italian	2019	482	28.2	11.8	1.0	2.7
Steak‡ & Cheese (includes American Style Processed Cheese)	1483	355	7.6	3.7	9.8	1.7
SUBWAY MELT™ (includes American Style Processed Cheese)	1582	375	16.2	4.7	7.8	2.2
Tuna (with Lite Mayonnaise)	1459	350	11.6	1.8	7.3	1.6
Veggie Patty*	1592	381	10.7	3.8	6.8	1.8

KIDS' PAK™ MINI SUBS	Energy (kJ)	Energy (kcal)	Fat (g)	of which Saturates (g)	Sugars (g)	Salt (g)
Ham‡	780	182	3.6	1.8	4.9	0.9
Turkey Breast‡	772	184	2.1	0.8	4.7	0.9
VEGGIE DELITE®	655	157	1.5	0.7	4.5	0.4

LOW FAT SALADS per portion	Energy (kJ)	Energy (kcal)	Fat (g)	of which Saturates (g)	Sugars (g)
Chicken Breast	573	137	2.3	0.5	6.4
Chicken Tikka	591	141	2.3	0.5	6.8
Ham‡	507	121	1.1	0.5	6.8
Chicken Teriyaki	693	165	2.4	0.7	6.4
Turkey Breast‡	682	163	1.5	0.4	6.4
Turkey Breast‡ & Ham‡	515	124	2.9	0.5	6.7
VEGGIE DELITE®	247	62	0.9	0.2	6.1

SALADS per portion	Energy (kJ)	Energy (kcal)	Fat (g)	of which Saturates (g)	Sugars (g)
Big Beef Melt‡	979	234	14.0	7.3	7.3
Chicken & Bacon Ranch Melt	1307	314	19.4	7.5	6.6
Chicken Pizziola (includes American Style Processed Cheese)	1147	274	13.1	5.3	9.2
Italian B.M.T.®	1019	244	16.4	8.5	0.7
Meatball Marinara	1131	270	14.8	8.3	12.8
Spicy Italian	1212	314	24.9	10.2	0.4
Steak‡ & Cheese (includes American Style Processed Cheese)	777	186	8.7	2.9	6.4
SUBWAY MELT™ (includes American Style Processed Cheese)	888	200	8.9	3.9	7.2
Tuna (with Lite Mayonnaise)	785	187	10.5	1.8	6.7
Veggie Patty*	888	212	9.4	2.2	7.4

BREADS per 6-inch	Energy (kJ)	Energy (kcal)	Fat (g)	of which Saturates (g)	Sugars (g)
9-Inch Italian (White) Bread	829	195	1.9	0.9	4.9
9-Inch 9-Grain Wheat Bread	659	265	3.1	1.3	4.7
9-Inch 9-Grain Honey Oat	914	216	2.3	1.8	8.3
9-Inch Hearty Italian Bread	875	209	2.0	0.9	5.0
9-Inch Italian Herb & Cheese	1014	242	4.8	2.7	5.1
Flatbread	950	230	3.6	0.3	6.6

Yo! Sushi

ROLLS. Large & small nori rolls with rice

Description	Energy	Each portion contains: Fat	Saturates	Sugars	Salt	Cereals Containing Gluten	Crustaceans	Eggs	Fish	Peanuts	Soya	Milk	Nuts	Celery	Mustard	Sesame Seeds	Sulphur Dioxide	Lupin	Molluscs
Crispy Salmon Skin Roll	118kcal	2.6g	0.4g	2.7g	0.38g				✔							✔			
California Roll	142kcal	0.3g	0.4g	2.6g	0.88g	✔		✔	✔		✔				✔	✔			
																			Contains Wheat
Spicy Chicken Roll	150kcal	3.2g	0.4g	2.6g	0.87g	✔		✔								✔			
																			Contains Wheat
Seared Beef Roll	161 kcal	1.7g	0.4g	9.4g	1.2g	✔					✔					✔			
																			Contains Wheat
Smoked Salmon & Cream Cheese Roll	220kcal	9g	7.4g	2.7g	1.4g				✔			✔							
YO! Roll	140kcal	4.7g	0.7g	2.7g	0.66g	✔		✔	✔		✔				✔				
																			Contains Wheat
Tuna Roll	170kcal	5.6g	0.3g	5.5g	1.3g	✔		✔			✔				✔				
																			Contains Wheat
Spicy Tuna Roll	182kcal	8.8g	0.4g	3.9g	0.71g			✔	✔		✔				✔	✔			
Eel Roll	137kcal	3.2g	0.4g	2.7g	1.0g	✔	✔	✔			✔				✔				
																			Contains Wheat

Zizzi

• CLASSIC PIZZA •

Per serving if not stated otherwise in the menu dish name

MENU ITEM	Energy		Total Fat (g)	Saturated Fat (g)	Total Sugars (g)	Salt (g)
	Kcal	KJ				
MARGHERITA CLASSIC	853	3567	39	13	12	3.6
PEPPERONI CAMPAGNA CLASSIC	970	3620	39	12	16	4.1
WINTER ZUCCA CLASSIC	1170	4926	47	20	22	4.3
ITALIAN HOT CLASSIC	920	3858	38	18	9.4	4.2
POLPETTE CLASSIC	1204	5106	57	25	16	4.7

• SKINNY PIZZA •

Per serving if not stated otherwise in the menu dish name

MENU ITEM	Energy		Total Fat (g)	Saturated Fat (g)	Total Sugars (g)	Salt (g)
	Kcal	KJ				
SKINNY KING PRAWN DIAVOLA	480	2050	13	6.3	5.6	3.3
SKINNY POLLO ROSSO	511	2129	16	7.2	4.3	2.7
SKINNY PRIMAVERA	521	2192	18	4.8	14	2.5

• SALADS •

Per serving if not stated otherwise in the menu dish name

MENU ITEM	Energy		Total Fat (g)	Saturated Fat (g)	Total Sugars (g)	Salt (g)
	Kcal	KJ				
SUPER ZUCCA SALAD	572	2408	31	8.4	14	1.9
CHICKEN & PROSCIUTTO SALAD	397	1660	23	3.9	3.8	2.4
GREEN GODDESS SALAD (BROCCOLI)	266	1114	16	1.9	4.0	0.7
GREEN GODDESS SALAD (SALMON)	395	2491	35	5.4	3.2	1.4

*Unfortunately we do not hold the nutritional data for the Super Zucca Salad with chicken.

• RUSTICA PIZZA •

Per serving if not stated otherwise in the menu dish name

MENU ITEM	Energy		Total Fat (g)	Saturated Fat (g)	Total Sugars (g)	Salt (g)
	Kcal	KJ				
PICCANTE RUSTICA	1328	5551	58	25	17	5.5
CARNOSO RUSTICA	1275	5320	64	26	6.9	6.0
PULLED PORK ROMA RUSTICA	1542	6436	84	37	35	4.6
PRIMAVERA RUSTICA	1309	5461	68	26	13	5.4
SOFIA RUSTICA	1308	5575	51	23	7.7	7.1
MARGHERITA RUSTICA	883	3690	29	10	10	3.5
PEPPERONI CAMPAGNA RUSTICA	1039	4344	39	17	12	5.5
WINTER ZUCCA RUSTICA	1404	5790	69	28	10	4.7
ITALIAN HOT RUSTICA	1130	4707	54	26	9.1	5.0
POLLO ROSSO RUSTICA	1408	5916	63	34	13	5.1
POLPETTE RUSTICA	1514	6393	70	30	10	10.1

• SIDES •

Per serving if not stated otherwise in the menu dish name

MENU ITEM	Energy		Total Fat (g)	Saturated Fat (g)	Total Sugars (g)	Salt (g)
	Kcal	KJ				
TENDERSTEM BROCCOLI	34	152	2.6	0.3	0.1	0.18
MIXED LEAF, TOMATO & SPRING ONION SALAD	69	269	5.5	0.8	1.8	0.08
RICHEST & GRANA PADANO SALAD	133	558	11	3.2	0.1	0.26
ITALIAN RAISED SLAW	23	101	0.3	0.1	3.0	0.09
TUSCAN POTATOES	235	961	6.5	2.1	2.9	0.26
GREEN BEANS	28	69	0.1	0	0.3	0.03
BUFALA MOZZARELLA, TOMATO & BASIL SALAD	268	1108	22	9.6	3.2	0.68
BUTTERED MASH	279	1168	18	12	2.7	1.3

CALIBER Menu 1 (LB15) – Dietary guidelines

	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Breakfast	Porridge with semi-skimmed milk 1 slice of wholemeal toast with polyunsaturated reduced fat spread and jam Tea with semi-skimmed milk	Overnight oats with mixed berries and chia seeds Tea with semi-skimmed milk	Porridge with semi-skimmed milk 1 slice of wholemeal toast with polyunsaturated reduced fat spread and honey Tea with semi-skimmed milk	Porridge with semi-skimmed milk 1 slice of wholemeal toast with polyunsaturated reduced fat spread and jam Tea with semi-skimmed milk	Overnight oats with raspberries and almonds Coffee with skimmed milk	Porridge with semi-skimmed milk 1 slice of wholemeal toast with polyunsaturated reduced fat spread and honey Tea with semi-skimmed milk	Cornflakes with semi-skimmed milk 1 slice of wholemeal toast with polyunsaturated reduced fat spread and jam Orange juice Tea with semi-skimmed milk
Lunch	Broccoli, pea and basil soup with 2 slices of wholemeal bread with polyunsaturated reduced fat spread	Fennel, cucumber and dill salad with roasted chickpeas and a wholemeal roll	White bean salad with garlic and kale with ciabatta bread	Left-over quinoa and salmon cakes on wholemeal roll with lettuce, tomato, cucumber and crème fraiche	Polenta with tomato, mozzarella, parma ham and pesto	Courgette, feta and oregano frittata with mixed salad	Chicken breasts stuffed with spinach and goats cheese with potatoes, carrots and courgette and garlic gratin
Dinner	Mushroom and barley risotto with parmesan, Ciabatta bread and rocket and cherry tomato salad	Left-over Broccoli, pea and basil soup with 2 slices of wholemeal bread with polyunsaturated reduced fat spread	Quinoa and salmon cakes with sweet baked potato, low-fat crème fraiche and rocket	Roast lamb and summer vegetables	Italian hake bake with sautéed cherry tomatoes and herbs, steamed broccoli and brown rice	Lemon-Parsley bean salad with pitta bread	Left-over Courgette, feta and oregano frittata with mixed salad
Snacks	2 slices of wholemeal toast with marmite 1 banana, 1 apple, 2 biscuits	1 pears, Grapes, 1 small handful of walnuts, 1 small pot of low-fat Greek yoghurt 2 biscuits	1 banana, Strawberries, 1 Orange 1 small handful of cashew nuts Crackers with hummus and veg sticks 2 biscuits	2 apricots, 1 banana, 1 small slice of reduced fat cheese, Roasted chickpeas, 2 biscuits	Strawberries, 2 apricots, 1 small handful of Brazil nuts, 1 apple 2 biscuits	1 Kiwi, 1 peach, 1 apple, 2 biscuits 1 small pot of low-fat Greek yoghurt with 1 tablespoon of chia seeds	2 apricots 1 nectarine Strawberries 1 small handful of Brazil nuts
Drinks	Coffee (no sugar) with semi-skimmed milk; Tea (no sugar) with semi-skimmed milk; Water (unflavoured or flavoured with citrus fruit, mint)						

8.3: Appendix 3: Fibre intake and Adverse events questionnaire

Check list of fibre-rich foods consumed during intervention

CALIBER (Carbohydrates, lipids and biomarkers of traditional and emerging cardiometabolic risk factors): A pilot study in normal-weight and overweight adults

Participant ID **Date**

Over the past 4 weeks, did you consume any of the following foods?

Tick all that apply

Food name	Consumed
Almonds	
Apples	
Asparagus	
Aubergines	
Avocado	
Bananas	
Beans, baked in tomato sauce (tinned)	
Beans, broad	
Beans, butter	
Beans, green	
Beans, red kidney	
Beans, soy/edamame	
Beetroot	
Blackberries	

Brazil nuts	
Broccoli	
Broccoli, Purple sprouting	
Brussel sprouts	
Butternut squash	
Cabbage, green	
Cabbage, red	
Cabbage, white	
Cabbage, spring greens	
Carrots	
Cauliflower	
Celery	
Chia seeds	
Chickpeas, canned, re-heated	
Coconut, desiccated	
Coconut, fresh	
Courgette	
Cucumber	
Fennel, Florence	
Flaxseeds (Linseeds)	
Hazelnuts	
High fibre breakfast cereal	
Kale, curly	
Kohlrabi	
Leeks	
Lentils, green or brown	
Lentils, red	
Lettuce, cos	
Lettuce, iceberg	
Lettuce, romaine	
Mushrooms, oyster	

Mushrooms, Portobello	
Mushrooms, shitake	
Mushrooms, white	
Oat bran	
Olives, green in brine	
Onions	
Oranges	
Parsley	
Parsnip	
Peanuts	
Peanut butter, smooth	
Pears	
Peas, green	
Pecan nuts	
Pepper, green	
Pepper, red	
Pepper, yellow	
Pistachios	
Plums	
Porridge	
Potatoes	
Prunes	
Pumpkin	
Pumpkin seeds	
Radishes	
Raspberries, red	
Rocket	
Spinach, baby	
Spinach, frozen	
Spring onion	
Strawberries	

Sunflower seeds	
Sweetcorn	
Sweet potato	
Tomatoes	
Turnips	
Walnuts	
Watercress	
Wholegrain bread	
Wholegrain pasta	

Adverse events (AE) structured 4-weekly interview

CALIBER (Carbohydrates, lipids and biomarkers of traditional and emerging cardiometabolic risk factors): A pilot study in normal-weight and overweight adults

Participant ID Date

Over the past 4 weeks have you experienced any of the following side effects that you would say were a direct result of your participation in this study, such as

Impaired cognition/'brain fog'	Yes ☐	No ☐
Do you remember on which date (or thereabout) this occurred?		
Can you give further details of what happened?		
Can you remember how long this lasted?		
How severe would you say these effects were?		
What did you do to ameliorate these effects?		

Dizziness	Yes ☐	No ☐
Do you remember on which date (or thereabout) this occurred?		
Can you give further details of what happened?		
Can you remember how long this lasted?		
How severe would you say these effects were?		
What did you do to ameliorate these effects?		

Constipation	Yes ☐	No ☐
Do you remember on which date (or thereabout) this occurred?		
Can you give further details of what happened?		
Can you remember how long this lasted?		
How severe would you say these effects were?		
What did you do to ameliorate these effects?		

Headaches or other flu-like symptoms	Yes ☐	No ☐
Do you remember on which date (or thereabout) this occurred?		
Can you give further details of what happened?		
Can you remember how long this lasted?		
How severe would you say these effects were?		
What did you do to ameliorate these effects?		

Muscle cramps	Yes ☐	No ☐
Do you remember on which date (or thereabout) this occurred?		
Can you give further details of what happened?		
Can you remember how long this lasted?		
How severe would you say these effects were?		
What did you do to ameliorate these effects?		

Bad breath	Yes ☐	No ☐
Do you remember on which date (or thereabout) this occurred?		
Can you give further details of what happened?		
Can you remember how long this lasted?		
How severe would you say these effects were?		
What did you do to ameliorate these effects?		

General weakness	Yes ☐	No ☐
Do you remember on which date (or thereabout) this occurred?		
Can you give further details of what happened?		
Can you remember how long this lasted?		
How severe would you say these effects were?		
What did you do to ameliorate these effects?		

Are there any other comments that you would like to make regarding your participation in the CALIBER study over the past 4 weeks?

END OF INTERVIEW

For the researcher only:

Reported as adverse event? (Y/N)	Yes ☐	No ☐
AE reference no and date	*Please note that each occurrence reported above will create a separate AE record.*	

8.4: Appendix 4: Semi-structured interview for AAS use

Anabolic steroid use in male bodybuilders: Interview Schedule

Steroid and other IPED use questions

1. Can you tell me a bit about your history with using steroids? (At what age did you first use, and why? Have you used regularly since you first started, or had long breaks?)

2. Which oral steroids or other oral IPEDs have you used in the past year? *(refer to table 1)*

3. Which steroids or other IPEDs have you injected in the past year? *(refer to table 2)*

4. Can you tell me a bit about your patterns of steroid use in the past year? (Ask whether use was continuous or in cycles: the length of 'on' cycles, the number of 'on' cycles in the year, the time between 'on' cycles).

5. Can you tell me about the amounts of steroids that you used in the past year on average during your 'on' cycles? (Ask: how often they were using their steroids? How much [dose, strength] were they using each time?)

6. What would you estimate your maximum testosterone intake was at any time during the past year? (Ask: for approximate testosterone equivalent in mg during a week, when was that? How long did you use that amount for?)

Other drug use questions

7. In the past year have you taken any other (recreational) drugs? *(refer to table 3)*

8. Why did you take them? (e.g. for fun, for work, competition related?)

9. Do you drink alcohol? (How often? How much at a time?)

Table 1: oral steroids and IPEDs list

ORAL STEROIDS	**Common**
Methandrostenolone (Dianabol)	D-Bol
Mesterolone (Proviron)	Pro-V
Oxandrolone (Anavar)	Anavar
Oxymetholone (Anapolan 50)	Oxies
Stanozolol (winstrol)	Winnie

ESTROGEN CONTROL and POST CYCLE	
Nolvadex (Tamoxifen citrate)	Tamoxies
Clomid (Clomiphene citrate)	Clomid
Arimidex	Arimidex
Letrozole	Letro

FAT LOSS and OTHERS	
Ephedrine, Caffeine and Aspirin (T5)	T5
Clenbuterol	Clen
Dinitrophenol (DNP)	DNP
Levothyroxine (T4)	T4
Liothyronine (T3)	T3
Ephedrine	Ephedrine
Pre-workout (stimulant type)	Pre-workout
Diuretics (ie: spironolactone)	
Prohormones/Designer steroids (ie: Superdrol,	
Viagra/Cialis	Viagra
Other oral IPEDs:	

Table 2: Injectable steroids and IPEDs list

INJECTABLE STEROIDS	**Common**
Testosterone Propionate	Test P
Testosterone Cypionate	Test Cyp
Testosterone Enanthate	Test E
Testosterone Suspension	Test
Sustanon	Sus 250
Trenbolone Acetate (Tren Ace)	Tren Ace
Trenbolone Enanthate (Tren E)	Tren E
Winstrol (Stanozolol)	Winnie
Equipoise (Boldenone)	EQ
Masteron (Drostanolone)	Mast
Methenolone (Primpobolan)	Primo
Deca-Durabolin (Nandrolone)	Deca
Blend of steroids in one vial (e.g. Fast Rip, Tri-Tren	
PEPTIDES and OTHER HORMONES	
HGH (Human Growth Hormone)	HGH
IGF (Insulin-like growth factor 1)	IGF
CJC 1295	CJC
MGF (*Mechano Growth Factor)*	MGF
GHRP (Growth hormone-releasing peptide) 2or 6	GHRP's
Insulin	Slin
Melanotan (I or II)	Tan
ESTROGEN CONTROL and POST CYCLE	
HCG (*Human chorionic gonadotropin)*	HCG
Other injectable IPEDs:	

Table 3: Recreational drug use

	Used in past year	Used in past month
Amphetamine (Speed)		
Ecstasy (MDMA)		
Cocaine		
Crack		
Heroin		
Mephedrone (M-Cat)/ Other Stimulant		
Cannabis		
GHB/GBL		
Ketamine		
Poppers		
Synthetic cannabinoids		
Prescribed painkiller medication		
Painkiller medication purchased over the counter		
Other (please state):		

8.5: Appendix 5: Journal article: Reviews in Endocrine and Metabolic disorders (2020)

Reviews in Endocrine and Metabolic Disorders
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How the love of muscle can break a heart: Impact of anabolic androgenic steroids on skeletal muscle hypertrophy, metabolic and cardiovascular health

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Abstract
It is estimated 6.4% of males and 1.6% of females globally use anabolic-androgenic steroids (AAS), mostly for appearance and performance enhancing reasons. In combination with resistance exercise, AAS use increases muscle protein synthesis resulting in skeletal muscle hypertrophy and increased performance. Primarily through binding to the androgen receptor, AAS exert their hypertrophic effects via genomic, non-genomic and anti-catabolic mechanisms. However, chronic AAS use also has a detrimental effect on metabolism ultimately increasing the risk of cardiovascular disease (CVD). Much research has focused on AAS effects on blood lipids and lipoproteins, with abnormal concentrations of these associated with insulin resistance, hypertension and increased visceral adipose tissue (VAT). This clustering of interconnected abnormalities is often referred as metabolic syndrome (MetS). Therefore, the aim of this review is to explore the impact of AAS use on mechanisms of muscle hypertrophy and markers of MetS. AAS use markedly decreases high-density lipoprotein cholesterol (HDL-C) and increases low-density lipoprotein cholesterol (LDL-C). Chronic AAS use also appears to cause higher fasting insulin levels and impaired glucose tolerance and possibly higher levels of VAT; however, research is currently lacking on the effects of AAS use on glucose metabolism. While cessation of AAS use can restore normal lipid levels, it may lead to withdrawal symptoms such as depression and hypogonadism that can increase CVD risk. Research is currently lacking on effective treatments for withdrawal symptoms and further long-term research is warranted on the effects of AAS use on metabolic health in males and females.

Keywords Anabolic-androgenic steroids · Metabolic syndrome · High-density lipoprotein cholesterol · Low-density lipoprotein cholesterol · Insulin resistance · Cardiovascular disease

1 Introduction

The fine margins of winning and losing in athletic competitions has always encouraged innovative techniques to help athletes gain a competitive advantage with little regard to the potential negative consequences. Although research into sex hormones existed in the early 19th century, it was only in the 1930s when the anabolic effects of testosterone were demonstrated [1]. Shortly thereafter, the hormone started to be used by competitive athletes to increase muscle mass and performance, however, the British Association of Sports Medicine and the American College of Sports Medicine continued to deny its potential benefits until the 1970s [2, 3]. The use of testosterone and its derivatives were later banned by the International Olympics Committee in 1974 [4]. Due to advancements in technology and pharmacology, a range of anabolic androgenic steroids (AAS) (Table 1, [5, 6]) began to be commonly used by the recreational gym-user in the 1980s, primarily by young men to improve body image [1, 7]. Due to this rise in use and the associated adverse effects of AAS, many countries changed their legislation to incorporate AAS to regulate its use and distribution in the 1990s [8–10]. The world anti-doping agency was created in 1999 to protect

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athletes from the detrimental health risks of AAS use and to ensure maintenance of the integrity of sport globally [4, 11].

It is currently estimated that 6.4% of males and 1.6% of females use AAS globally, with recreational sportspeople being the highest users [12]. Although it is common for individuals to use AAS for multiple reasons, the greatest motivation to use AAS is primarily to improve body image, while competitive bodybuilding and athletic performance (non-bodybuilding) are secondary and tertiary respectively [12–15]. The Middle East has relatively significantly high levels of AAS use while use in South America, Europe, North America, Oceania and Africa ranges from 5–2% of the population, highlighting the global issue at hand [12]. However, the significantly higher prevalence rates in the Middle East may be due to the majority of studies relying on self-reports from athletes rather than general populations [12]

1.1 Effects on skeletal muscle

Testosterone and its AAS derivatives increase muscle protein synthesis (MPS) and accretion, satellite cell activation and possibly decrease catabolic pathways via genomic and non-genomic mechanisms (Fig. 1) [16]. Genomic actions of AAS occur when androgens bind to the nuclear androgen receptor (AR) and translocate to the cell nucleus, binding to specific DNA sequences resulting in enhanced transcription of target anabolic genes [17, 18]. AAS also exert non-genomic actions by binding of the membrane-located AR and additional membrane receptors such as endothelial growth factor receptor (EGFR) and sex hormone-binding globulin receptor (SHBGR) that also alter anabolic/catabolic signalling pathways [17, 18]. Resistance exercise also increases muscle MPS and satellite cell activation resulting in skeletal muscle hypertrophy [19, 20]. Although testosterone administration and resistance exercise alone may increase skeletal muscle hypertrophy, the combination of both results in enhanced skeletal muscle hypertrophy [21]. As a result, AAS are commonly used in conjunction with exercise to increase muscle mass and improve perceived body image [1, 23].

Table 1 List of injectable and oral AAS and typical doses used

Injectable AAS	Typical weekly dose
Boldenone Undecanoate	200–400 mg
Drostanolone Propionate	300–450 mg
Methenolone Enanthate	200–400 mg
Nandrolone Decanoate	200–400 mg
Stanozolol	150–700 mg
Testosterone Cypionate	200–600 mg
Testosterone Enanthate	200–600 mg
Testosterone Propionate	150–300 mg
Testosterone Suspension	150–700 mg
Trenbolone Acetate	150–300 mg
Trenbolone Enanthate	200–300 mg
Trenbolone Hexahydrobenzylcarbonate	150–230 mg
Oral AAS	**Daily dose**
4-chlorodehydromethyltestosterone	20–80 mg
Fluoxymesterone	20–40 mg
Methandrostenolone, Methandienone	20–40 mg
Mesterolone	50–100 mg
Oxandrolone	20–40 mg
Oxymetholone	50–100 mg
Stanozolol	20–50 mg
Testosterone Undecanoate	80–160 mg

1.2 Effects on metabolic health

Regular exercise is undoubtedly beneficial for mental, physical and metabolic health [22]. However, the potential benefits acquired from regular exercise may be reduced with chronic AAS use as AAS users are at a higher risk of developing cardiovascular disease (CVD), psychological disorders, neuroendocrine disorders, sex-specific disorders (aromatisation and hypogonadism in males and virilisation in females) and a range of other disorders (Table 2) [7, 23–26]. Long term AAS use has been shown to result in premature death due to cardiovascular events; however, due to AAS use only being prevalent since the 1980s, long term longitudinal studies, on their impact, are scarce [27]. Furthermore, the direct impact of AAS use on health is difficult to determine as users reportedly use other substances to complement their AAS use while also using a variety of AAS types, doses and cycles [13, 28]. AAS-related polysubstance use also includes other anabolic agents such as insulin-like growth factor-I (IGF-I) and growth hormone (hGH); drugs to prevent AAS-related adverse effects, other image enhancing drugs (clenbuterol, diuretics and thyroid hormones) and psychoactive drugs [13, 28]. The chemical interactions of AAS-related polysubstance use may also elicit additional adverse health outcomes. Quantifying the adverse effects of these drugs is further complicated by the prevalence of adulterated products, an inevitable consequence of the illicit market [29].

Metabolic syndrome (MetS) is the constellation of the often interrelated metabolic abnormalities that lead to increased risk of CVD, which are the number one cause of death globally [30, 31]. It is most commonly associated with sedentary/obese populations and is defined by having a combination of some, but not all, of high triglycerides (TG), low high-density lipoprotein cholesterol (HDL-C), elevated blood glucose, hypertension and elevated waist circumference [30, 32]. Insulin resistance (IR), visceral adipose tissue (VAT) and small dense low-density lipoprotein cholesterol (sdLDL-C) also highly correlate with MetS [30]. Although AAS users are highly

active, they are also at risk of CVD as AAS use has been reported to increase the risk of sudden cardiac arrest as a result of cardiac remodelling and abnormal cardiac function [33–35]. The use of AAS reportedly results in polycythaemia, reduced left ventricular and diastolic function and accelerated atherosclerosis compared to non-use [24, 36]. AAS use may affect blood pressure (BP) and metabolism which ultimately increases CVD risk in addition to altered cardiac function [33]. Furthermore, AAS use can increase low-density lipoprotein cholesterol (LDL-C) and decrease high-density lipoprotein cholesterol (HDL-C) increasing the risk of developing atherosclerosis and hence CVD [33], particularly given that AAS use could result in lower insulin sensitivity and higher levels of VAT compared to matched controls [37]. Therefore, although most AAS users have high levels of activity and low adiposity, they can also share similar metabolic characteristics of obese/sedentary populations such as the MetS, thereby increasing risk of CVD.

1.3 AAS withdrawal

While increasing levels of lean mass has an inverse relationship with CVD risk, AAS use has such a deleterious effect on health that it is not recommended to use for appearance or performance reasons [7, 38]. Discontinuing AAS use can be a difficult process as immediate cessation may also have a detrimental effect on health and wellbeing. The withdrawal effects of AAS can cause hypogonadism, depression and fatigue, reduced libido, leading to relapse and AAS dependency [7, 39]. Current evidence on successful treatments for cessation of AAS use are scarce and further research is required, but potential strategies for males include testosterone replacement therapy (TRT), selective estrogen receptor modulators (SERM), human chorionic gonadotropin (hCG) and aromatase inhibitors [40, 41]. As a result, up to date guidance and information on the risks of commencing and ceasing AAS use along with effective treatments for withdrawal symptoms are required to prevent adverse health outcomes.

2 Aim and scope

Although previous reviews have focused on the effects of AAS use on blood lipid and lipoproteins profiles [33, 42], the effects on overall metabolism have yet to be reviewed. Abnormal lipid metabolism is commonly associated with impaired glucose metabolism, hypertension and VAT accumulation and this may also be the case in AAS users [30]. Therefore, the objectives of this review are to: 1, highlight the mechanisms by which AAS exert their hypertrophic effects on skeletal muscle; 2, explore the impact of AAS use on lipid, lipoprotein and glucose metabolism, all indicators of MetS and 3, explore the negative effects of AAS withdrawal and potential treatments. With the substantial levels of AAS use [7, 12], better knowledge of these interrelated mechanisms and issues may lead to targeted interventions to reduce the potential harm that may be associated with AAS use.

3 Mechanism of action on skeletal muscle

3.1 Genomic-mediated mechanisms

The primary action of AAS is to bind to the nuclear AR located in the cytoplasm which results in their translocation to the nucleus following disassociation of the AR complex with chaperone (Hsp90, Hsp70) and co-chaperone proteins (Hsp organising protein (Hop)) [43, 44]. At the nucleus the androgen/AR complex moderates gene transcription by binding to the ARE of the DNA [17, 45]. Transcription is altered further by the recruitment co-activators such as cAMP response element-binding protein (CREB)-binding protein (CBP)/p300 and steroid receptor coactivator (Src) 1, 2 and 3 [17, 45, 46]. This, in turn, upregulates expression of genes related to protein accretion and anabolism such as IGF-I, nutrient sensing, storage and transporting (Lipin, GLUT3 and SAT2) and satellite cell differentiation (myogenin), while also increasing satellite cell number [47–50]. ARE binding may also downregulate genes involved in muscle atrophy such as I-Kappa kinase alpha (IKKα) [47, 51] (Fig. 1). The transactivation domain of the AR is susceptible to a CAG repeat polymorphism within the first exon, which may regulate AR activity. The number of CAG repeats typically ranges from 11 to 31 triplets in length and is inversely associated with transactivational activity of the AR [43]. An increase in CAG repeats is associated with elevated testosterone levels, perhaps due to decreased AR activity, which may affect hypothalamic-

Table 2 Diseases associated with chronic AAS use

Cardiovascular	Psychological	Neuroendocrine	Other
Cardiomyopathy	Depression	Neurotoxicity	Hepatoxicity
Coronary heart disease	Mood disorders	Reduced grey matter	Hypogonadism (males)
Sudden cardiac death	Substance abuse	Thinner and smaller cortices	Virilisation (females)
Stroke	Dependence	Cognitive impairment	Acne
Myocardial infarction			Fertility
Hypertension			Cancer

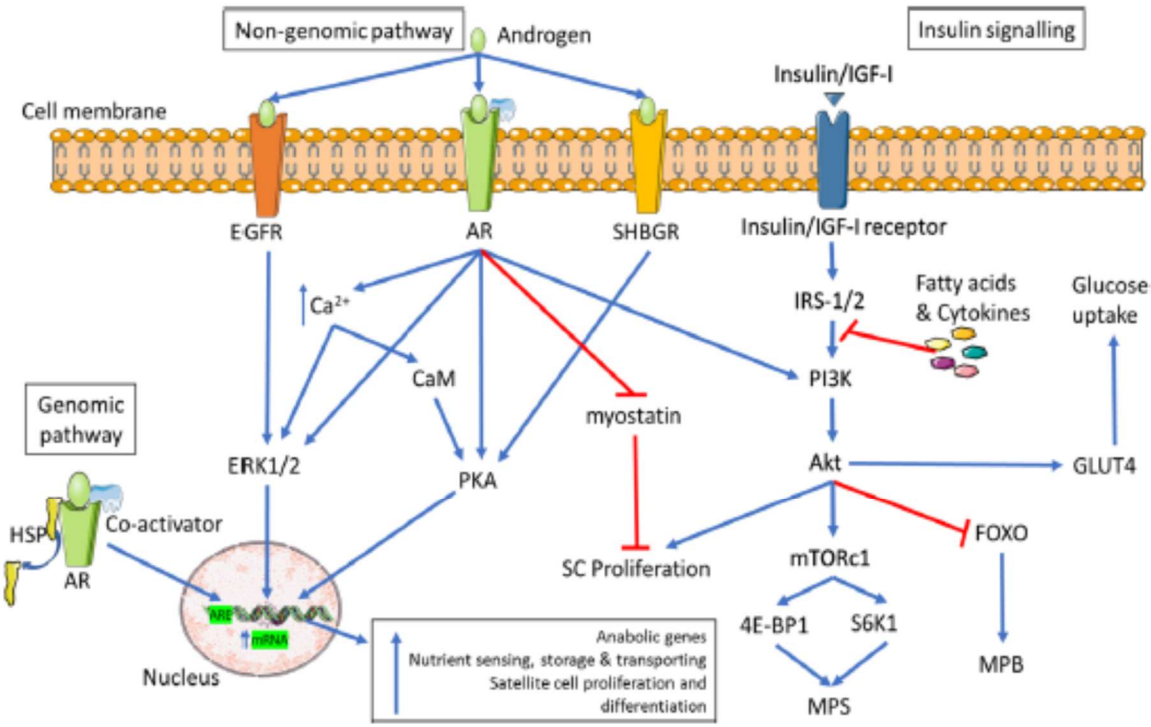

Fig. 1 Genomic and non-genomic mechanisms of AAS induced skeletal muscle hypertrophy and mechanisms of insulin signalling and resistance. Genomic pathway: Androgen binding of the AR complex causes translocation to the nucleus following dissociation of heat shock proteins (HSP). The androgen/AR complex regulates gene transcription on the androgen response element (ARE) of DNA. Non-genomic pathway: In addition to the AR, androgens can activate other membrane-bound receptors such as EGFR and SHBGR. This causes an increase in intracellular calcium (Ca2+), activation of several second messenger signalling such as extracellular regulated kinases 1/2 (ERK 1/2), protein kinase A (PKA), calmodulin (CaM) and phosphatidylinositol-3-phosphate kinase (PI3K)/Akt/mTORc1 pathways and deactivation of myostatin pathway. Activation of these genomic and non-genomic pathways leads to skeletal muscle hypertrophy via upregulating gene transcription of anabolic genes, nutrient sensing, storage and transporting. While also upregulating satellite cell proliferation, differentiation, MPS and inhibiting muscle protein breakdown (MPB). Insulin/IGF-1 signalling pathway: Insulin/IGF-1 bind to the insulin/IGF-1 receptor on the cell membrane inflicting tyrosine phosphorylation. The now activated receptor causes phosphorylation of insulin receptor substrate-1/2 (IRS-1/2) activating the PI3K/Akt signalling cascade leading to satellite cell proliferation; MPS via mTORc1, 4E-binding protein 1 (4E BP1) and p70 S6 kinase 1 (S6K1) activation; glucose uptake via GLUT4 translocation and inhibition of forkhead O transcription factor (FOXO) leading to reduced MPB. Abnormal levels of circulating fatty acids and inflammatory cytokines result in serine/threonine phosphorylation of IRS-1 causing insulin resistance.

pituitary feedback regulation although no association has been observed with muscle mass in young males (25–45 years) [52]. There is currently a lack of evidence on the response of AAS in relation to the amount of AR CAG repeats.

3.2 Non-genomic mediated adaptations

Non-genomic actions of AAS are characterised by the speed in which they exert their effects (within minutes) thus indicating activities independent of transcription [17, 53]. AAS have been reported to exert non-genomic effects via membrane-located receptors; membrane-located AR, EGFR, and SHBGR [17]. Binding of these receptors leads to an increase in intracellular calcium and activation of several second messenger signalling cascades including; mitogen-activated protein kinases (MAPK), ERK 1/2, PKA, PI3K/Akt and CaM pathways [17, 54, 55]. Activation of PI3K/Akt by testosterone, triggers mTORc1, a key regulator of protein turnover via activation of the eukaryotic initiation factor 4E-BPs and S6K1 [19, 56]. Resistance exercise also activates S6K1 via mTORc1, increasing MPS and muscle hypertrophy [57, 58]. The combination of testosterone and resistance exercise further increases mTORc1, 4E-BP1 and S6K1 activation compared to either alone [59]. These signalling cascades upregulate transcription, satellite cell proliferation, muscle protein synthesis and reduce apoptosis ultimately resulting in skeletal muscle hypertrophy [17, 54, 55, 60] (Fig. 1).

a placebo-controlled group [75]. Additionally, the testosterone trials involved 7 coordinated placebo-controlled trials with the aim of increasing deficient testosterone levels to normal levels in 788 males aged ≥ 65 years old with transdermal TRT for one year [76]. TRT resulted in improving 6-minute walking distance in addition to increasing sexual function, mood and bone mineral density [77].

Nonetheless, TRT is only recommended for individuals who exhibit symptoms of testosterone deficiency (reduced libido, gynecomastia, depression, low bone mineral density, decreased energy, low muscle mass/strength and poor cardiovascular health profile) and low testosterone serum levels (< 12 nmol/l) [78, 79]. The risks associated with TRT include infertility (in young), cardiovascular disease and prostate cancer and therefore, should be assessed on a case by case base by a clinician [79, 80]. It has also been reported that an important motivation to take AAS was to "slow the ageing process" in older males, which may exhibit greater health risks compared to clinically prescribed TRT [81].

In summary, chronic AAS use increases skeletal muscle anabolism, which results in skeletal muscle hypertrophy, improved function and body composition via genomic, non-genomic and anti-catabolic signalling pathways. Nevertheless, the use of AAS has negative consequences on overall metabolic health through altered lipid metabolism and therefore an increase in CVD risk. With CVD being the number one cause of deaths globally, the potential clinical benefits of AAS use on skeletal muscle are far outweighed by the negative outcomes on cardiovascular health [31].

4 Impact on metabolic health

4.1 Lipid metabolism

Dyslipidaemia is associated with an increased CVD risk and is underpinned by high levels of triglycerides > 150 mg/dL, LDL-C > 116 mg/dL and/or low levels of HDL-C < 40 mg/dL in males and < 50 mg/dL in females [82, 83]. Although only triglycerides and HDL-C are considered components of MetS, sdLDL-C is considered an additional element to this disease [30]. LDL can be separated into 4 groups: large and buoyant (lbLDL), intermediate size and density (idLDL), small and dense (sdLDL) and very small and dense (vsdLDL) [84]. High circulating particles of sdLDL and vsdLDL indicate a greater risk of CVD events compared to total LDL alone [84, 85]. Cholesterol is primarily synthesized in the liver and circulates around the body as very-low-density lipoprotein (VLDL) (rich in TG) of which apolipoprotein B100 (ApoB) is the major apolipoprotein (Fig. 3) [86]. Upon interaction with lipases at various tissues, the VLDL containing TG are hydrolysed, and free fatty acids are released for energy or subsequent storage as adipose tissue [87]. The remaining lipoprotein is now cholesterol-rich, TG poor LDL (or LDL-C). This LDL will bind to the hepatic LDL receptor to increase LDL-C clearance [87]. With dysregulated metabolism, as observed in MetS, there is an increase in circulating sdLDL which, has a lower affinity for the LDL receptor, therefore, having a reduced clearance rate, subsequently increasing circulating levels and CVD risk [88]. The sdLDL can also penetrate the arterial wall easier compared to lbLDL due to its small size increasing the risk of trapping ApoB depositing atherogenic cholesterol and increasing the risk of a CVD event [89]. High-density lipoprotein (HDL), particularly subfraction HDL_2 transports cholesterol away from peripheral tissue, including arterial lesions, to the liver to be excreted, through a process of reverse cholesterol transport, thereby reducing CVD risk [90, 91]. HDL of which apolipoprotein A1 (ApoA1) is the major apolipoprotein, also has an anti-inflammatory and antioxidant effect on the vascular system further reducing the potential of CVD [92]. Use of AAS has shown a reduction in HDL-C of ≥ 70% and increased LDL-C levels of > 20% [33]. Testosterone has been reported to significantly decrease HDL-C, although with differential dose and time responses. Increasing doses of Test E for 20 weeks in resistance-trained males has been reported to have an inverse dose-response relationship with HDL-C and Apo A1 but only 600 mg/wk was significantly ($P < 0.001$) different to baseline levels [93]. However, in contrast, 150 mg/wk for 2 weeks and a 300 mg dose of testosterone cypionate on week 3 resulted in the largest decrease in HDL-C but no further decrease was observed with 600 mg/wk for a further 4 weeks [94]. Furthermore, 3 weeks of 600 mg/wk Test E administration in inactive ageing males resulted in significant decreases in HDL-C, particularly HDL_2 [95]. Although 200 mg/wk of Test E in resistance training males showed significant decreases in HDL-C after 6 weeks, no effect was observed on HDL_2 [96]. In healthy males, 200 mg/wk of Test E administration for 12 months had dramatic significant (*mean*:1.15 mmol/L to 0.09 mmol/L, $P < 0.05$) decrease in fasting HDL-C levels. Interestingly, neither study observed significant deleterious changes in LDL-C or TG levels in fact, Thompson et al. (1989) reported a significant ($P < 0.05$) decrease in LDL-C.

Nandrolone administration has reported contrasting effects on lipid metabolism. HDL-C has been reported to significantly decrease after a 200 mg starting dose of nandrolone and a further 100 mg/wk for a total of 8 weeks in male bodybuilders [97]. Although in a similar design and population, 200 mg/wk of nandrolone for 8 weeks resulted in no significant change in HDL-C [98]. In healthy adults, 100 mg/wk for 6 weeks resulted in no change in HDL-C [99]. No effect was observed on LDL-C, TGs, Apo A1 or Apo B levels in the above studies [97–99]. Nandrolone administration (200 mg/wk) for 6 months in ageing males undergoing haemodialysis resulted in significantly reduced HDL-C and increased apo B levels

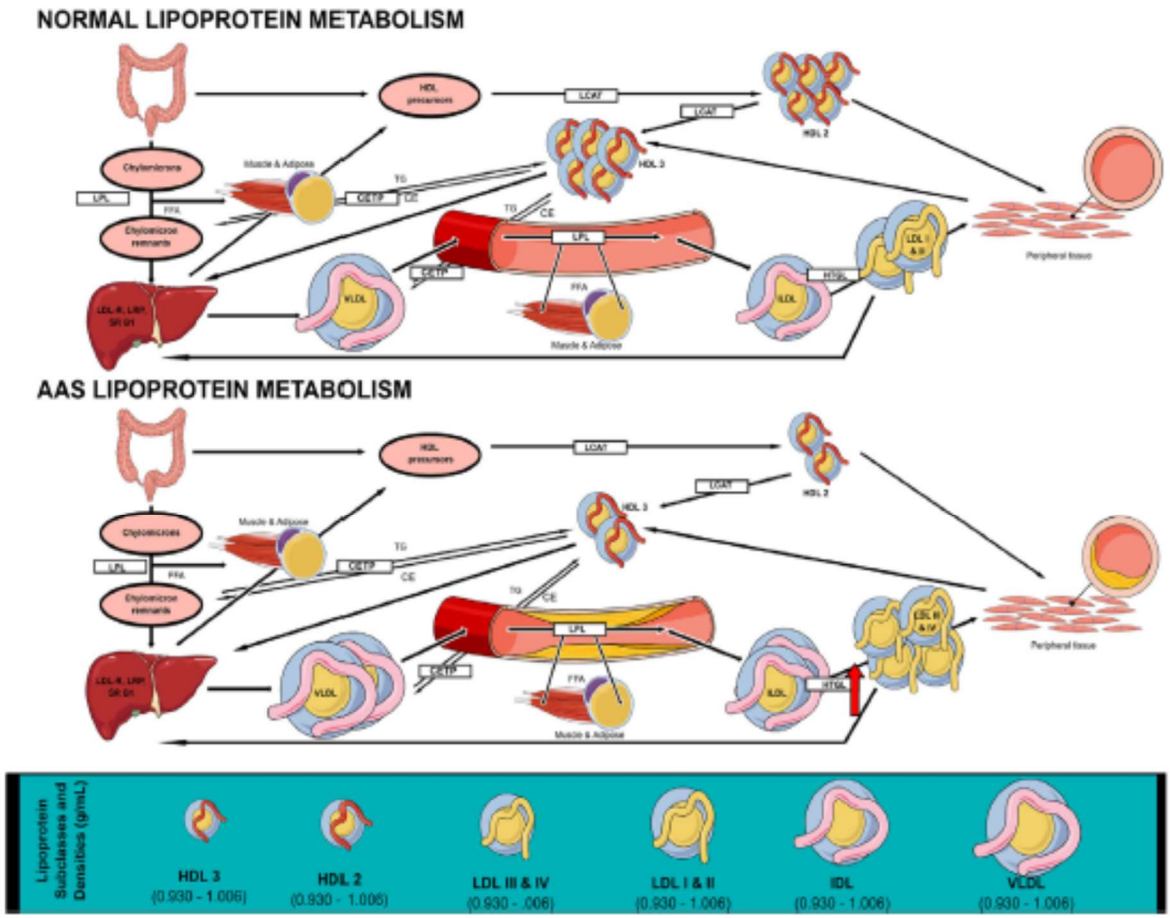

Fig. 3 Normal and AAS-influenced lipoprotein metabolism. During normal lipoprotein metabolism, intestinally produced chylomicrons carrying dietary lipids are hydrolysed by lipoprotein lipase (LPL). FFA are liberated and taken up by the liver, muscle and adipose tissue. Resulting chylomicron remnants are taken up by the liver via low-density lipoprotein receptor (LDL-R) and the LDL receptor-related protein (LRP). Meanwhile, hepatically produced VLDL transport cholesterol esters (CE) and TG through blood vessels, during which they undergo hydrolysis, releasing FFA which are taken up by peripheral tissues. This loss of TG means VLDL particles decrease in size (and therefore density) and become cholesterol-enriched and known as idLDL. Due to the action of HGTL, IDL particles become even smaller and known as LDL. LDL particles have an increased propensity to deposit cholesterol in peripheral tissues; however, they primarily transport cholesterol to the liver, where they are taken up by the LDL-R. The intestine also produces precursors which contribute towards the production of HDL. Small HDL3 particles acquire CE and TG and form larger HDL2 particles which, with the assistance of lecithin–cholesterol acyltransferase (LCAT), subsequently exchange CE for even more TG with VLDL particles and chylomicrons, before travelling to the liver where they are taken up by scavenger receptor B1 (SR-B1) or LDL-R. During AAS-influenced lipoprotein metabolism HGTL is upregulated, resulting in a preponderance of more atherogenic small, dense LDL III and IV particles, as opposed the larger and more buoyant LDL I and II particles found in normal lipoprotein metabolism. There is also a severe decrease in the number of HDL 2 and 3 particles overall, which are generally regarded as being atheroprotective.

but had no effect on TG or Apo A1. [100]. In post-menopausal women, 50 mg/wk of nandrolone for 3 weeks significantly decreased HDL-C and Apo A1 levels [101]. In male bodybuilders, 42 mg/wk of oral stanozolol administration has shown to significantly reduce HDL-C, ApoA1 and TGs after 6 weeks while also increasing LDL-C [96]. In healthy males, one intramuscular injection of 50 mg of stanozolol resulted in a significant reduction and increase in HDL-C and LDL-C levels respectively 28 days later [102]. Both returned to baseline levels after 56 days [102]. Similar results have been observed in postmenopausal females with osteoporosis, as 42 mg/wk of oral stanozolol resulted in significant reductions in HDL-C and ApoA1 levels after 2 weeks and was maintained until the end of the treatment at 6 weeks along with an increase in LDL-C levels [103]. No change was observed in TG levels [103].

Differences in study designs, populations and lack of dietary control in some studies has resulted in differing responses in lipid metabolism with AAS administration. Nonetheless, increasing doses of testosterone administration has a large

negative impact on HDL-C with no adverse effect on other lipid markers. Although inconsistent, the negative effects of nandrolone administration are primarily observed on HDL-C levels, however, nandrolone does appear to consistently reduce lipoprotein(a) (Lp (a)) levels [98, 100, 101], an independent risk factor of CVD [104, 105], yet further research is warranted on the potential benefits of nandrolone, if any. In contrast, stanozolol administration may have a greater deleterious effect on lipid metabolism as it has shown to negatively affect LDL-C and HDL-C levels.

Individuals who use AAS for appearance and performance reasons typically do not use one type of AAS but rather administer a polypharmacy regime which may lead to different implications on lipid metabolism.

Early studies reported that after 8 weeks of AAS administration, HDL-C and LDL-C significantly ($P<0.01$) decreased by 49% and increased by 31% respectively [106]. Similarly, this suppression on HDL-C, particularly HDL_2, is maintained after 14 weeks of self-administration [98]. However, Bonetti et al.. only reported a significant ($P<0.05$) decrease in HDL-C after 18 months [107]. The method of using self-administrating participants results in a variety of AAS dosages, types and cycles being used which may lead to different health outcomes thereby making comparisons between studies difficult. Critically, however, although they may be less controlled, they may be more representative of the population compared to randomised controlled trials as it replicates the AAS and AAS-related polysubstance methods used by this unique population. A more recent cross-sectional study reported similar results in which current users of AAS, displayed 45% lower HDL-C, and 26% and 35% higher LDL-C and TG levels vs. non-AAS using controls (all $P<0.01$) [37]. A case study of prolonged AAS use in a 35-year-old male demonstrated an almost 100% decrease in HDL-C and a 100% increase in LDL-C during 5 years of AAS cycling [108]. Similarly, in females, HDL-C is shown to be significantly depressed with chronic AAS use compared to healthy controls. While AAS use may also exhibit an increase in plasma TG, data remains equivocal as this was only reported by Moffatt et al. [109–111]. In addition to small sample sizes in female studies, the variety in AAS use, type, dose and frequency might explain the differences in results. Although, the lipoprotein profile is undoubtedly impacted by chronic AAS use and therefore highlights the increased risk of future CVD incidence, due to the uncontrolled self-administration of AAS and other anabolic substances the severity in which it impacts health can be variable. Conversely, AAS polypharmacy is also reported to improve Lp (a) levels, similar to the effect of nandrolone administration alone [98]. Self-administration of a variety of AAS resulted in a significant ($P<0.05$) decrease in Lp (a) after 8 weeks and was maintained after 14 weeks [98]. However, 24 months of AAS use did not result in a significant decrease (mean ± SD; 179 ± 117 vs. 137 ± 80 mg/dL, $P>0.05$) in Lp (a) [107]. Although non-significant, it may be clinically significant as Lp(a) levels > 180 mg/dL are considered high risk of CVD [82]. The implications of AAS induced improvements in Lp(a) concerning CVD risk are unclear and warrant further investigation. Use of AAS also doesn't appear to negatively impact TG levels in males as only one cross-sectional study reported significantly ($P<0.01$) higher TGs (although not clinically significant < 1.7 mmol/L) with AAS use, yet self-administration studies showed no significant negative effect [98, 106, 107]. The mechanisms by which AAS negatively impact lipid metabolism are not fully understood, but the upregulated activity of hepatic triglyceride lipase (HTGL) has been implicated (Fig. 3) [95, 96]. Phospholipase activity of HTGL catabolises HDL-C and its removal from the plasma and conversion of idLDL to sdLDL [42, 112]. Research of the impact of AAS use on LDL density are limited with most focusing on total LDL-C however, one randomised controlled trial investigated the short-term (3 weeks) effects of TestE administration on cholesterol associated with LDL density by density gradient ultracentrifugation (DGUC) [95]. In older eugonadal males (mean 71 years old), 600 mg of TestE increased sdLDL-C indicating an increase in CVD risk [85, 95]. Unpublished data, by the authors, showed no significant ($P>0.05$) difference in a cross-section of AAS using males and healthy controls in sdLDL-C. Further research is warranted on the effect of AAS use on LDL density and its associated CVD risk. The type of AAS and route of administration also has an impact on the effect of HTGL activity and lipoprotein levels. Orally administered stanozolol showed a significant ($P<0.05$) increase in HTGL activity, leading to a significant ($P<0.05$) increase and decrease in LDL-C and HDL_2-C respectively whereas injected TestE showed no significant ($P>0.05$) change in HDL_2-C after 6 weeks, but a significant ($P<0.05$) decrease in LDL-C [96]. The slower liver clearance rate of orally administered AAS compared to injected AAS could have a greater detrimental effect on metabolic health and also increase the risk of hepatoxicity [113–115]. Interestingly, the effect of AAS on the lipoprotein profile is reversible, as former users of AAS with long term discontinuation of at least one year, are reported to have healthy lipoprotein levels [37, 116]. The reversible effects may be seen as

early as 10 weeks of AAS cessation as shown by a case study in a 35-year-old male [108].

4.2 Glucose metabolism and VAT

Key features associated with MetS are IR and VAT [30]. IR is the precursor of the development of Type 2 diabetes (T2D), with lipid accumulation and inflammation being implicated as the primary triggers [117–119]. IR can be measured by the hyperinsulinemic-euglycemic clamp with IR being defined as a glucose disposal rate below 5.6 mg/kgFFM + 17.7/min [120]. Skeletal muscle is the largest tissue for insulin-induced glucose uptake [121]. Insulin binds to the insulin receptor on the cell membrane causing its tyrosine phosphorylation of the receptor (Fig. 1). The now activated insulin receptor causes phosphorylation of insulin receptor substrate-1 (IRS-1) on tyrosine residues, which allows the recruitment of the Type IA phosphatidylinositol 3' kinase (PI3K). PI3K catalyses the formation of PI(4,5)-bisphosphate to PI(3,4,5)-trisphosphate thus recruiting 3' phosphoinositide-dependent kinase-1 (PDK-1). PDK-1 phosphorylates protein kinase B (PKB) (also known as Akt) and the atypical protein kinase C (PKC) [118, 119, 122, 123]. Akt phosphorylates 160-kDa substrate of Akt (AS160) which stimulates translocation of GLUT4 storage vesicles to fuse at the cell surface to release GLUT4 into the plasma membrane allowing cellular glucose uptake [124, 125]. However, within IR tissue this signalling cascade is diminished possibly due to increased circulating fatty acids, inflammatory cytokines and/or reactive oxygen species (ROS) which result in serine/threonine phosphorylation of IRS-1. This reduces Akt activity and glucose uptake and negatively affects other downstream signalling such as protein synthesis and apoptosis (Fig. 1) [119, 122, 126]. Acute testosterone administration has shown to activate the PI3K/Akt pathway and GLUT4 translocation *in vitro* indicating an increase in cellular glucose uptake [54]. However, supraphysiological levels of testosterone and nandrolone have been reported to significantly ($P < 0.05$) diminish the response of insulin-induced glucose uptake in rodents [127, 128]. Rodents also showed impairments in gluconeogenesis, most likely due to the high fasting insulin levels [128]. In contrast, increasing doses of testosterone (25–600 mg/wk) for 20 weeks had no significant effect on insulin sensitivity in resistance-trained males [93]. Additionally, in a double-blind crossover design, 300 mg/wk of Test E and nandrolone administration for 6 weeks did not affect glucose tolerance or fasting insulin levels in healthy males [129]. Although research is lacking, females who use AAS for performance are reported to display reduced insulin sensitivity [130]. In healthy females, up to 12 days of methyltestosterone dosing (5 mg), showed a significant ($P < 0.05$) reduction in whole-body insulin sensitivity [130]. Similarly, in postmenopausal females, 120 mg of testosterone undecanoate per week resulted in a significant decrease in insulin sensitivity [131]. Hyperandrogenism in females is a significant risk factor in developing polycystic ovary syndrome (PCOS) and PCOS increases the risk of developing MetS although the risk of developing CVD is currently unclear [132–135]. Interestingly, muscle strength determined by bench press and handgrip test was shown to be significantly ($P < 0.05$) higher in females with PCOS compared to healthy controls, further indicating that hyperandrogenism may be implicated in PCOS and MetS [136].

Although individual AAS use may not result in reduced insulin sensitivity in males [93, 129], limited research suggests chronic AAS polysubstance use may be detrimental to glucose metabolism as shown by Cohen et al. [137]. Powerlifting steroid users (PS) were shown to have similar fasting glucose levels as non-using powerlifters (NP) and sedentary participants; however, they had significantly ($P < 0.05$) higher fasting insulin levels that were similar to those observed in obese participants [137]. An oral glucose tolerance test (OGTT) also revealed the PS to have a significant ($P < 0.05$) 2-fold increase in post-glucose glycaemia compared to NP, which was a similar increase to the obese group. Post-glucose insulinaemia in the PS group was also significantly ($P < 0.01$) higher compared to all groups, with it being at least 2-fold higher compared to obese participants [137]. The authors only report participants use of AAS although insulin is commonly used for its anabolic potential and may have also been used by participants which may have impacted the results. More recently in males, an OGTT between healthy controls, steroid-using bodybuilders and former steroid-using bodybuilders (mean discontinuation of 2.5 years) revealed that current and former AAS users had significantly ($P < 0.05$) impaired glucose tolerance compared to healthy controls [37]. Reduced insulin sensitivity in former AAS users, was associated with higher % body fat, which may be due to reduced testosterone levels compared to healthy controls [37, 138].

Chronic AAS use suppresses the hypothalamic-pituitary-testicular (HPT) axis resulting in reduced endogenous testosterone production [39]. Low testosterone levels reduce insulin sensitivity and increases risk of developing MetS and CVD [139]. Interestingly, although current users of AAS had significantly ($P < 0.001$) lower % body fat compared to healthy controls and former users, they had significantly ($P < 0.05$) greater levels of VAT and reduced adiponectin and leptin levels which are all independent predictors of IR, T2D and MetS [37, 140–143].

However, a randomised controlled trial of the dose-response of TestE for 20 weeks showed significant

($P < 0.05$) decreases in VAT with higher doses, indicating supraphysiological doses do not increase VAT [144]. Although cross-sectional studies cannot determine causation, it may be more representative of long term AAS and AAS-associated polysubstance use in this case. Individuals typically use a range of AAS types and other complementary drugs; doses, cycles, methods of administration and for years rather than weeks or one AAS which may explain the differences in results.

Accumulation of VAT is an important indicator of glucose tolerance, MetS and CVD risk, much more so than subcutaneous adipose tissue (SAT) [143, 145, 146]. In healthy adipose tissue, when surplus energy is consumed, the energy is stored in SAT. However, in unhealthy or IR adipose tissue, the excess energy will be deposited in VAT and a variety of organs including muscle tissue [147, 148]. The lipolytic rate of VAT is increased compared to SAT due to the increased effect of pro-lipolytic catecholamines and decreased effect of anti-lipolytic insulin. This increases the flux of FFA to the liver, which may further increase hepatic IR [146]. Though, with AAS use, VAT was associated with lower lipolysis rates as determined by lower levels of plasma glycerol [37]. The unusual lower lipolytic activity may be attributable to reduced activity of catecholamines due to AAS compounds such as nandrolone downregulating β3-adrenoceptor expression [149]. In addition to being involved in lipid storage and mobilisation, adipocytes are also an endocrine tissue, releasing cytokines and adipokines. An increase in VAT leads to a pro-inflammatory state as shown by an increase in C-reactive protein (CRP) and tumour necrosis factor-alpha (TNF-a) which may further increase IR [150, 151]. As skeletal muscle is the largest tissue for glucose disposal, increases in muscle mass should improve insulin sensitivity; paradoxically, these results indicate that chronic AAS may cause tissue IR. This may be due to an imbalance of regulatory adipokines and cytokines from increased VAT levels and circulating lipids leading to a decreased/delayed stimulus of the PI3K/Akt signalling cascade in response to glucose ingestion, as also observed in T2D individuals [119]. This dysregulated metabolism leads to a continuous cycle of VAT and IR that potentiate each other. Furthermore, nutrient overload is reported to increase IR via mTORc1 dependent pathway. Chronic activation of S6K1 mediated by mTORc1, inflicts serine phosphorylation of IRS1 leading to reduced insulin sensitivity [152, 153]. For example, chronic high glucose concentrations in murine skeletal muscle cells (C2C12 myoblasts) induce IR and reduced Akt stimulation; however, inhibition of mTOR/S6K1 signalling with rapamycin restored insulin induced Akt stimulation [154]. It may be possible that chronic AAS use, leading to hyperactivation of mTORc1/S6K1 signalling may cause IR (Fig. 4). Estradiol has shown to be significantly ($P < 0.01$) higher with AAS use compared to healthy controls and may also be a cause of IR in this population. The conversion of testosterone to estradiol resulting in a decrease in the testosterone to estradiol ratio has been implicated in the development of MetS in older males [155]. Additionally, estradiol is reported to bind to insulin and the insulin receptor further highlighting its potential role in inducing IR (Fig. 4) [156].

Research is currently lacking on the prevalence of increased levels of VAT with AAS use, most likely due to AAS typically reducing fat and to its dysregulation of insulin sensitivity. This field of research warrants further investigation.

4.3 Hypertension

Hypertension is highly associated with MetS and CVD risk [30, 157]. Hypertension is caused by an increase in vascular stiffness due to degenerative changes in the extracellular matrix (ECM) derived from an imbalance of arterial scaffolding proteins such as elastin and collagen [158]. Chronic low-grade inflammation, underpinned by cytokines such as CRP, TNF-a and interleukin-6, induced by ageing, T2D or an atherogenic lipid profile results in endothelial and smooth muscle cell proliferation, hypertrophy, remodelling and apoptosis [159, 160]. This vascular proinflammatory state characterised by angiotensin II results in upregulation of matrix metalloproteinases (MMPs) leading to degradation of elastin fibres and increased collagen deposition resulting in ECM remodelling and arterial stiffening [161, 162]. Furthermore, metabolic disorders such as T2D can cause disruption of vasodilation (nitric oxide) and vasoconstriction (endothelin) regulators resulting in hypertension [160].

Hypertension, as a result of chronic AAS use, is under debate due to conflicting data [33]. Although further research is required, there is some evidence to suggest that chronic AAS use in males may lead to increased BP [33, 163–165]. By contrast, early studies suggested that AAS use did not have a detrimental effect on BP even with 24-hour monitoring [97, 106, 166]. Short-term (< 8 weeks) testosterone (200 mg/wk) and nandrolone (100–200 mg/wk) administration resulted in no change in systolic or diastolic BP [97, 167] however, long term-controlled studies are lacking.

Lenders et al., reported AAS polysubstance use to have a significant ($P < 0.05$) increase in systolic BP (SBP) after an average AAS use of 5 months although the increase was not clinically relevant (118 ± 2.2 to 121 ± 2.4 mmHg) [106]. Nevertheless, more recent studies have shown chronic AAS polysubstance use to have significantly ($P < 0.05$) higher SBP compared to healthy controls and former AAS users [163, 165]. These results may be clinically relevant as mean SBP was reported to be 132 mmHg and 138 mmHg in current AAS users [163, 165]. Current and former AAS users were also reported to have significantly ($P < 0.05$) increased aortic

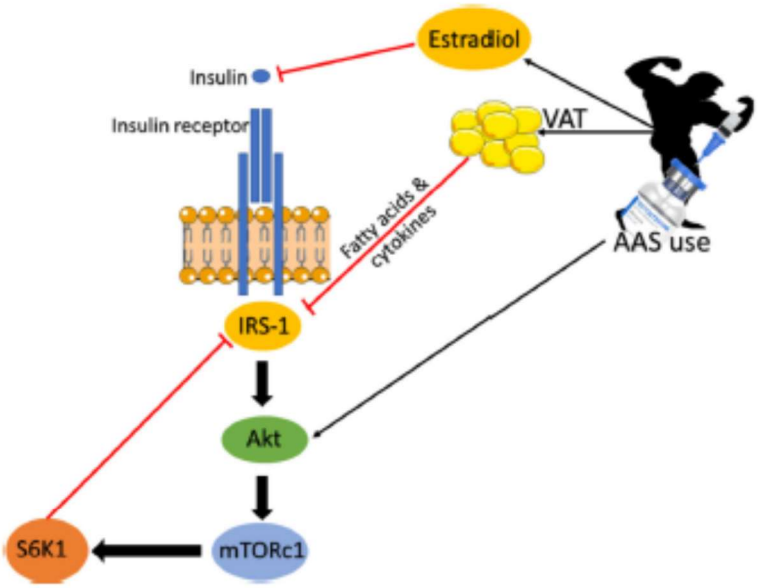

Fig. 4 Potential mechanisms of insulin resistance with chronic anabolic steroid use. Chronic upregulation of S6K1 via activation of PI3K/Akt signalling cascade by AAS may reduce insulin sensitivity due to inhibition of IRS-1 by S6K1 as seen with nutrient overload models. Furthermore, chronic AAS use may lead to an increase in VAT increasing circulating fatty acids and/or inflammatory cytokines causing inhibition of IRS-1 and reducing insulin sensitivity. Aromatisation of testosterone may lead to increasing levels of Estradiol causing IR by binding to insulin and the insulin receptor.

stiffness. These higher levels in aortic stiffness and SBP were associated with the significantly ($P < 0.05$) lower mid-regional pro-atrial natriuretic peptide (MR-proANP) in AAS users [163]. ANPs regulate vasodilation, reduce renin-angiotensin-aldosterone system activity and sympathetic nerve activity; yet, high levels of MR-proANP are associated with hypertension and incidence of mortality [168, 169]. The conflicting results regarding hypertension with AAS use may be partly due to differences in study designs. Repeated measure designs as implemented in the early studies are more indicative of causal effects compared to the most recent cross-sectional studies; however, the cross-sectional studies have larger sample sizes and potentially greater power but only association can be conferred. The lack of control on AAS type and quantity also makes it difficult to compare findings. Nonetheless, chronic AAS use may have detrimental effects on the vasculature and consequently causing hypertension and increased risk of CVD, but more long-term controlled studies are required.

5 Reducing CVD risk

In addition to increased LDL-C, research indicates that AAS users may develop MetS due to having low HDL-C, IR, possible hypertension and increased VAT. Considering this, they share a similar metabolic phenotype to sedentary/obese populations and have an increased risk of CVD incidence [30, 32, 33, 37, 98]. As MetS is typically associated with obese/sedentary populations, treatments include inducing weight loss through improving lifestyle behaviours (exercise and nutrition) and bariatric surgery or pharmaceutical medication to alter negative metabolic function [170]. However, these interventions would not apply to AAS users due to their already low body fat and high physical activity [170].

To reduce MetS and CVD risk, cessation of AAS use is highly recommended as it has been shown to at least improve the lipoprotein profile, yet may have lasting effects on insulin sensitivity, BP and VAT levels [37, 106, 108, 163]. Unfortunately, total cessation can lead to withdrawal symptoms such as hypogonadism in males, infertility and depression [7, 39]. Suppression of the HPT axis results in low endogenous testosterone production leading to decreased sexual function, such as erectile dysfunction and reduced libido and may be dependent on the dose and duration of AAS use [171]. These symptoms promote relapse and AAS dependency and must be treated accordingly with pharmaceutical and cognitive behaviour therapies to help with AAS cessation and prevent relapse [172]. AAS use may also lead to gynecomastia, due to an increase in the estrogen to testosterone ratio via an increase in aromatase activity resulting in the conversion of testosterone to estradiol [173]. Therefore, use of estrogen receptor antagonists are typically used in conjunction with AAS, particularly during times of AAS cessation [41, 173]. On cessation of AAS, pharmaceuticals such as hCG, aromatase inhibitors and SERMs can reduce withdrawal symptoms although current evidence is lacking on its benefits [7, 41, 174]. Hypogonadism is also associated with MetS, therefore, total cessation of AAS use may not improve MetS symptoms and CVD risk [175, 176]. It may be feasible to decrease AAS use instead of total cessation as low testosterone (40–80 mg per day) treatment in males with hypogonadism has been shown to improve MetS markers [177]. However, further clinical trials in AAS users, who wish to stop, are required before any true treatment can be recommended. Additionally, many users of AAS

rely on information from websites or online forums for post-cycle therapy which may lead to mismanagement of AAS withdrawal symptoms [178]. It's imperative that users of AAS seek professional advice while it is also equally important that clinicians aim to help in a non-judgemental way to reduce the likelihood of permanent adverse effects of AAS use. There is currently a lack of research on the effects of AAS withdrawal in females although they are likely to improve their lipoprotein profile but may also require treatment for depressive symptoms and amenorrhea [179–181].

6 Conclusion

CVD is the number one cause of deaths globally, with the obesity epidemic being a major contributor. The increasing prevalence of AAS use, particularly in young males, will exacerbate the current CVD rates. Chronic use of AAS leads to increased skeletal muscle hypertrophy and improved performance by binding to the AR. Activation of the AR by AAS leads to enhanced gene transcription, second messenger signalling, and satellite cell activation leading to increased muscle protein accretion and synthesis and possibly decreased catabolism. However, chronic AAS use not only leads to impaired cardiac function but also MetS and associated dysregulated metabolic health (IR, dyslipidaemia, VAT and BP) which is more commonly related with the sedentary/obese population. Effective management of AAS and AAS-related polypharmacy use in the first place, together with appropriate guidance on AAS cessation is key, both of which may be managed by education and psychological interventions to ultimately improve health. Therefore, further research is warranted on the long-term effects of AAS use and cessation on markers of metabolic health to provide accurate information on the potential harms in males and females. Further research is also required for treatments to aid AAS cessation and combat adverse metabolic health in this population.

Author contributions Deaglan McCullough, Claire E. Stewart and Ian G. Davies conceived and designed the review. The first draft of the manuscript was written by Deaglan McCullough and all authors critically revised all versions of the manuscript. All authors read and approved the final manuscript.

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Data availability Not applicable.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Consent for publication All the authors have read and approved the revised manuscript, and they are willing to publish it.

Code availability Not applicable.

References

1. Kanayama G, Pope HG. History and epidemiology of anabolic androgens in athletes and non-athletes. Mol Cell Endocrinol. 2018;464:4–13. https://doi.org/10.1016/j.mce.2017.02.039.
2. Hoberman JM, Yesalis CE. The history of synthetic testosterone. Sci Am. 1995;272(2):76–81.
3. Taylor WN. Anabolic steroids and the athlete. 2nd ed. In: Taylor WN editor. Google books. 2nd ed. Jefferson: McFarland & Company Inc.; 2001. 111–114 p.
4. Bird SR, Goebel C, Burke LM, Greaves RF. Doping in sport and exercise: anabolic, ergogenic, health and clinical issues. Ann Clin Biochem. 2016;53(2):196–221.
5. Kicman AT. Pharmacology of anabolic steroids. Br J Pharmacol. 2008;154(3):502–21.
6. De Souza GL, Hallak J. Anabolic steroids and male infertility: A comprehensive review. BJU Int. 2011;108(11):1860–5.
7. Goldman AL, Pope HG, Bhasin S. The health threat posed by the hidden epidemic of anabolic steroid use and body image disorders among young men. J Clin Endocrinol Metab. 2019;104(4):1069–74.
8. ACMD. Advisory Council on the Misuse of Drugs: Consideration of the anabolic steroids. London: Home Office. 2010.
9. Drug Enforcement Agency. Controlled substances - Alphabetical order [Internet]. Drug Enforcement Agency. 2019. https://www.deadiversion.usdoj.gov/schedules/orangebook/c_cs_alpha.pdf, https://www.dea.gov/drug-scheduling.
10. Council of Europe. Anti-Doping Convention [Internet]. 2020 [cited 2020 May 10]. https://www.coe.int/en/web/conventions/full-list/-/conventions/treaty/135/signatures?p_auth=qSBSK9FU.
11. World Anti-Doping Agency. What is Prohibited | World Anti-Doping Agency [Internet]. 2020 [cited 2020 May 10]. https://www.wada-ama.org/en/content/what-is-prohibited.
12. Sagoe D, Molde H, Andreassen CS, Torsheim T, Pallesen S. The global epidemiology of anabolic-androgenic steroid use: A meta-analysis and meta-regression analysis. Ann Epidemiol. 2014;24(5):383–98. https://doi.org/10.1016/j.annepidem.2014.01.009.
13. Begley E, McVeigh J, Hope V. Image and Performance Enhancing Drugs: 2016 National Survey Results [Internet]. 2017 [cited 2020 May 18]. http://www.ipedinfo.co.uk/resources/downloads/2016 National IPED Info Survey report FINAL.pdf
14. Parkinson AB, Evans NA. Anabolic androgenic steroids: A survey of 500 users. Med Sci Sports Exerc. 2006;38(4):644–51.
15. Dunn M, Mazanov J, Sitharthan G. Predicting future anabolic-androgenic steroid use intentions with current substance use: Findings from an internet-based survey. Clin J Sport Med. 2009;19(3):222–7.

8.6: Appendix 6: Conference abstract:

Proceedings of the Nutrition Society (2019)

Proceedings of the Nutrition Society (2019), **78 (OCE1)**, E16 doi:10.1017/S002966511900020X

Winter Meeting, 4–5 December 2018, Optimal diet and lifestyle strategies for the management of cardio-metabolic risk

The effect of dietary carbohydrate manipulation on low-density lipoprotein-cholesterol and its associated cardiometabolic risk

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Cardiometabolic (CM) risk is typically increased with elevated low-density lipoprotein-cholesterol (LDL-C) and insulin resistance (IR)[1,2]. A low carbohydrate, high fat (LCHF) diet has been shown to increase LDL-C albeit improving other CM risk factors such as high-density lipoprotein-cholesterol and triglycerides[3]. There are several subclasses of LDL, in which some may be more atherogenic such as small-dense LDL (sdLDL)[4]. Few studies have compared a LCHF diet to a high carbohydrate, lower fat diet under *ad libitum* conditions, particularly their effect on sdLDL:LDL-C ratio. The current feasibility study intends to address such gap. Furthermore, to the authors' knowledge, the effect of the new reduced sugar UK Eatwell guide on CM health is yet to be investigated. Therefore, the aims of this investigation was to measure the effect of a low carbohydrate (LC) diet vs. a high carbohydrate (HC) diet on LDL-C, sdLDL-C and IR in 16 (9 males, 7 females) healthy Caucasian adults aged 19-64.

The study received ethical approval from Liverpool John Moores University Research Ethics Committee (16/ELS/029) and was registered with ClinicalTrials.gov (Ref. NCT03257085). Participants were randomly assigned to either a HC diet (the UK Eatwell guidelines; ≥50 % of energy from carbohydrates) (n = 8, 5 males, 3 females), or a LC diet (consume <50 g/day of carbohydrates) (n = 8, 4 males, 4 females) for 8 weeks. At 0, 4 and 8 weeks blood was collected after a 12 hour fast, processed for plasma and stored at -80°C. Plasma was analysed by an automated chemistry analyser (Daytona, Randox Laboratories Ltd, UK) for LDL-C, sdLDL-C and glucose levels. Insulin levels were measured using immunoassay technology (Randox Evidence Investigator™ Metabolic Syndrome Arrays I). The homeostatic model assessment (HOMA) was used to calculate IR. Statistical analysis was undertaken using IBM SPSS 24®. Normally distributed data underwent a 2 × 3 mixed ANOVA to investigate significant differences for effect of time and interaction effect. Spearman's correlation was used to analyse the association between variables.

LDL-C non-significantly ($P = 0.141$) increased by 0.22 (mmol/L) within the LC group whereas the HC group remained unchanged. Within the LC group sdLDL-C levels decreased by 0.14 (mmol/L); however, sdLDL-C in the HC group increased by 0.07 (mmol/L) resulting in a significant interaction effect ($P = 0.026$). The ratio of sdLDL:LDL-C therefore decreased by 0.06 in the LC group and increased by 0.01 in the HC group resulting in a significant interaction effect ($P = 0.003$). HOMA significantly improved ($P = 0.008$) similarly in both groups but the change in HOMA was only significantly ($R^2 = 0.988$, $P = 0.008$) associated with the change in sdLDL:LDL-C within the LC group.

In conclusion, the study provided preliminary evidence showing that a LC diet may improve CM health via positive changes in LDL composition with an associated reduction in IR. Although the HC diet improved IR, the unfavourable changes in LDL size may indicate only a partial improvement in CM health. Further research is required on how dietary carbohydrate manipulation can improve LDL composition and overall CM health. The use of sdLDL:LDL-C ratio may be of more importance when assessing improvements in CM health compared to LDL-C alone.

1. Fulcher J, O'Connell R, Voysey M, *et al. Lancet*, 2015; **385**, 1397–405.
2. Gast KB, Tjeerdema N, Stijnen T, *et al. PloS one.* 2012 **28**, 7(12):e52036.
3. Mansoor N, Vinknes KJ, Veierød MB *et al. Brit J Nutr.* 2016 **115**, 466–479.
4. Hirayama S & Miida T. *Clin Chim Acta.* 2012 **414**, 215–224.

8.7: Appendix 7: Conference abstracts:

Proceedings of the Nutrition Society (2020)

Proceedings of the Nutrition Society (2020), **79 (OCE2)**, E530 doi:10.1017/S0029665120004796

The 13th European Nutrition Conference, FENS 2019, was held at the Dublin Convention Centre, 15–18 October 2019

The effect of a low carbohydrate high fat diet on emerging biochemical markers of cardiometabolic risk

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Abstract

Worldwide, cardiovascular disease (CVD) is the number 1 cause of mortality and is associated with insulin resistance (IR). Emerging biomarkers such as FGF21 and adiponectin are associated with cardiometabolic risk. Low carbohydrate, high fat (LCHF) diets have been reported to reduce cardiometabolic risk markers; however, few studies have compared a LCHF diet vs. a high carbohydrate (HC), lower fat diet under *ad libitum* conditions on adiponectin and FGF21. The purpose of this study was to investigate the effects of an *ad libitum* LCHF vs. HC diet on IR, FGF21 and adiponectin in 16 healthy adults. Ethical approval: Liverpool John Moores University Research Ethics Committee (16/ELS/029); registered with ClinicalTrials.gov (Ref. NCT03257085). Participants were randomly assigned to a HC diet (n = 8, the UK Eatwell guidelines; ≥ 50% of energy from carbohydrates) or a LCHF diet (n = 8, consume < 50 g/day of carbohydrates). All provided plasma samples at 0, 4 and 8 weeks. FGF21 (R&D Systems) was analysed via ELISA and adiponectin, insulin and glucose were analysed via immunoassay technology (Randox Evidence Investigator™ Metabolic Syndrome Arrays I & II). Mann Whitney, Friedmans, Wilcoxon tests and 2×3 ANOVA (IBM SPSS 25®) were undertaken to investigate significant differences between and within groups. The homeostatic model assessment (HOMA) was used to calculate IR. FGF21 significantly ($P = 0.04$) decreased (*Mdn, IQR*:148.16, 78.51–282.02 to 99.4, 39.87–132.29 pg/ml) after 4 weeks and significantly ($P = 0.02$) increased (*Mdn, IQR*:167.38, 80.82–232.89 pg/ml) by 8 weeks vs. baseline with LCHF. No significant differences ($P > 0.05$) were observed between groups. Adiponectin was significantly ($P = 0.03$) different at week 4 only between groups. Adiponectin increased after 4 weeks (*Mdn, IQR*:13.44, 9.12–25.47 to 16.64, 11.96–21.51 ng/ml) but was only significantly ($P = 0.03$) different by 8 weeks vs. baseline in the HC group (*Mdn, IQR*:16, 10.8–27.43 ng/ml). Adiponectin remained unchanged ($P = 0.96$) in the LCHF group. HOMA significantly decreased with both diets after 8 weeks only (*mean* ± SD, LCHF: 2.9 ± 1.3 to 1.8 ± 0.8, HC: 2.5 ± 0.6 to 1.9 ± 0.6, $P = 0.008$) but was not significantly ($P = 0.60$) different between groups. These preliminary data reveal that while both diets improved insulin sensitivity, they may do so by different mechanisms. Future studies are warranted to investigate further, how a LCHF vs. HC diet affects FGF21 and adiponectin, and the subsequent regulation of IR. Furthermore, studies that extend these findings by determining the impact of LCHF vs. HC on peripheral metabolism to determine potential nutrition-mediated mechanisms of metabolic adaptation are warranted.

Conflict of Interest

There is no conflict of interest.

8.8: Appendix 8: Conference abstracts:

Proceedings of the Nutrition Society (2020)

Proceedings of the Nutrition Society (2020), **79 (OCE2)**, E677 doi:10.1017/S0029665120006266

The 13th European Nutrition Conference, FENS 2019, was held at the Dublin Convention Centre, 15–18 October 2019

The effect of a low carbohydrate high fat diet on apolipoproteins and cardiovascular risk

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Abstract

Apolipoproteins (apo) regulate lipoprotein characteristics and lipid metabolism. ApoC-III is a regulator of triglyceride-rich lipoprotein (TRL) metabolism and apolipoproteins are important biomarkers for cardiovascular disease (CVD) risk prediction. A low carbohydrate high fat (LCHF) diet improves cardiometabolic risk, especially via reduction of TRL. However, few studies have compared a LCHF vs. a high carbohydrate (HC), lower fat diet under *ad libitum* conditions on apoC-III levels. The objectives of this investigation were to measure the effect of a LCHF vs. a HC diet on apoC-III, apoA1, apoB and apoB/apoA1 in 16 healthy Caucasian adults aged 19–64. Ethical approval: Liverpool John Moores University Research Ethics Committee (16/ELS/029); registered with ClinicalTrials.gov (Ref. NCT03257085). Participants randomly assigned to a HC diet (UK Eatwell guidelines; ≥ 50% of energy from carbohydrates) (n = 8), or a LCHF diet (consume < 50 g/day of carbohydrates) (n = 8) provided plasma samples at 0, 4 and 8 weeks. ApoA1 and apoB were analysed by an automated chemistry analyser (Daytona, Randox Laboratories Ltd, UK). ApoC-III was analysed via ELISA (Thermo Fisher Ltd, USA). Factorial 2×3 ANOVA and ANCOVA (IBM SPSS 25®) were undertaken to investigate significant differences and to control for variables influenced by baseline measures and visceral adipose tissue (VAT). Results show 0, 4, and 8 weeks respectively: ApoC-III (LCHF: 19.12 ± 9.14, 16.05 ± 7.95, 15.11 ± 3.17 mg/dl; HC: 22.13 ± 8.38, 28.22 ± 13.85, 22.22 ± 7.7 mg/dl) showed no significant ($P = 0.319$) change. No significant ($P = 0.23$) change was also observed in ApoB (LCHF: 107.25 ± 20.35, 111.38 ± 24.81, 111.43 ± 19.93 mg/dl; HC: 94.38 ± 20.79, 105.00 ± 20.13, 99.00 ± 29.09 mg/dl). Similarly apoA1 (LCHF: 158.71 ± 14.27, 166.50 ± 23.09, 173.00 ± 29.42 mg/dl; HC: 164.71 ± 30.25, 172.50 ± 29.44, 174.00 ± 32.83 mg/dl) showed no significant change ($P = 0.76$). This resulted in a relatively unchanged apoB/A1 throughout the study in both diets ($P = 0.30$). No significant ($P > 0.05$) differences were found after 4 weeks or between groups also. ANCOVA revealed a trend ($P = 0.06$) in apoC-III for a difference between groups (LCHF: Δ-6.6 mg/dl vs. HC: Δ1.2 mg/dl) after 8 weeks but no significant ($P > 0.05$) changes in other apolipoproteins were detected. These preliminary data reveal that a LCHF diet does not improve the apolipoprotein profile; however, when accounting for other metabolic risk factors (i.e. VAT) there was a trend towards lowering apoC-III levels ($P = 0.06$). Modulation of apoC-III may lead to improved lipid metabolism, but higher-powered studies are warranted before any improvement on CVD risk can be inferred.

Conflict of Interest

There is no conflict of interest.

References

Abdelmoez, A. M., Puig, L. S., Smith, J. A. B., Gabriel, B. M., Savikj, M., Dollet, L., Chibalin, A. V., Krook, A., Zierath, J. R. and Pillon, N. J. (2020). Comparative profiling of skeletal muscle models reveals heterogeneity of transcriptome and metabolism. *American Journal of Physiology - Cell Physiology*, 318(3), C615–C626. doi: 10.1152/ajpcell.00540.2019.

Abdul-Ghani, M. A. and DeFronzo, R. A. (2010). Pathogenesis of Insulin Resistance in Skeletal Muscle. *Journal of Biomedicine and Biotechnology*, 2010, 1–19. doi: 10.1155/2010/476279.

Achar, S., Rostamian, A. and Narayan, S. M. (2010). Cardiac and metabolic effects of anabolic-androgenic steroid abuse on lipids, blood pressure, left ventricular dimensions, and rhythm. *American Journal of Cardiology*. Elsevier Inc., 106(6), 893–901. doi: 10.1016/j.amjcard.2010.05.013.

ACMD (2010). Consideration of the anabolic steroids., (September), 72.

Adan, A., Alizada, G., Kiraz, Y., Baran, Y. and Nalbant, A. (2017). Flow cytometry: basic principles and applications. *Critical Reviews in Biotechnology*, 37(2), 163–176. doi: 10.3109/07388551.2015.1128876.

Addison, O., Marcus, R. L., Lastayo, P. C. and Ryan, A. S. (2014). Intermuscular fat: A review of the consequences and causes. *International Journal of Endocrinology*. Hindawi Publishing Corporation, 2014, 34–36. doi: 10.1155/2014/309570.

Ahl, S., Guenther, M., Zhao, S., James, R., Marks, J., Szabo, A. and Kidambi, S. (2015). Adiponectin levels differentiate metabolically healthy vs unhealthy among obese and nonobese white individuals. *Journal of Clinical Endocrinology and Metabolism*, 100(11), 4172–4180. doi: 10.1210/jc.2015-2765.

Al-bayati, A., Brown, A. and Walker, M. (2019). Impaired enhancement of insulin action in cultured skeletal muscle cells from insulin resistant type 2 diabetic patients in response to contraction using electrical pulse stimulation. *Journal of Diabetes and its Complications*. Elsevier Inc., 33(12), 107412. doi: 10.1016/j.jdiacomp.2019.107412.

Al-Shanti, N. and Stewart, C. E. (2008). PD98059 enhances C2 myoblast differentiation through p38 MAPK activation: A novel role for PD98059. *Journal of Endocrinology*, 198(1), 243–252. doi: 10.1677/JOE-08-0151.

Alberti, K. G. M. M. M. M., Eckel, R. H., Grundy, S. M., Zimmet, P. Z., Cleeman, J. I., Donato, K. A., Fruchart, J. C., James, W. P. T., Loria, C. M. and Smith, S. C. (2009). Harmonizing the metabolic syndrome: A joint interim statement of the international diabetes federation task force on epidemiology and prevention; National heart, lung, and blood institute; American heart association; World heart federation; International . *Circulation*, 120(16), 1640–1645. doi: 10.1161/CIRCULATIONAHA.109.192644.

Alessi, D. R., James, S. R., Downes, C. P., Holmes, A. B., Gaffney, P. R. J., Reese, C. B. and Cohen, P. (1997). Characterization of a 3-phosphoinositide-dependent protein kinase which phosphorylates and activates protein kinase Bα. *Current Biology*, 7(4), 261–269. doi: 10.1016/s0960-9822(06)00122-9.

Alessi, M. C. and Juhan-Vague, I. (2006). PAI-1 and the metabolic syndrome: Links, causes, and consequences. *Arteriosclerosis, Thrombosis, and Vascular Biology*, 26(10), 2200–2207. doi: 10.1161/01.ATV.0000242905.41404.68.

Alsiö, J., Birgner, C., Björkblom, L., Isaksson, P., Bergström, L., Schiöth, H. B. and Lindblom, J. (2009). Impact of nandrolone decanoate on gene expression in endocrine systems related to the adverse effects of anabolic androgenic steroids. *Basic and Clinical Pharmacology and Toxicology*, 105(5), 307–314. doi: 10.1111/j.1742-7843.2009.00439.x.

Altman, D. G. and Bland, M. J. (1999). Treatment allocation in controlled trials: Why randomise?. *BMJ*. British Medical Journal Publishing Group, 318(7192), 1209. doi: 10.1136/bmj.318.7192.1209.

Anderson, L. J., Tamayose, J. M. and Garcia, J. M. (2018). Use of growth hormone, IGF-I, and insulin for anabolic purpose: Pharmacological basis, methods of detection, and adverse effects. *Molecular and Cellular Endocrinology*. Elsevier Ireland Ltd, 464, 65–74. doi: 10.1016/j.mce.2017.06.010.

Di Angelantonio, E., Sarwar, N., Perry, P. and Al., E. (2009). Major Lipids, Apolipoproteins, and Risk of Vascular Disease. *JAMA*, 302(18), 1993–2000. doi: 10.1001/jama.2009.1619.

Annuzzi, G., Bozzetto, L., Costabile, G., Giacco, R., Mangione, A., Anniballi, G., Vitale, M., Vetrani, C., Cipriano, P., Della Corte, G., Pasanisi, F., Riccardi, G. and Rivellese, A. A. (2014). Diets naturally rich in polyphenols improve fasting and postprandial dyslipidemia and reduce oxidative stress: A randomized controlled trial. *American Journal of Clinical Nutrition*, 99(3), 463–471. doi: 10.3945/ajcn.113.073445.

Antinozzi, C., Marampon, F., Corinaldesi, C., Vicini, E., Sgrò, P., Vannelli, G. B., Lenzi, A., Crescioli, C. and Di Luigi, L. (2017). Testosterone insulin-like effects: an in vitro study on the short-term metabolic effects of testosterone in human skeletal muscle cells. *Journal of Endocrinological Investigation*. Springer International Publishing, 40(10), 1133–1143. doi: 10.1007/s40618-017-0686-y.

Arnett, D. K., Blumenthal, R. S., Albert, M. A., Buroker, A. B., Goldberger, Z. D., Hahn, E. J., Himmelfarb, C. D., Khera, A., Lloyd-Jones, D., McEvoy, J. W., Michos, E. D., Miedema, M. D., Muñoz, D., Smith, S. C., Virani, S. S., Williams, K. A., Yeboah, J. and Ziaeian, B. (2019). 2019 ACC/AHA Guideline on the Primary Prevention of Cardiovascular Disease: A Report of the American College of Cardiology/American Heart Association Task Force on Clinical Practice Guidelines. *Journal of the American College of Cardiology*, 74(10), e177–e232. doi: 10.1016/j.jacc.2019.03.010.

Arpón, A., Milagro, F. I., Santos, J. L., García-Granero, M., Riezu-Boj, J. I. and Martínez, J. A. (2019). Interaction among sex, aging, and epigenetic processes concerning visceral fat, insulin resistance, and dyslipidaemia. *Frontiers in Endocrinology*, 10(JULY), 1–9. doi: 10.3389/fendo.2019.00496.

Atlantis, E., Martin, S. A., Haren, M. T., Taylor, A. W. and Wittert, G. A. (2009). Inverse associations between muscle mass, strength, and the metabolic syndrome. *Metabolism: Clinical and Experimental*. Elsevier Inc., 58(7), 1013–1022. doi: 10.1016/j.metabol.2009.02.027.

Baggish, A. L., Weiner, R. B., Kanayama, G., Hudson, J. I., Lu, M. T., Hoffmann, U. and Pope, H. G.

(2017). Cardiovascular toxicity of illicit anabolic-androgenic steroid use. *Circulation*, 135(21), 1991–2002. doi: 10.1161/CIRCULATIONAHA.116.026945.

Balsan, G. A., Vieira, J. L. da C., Oliveira, A. M. de and Portal, V. L. (2015). Relationship between adiponectin, obesity and insulin resistance. *Revista da Associação Médica Brasileira*, 61(1), 72–80. doi: 10.1590/1806-9282.61.01.072.

Banerjee, A., Chitnis, U., Jadhav, S., Bhawalkar, J. and Chaudhury, S. (2009). Hypothesis testing, type I and type II errors. *Industrial Psychiatry Journal*. Medknow, 18(2), 127. doi: 10.4103/0972-6748.62274.

Barnett, A. G., van der Pols, J. C. and Dobson, A. J. (2005). Regression to the mean: What it is and how to deal with it. *International Journal of Epidemiology*, 34(1), 215–220. doi: 10.1093/ije/dyh299.

Barter, P. J., Nicholls, S., Rye, K. A., Anantharamaiah, G. M., Navab, M. and Fogelman, A. M. (2004). Antiinflammatory properties of HDL. *Circulation Research*, 95(8), 764–772. doi: 10.1161/01.RES.0000146094.59640.13.

Basualto-Alarcón, C., Jorquera, G., Altamirano, F., Jaimovich, E. and Estrada, M. (2013). Testosterone signals through mTOR and androgen receptor to induce muscle hypertrophy. *Medicine and Science in Sports and Exercise*, 45(9), 1712–1720. doi: 10.1249/MSS.0b013e31828cf5f3.

Bazzano, L., Hu, T., K, R., L, Y., C, B. and Y, L. (2015). A Randomized Trial Comparing a Very Low Carbohydrate Diet and a Calorie-Restricted Low Fat Diet on Body Weight and Cardiovascular Risk Factors in Healthy Women. *Ann Intern Med*, 88(3), 1617–1623. doi: 10.7326/M14-0180.Effects.

Beavers, K. M., Ambrosius, W. T., Rejeski, W. J., Burdette, J. H., Walkup, M. P., Sheedy, J. L., Nesbit, B. A., Gaukstern, J. E., Nicklas, B. J. and Marsh, A. P. (2017). Effect of Exercise Type During Intentional Weight Loss on Body Composition in Older Adults with Obesity. *Obesity*, 25(11), 1823–1829. doi: 10.1002/oby.21977.

Begley, E., McVeigh, J. and Hope, V. (2017). *Image and Performance Enhancing Drugs: 2016 National Survey Results*. Available at: http://www.ipedinfo.co.uk/resources/downloads/2016 National IPED Info Survey report FINAL.pdf (Accessed: 20 May 2020).

Bennett, N. C., Gardiner, R. A., Hooper, J. D., Johnson, D. W. and Gobe, G. C. (2010). Molecular cell biology of androgen receptor signalling. *International Journal of Biochemistry and Cell Biology*. Elsevier Ltd, 42(6), 813–827. doi: 10.1016/j.biocel.2009.11.013.

Bhasin, S., Storer, T. W., Berman, N., Callegari, C., Clevenger, B., Phillips, J., Bunnell, T. J., Tricker, R., Shirazi, A. and Casaburi, R. (1996). The effects of supraphysiologic doses of testosterone on muscle size and strength in normal men. *New England Journal of Medicine*, 335(1), 1–7. doi: 10.1056/NEJM199607043350101.

Bhasin, S., Woodhouse, L., Casaburi, R., Singh, A. B., Bhasin, D., Berman, N., Chen, X., Yarasheski, K. E., Magliano, L., Dzekov, C., Dzekov, J., Bross, R., Phillips, J., Sinha-Hikim, I., Shen, R. and Storer, T. W. (2001). Testosterone dose-response relationships in healthy young men. *American Journal of*

Physiology - Endocrinology and Metabolism, 281(6 44-6), 1172–1181. doi: 10.1152/ajpendo.2001.281.6.e1172.

Bjørnebekk, A., Walhovd, K. B., Jørstad, M. L., Due-Tønnessen, P., Hullstein, I. R. and Fjell, A. M. (2017). Structural Brain Imaging of Long-Term Anabolic-Androgenic Steroid Users and Nonusing Weightlifters. *Biological Psychiatry*. Elsevier Inc., 82(4), 294–302. doi: 10.1016/j.biopsych.2016.06.017.

Bland, J. M. and Altman, D. G. (2011). Comparisons against baseline within randomised groups are often used and can be highly misleading. *Trials*, 12. doi: 10.1186/1745-6215-12-264.

Blau, H. M., Chiu, C. P. and Webster, C. (1983). Cytoplasmic activation of human nuclear genes in stable heterocaryons. *Cell*, 32(4), 1171–1180. doi: 10.1016/0092-8674(83)90300-8.

Blüher, M. (2019). Obesity: global epidemiology and pathogenesis. *Nature Reviews Endocrinology*, 15(5), 288–298. doi: 10.1038/s41574-019-0176-8.

Bonetti, A., Tirelli, F., Catapano, A., Dazzi, D., Dei Cas, A., Solito, F., Ceda, C., Reverberi, C., Monica, C., Pipitone, S., Elia, G., Spattini, M. and Magnati, G. (2008). Side effects of anabolic androgenic steroids abuse. *International Journal of Sports Medicine*, 29(8), 679–687. doi: 10.1055/s-2007-965808.

Bonita, R., Winkelmann, R., Douglas, K. A. and de Courten, M. (2003). The WHO Stepwise Approach to Surveillance (Steps) of Non-Communicable Disease Risk Factors.in *Global Behavioral Risk Factor Surveillance*. Springer US, 9–22. doi: 10.1007/978-1-4615-0071-1_3.

Boucher, J., Kleinridders, A., Kahn, C. R., Kahn, J. ´re´mie B. A. K. and C. R., Boucher, J., Kleinridders, A., Kahn, C. R., Kahn, J. ´re´mie B. A. K. and C. R., Boucher, J., Kleinridders, A. and Kahn, C. R. (2014). Insulin Receptor Signaling in Normal. *Cold Spring Harb Perspect Biol 2014*, 6, a009191. doi: 10.1101/cshperspect.a009191.

Brinkworth, G. D., Noakes, M., Buckley, J. D., Keogh, J. B. and Clifton, P. M. (2009). Long-term effects of a very-low-carbohydrate weight loss diet compared with an isocaloric low-fat diet after 12 mo. *American Journal of Clinical Nutrition*, 90(1), 23–32. doi: 10.3945/ajcn.2008.27326.

British Heart Foundation (2020). UK Factsheet. *British Heart Foundation*, (January), 1–21.

Brøns, C. and Grunnet, L. G. (2017). Skeletal muscle lipotoxicity in insulin resistance and type 2 diabetes: A causal mechanism or an innocent bystander?. *European Journal of Endocrinology*, 176(2), R67–R78. doi: 10.1530/EJE-16-0488.

Bruun, J. M., Lihn, A. S., Verdich, C., Pedersen, S. B., Toubro, S., Astrup, A. and Richelsen, B. (2003). Regulation of adiponectin by adipose tissue-derived cytokines: in vivo and in vitro investigations in humans. *American Journal of Physiology - Endocrinology And Metabolism*, 285(3), E527–E533. doi: 10.1152/ajpendo.00110.2003.

Brzeszczyńska, J., Meyer, A., McGregor, R., Schilb, A., Degen, S., Tadini, V., Johns, N., Langen, R., Schols, A., Glass, D. J., Roubenoff, R., Ross, J. A., Fearon, K. C. H., Greig, C. A. and Jacobi, C. (2018). Alterations in the in vitro and in vivo regulation of muscle regeneration in healthy ageing and the

influence of sarcopenia. *Journal of Cachexia, Sarcopenia and Muscle*, 9(1), 93–105. doi: 10.1002/jcsm.12252.

Bueno, N. B., De Melo, I. S. V., De Oliveira, S. L. and Da Rocha Ataide, T. (2013). Very-low-carbohydrate ketogenic diet v. low-fat diet for long-term weight loss: A meta-analysis of Randomised controlled trials. *British Journal of Nutrition*, 110(7), 1178–1187. doi: 10.1017/S0007114513000548.

Cantor, J. R., Abu-Remaileh, M., Kanarek, N., Freinkman, E., Gao, X., Louissaint, A., Lewis, C. A. and Sabatini, D. M. (2017). Physiologic Medium Rewires Cellular Metabolism and Reveals Uric Acid as an Endogenous Inhibitor of UMP Synthase. *Cell*. Elsevier, 169(2), 258-272.e17. doi: 10.1016/j.cell.2017.03.023.

Carrageta, D. F., Oliveira, P. F., Alves, M. G. and Monteiro, M. P. (2019). Obesity and male hypogonadism: Tales of a vicious cycle. *Obesity Reviews*, 20(8), 1148–1158. doi: 10.1111/obr.12863.

Carson, B. P., Patel, B., Amigo-Benavent, M., Pauk, M., Kumar Gujulla, S., Murphy, S. M., Kiely, P. A. and Jakeman, P. M. (2018). Regulation of muscle protein synthesis in an in vitro cell model using ex vivo human serum. *Experimental Physiology*, 103(6), 783–789. doi: 10.1113/EP086860.

Cartee, G. D. (2015). Roles of TBC1D1 and TBC1D4 in insulin- and exercise-stimulated glucose transport of skeletal muscle. *Diabetologia*, 58(1), 19–30. doi: 10.1007/s00125-014-3395-5.

De Carvalho, F. G., Justice, J. N., de Freitas, E. C., Kershaw, E. E. and Sparks, L. M. (2019). Adipose Tissue Quality in Aging: How Structural Skeletal Muscle Quality?. *Nutrients*, 11, 2553.

Chang, S., Münster, A. M. B., Gram, J. and Sidelmann, J. J. (2018). Anabolic Androgenic Steroid Abuse: The Effects on Thrombosis Risk, Coagulation, and Fibrinolysis. *Seminars in Thrombosis and Hemostasis*, 44(8), 734–746. doi: 10.1055/s-0038-1670639.

Cheng, K. K. Y., Lam, K. S. L., Wang, B. and Xu, A. (2014). Signaling mechanisms underlying the insulin-sensitizing effects of adiponectin. *Best Practice and Research: Clinical Endocrinology and Metabolism*. Elsevier Ltd, 28(1), 3–13. doi: 10.1016/j.beem.2013.06.006.

Cheung, A. S. and Grossmann, M. (2018). Physiological basis behind ergogenic effects of anabolic androgens. *Molecular and Cellular Endocrinology*. Elsevier Ireland Ltd, 464(November 2016), 14–20. doi: 10.1016/j.mce.2017.01.047.

Ching, J. K., Rajguru, P., Marupudi, N., Banerjee, S. and Fisher, J. S. (2010). A role for AMPK in increased insulin action after serum starvation. *American Journal of Physiology - Cell Physiology*, 299(5), 1171–1179. doi: 10.1152/ajpcell.00514.2009.

Christodoulides, C., Dyson, P., Sprecher, D., Tsintzas, K. and Karpe, F. (2009). Circulating fibroblast growth factor 21 is induced by peroxisome proliferator-activated receptor agonists but not ketosis in man. *Journal of Clinical Endocrinology and Metabolism*, 94(9), 3594–3601. doi: 10.1210/jc.2009-0111.

Chung, T., Kelleher, S., Liu, P. Y., Conway, A. J., Kritharides, L. and Handelsman, D. J. (2007). Effects of testosterone and nandrolone on cardiac function: A randomized, placebo-controlled study. *Clinical*

Endocrinology, 66(2), 235–245. doi: 10.1111/j.1365-2265.2006.02715.x.

Clarke, E. M., Thompson, R. C., Allam, A. H., Wann, L. S., Lombardi, G. P., Sutherland, M. L., Sutherland, J. D., Cox, S. L., Soliman, M. A. T., Abd el-Maksoud, G., Badr, I., Miyamoto, M. I., Frohlich, B., Nur el-din, A. H., Stewart, A. F. R., Narula, J., Zink, A. R., Finch, C. E., Michalik, D. E. and Thomas, G. S. (2014). Is atherosclerosis fundamental to human aging? Lessons from ancient mummies. *Journal of Cardiology*. Japanese College of Cardiology, 63(5), 329–334. doi: 10.1016/j.jjcc.2013.12.012.

Cogan, K. E., Carson, B. P., Patel, B., Amigo-Benavent, M., Jakeman, P. M. and Egan, B. (2019). Regulation of GLUT4 translocation in an in vitro cell model using postprandial human serum ex vivo. *Experimental Physiology*, 104(6), 800–807. doi: 10.1113/EP087356.

Cohen, J. C., Faber, W. M., Benade, A. J. S. and Noakes, T. D. (1986). Altered serum lipoprotein profiles in male and female power lifters ingesting anabolic steroids. *Physician and Sportsmedicine*, 14(6), 131–136. doi: 10.1080/00913847.1986.11709105.

Cohen, J. C. and Hickman, R. (1987). Insulin resistance and diminished glucose tolerance in powerlifters ingesting anabolic steroids. *Journal of Clinical Endocrinology and Metabolism*, 64(5), 960–963. doi: 10.1210/jcem-64-5-960.

Committee on Medical Aspects of Food Policy (COMA) (1991). *Dietary Reference Values for Food Energy and Nutrients for the United Kingdom London: HMSO*.

Cooper, G. M. (2019). *The Cell. A Molecular Approach*. 8th edn *Journal of Chemical Information and Modeling*. 8th edn. New York: Oxford University Press.

Council of Europe (2020). *Anti-Doping Convention*. Available at: https://www.coe.int/en/web/conventions/full-list/-/conventions/treaty/135/signatures?p_auth=qSBSK9FU (Accessed: 10 May 2020).

Coviello, A. D., Legro, R. S. and Dunaif, A. (2006). Adolescent girls with polycystic ovary syndrome have an increased risk of the metabolic syndrome associated with increasing androgen levels independent of obesity and insulin resistance. *Journal of Clinical Endocrinology and Metabolism*, 91(2), 492–497. doi: 10.1210/jc.2005-1666.

Cram, S. L. (2002). Flow cytometry: An overview. *Methods in Cell Science*, 24, 1–9.

Crossland, H., Smith, K., Atherton, P. J. and Wilkinson, D. J. (2017). A novel puromycin decorporation method to quantify skeletal muscle protein breakdown: A proof-of-concept study. *Biochemical and Biophysical Research Communications*. Elsevier Ltd, 494(3–4), 608–614. doi: 10.1016/j.bbrc.2017.10.085.

Czech, M. P. (2017). Insulin action and resistance in obesity and type 2 diabetes. *Nature Medicine*, 23(7), 804–814. doi: 10.1038/nm.4350.

D'Andrea, A., Radmilovic, J., Caselli, S., Carbone, A., Scarafile, R., Sperlongano, S., Tocci, G., Formisano, T., Martone, F., Liccardo, B., D'Alto, M., Bossone, E., Galderisi, M. and Golino, P. (2018).

Left atrial myocardial dysfunction after chronic abuse of anabolic androgenic steroids: a speckle tracking echocardiography analysis. *International Journal of Cardiovascular Imaging*. Springer Netherlands, 34(10), 1549–1559. doi: 10.1007/s10554-018-1370-9.

D'Elia, L., Strazzullo, P., Iacone, R., Russo, O. and Galletti, F. (2019). Leptin levels predict the development of insulin resistance in a sample of adult men–The Olivetti Heart Study. *Nutrition, Metabolism and Cardiovascular Diseases*. Elsevier B.V, 29(1), 39–44. doi: 10.1016/j.numecd.2018.10.003.

Davey, R. A. and Grossmann, M. (2016). Androgen Receptor Structure, Function and Biology: From Bench to Bedside. *The Clinical biochemist. Reviews*, 37(1), 3–15. Available at: http://www.ncbi.nlm.nih.gov/pubmed/27057074%0Ahttp://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC4810760.

Davies, I. G., Graham, J. M. and Griffin, B. A. (2003). Rapid Separation of LDL Subclasses by Iodixanol Gradient Ultracentrifugation. *Clinical Chemistry*, 49(11), 1865–1872. doi: 10.1373/clinchem.2003.023366.

Defronzo, R. A., Jacot, E., Jequier, E., Maeder, E., Wahren, J. and Felber, J. P. (1981). The Effect of Insulin on the Disposal of Intravenous Glucose. *Diabetes*, 590(14), 1000–1007. doi: 10.1113/jphysiol.2012.235127.

DeFronzo, R. A. and Tripathy, D. (2009). Skeletal muscle insulin resistance is the primary defect in type 2 diabetes. *Diabetes care*, 32 Suppl 2. doi: 10.2337/dc09-S302.

DeMarco, V. G., Aroor, A. R. and Sowers, J. R. (2014). The pathophysiology of hypertension in patients with obesity. *Nature Reviews Endocrinology*. Nature Publishing Group, 10(6), 364–376. doi: 10.1038/nrendo.2014.44.

Deng, Q., Zhang, Z., Wu, Y., Yu, W. Y., Zhang, J., Jiang, Z. M., Zhang, Y., Liang, H. and Gui, Y. T. (2017). Non-Genomic Action of Androgens is Mediated by Rapid Phosphorylation and Regulation of Androgen Receptor Trafficking. *Cellular Physiology and Biochemistry*, 43(1), 223–236. doi: 10.1159/000480343.

Després, J.-P. P., Lemieux, I., Bergeron, J., Pibarot, P., Mathieu, P., Larose, E., Rodés-Cabau, J., Bertrand, O. F. and Poirier, P. (2008). Abdominal Obesity and the Metabolic Syndrome: Contribution to global cardiometabolic risk. *Arteriosclerosis, Thrombosis, and Vascular Biology*, 28(6), 1039–1049. doi: 10.1161/ATVBAHA.107.159228.

Després, J. P. and Lemieux, I. (2006). Abdominal obesity and metabolic syndrome. *Nature*, 444(7121), 881–887. doi: 10.1038/nature05488.

Diamond, M. P., Grainger, D., Diamond, M. C., Sherwin, R. S. and Defronzo, R. A. (1998). Effects of methyltestosterone on insulin secretion and sensitivity in women. *Journal of Clinical Endocrinology and Metabolism*, 83(12), 4420–4425. doi: 10.1210/jc.83.12.4420.

Diffenderfer, M. R. and Schaefer, E. J. (2014). The composition and metabolism of large and small LDL. *Current Opinion in Lipidology*, 25(3), 221–226. doi: 10.1097/MOL.0000000000000067.

Domouzoglou, E. M., Naka, K. K., Vlahos, A. P., Papafaklis, M. I., Michalis, L. K., Tsatsoulis, A. and Maratos-Flier, E. (2015). Fibroblast growth factors in cardiovascular disease: The emerging role of FGF21. *American Journal of Physiology - Heart and Circulatory Physiology*, 309(6), H1029–H1038. doi: 10.1152/ajpheart.00527.2015.

Drake, I., Sonestedt, E., Ericson, U., Wallström, P. and Orho-Melander, M. (2018). A Western dietary pattern is prospectively associated with cardio-metabolic traits and incidence of the metabolic syndrome. *British Journal of Nutrition*, 119(10), 1168–1176. doi: 10.1017/S000711451800079X.

Drug Enforcement Agency (2019). *Controlled Substances - Alphabetical Order Drug Enforcement Agency*. Available at: https://www.deadiversion.usdoj.gov/schedules/orangebook/c_cs_alpha.pdf (Accessed: 10 May 2020).

Dugdale, H. F., Hughes, D. C., Allan, R., Deane, C. S., Coxon, C. R., Morton, J. P., Stewart, C. E. and Sharples, A. P. (2018). The role of resveratrol on skeletal muscle cell differentiation and myotube hypertrophy during glucose restriction. *Molecular and Cellular Biochemistry*. Springer US, 444(1–2), 109–123. doi: 10.1007/s11010-017-3236-1.

Dumont, N. A., Wang, Y. X. and Rudnicki, M. A. (2015). Intrinsic and extrinsic mechanisms regulating satellite cell function. *Development (Cambridge)*, 142(9), 1572–1581. doi: 10.1242/dev.114223.

Dunn, M., Mazanov, J. and Sitharthan, G. (2009). Predicting future anabolic-androgenic steroid use intentions with current substance use: Findings from an internet-based survey. *Clinical Journal of Sport Medicine*, 19(3), 222–227. doi: 10.1097/JSM.0b013e31819d65ad.

Dunn, M., Mckay, F. H. and Iversen, J. (2014). Steroid users and the unique challenge they pose to needle and syringe program workers. *Drug and Alcohol Review*, 33(1), 71–77. doi: 10.1111/dar.12085.

Duran, E. K., Aday, A. W., Cook, N. R., Buring, J. E., Ridker, P. M. and Pradhan, A. D. (2020). Triglyceride-Rich Lipoprotein Cholesterol, Small Dense LDL Cholesterol, and Incident Cardiovascular Disease. *Journal of the American College of Cardiology*, 75(17), 2122–2135. doi: 10.1016/j.jacc.2020.02.059.

Ebbeling, C. B., Bielak, L., Lakin, P. R., Klein, G. L., Wong, J. M. W., Luoto, P. K., Wong, W. W. and Ludwig, D. S. (2020). Energy Requirement Is Higher During Weight-Loss Maintenance in Adults Consuming a Low- Compared with High-Carbohydrate Diet. *The Journal of Nutrition*. Oxford University Press, 1–7. doi: 10.1093/jn/nxaa150.

Ebbeling, C. B., Feldman, H. A., Klein, G. L., Wong, J. M. W., Bielak, L., Steltz, S. K., Luoto, P. K., Wolfe, R. R., Wong, W. W. and Ludwig, D. S. (2018). Effects of a low carbohydrate diet on energy expenditure during weight loss maintenance: randomized trial. *BMJ (Clinical research ed.)*, 363, k4583. doi: 10.1136/bmj.k4583.

Eckardt, K., Taube, A. and Eckel, J. (2011). Obesity-associated insulin resistance in skeletal muscle: Role of lipid accumulation and physical inactivity. *Reviews in Endocrine and Metabolic Disorders*, 12(3), 163–172. doi: 10.1007/s11154-011-9168-2.

Egan, B. and Zierath, J. R. (2013). Exercise Metabolism and the Molecular Regulation of Skeletal Muscle Adaptation. *Cell Metabolism*. Elsevier Inc., 17(2), 162–184. doi: 10.1016/j.cmet.2012.12.012.

Esser, N., Legrand-Poels, S., Piette, J., Scheen, A. J. and Paquot, N. (2014). Inflammation as a link between obesity, metabolic syndrome and type 2 diabetes. *Diabetes Research and Clinical Practice*, 105(2), 141–150. doi: 10.1016/j.diabres.2014.04.006.

Esteghamati, Alireza, Noshad, S., Khalilzadeh, O., Morteza, A., Nazeri, A., Meysamie, A., Esteghamati, Abdolreza and Nakhjavani, M. (2011). Contribution of Serum Leptin to Metabolic Syndrome in Obese and Nonobese Subjects. *Archives of Medical Research*, 42(3), 244–251. doi: 10.1016/j.arcmed.2011.05.005.

Estrada, M., Espinosa, A., Müller, M. and Jaimovich, E. (2003). Testosterone stimulates intracellular calcium release and mitogen-activated protein kinases via a G protein-coupled receptor in skeletal muscle cells. *Endocrinology*, 144(8), 3586–3597. doi: 10.1210/en.2002-0164.

Ettehad, D., Emdin, C. A., Kiran, A., Anderson, S. G., Callender, T., Emberson, J., Chalmers, J., Rodgers, A. and Rahimi, K. (2016). Blood pressure lowering for prevention of cardiovascular disease and death: A systematic review and meta-analysis. *The Lancet*. Elsevier Ltd, 387(10022), 957–967. doi: 10.1016/S0140-6736(15)01225-8.

European Commission (no date). *Food-Based Dietary Guidelines in Europe | EU Science Hub*. Available at: https://ec.europa.eu/jrc/en/health-knowledge-gateway/promotion-prevention/nutrition/food-based-dietary-guidelines (Accessed: 7 May 2020).

Evans-Brown, M., Kimergård, A. and McVeigh, J. (2009). Elephant in the room? The methodological implications for public health research of performance-enhancing drugs derived from the illicit market. *Drug Testing and Analysis*, 1(7), 323–326. doi: 10.1002/dta.74.

Fabiani, R., Naldini, G. and Chiavarini, M. (2019). Dietary patterns and metabolic syndrome in adult subjects: A systematic review and meta-analysis. *Nutrients*, 11(9), 1–36. doi: 10.3390/nu11092056.

Faghihnia, N., Tsimikas, S., Miller, E. R., Witztum, J. L. and Krauss, R. M. (2010). Changes in lipoprotein(a), oxidized phospholipids, and LDL subclasses with a low-fat high-carbohydrate diet. *Journal of Lipid Research*, 51(11), 3324–3330. doi: 10.1194/jlr.M005769.

Fan, J., Liu, Y., Yin, S., Chen, N., Bai, X., Ke, Q., Shen, J. and Xia, M. (2019). Small dense LDL cholesterol is associated with metabolic syndrome traits independently of obesity and inflammation. *Nutrition and Metabolism*. Nutrition & Metabolism, 16(1), 1–9. doi: 10.1186/s12986-019-0334-y.

Fardouly, J. and Vartanian, L. R. (2016). Social Media and Body Image Concerns: Current Research and Future Directions. *Current Opinion in Psychology*. Elsevier Ltd, 9, 1–5. doi:

10.1016/j.copsyc.2015.09.005.

Fazeli, P. K., Lun, M., Kim, S. M., Bredella, M. A., Wright, S., Zhang, Y., Lee, H., Catana, C., Klibanski, A., Patwari, P. and Steinhauser, M. L. (2015). FGF21 and the late adaptive response to starvation in humans. *Journal of Clinical Investigation*, 125(12), 4601–4611. doi: 10.1172/JCI83349.

Fazleen, N. E., Whittaker, M. and Mamun, A. (2018). Risk of metabolic syndrome in adolescents with polycystic ovarian syndrome: A systematic review and meta-analysis. *Diabetes and Metabolic Syndrome: Clinical Research and Reviews*. Diabetes India, 12(6), 1083–1090. doi: 10.1016/j.dsx.2018.03.014.

Feinman, R. D., Pogozelski, W. K., Astrup, A., Bernstein, R. K., Fine, E. J., Westman, E. C., Accurso, A., Frassetto, L., Gower, B. A., McFarlane, S. I., Nielsen, J. V., Krarup, T., Saslow, L., Roth, K. S., Vernon, M. C., Volek, J. S., Wilshire, G. B., Dahlqvist, A., Sundberg, R., Childers, A., Morrison, K., Manninen, A. H., Dashti, H. M., Wood, R. J., Wortman, J. and Worm, N. (2015). Dietary carbohydrate restriction as the first approach in diabetes management: Critical review and evidence base. *Nutrition*. Elsevier Inc, 31(1), 1–13. doi: 10.1016/j.nut.2014.06.011.

Ference, B. A., Ginsberg, H. N., Graham, I., Ray, K. K., Packard, C. J., Bruckert, E., Hegele, R. A., Krauss, R. M., Raal, F. J., Schunkert, H., Watts, G. F., Borén, J., Fazio, S., Horton, J. D., Masana, L., Nicholls, S. J., Nordestgaard, B. G., Van De Sluis, B., Taskinen, M.-R. R., Tokgözoğlu, L., Landmesser, U., Laufs, U., Wiklund, O., Stock, J. K., Chapman, M. J., Catapano, A. L., Watt, G. F., Borén, J., Fazio, S., Horton, J. D., Masana, L., Nicholls, S. J., Nordestgaard, B. G., Van De Sluis, B., Taskinen, M.-R. R., Tokgözoğlu, L., Landmesser, U., Laufs, U., Wiklund, O., Stock, J. K., Chapman, M. J., Catapano, A. L., Watts, G. F., Borén, J., Fazio, S., Horton, J. D., Masana, L., Nicholls, S. J., Nordestgaard, B. G., Van De Sluis, B., Taskinen, M.-R. R., Tokgözoğlu, L., Landmesser, U., Laufs, U., Wiklund, O., Stock, J. K., Chapman, M. J., Catapano, A. L., Watt, G. F., Borén, J., Fazio, S., Horton, J. D., Masana, L., Nicholls, S. J., Nordestgaard, B. G., Van De Sluis, B., Taskinen, M.-R. R., Tokgözoğlu, L., Landmesser, U., Laufs, U., Wiklund, O., Stock, J. K., Chapman, M. J. and Catapano, A. L. (2017). Low-density lipoproteins cause atherosclerotic cardiovascular disease. 1. Evidence from genetic, epidemiologic, and clinical studies. A consensus statement from the European Atherosclerosis Society Consensus Panel. *European Heart Journal*, 38(32), 2459–2472. doi: 10.1093/eurheartj/ehx144.

Ferenchick, G. S., Hirokawa, S., Mammen, E. F. and Schwartz, K. A. (1995). Anabolic-Androgenic steroid abuse in weight lifters: Evidence for activation of the hemostatic system. *American Journal of Hematology*, 49(4), 282–288. doi: 10.1002/ajh.2830490405.

Ferrando, A. A., Tipton, K. D., Doyle, D., Phillips, S. M., Cortiella, J. and Wolfe, R. R. (1998). Testosterone injection stimulates net protein synthesis but not tissue amino acid transport. *American Journal of Physiology - Endocrinology and Metabolism*, 275(5 38-5), 864–871. doi: 10.1152/ajpendo.1998.275.5.e864.

Fisher, F. M. and Maratos-Flier, E. (2016). Understanding the Physiology of FGF21. *Annual Review of Physiology*, 78(1), 223–241. doi: 10.1146/annurev-physiol-021115-105339.

Fleming, K. K. and Hubel, A. (2006). Cryopreservation of hematopoietic and non-hematopoietic stem cells. *Transfusion and Apheresis Science*, 34(3), 309–315. doi: 10.1016/j.transci.2005.11.012.

Flück, M. and Hoppeler, H. (2003). Molecular basis of skeletal muscle plasticity--from gene to form and function. *Reviews of physiology, biochemistry and pharmacology*, 146, 159–216. doi: 10.1007/s10254-002-0004-7.

Foster, G. D., Wyatt, H. R., Hill, J. O., Makris, A. P., Rosenbaum, D. L., Brill, C., Stein, R. I., Mohammed, S., Miller, B., Rader, D. J., Zemel, B., Wadden, T. A., Tenhave, T., Newcomb, C. W. and Klein, S. (2010). Weight and metabolic outcomes after 2 years on a low-carbohydrate versus low-fat diet. *Obstetrical and Gynecological Survey*, 65(12), 769–770. doi: 10.1097/OGX.0b013e31821342ba.

Foulstone, E. J., Huser, C., Crown, A. L., Holly, J. M. P. and Stewart, C. E. H. (2004). Differential signalling mechanisms predisposing primary human skeletal muscle cells to altered proliferation and differentiation: Roles of IGF-I and TNFα. *Experimental Cell Research*, 294(1), 223–235. doi: 10.1016/j.yexcr.2003.10.034.

Frankenfeld, S. P., de Oliveira, L. P., Ignacio, D. L., Coelho, R. G., Mattos, M. N., Ferreira, A. C. F., Carvalho, D. P. and Fortunato, R. S. (2014). Nandrolone decanoate inhibits gluconeogenesis and decreases fasting glucose in Wistar male rats. *Journal of Endocrinology*, 220(2), 143–153. doi: 10.1530/JOE-13-0259.

Freeman, M. W. and Walford, G. A. (2015). Lipoprotein Metabolism and the Treatment of Lipid Disorders. *Endocrinology: Adult and Pediatric*. Elsevier, 1–2, 715-736.e7. doi: 10.1016/B978-0-323-18907-1.00041-X.

Freiberg, J. J., Tybjaerg-Hansen, A., Jensen, J. S. and Nordestgaard, B. G. (2008). Nonfasting Triglycerides and Risk of Ischemic Stroke in the General Population. *JAMA*, 300(18), 2142. doi: 10.1001/jama.2008.621.

Freshney, R. I. (2015). *Culture of Animal Cells : A Manual of Basic Technique and Specialized Applications*. 7th edn. Edited by A. Capes-Davis, C. Gregory, and S. Przyborski. John Wiley and Sons Ltd.

Frontera, W. R. and Ochala, J. (2015). Skeletal Muscle: A Brief Review of Structure and Function. *Behavior Genetics*, 45(2), 183–195. doi: 10.1007/s00223-014-9915-y.

Frost, R. A. and Lang, C. H. (2007). Protein kinase B/Akt: A nexus of growth factor and cytokine signaling in determining muscle mass. *Journal of Applied Physiology*, 103(1), 378–387. doi: 10.1152/japplphysiol.00089.2007.

Frühbeck, G., Catalán, V., Rodríguez, A., Ramírez, B., Becerril, S., Salvador, J., Colina, I. and Gómez-Ambrosi, J. (2019). Adiponectin-leptin ratio is a functional biomarker of adipose tissue inflammation. *Nutrients*, 11(2), 1–13. doi: 10.3390/nu11020454.

Frühbeck, G., Catalán, V., Rodríguez, A., Ramírez, B., Becerril, S., Salvador, J., Portincasa, P., Colina,

I. and Gómez-Ambrosi, J. (2017). Involvement of the leptin-adiponectin axis in inflammation and oxidative stress in the metabolic syndrome. *Scientific Reports*, 7(1), 1–8. doi: 10.1038/s41598-017-06997-0.

Fuster, V., Moreno, P. R., Fayad, Z. A., Corti, R. and Badimon, J. J. (2005). Atherothrombosis and high-risk plaque: Part I: Evolving concepts. *Journal of the American College of Cardiology*. Elsevier Masson SAS, 46(6), 937–954. doi: 10.1016/j.jacc.2005.03.074.

Galmés-Pascual, B. M., Martínez-Cignoni, M. R., Morán-Costoya, A., Bauza-Thorbrügge, M., Sbert-Roig, M., Valle, A., Proenza, A. M., Lladó, I. and Gianotti, M. (2020). 17B-Estradiol Ameliorates Lipotoxicity-Induced Hepatic Mitochondrial Oxidative Stress and Insulin Resistance. *Free Radical Biology and Medicine*. Elsevier B.V., 150(November 2019), 148–160. doi: 10.1016/j.freeradbiomed.2020.02.016.

Gao, X., Zhang, W., Wang, Y., Pedram, P., Cahill, F., Zhai, G., Randell, E., Gulliver, W. and Sun, G. (2016). Serum metabolic biomarkers distinguish metabolically healthy peripherally obese from unhealthy centrally obese individuals. *Nutrition & metabolism*. Nutrition & Metabolism, 13, 33. doi: 10.1186/s12986-016-0095-9.

Gardner, C. D., Trepanowski, J. F., Gobbo, L. C. D., Hauser, M. E., Rigdon, J., Ioannidis, J. P. A., Desai, M. and King, A. C. (2018). Effect of low-fat VS low-carbohydrate diet on 12-month weight loss in overweight adults and the association with genotype pattern or insulin secretion the DIETFITS randomized clinical trial. *JAMA - Journal of the American Medical Association*, 319(7), 667–679. doi: 10.1001/jama.2018.0245.

Garofalo, R. S., Mcneish, J. D., Coleman, K. G., Garofalo, R. S., Orena, S. J., Rafidi, K., Torchia, A. J., Stock, J. L., Hildebrandt, A. L., Coskran, T., Black, S. C., Brees, D. J., Wicks, J. R., Mcneish, J. D. and Coleman, K. G. (2003). Severe diabetes , age-dependent loss of adipose tissue , and mild growth deficiency in mice lacking Akt2 / PKB b Find the latest version : Severe diabetes , age-dependent loss of adipose tissue , and mild growth deficiency in mice lacking Akt2 / PKB β., 112(2), 197–208. doi: 10.1172/JCI200316885.Introduction.

Gastaldelli, A. (2011). Role of beta-cell dysfunction, ectopic fat accumulation and insulin resistance in the pathogenesis of type 2 diabetes mellitus. *Diabetes Research and Clinical Practice*. Elsevier Ireland Ltd, 93(SUPPL. 1), S60–S65. doi: 10.1016/S0168-8227(11)70015-8.

Gaster, M. (2019). The diabetic phenotype is preserved in myotubes established from type 2 diabetic subjects: a critical appraisal. *Apmis*, 127(1), 3–26. doi: 10.1111/apm.12908.

German, J. P., Wisse, B. E., Thaler, J. P., Oh-I, S., Sarruf, D. A., Ogimoto, K., Kaiyala, K. J., Fischer, J. D., Matsen, M. E., Taborsky, G. J., Schwartz, M. W. and Morton, G. J. (2010). Leptin deficiency causes insulin resistance induced by uncontrolled diabetes. *Diabetes*, 59(7), 1626–1634. doi: 10.2337/db09-1918.

Gjuladin-Hellon, T., Davies, I. G., Penson, P. and Baghbadorani, R. A. (2019). Effects of carbohydrate-

restricted diets on low-density lipoprotein cholesterol levels in overweight and obese adults: A systematic review and meta-analysis. *Nutrition Reviews*, 77(3), 161–180. doi: 10.1093/nutrit/nuy049.

Glazer, G. (1991). Atherogenic Effects of Anabolic Steroids on Serum Lipid Levels: A Literature Review. *Archives of Internal Medicine*, 151(10), 1925–1933. doi: 10.1001/archinte.1991.00400100013003.

Glazer, G. and Suchman, A. L. (1994). Lack of demonstrated effect of nandrolone on serum lipids. *Metabolism*, 43(2), 204–210. doi: 10.1016/0026-0495(94)90246-1.

Goldman, A. L., Pope, H. G. and Bhasin, S. (2019). The health threat posed by the hidden epidemic of anabolic steroid use and body image disorders among young men. *Journal of Clinical Endocrinology and Metabolism*, 104(4), 1069–1074. doi: 10.1210/jc.2018-01706.

Gómez-Ambrosi, J., Gallego-Escuredo, J. M., Catalán, V., Rodríguez, A., Domingo, P., Moncada, R., Valentí, V., Salvador, J., Giralt, M., Villarroya, F. and Frühbeck, G. (2017). FGF19 and FGF21 serum concentrations in human obesity and type 2 diabetes behave differently after diet- or surgically-induced weight loss. *Clinical Nutrition*, 36(3), 861–868. doi: 10.1016/j.clnu.2016.04.027.

Gonzalez, E. and McGraw, T. E. (2009). The Akt kinases: Isoform specificity in metabolism and cancer. *Cell Cycle*, 8(16), 2502–2508. doi: 10.4161/cc.8.16.9335.

Goodpaster, B. H. and Sparks, L. M. (2017). Metabolic Flexibility in Health and Disease. *Cell Metabolism*, 25(5), 1027–1036. doi: 10.1016/j.cmet.2017.04.015.

Gordon, B. A., Benson, A. C., Bird, S. R. and Fraser, S. F. (2009). Resistance training improves metabolic health in type 2 diabetes: A systematic review. *Diabetes Research and Clinical Practice*, 83(2), 157–175. doi: 10.1016/j.diabres.2008.11.024.

Gower, B. A. and Goss, A. M. (2015). A Lower-Carbohydrate , Higher-Fat Diet Reduces Abdominal and Intermuscular Fat and Increases Insulin Sensitivity in Adults at Risk of. *Journal of Nutrition*, 145(1), 177–183. doi: 10.3945/jn.114.195065.177S.

Greenland, S., Senn, S. J., Rothman, K. J., Carlin, J. B., Poole, C., Goodman, S. N. and Altman, D. G. (2016). Statistical tests, P values, confidence intervals, and power: a guide to misinterpretations. *European Journal of Epidemiology*. Springer Netherlands, 31(4), 337–350. doi: 10.1007/s10654-016-0149-3.

Griffiths, S., Murray, S. B., Krug, I. and McLean, S. A. (2018). The Contribution of Social Media to Body Dissatisfaction, Eating Disorder Symptoms, and Anabolic Steroid Use among Sexual Minority Men. *Cyberpsychology, Behavior, and Social Networking*, 21(3), 149–156. doi: 10.1089/cyber.2017.0375.

Griggs, R. C., Kingston, W., Jozefowicz, R. F., Herr, B. E., Forbes, G. and Halliday, D. (1989). Effect of testosterone on muscle mass and muscle protein synthesis. *Journal of Applied Physiology*, 66(1), 498–503. doi: 10.1152/jappl.1989.66.1.498.

Grundy, S. M. (2016). Metabolic syndrome update. *Trends in Cardiovascular Medicine*. Elsevier, 26(4), 364–373. doi: 10.1016/j.tcm.2015.10.004.

Gu, Y., Zhao, A., Huang, F., Zhang, Y., Liu, J., Wang, C., Jia, Wei, Xie, G. and Jia, Weiping (2013). Very low carbohydrate diet significantly alters the serum metabolic profiles in obese subjects. *Journal of Proteome Research*, 12(12), 5801–5811. doi: 10.1021/pr4008199.

Gupte, A. A., Pownall, H. J. and Hamilton, D. J. (2015). Estrogen: An Emerging Regulator of Insulin Action and Mitochondrial Function. *Journal of Diabetes Research*, 2015, 1–9. doi: 10.1155/2015/916585.

Haeusler, R. A., McGraw, T. E. and Accili, D. (2018). Metabolic Signalling: Biochemical and cellular properties of insulin receptor signalling. *Nature Reviews Molecular Cell Biology*. Nature Publishing Group, 19(1), 31–44. doi: 10.1038/nrm.2017.89.

Hall, K. D., Bemis, T., Brychta, R., Chen, K. Y., Courville, A., Crayner, E. J., Goodwin, S., Guo, J., Howard, L., Knuth, N. D., Miller, B. V., Prado, C. M., Siervo, M., Skarulis, M. C., Walter, M., Walter, P. J. and Yannai, L. (2015). Calorie for calorie, dietary fat restriction results in more body fat loss than carbohydrate restriction in people with obesity. *Cell Metabolism*. Elsevier Inc., 22(3), 427–436. doi: 10.1016/j.cmet.2015.07.021.

Hall, K. D., Chen, K. Y., Guo, J., Lam, Y. Y., Leibel, R. L., Mayer, L. E. S., Reitman, M. L., Rosenbaum, M., Smith, S. R., Walsh, B. T. and Ravussin, E. (2016). Energy expenditure and body composition changes after an isocaloric ketogenic diet in overweight and obese men 1 , 2., (C), 1–10. doi: 10.3945/ajcn.116.133561.

Hall, K. D. and Guo, J. (2017). Obesity Energetics: Body Weight Regulation and the Effects of Diet Composition. *Gastroenterology*. Elsevier, Inc, 152(7), 1718-1727.e3. doi: 10.1053/j.gastro.2017.01.052.

Hall, K. D., Guo, J., Chen, K. Y., Leibel, R. L., Reitman, M. L., Rosenbaum, M., Smith, S. R. and Ravussin, E. (2019). Methodologic considerations for measuring energy expenditure differences between diets varying in carbohydrate using the doubly labeled water method. *American Journal of Clinical Nutrition*. Oxford University Press, 109(5), 1328–1334. doi: 10.1093/ajcn/nqy390.

Hamdi, M. M. and Mutungi, G. (2010). Dihydrotestosterone activates the MAPK pathway and modulates maximum isometric force through the EGF receptor in isolated intact mouse skeletal muscle fibres. *Journal of Physiology*, 588(3), 511–525. doi: 10.1113/jphysiol.2009.182162.

Hardie, D. G., Ross, F. A. and Hawley, S. A. (2012). AMPK: A nutrient and energy sensor that maintains energy homeostasis. *Nature Reviews Molecular Cell Biology*. Nature Publishing Group, 13(4), 251–262. doi: 10.1038/nrm3311.

Haren, M. T., Siddiqui, A. M., Armbrecht, H. J., Kevorkian, R. T., Kim, M. J., Haas, M. J., Mazza, A., Kumar, V. B., Green, M., Banks, W. A. and Morley, J. E. (2011). Testosterone modulates gene expression pathways regulating nutrient accumulation, glucose metabolism and protein turnover in mouse skeletal muscle. *International Journal of Andrology*, 34(1), 55–68. doi: 10.1111/j.1365-2605.2010.01061.x.

Hartgens, F. and Kuipers, H. (2004). Effects of androgenic-anabolic steroids in athletes. *Sports Medicine*, 34(8), 513–554. doi: 10.2165/00007256-200434080-00003.

Hartgens, F., Rietjens, G., Keizer, H. A., Kuipers, H. and Wolffenbuttel, B. H. R. (2004). Effects of androgenic-anabolic steroids on apolipoproteins and lipoprotein (a). *British Journal of Sports Medicine*, 38(3), 253–259. doi: 10.1136/bjsm.2003.000199.

van Hees, V. T., Gorzelniak, L., Dean León, E. C., Eder, M., Pias, M., Taherian, S., Ekelund, U., Renström, F., Franks, P. W., Horsch, A. and Brage, S. (2013). Separating Movement and Gravity Components in an Acceleration Signal and Implications for the Assessment of Human Daily Physical Activity. *PLoS ONE*, 8(4), 1–10. doi: 10.1371/journal.pone.0061691.

Herbst, K. L., Amory, J. K., Brunzell, J. D., Chansky, H. A. and Bremner, W. J. (2003). Testosterone administration to men increases hepatic lipase activity and decreases HDL and LDL size in 3 wk. *American Journal of Physiology - Endocrinology and Metabolism*, 284(6 47-6), 1112–1118.

Herzig, S. and Shaw, R. J. (2018). AMPK: guardian of metabolism and mitochondrial homeostasis. *Nature Reviews Molecular Cell Biology*, 19(2), 121–135. doi: 10.1038/nrm.2017.95.AMPK.

Hite, A. H., Berkowitz, V. G. and Berkowitz, K. (2011). Low-carbohydrate diet review: Shifting the paradigm. *Nutrition in Clinical Practice*, 26(3), 300–308. doi: 10.1177/0884533611405791.

Hobbs, C. J., Jones, R. E. and Plymate, S. R. (1996). Nandrolone, a 19-Nortestosterone, Enhances insulin-independent glucose uptake in normal men. *journal of clinical end*, 81(4), 3–6.

Hoefner, D. M., Hodel, S. D., O'Brien, J. F., Branum, E. L., Sun, D., Meissner, I. and McConnell, J. P. (2001). Development of a rapid, quantitative method for LDL subfractionation with use of the quantimetrix lipoprint LDL system. *Clinical Chemistry*, 47(2), 266–274. doi: 10.1093/clinchem/47.2.266.

Holmang, A. and Bjorntorp, P. (1992). The effects of testosterone on insulin sensitivity in male rats. *Acta Physiologica Scandinavica*, 146(4), 505–510. doi: 10.1111/j.1748-1716.1992.tb09452.x.

Hoogeveen, R. C., Gaubatz, J. W., Sun, W., Dodge, R. C., Crosby, J. R., Jiang, J., Couper, D., Virani, S. S., Kathiresan, S., Boerwinkle, E. and Ballantyne, C. M. (2014). Small dense low-density lipoprotein-cholesterol concentrations predict risk for coronary heart disease: The Atherosclerosis Risk in Communities (ARIC) study. *Arteriosclerosis, Thrombosis, and Vascular Biology*, 34(5), 1069–1077. doi: 10.1161/ATVBAHA.114.303284.

Hooper, L., Martin, N., Abdelhamid, A. and Davey Smith, G. (2015). Reduction in saturated fat intake for cardiovascular disease. *Cochrane Database of Systematic Reviews*. John Wiley and Sons Ltd. doi: 10.1002/14651858.CD011737.

Hung, J., McQuillan, B. M., Thompson, P. L. and Beilby, J. P. (2008). Circulating adiponectin levels associate with inflammatory markers, insulin resistance and metabolic syndrome independent of obesity. *International Journal of Obesity*, 32(5), 772–779. doi: 10.1038/sj.ijo.0803793.

Huntriss, R., Campbell, M. and Bedwell, C. (2018). The interpretation and effect of a low-carbohydrate

diet in the management of type 2 diabetes: A systematic review and meta-analysis of randomised controlled trials. *European Journal of Clinical Nutrition*. Springer US, 72(3), 311–325. doi: 10.1038/s41430-017-0019-4.

Hyde, P. N., Krauss, R. M. and Volek, J. S. (2019). Dietary carbohydrate restriction improves metabolic syndrome independent of weight loss Graphical abstract Clinical Medicine Metabolism Find the latest version: C L I N I C A L M E D I C I N E., 4(12). doi: 10.1172/jci.insight.128308.

Hyde, P. N., Sapper, T. N., Crabtree, C. D., LaFountain, R. A., Bowling, M. L., Buga, A., Fell, B., McSwiney, F. T., Dickerson, R. M., Miller, V. J., Scandling, D., Simonetti, O. P., Phinney, S. D., Kraemer, W. J., King, S. A., Krauss, R. M. and Volek, J. S. (2019). Dietary carbohydrate restriction improves metabolic syndrome independent of weight loss. *JCI Insight*, 4(12), 1–16. doi: 10.1172/jci.insight.128308.

Idzikowska, K. and Zielińska, M. (2018). Midregional pro-atrial natriuretic peptide, an important member of the natriuretic peptide family: potential role in diagnosis and prognosis of cardiovascular disease. *Journal of International Medical Research*, 46(8), 3017–3029. doi: 10.1177/0300060518786907.

Ihalainen, J. K., Inglis, A., Mäkinen, T., Newton, R. U., Kainulainen, H., Kyröläinen, H. and Walker, S. (2019). Strength training improves metabolic health markers in older individual regardless of training frequency. *Frontiers in Physiology*, 10(FEB), 1–12. doi: 10.3389/fphys.2019.00032.

Ivanova, E. A., Myasoedova, V. A., Melnichenko, A. A., Grechko, A. V. and Orekhov, A. N. (2017). Small Dense Low-Density Lipoprotein as Biomarker for Atherosclerotic Diseases. *Oxidative Medicine and Cellular Longevity*. Elsevier Ltd, 2017(2), 1–10. doi: 10.1155/2017/1273042.

Izumiya, Y., Bina, H. A., Ouchi, N., Akasaki, Y., Kharitonenkov, A. and Walsh, K. (2008). FGF21 is an Akt-regulated myokine. *FEBS Letters*, 582(27), 3805–3810. doi: 10.1016/j.febslet.2008.10.021.

Jaiswal, N., Gavin, M. G., Quinn, W. J., Luongo, T. S., Gelfer, R. G., Baur, J. A. and Titchenell, P. M. (2019). The role of skeletal muscle Akt in the regulation of muscle mass and glucose homeostasis. *Molecular Metabolism*. Elsevier GmbH, 28(August), 1–13. doi: 10.1016/j.molmet.2019.08.001.

Jebb, S. a, Lovegrove, J. a, Griffin, B. a, Frost, G. S., Moore, C. S., Chatfield, M. D., Bluck, L. J., Williams, C. M., Ab, T. and Group, S. (2010). Effect of changing the amount and type of fat and carbohydrate on insulin sensitivity and cardiovascular risk : the RISCK trial. *The Americal Journal of Clinical Nutrition*, 92, 748–758. doi: 10.3945/ajcn.2009.29096.1.

Jenkins, D. J. A., Kendall, C. W. C., Augustin, L. S. A., Mitchell, S., Sahye-Pudaruth, S., Blanco Mejia, S., Chiavaroli, L., Mirrahimi, A., Ireland, C., Bashyam, B., Vidgen, E., De Souza, R. J., Sievenpiper, J. L., Coveney, J., Leiter, L. A. and Josse, R. G. (2012). Effect of legumes as part of a low glycemic index diet on glycemic control and cardiovascular risk factors in type 2 diabetes mellitus: A randomized controlled trial. *Archives of Internal Medicine*, 172(21), 1653–1660. doi: 10.1001/2013.jamainternmed.70.

Jeon, J. Y., Choi, S. E., Ha, E. S., Kim, T. H., Jung, J. G., Han, S. J., Kim, H. J., Kim, D. J., Kang, Y.

and Lee, K. W. (2016). Association between insulin resistance and impairment of FGF21 signal transduction in skeletal muscles. *Endocrine*. Springer US, 53(1), 97–106. doi: 10.1007/s12020-015-0845-x.

Jiang, Q., Lou, K., Hou, L., Lu, Y., Sun, L., Tan, S. C., Low, T. Y., Kord-Varkaneh, H. and Pang, S. (2020). The effect of resistance training on serum insulin-like growth factor 1(IGF-1): A systematic review and meta-analysis. *Complementary Therapies in Medicine*. Elsevier, 50(February), 102360. doi: 10.1016/j.ctim.2020.102360.

Jones, T. H., Arver, S., Behre, H. M., Buvat, J., Meuleman, E., Moncada, I., Morales, A. M., Volterrani, M., Yellowlees, A., Howell, J. D. and Channer, K. S. (2011). Testosterone replacement in hypogonadal men with Type 2 diabetes and/or metabolic syndrome (the TIMES2 study). *Diabetes Care*, 34(4), 828–837. doi: 10.2337/dc10-1233.

Kahn, N. N., Sinha, A. K., Spungen, A. M. and Bauman, W. A. (2006). Effects of oxandrolone, an anabolic steroid, on hemostasis. *American Journal of Hematology*, 81(2), 95–100. doi: 10.1002/ajh.20532.

Kalampouka, I., van Bekhoven, A. and Elliott, B. T. (2018). Differing effects of younger and older human plasma on C2C12 myocytes in vitro. *Frontiers in Physiology*, 9(FEB), 1–7. doi: 10.3389/fphys.2018.00152.

Kanayama, G., Hudson, J. I., Deluca, J., Isaacs, S., Baggish, A., Weiner, R., Bhasin, S. and Pope, H. G. (2015). Prolonged hypogonadism in males following withdrawal from anabolic-androgenic steroids: An under-recognized problem. *Addiction*, 110(5), 823–831. doi: 10.1111/add.12850.

Kanayama, G. and Pope, H. G. (2018). History and epidemiology of anabolic androgens in athletes and non-athletes. *Molecular and Cellular Endocrinology*. Elsevier Ireland Ltd, 464, 4–13. doi: 10.1016/j.mce.2017.02.039.

Kang, Y. E., Kim, J. M., Joung, K. H., Lee, J. H., You, B. R., Choi, M. J., Ryu, M. J., Ko, Y. B., Lee, M. A., Lee, J., Ku, B. J., Shong, M., Lee, K. H. and Kim, H. J. (2016). The roles of adipokines, proinflammatory cytokines, and adipose tissue macrophages in obesity-associated insulin resistance in modest obesity and early metabolic dysfunction. *PLoS ONE*, 11(4), 1–14. doi: 10.1371/journal.pone.0154003.

Karakas, M., Zierer, A., Herder, C., Baumert, J., Meisinger, C., Koenig, W. and Thorand, B. (2010). Leptin, adiponectin, their ratio and risk of coronary heart disease: Results from the MONICA/KORA Augsburg Study 1984-2002. *Atherosclerosis*. Elsevier Ireland Ltd, 209(1), 220–225. doi: 10.1016/j.atherosclerosis.2009.08.020.

Karlsson, H. K. R., Zierath, J. R., Kane, S., Krook, A., Lienhard, G. E. and Wallberg-henriksson, H. (2005). Insulin-Stimulated Phosphorylation of the Akt Substrate Diabetic Subjects. *Diabetes*, 54, 1692–1697. doi: 10.2337/DIABETES.54.6.1692.

Karlsson, T., Rask-Andersen, M., Pan, G., Höglund, J., Wadelius, C., Ek, W. E. and Johansson, Å.

(2019). Contribution of genetics to visceral adiposity and its relation to cardiovascular and metabolic disease. *Nature Medicine*. Springer US, 25(9), 1390–1395. doi: 10.1038/s41591-019-0563-7.

Karpe, F., Dickmann, J. R. and Frayn, K. N. (2011). Fatty acids, obesity, and insulin resistance: Time for a reevaluation. *Diabetes*, 60(10), 2441–2449. doi: 10.2337/db11-0425.

Katzmann, J. L. and Laufs, U. (2019). New Insights in the Control of Low-Density Lipoprotein Cholesterol to Prevent Cardiovascular Disease. *Current Cardiology Reports*. Current Cardiology Reports, 21(8). doi: 10.1007/s11886-019-1159-z.

Kaur, J. (2014). A comprehensive review on metabolic syndrome. *Cardiology Research and Practice*. Hindawi Publishing Corporation, 2014. doi: 10.1155/2014/943162.

Kazyken, D., Magnuson, B., Bodur, C., Acosta-Jaquez, H. A., Zhang, D., Tong, X., Barnes, T. M., Steinl, G. K., Patterson, N. E., Altheim, C. H., Sharma, N., Inoki, K., Cartee, G. D., Bridges, D., Yin, L., Riddle, S. M. and Fingar, D. C. (2019). AMPK directly activates mTORC2 to promote cell survival during acute energetic stress. *Science Signaling*, 12(585). doi: 10.1126/scisignal.aav3249.

Kelley, D. E., Goodpaster, B., Wing, R. R. and Simoneau, J. A. (1999). Skeletal muscle fatty acid metabolism in association with insulin resistance, obesity, and weight loss. *American Journal of Physiology - Endocrinology and Metabolism*, 277(6 40-6). doi: 10.1152/ajpendo.1999.277.6.e1130.

Kelley, D., Mitrakou, A., Marsh, H., Schwenk, F., Benn, J., Sonnenberg, G., Arcangeli, M., Aoki, T., Sorensen, J., Berger, M., Sonksen, P. and Gerich, J. (1988). Skeletal muscle glycolysis, oxidation, and storage of an oral glucose load. *Journal of Clinical Investigation*, 81(5), 1563–1571. doi: 10.1172/JCI113489.

Kelli, H. M., Kassas, I. and Lattouf, O. M. (2015). Diabetes & Metabolism Cardio Metabolic Syndrome : A Global Epidemic., 6(3). doi: 10.4172/2155-6156.100051.

Kelly, D. M. and Jones, T. H. (2013). Testosterone: A metabolic hormone in health and disease. *Journal of Endocrinology*, 217(3). doi: 10.1530/JOE-12-0455.

Keren, A., Tamir, Y. and Bengal, E. (2006). The p38 MAPK signaling pathway: A major regulator of skeletal muscle development. *Molecular and Cellular Endocrinology*, 252(1–2), 224–230. doi: 10.1016/j.mce.2006.03.017.

Keys, A., Aravanis, C., Blackburn, H. W., Van Buchem, F. S., Buzina, R., Djordjević, B. D., Dontas, A. S., Fidanza, F., Karvonen, M. J., Kimura, N., Lekos, D., Monti, M., Puddu, V. and Taylor, H. L. (1966). Epidemiological studies related to coronary heart disease: characteristics of men aged 40-59 in seven countries. *Acta medica Scandinavica. Supplementum*, 460, 1–392. Available at: http://www.ncbi.nlm.nih.gov/pubmed/5226858.

Khaleghi, M., Saleem, U., Morgenthaler, N. G., Turner, S. T., Bergmann, A., Struck, J., Mosley, T. H. and Kullo, I. J. (2009). Plasma midregional pro-atrial natriuretic peptide is associated with blood pressure indices and hypertension severity in adults with hypertension. *American Journal of*

Hypertension, 22(4), 425–431. doi: 10.1038/ajh.2009.8.

Kharitonenkov, A. and Larsen, P. (2011). FGF21 reloaded: Challenges of a rapidly growing field. *Trends in Endocrinology and Metabolism*. Elsevier Ltd, 22(3), 81–86. doi: 10.1016/j.tem.2010.11.003.

Khera, A. V., Cuchel, M., de la Llera-Moya, M., Rodrigues, A., Burke, M. F., Jafri, K., French, B. C., Phillips, J. A., Mucksavage, M. L., Wilensky, R. L., Mohler, E. R., Rothblat, G. H. and Rader, D. J. (2011). Cholesterol Efflux Capacity, High-Density Lipoprotein Function, and Atherosclerosis. *New England Journal of Medicine*, 364(2), 127–135. doi: 10.1056/NEJMoa1001689.

Kim, K. H. and Lee, M. S. (2014). FGF21 as a stress hormone: The roles of FGF21 in stress adaptation and the treatment of metabolic disease. *Diabetes and Metabolism Journal*, 38(4), 245–251. doi: 10.4093/dmj.2014.38.4.245.

Kim, S., Go, G. W. and Imm, J. Y. (2017). Promotion of Glucose Uptake in C2C12 Myotubes by Cereal Flavone Tricin and Its Underlying Molecular Mechanism. *Journal of Agricultural and Food Chemistry*, 65(19), 3819–3826. doi: 10.1021/acs.jafc.7b00578.

Kim, Y. J., Huh, I., Kim, J. Y., Park, S., Ryu, S. H., Kim, K. B., Kim, S., Park, T. and Kwon, O. (2017). Integration of traditional and metabolomics biomarkers identifies prognostic metabolites for predicting responsiveness to nutritional intervention against oxidative stress and inflammation. *Nutrients*, 9(3). doi: 10.3390/nu9030233.

Kirk, E. P. and Klein, S. (2009). Pathogenesis and pathophysiology of the cardiometabolic syndrome. *Journal of Clinical Hypertension*, 11(12), 761–765. doi: 10.1111/j.1559-4572.2009.00054.x.

Kjøbsted, R., Munk-Hansen, N., Birk, J. B., Foretz, M., Viollet, B., Björnholm, M., Zierath, J. R., Treebak, J. T. and Wojtaszewski, J. F. P. (2017). Enhanced muscle insulin sensitivity after contraction/exercise is mediated by AMPK. *Diabetes*, 66(3), 598–612. doi: 10.2337/db16-0530.

Klop, B., Elte, J. W. F. and Cabezas, M. C. (2013). Dyslipidemia in Obesity: Mechanisms and Potential Targets. *Nutrients*, 5(4), 1218–1240. doi: 10.3390/nu5041218.

Klöting, N. and Blüher, M. (2014). Adipocyte dysfunction, inflammation and metabolic syndrome. *Reviews in Endocrine and Metabolic Disorders*, 15(4), 277–287. doi: 10.1007/s11154-014-9301-0.

Knight, J. D. R. and Kothary, R. (2011). The myogenic kinome: Protein kinases critical to mammalian skeletal myogenesis. *Skeletal Muscle*, 1(1), 1–18. doi: 10.1186/2044-5040-1-29.

Kogure, G. S., Silva, R. C., Picchi Ramos, F. K., Miranda-Furtado, C. L., Lara, L. A. D. S., Ferriani, R. A. and Dos Reis, R. M. (2015). Women with polycystic ovary syndrome have greater muscle strength irrespective of body composition. *Gynecological Endocrinology*. Informa UK Ltd, 31(3), 237–242. doi: 10.3109/09513590.2014.982083.

Koh, T. J., Bryer, S. C., Pucci, A. M. and Sisson, T. H. (2005). Mice deficient in plasminogen activator inhibitor-1 have improved skeletal muscle regeneration. *American Journal of Physiology - Cell Physiology*, 289(1 58-1), 217–223. doi: 10.1152/ajpcell.00555.2004.

De Koning, L., Merchant, A. T., Pogue, J. and Anand, S. S. (2007). Waist circumference and waist-to-hip ratio as predictors of cardiovascular events: Meta-regression analysis of prospective studies. *European Heart Journal*, 28(7), 850–856. doi: 10.1093/eurheartj/ehm026.

Kouri, E. M., Pope, H. G. J., L.katz, D. and Oliva, P. (1995). Fat Free Mass Index in Users and Nonusers of Anabolic-Androgenic Steroids. *Clinical Journal of Sport Medicine*, 5, 223–228.

Kouri, E. M., Pope, H. G. and Oliva, P. S. (1996). Changes in lipoprotein-lipid levels in normal men following administration of increasing doses of testosterone cypionate. *Clinical Journal of Sport Medicine*, 152–157. doi: 10.1097/00042752-199607000-00003.

Krauss, R. M. and Blanche, P. J. (1992). Detection and quantitation of LDL subfractions. *Current Opinion in Lipidology*, 3, 377–383.

Krutzik, P. O., Irish, J. M., Nolan, G. P. and Perez, O. D. (2004). Analysis of protein phosphorylation and cellular signaling events by flow cytometry: Techniques and clinical applications. *Clinical Immunology*, 110(3), 206–221. doi: 10.1016/j.clim.2003.11.009.

Kuipers, H., Wijnen, J. A. G., Hartgens, F. and Willems, S. M. M. (1991). Influence of anabolic steroids on body composition, blood pressure, lipid profile and liver functions in body builders. *International Journal of Sports Medicine*, 12(4), 413–418. doi: 10.1055/s-2007-1024704.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, 227(5259), 680–685.

Lakhani, I., Gong, M., Wong, W. T., Bazoukis, G., Lampropoulos, K., Wong, S. H., Wu, W. K. K., Wong, M. C. S., Ong, K. L., Liu, T. and Tse, G. (2018). Fibroblast growth factor 21 in cardio-metabolic disorders: a systematic review and meta-analysis. *Metabolism: Clinical and Experimental*. Elsevier Inc., 83, 11–17. doi: 10.1016/j.metabol.2018.01.017.

Lamphere, L. and Lienhard, G. E. (1992). Components of Signaling Pathways for Insulin and Insulin-Like Growth Factor-I in Muscle Myoblasts and Myotubes. *Endocrinology*, 131(5), 2196–2202.

Lasa, A., Miranda, J., Bulló, M., Casas, R., Salas-Salvadó, J., Larretxi, I., Estruch, R., Ruiz-Gutiérrez, V. and Portillo, M. P. (2014). Comparative effect of two Mediterranean diets versus a low-fat diet on glycaemic control in individuals with type 2 diabetes. *European Journal of Clinical Nutrition*, 68(7), 767–772. doi: 10.1038/ejcn.2014.1.

Lassale, C., Tzoulaki, I., Moons, K. G. M., Sweeting, M., Boer, J., Johnson, L., Huerta, J. M., Agnoli, C., Freisling, H., Weiderpass, E., Wennberg, P., Van Der A, D. L., Arriola, L., Benetou, V., Boeing, H., Bonnet, F., Colorado-Yohar, S. M., Engström, G., Eriksen, A. K., Ferrari, P., Grioni, S., Johansson, M., Kaaks, R., Katsoulis, M., Katzke, V., Key, T. J., Matullo, G., Melander, O., Molina-Portillo, E., Moreno-Iribas, C., Norberg, M., Overvad, K., Panico, S., Quiros, J. R., Saieva, C., Skeie, G., Steffen, A., Stepien, M., Tjønneland, A., Trichopoulou, A., Tumino, R., Van Der Schouw, Y. T., Verschuren, W. M. M., Langenberg, C., Di Angelantonio, E., Riboli, E., Wareham, N. J., Danesh, J. and Butterworth, A. S. (2018). Separate and combined associations of obesity and metabolic health with coronary heart

disease: A pan-European case-cohort analysis. *European Heart Journal*, 39(5), 397–406. doi: 10.1093/eurheartj/ehx448.

Lavoie, H., Gagnon, J. and Therrien, M. (2020). ERK signalling: a master regulator of cell behaviour, life and fate. *Nature Reviews Molecular Cell Biology*. Springer US, 21(10), 607–632. doi: 10.1038/s41580-020-0255-7.

Lawman, H. G., Troiano, R. P., Perna, F. M., Wang, C.-Y., Fryar, C. D. and Ogden, C. L. (2016). Associations of Relative Handgrip Strength and Cardiovascular Disease Biomarkers in U.S. Adults, 2011–2012. *American Journal of Preventive Medicine*, 50(6), 677–683. doi: 10.1016/j.amepre.2015.10.022.

Lee, C. M. Y., Huxley, R. R., Wildman, R. P. and Woodward, M. (2008). Indices of abdominal obesity are better discriminators of cardiovascular risk factors than BMI: a meta-analysis. *Journal of Clinical Epidemiology*, 61(7), 646–653. doi: 10.1016/j.jclinepi.2007.08.012.

Lee, D. K. (2002). Androgen receptor enhances myogenin expression and accelerates differentiation. *Biochemical and Biophysical Research Communications*, 294(2), 408–413. doi: 10.1016/S0006-291X(02)00504-1.

Lehti, M., Donelan, E., Abplanalp, W., Al-Massadi, O., Habegger, K. M., Weber, J., Ress, C., Mansfeld, J., Somvanshi, S., Trivedi, C., Keuper, M., Ograjsek, T., Striese, C., Cucuruz, S., Pfluger, P. T., Krishna, R., Gordon, S. M., Silva, R. A. G. D. A. G. D., Luquet, S., Castel, J., Martinez, S., D'Alessio, D., Davidson, W. S., Hofmann, S. M., D'Alessio, D., Davidson, W. S. and Hofmann, S. M. (2013). High-density lipoprotein maintains skeletal muscle function by modulating cellular respiration in mice. *Circulation*, 128(22), 2364–2371. doi: 10.1161/CIRCULATIONAHA.113.001551.

Lenders, J. W. M., Demacker, P. N. M., Vos, J. A., Jansen, P. L. M., Hoitsma, A. J., van't Laar, A. and Thien, T. (1988). Deleterious Effects of Anabolic Steroids on Serum Lipoproteins, Blood Pressure, and Liver Function in Amateur Body Builders. *international journal of sports medicine*, 9, 19–23.

Lenzi, J. L., Teixeira, E. L., de Jesus, G., Schoenfeld, B. J. and de Salles Painelli, V. (2019). Dietary Strategies of Modern Bodybuilders During Different Phases of the Competitive Cycle. *Journal of Strength and Conditioning Research*, (April), 1. doi: 10.1519/jsc.0000000000003169.

Leontieva, O. V., Demidenko, Z. N. and Blagosklonny, M. V. (2014). Rapamycin reverses insulin resistance (IR) in high-glucose medium without causing IR in normoglycemic medium. *Cell Death and Disease*. Nature Publishing Group, 5(5), 1–7. doi: 10.1038/cddis.2014.178.

Leren, P. (1970). The Oslo Diet-Heart Study. Eleven-year report. *Circulation*, 42, 935–942.

Li, D. T., Habtemichael, E. N., Julca, O., Sales, C. I., Westergaard, X. O., DeVries, S. G., Ruiz, D., Sayal, B. and Bogan, J. S. (2019). GLUT4 storage vesicles: Specialized organelles for regulated trafficking. *Yale Journal of Biology and Medicine*, 92(3), 453–470.

Li, M., Rabkin, S. W., Min, L. and Simon W, R. (2018). Extremely Low HDL Cholesterol and Increased

LDL Cholesterol Induced by the use of Anabolic Steroids in a Body Builder: A Case Study. *International Journal of Sports and Exercise Medicine*, 4(4), 1–4. doi: 10.23937/2469-5718/1510109.

Li, Y., Xu, S., Zhang, X., Yi, Z. and Cichello, S. (2015). Skeletal intramyocellular lipid metabolism and insulin resistance. *Biophysics Reports*. Springer Berlin Heidelberg, 1(2), 90–98. doi: 10.1007/s41048-015-0013-0.

Lin, X., Zhang, X., Guo, J., Roberts, C. K., McKenzie, S., Wu, W. C., Liu, S. and Song, Y. (2015). Effects of exercise training on cardiorespiratory fitness and biomarkers of cardiometabolic health: A systematic review and meta-analysis of randomized controlled trials. *Journal of the American Heart Association*, 4(7), 1–28. doi: 10.1161/JAHA.115.002014.

Lipina, C. and Hundal, H. S. (2017). Lipid modulation of skeletal muscle mass and function. *Journal of Cachexia, Sarcopenia and Muscle*, 8(2), 190–201. doi: 10.1002/jcsm.12144.

Lippi, G., Guidi, G., Ruzzenente, O., Braga, V. and Adami, S. (1997). Effects of nandrolone decanoate (Decadurabolin) on serum Lp(a), lipids and lipoproteins in women with postmenopausal osteoporosis. *Scandinavian Journal of Clinical and Laboratory Investigation*, 57(6), 507–511. doi: 10.3109/00365519709084601.

Liu, C., Feng, X., Li, Qi, Wang, Y., Li, Qian and Hua, M. (2016). Adiponectin, TNF-α and inflammatory cytokines and risk of type 2 diabetes: A systematic review and meta-analysis. *Cytokine*, 86, 100–109. doi: 10.1016/j.cyto.2016.06.028.

Liu, G. Y. and Sabatini, D. M. (2020). mTOR at the nexus of nutrition, growth, ageing and disease. *Nature Reviews Molecular Cell Biology*. Springer US, 21(4), 183–203. doi: 10.1038/s41580-019-0199-y.

Liu, S. Q., Roberts, D., Kharitonenkov, A., Zhang, B., Hanson, S. M., Li, Y. C., Zhang, L. Q. and Wu, Y. H. (2013). Endocrine protection of ischemic myocardium by FGF21 from the liver and adipose tissue. *Scientific Reports*, 3. doi: 10.1038/srep02767.

Liu, Y., Lee, D.-C., Li, Y., Zhu, W., Zhang, R., Sui, X., Lavie, C. J. and Blair, S. N. (2019). Associations of Resistance Exercise with Cardiovascular Disease Morbidity and Mortality. *Medicine & Science in Sports & Exercise*, 51(3), 499–508. doi: 10.1249/MSS.0000000000001822.

Lunn, J. and Theobald, H. E. (2006). The health effects of dietary unsaturated fatty acids. *Nutrition Bulletin*, 31(3), 178–224. doi: 10.1111/j.1467-3010.2006.00571.x.

Ma, W., Huang, T., Zheng, Y., Wang, M., Bray, G. A., Sacks, F. M. and Qi, L. (2016). Weight-loss diets, adiponectin, and changes in cardiometabolic risk in the 2-year pounds lost trial. *Journal of Clinical Endocrinology and Metabolism*, 101(6), 2415–2422. doi: 10.1210/jc.2016-1207.

Mach, F., Baigent, C., Catapano, A. L., Koskinas, K. C., Casula, M., Badimon, L., Chapman, M. J., De Backer, G. G., Delgado, V., Ference, B. A., Graham, I. M., Halliday, A., Landmesser, U., Mihaylova, B., Pedersen, T. R., Riccardi, G., Richter, D. J., Sabatine, M. S., Taskinen, M. R., Tokgozoglu, L., Wiklund,

O., Mueller, C., Drexel, H., Aboyans, V., Corsini, A., Doehner, W., Farnier, M., Gigante, B., Kayikcioglu, M., Krstacic, G., Lambrinou, E., Lewis, B. S., Masip, J., Moulin, P., Petersen, S., Petronio, A. S., Piepoli, M. F., Pinto, X., Raber, L., Ray, K. K., Reiner, Z., Riesen, W. F., Roffi, M., Schmid, J. P., Shlyakhto, E., Simpson, I. A., Stroes, E., Sudano, I., Tselepis, A. D., Viigimaa, M., Vindis, C., Vonbank, A., Vrablik, M., Vrsalovic, M., Gomez, J. L. Z., Collet, J. P., Windecker, S., Dean, V., Fitzsimons, D., Gale, C. P., Grobbee, D. E., Halvorsen, S., Hindricks, G., Iung, B., Jüni, P., Katus, H. A., Leclercq, C., Lettino, M., Merkely, B., Sousa-Uva, M., Touyz, R. M., Nibouche, D., Zelveian, P. H., Siostrzonek, P., Najafov, R., Van De Borne, P., Pojskic, B., Postadzhiyan, A., Kypris, L., Spinar, J., Larsen, M. L., Eldin, H. S., Strandberg, T. E., Ferrières, J., Agladze, R., Laufs, U., Rallidis, L., Bajnok, L., Gudjonsson, T., Maher, V., Henkin, Y., Gulizia, M. M., Mussagaliyeva, A., Bajraktari, G., Kerimkulova, A., Latkovskis, G., Hamoui, O., Slapikas, R., Visser, L., Dingli, P., Ivanov, V., Boskovic, A., Nazzi, M., Visseren, F., Mitevska, I., Retterstøl, K., Jankowski, P., Fontes-Carvalho, R., Gaita, D., Ezhov, M., Foscoli, M., Giga, V., Pella, D., Fras, Z., De Isla, L. P., Hagström, E., Lehmann, R., Abid, L., Ozdogan, O., Mitchenko, O. and Patel, R. S. (2020). 2019 ESC/EAS Guidelines for the management of dyslipidaemias: Lipid modification to reduce cardiovascular risk. *European Heart Journal*, 41(1), 111–188. doi: 10.1093/eurheartj/ehz455.

MacLaren, D. and Morton, J. (2011). *Biochemistry for Sport and Exercise Metabolism*. West Sussex: John Wiley and Sons Ltd.

Maggio, M., Lauretani, F., Ceda, G. P., Bandinelli, S., Basaria, S., Paolisso, G., Giumelli, C., Luci, M., Najjar, S. S., Metter, E. J., Valenti, G., Guralnik, J. and Ferrucci, L. (2010). Estradiol and metabolic syndrome in older Italian men: The InCHIANTI study. *Journal of Andrology*, 31(2), 155–162. doi: 10.2164/jandrol.108.006098.

Mahmud, A. and Feely, J. (2005). Arterial stiffness is related to systemic inflammation in essential hypertension. *Hypertension*, 46(5), 1118–1122. doi: 10.1161/01.HYP.0000185463.27209.b0.

Malarkey, W. B., Strauss, R. H., Leizman, D. J., Liggett, M. and Demers, L. M. (1991). Endocrine effects in female weight lifters who self-administer testosterone and anabolic steroids. *American Journal of Obstetrics and Gynecology*. Mosby, 165(5), 1385–1390. doi: 10.1016/0002-9378(91)90374-z.

Malik, V. S. and Hu, F. B. (2007). Popular weight-loss diets: From evidence to practice. *Nature Clinical Practice Cardiovascular Medicine*, 4(1), 34–41. doi: 10.1038/ncpcardio0726.

Mammi, C., Calanchini, M., Antelmi, A., Cinti, F., Rosano, G. M. C., Lenzi, A., Caprio, M. and Fabbri, A. (2012). Androgens and adipose tissue in males: A complex and reciprocal interplay. *International Journal of Endocrinology*, 2012. doi: 10.1155/2012/789653.

Manco, M., Greco, A. V., Capristo, E., Gniuli, D., De Gaetano, A. and Gasbarrini, G. (2000). Insulin resistance directly correlates with increased saturated fatty acids in skeletal muscle triglycerides. *Metabolism: Clinical and Experimental*, 49(2), 220–224. doi: 10.1016/S0026-0495(00)91377-5.

Manini, T. M. (2010). Energy expenditure and aging. *Ageing Research Reviews*, 9(1), 1–11. doi: 10.1016/j.arr.2009.08.002.

Manning, B. D. and Toker, A. (2017). AKT/PKB Signaling: Navigating the Network. *Cell*. Elsevier Inc., 169(3), 381–405. doi: 10.1016/j.cell.2017.04.001.

Mansoor, N., Vinknes, K. J., Veierod, M. B. and Retterstol, K. (2016). Effects of low-carbohydrate diets v. low-fat diets on body weight and cardiovascular risk factors a meta-analysis of randomised controlled trials. *British Journal of Nutrition*, 115(3), 466–479. doi: 10.1017/S0007114515004699.

Martín-Timón, I. (2014). Type 2 diabetes and cardiovascular disease: Have all risk factors the same strength?. *World Journal of Diabetes*, 5(4), 444. doi: 10.4239/wjd.v5.i4.444.

Martin, B. C., Warram, J. H., Krolewski, A. S., Soeldner, J. S., Kahn, C. R., Martin, B. C. and Bergman, R. N. (1992). Role of glucose and insulin resistance in development of type 2 diabetes mellitus: results of a 25-year follow-up study. *The Lancet*, 340(8825), 925–929. doi: 10.1016/0140-6736(92)92814-V.

Martin, N. M., Abu Dayyeh, B. K. and Chung, R. T. (2008). Anabolic steroid abuse causing recurrent hepatic adenomas and hemorrhage. *World Journal of Gastroenterology*, 14(28), 4573–4575. doi: 10.3748/wjg.14.4573.

Mashili, F. L., Austin, R. L., Deshmukh, A. S., Fritz, T., Caidahl, K., Bergdahl, K., Zierath, J. R., Chibalin, A. V., Moller, D. E., Kharitonenkov, A. and Krook, A. (2011). Direct effects of FGF21 on glucose uptake in human skeletal muscle: Implications for type 2 diabetes and obesity. *Diabetes/Metabolism Research and Reviews*, 27(3), 286–297. doi: 10.1002/dmrr.1177.

Mauro, A. (1961). Satellite cell of skeletal muscle fibers. *The Journal of biophysical and biochemical cytology*, 9, 493–495. doi: 10.1083/jcb.9.2.493.

McArdle, W. D., Katch, F. I. and Katch, V. L. (2015). *Exercise Physiology. Nutrition, Energy and Human Performance*. 8th edn *Journal of Chemical Information and Modeling*. 8th edn. Philadelphia: Wolters Kluwer Health.

McCullough, D., Harrison, T., Lane, K., Boddy, L., Amirabdollahian, F., Schmidt, M., Enright, K., Stewart, C. and Davies, I. (2020). The effect of a low carbohydrate high fat diet on emerging biochemical markers of cardiometabolic risk. *Proceedings of the Nutrition Society*, 79(OCE2), 2020. doi: 10.1017/s0029665120004796.

McVeigh, J. and Begley, E. (2017). Anabolic steroids in the UK: an increasing issue for public health. *Drugs: Education, Prevention and Policy*. Informa UK Limited, trading as Taylor 8 Francis Group, 24(3), 278–285. doi: 10.1080/09687637.2016.1245713.

Medina-Remón, A., Kirwan, R., Lamuela-Raventós, R. M. and Estruch, R. (2018). Dietary patterns and the risk of obesity, type 2 diabetes mellitus, cardiovascular diseases, asthma, and neurodegenerative diseases. *Critical Reviews in Food Science and Nutrition*. Taylor & Francis, 58(2), 262–296. doi: 10.1080/10408398.2016.1158690.

Meng, Y., Bai, H., Wang, S., Li, Z., Wang, Q. and Chen, L. (2017). Efficacy of low carbohydrate diet for type 2 diabetes mellitus management: A systematic review and meta-analysis of randomized controlled

trials. *Diabetes Research and Clinical Practice*, 131, 124–131. doi: 10.1016/j.diabres.2017.07.006.

Meriggiola, M. C., Marcovina, S., Paulsen, C. A. and Bremner, W. J. (1995). Testosterone enanthate at a dose of 200 mg/week decreases HDL-cholesterol levels in healthy men. *International Journal of Andrology*, 18(5), 237–242. doi: 10.1111/ijan.1995.18.5.237.

Mertens, I., Verrijken, A., Michiels, J. J., Van Der Planken, M., Ruige, J. B. and Van Gaal, L. F. (2006). Among inflammation and coagulation markers, PAI-1 is a true component of the metabolic syndrome. *International Journal of Obesity*, 30(8), 1308–1314. doi: 10.1038/sj.ijo.0803189.

Meshkani, R. and Adeli, K. (2009). Hepatic insulin resistance, metabolic syndrome and cardiovascular disease. *Clinical Biochemistry*. The Canadian Society of Clinical Chemists, 42(13–14), 1331–1346. doi: 10.1016/j.clinbiochem.2009.05.018.

Moffatt, R. J., Wallace, M. B. and Sady, S. P. (1990). Effects of anabolic steroids on lipoprotein profiles of female weight lifters. *Physician and Sportsmedicine*, 18(9). doi: 10.1080/00913847.1990.11710139.

Morigny, P., Houssier, M., Mouisel, E. and Langin, D. (2016). Adipocyte lipolysis and insulin resistance. *Biochimie*. Elsevier Ltd, 125, 259–266. doi: 10.1016/j.biochi.2015.10.024.

Muka, T., Nano, J., Jaspers, L., Meun, C., Bramer, W. M., Hofman, A., Dehghan, A., Kavousi, M., Laven, J. S. E. and Franco, O. H. (2017). Associations of steroid sex hormones and sex hormone-binding globulin with the risk of type 2 diabetes in women: A population-based cohort study and meta-Analysis. *Diabetes*, 66(3), 577–586. doi: 10.2337/db16-0473.

Mukund, K. and Subramaniam, S. (2020). Skeletal muscle: A review of molecular structure and function, in health and disease. *Wiley Interdisciplinary Reviews: Systems Biology and Medicine*, 12(1), 1–46. doi: 10.1002/wsbm.1462.

Mulligan, A. A., Luben, R. N., Bhaniani, A., Parry-Smith, D. J., O'Connor, L., Khawaja, A. P., Forouhi, N. G. and Khaw, K. T. (2014). A new tool for converting food frequency questionnaire data into nutrient and food group values: FETA research methods and availability. *BMJ Open*, 4(3). doi: 10.1136/bmjopen-2013-004503.

Muraleedharan, V. and Jones, T. H. (2010). Review: Testosterone and the metabolic syndrome. *Therapeutic Advances in Endocrinology and Metabolism*, 1(5), 207–223. doi: 10.1177/2042018810390258.

Natarajan, L., Pu, M., Fan, J., Levine, R. A., Patterson, R. E., Thomson, C. A., Rock, C. L. and Pierce, J. P. (2010). Measurement error of dietary self-report in intervention trials. *American Journal of Epidemiology*, 172(7), 819–827. doi: 10.1093/aje/kwq216.

Nathan, D. M., Davidson, M. B., DeFronzo, R. A., Heine, R. J., Henry, R. R., Pratley, R., Zinman, B. and Kahn, R. (2007). Impaired fasting glucose and impaired glucose tolerance: Implications for care. *Diabetes Care*, 30(3), 753–759. doi: 10.2337/dc07-9920.

Newgard, C. B. (2017). Metabolomics and Metabolic Diseases: Where Do We Stand?. *Cell Metabolism*.

Elsevier Inc., 25(1), 43–56. doi: 10.1016/j.cmet.2016.09.018.

Nguyen, N. T., Magno, C. P., Lane, K. T., Hinojosa, M. W. and Lane, J. S. (2008). Association of Hypertension, Diabetes, Dyslipidemia, and Metabolic Syndrome with Obesity: Findings from the National Health and Nutrition Examination Survey, 1999 to 2004. *Journal of the American College of Surgeons*. American College of Surgeons, 207(6), 928–934. doi: 10.1016/j.jamcollsurg.2008.08.022.

Niedfeldt, M. W. (2018). Anabolic Steroid Effect on the Liver. *Current Sports Medicine Reports*, 17(3), 97–102. doi: 10.1249/JSR.0000000000000467.

Norde, M. M., Collese, T. S., Giovannucci, E. and Rogero, M. M. (2020). A posteriori dietary patterns and their association with systemic low-grade inflammation in adults: a systematic review and meta-analysis. *Nutrition Reviews*, 0(0), 1–20. doi: 10.1093/nutrit/nuaa010.

Nordestgaard, B. G., Benn, M., Schnohr, P. and Tybjærg-Hansen, A. (2007). Nonfasting triglycerides and risk of myocardial infarction, ischemic heart disease, and death in men and women. *Journal of the American Medical Association*, 298(3), 299–308. doi: 10.1001/jama.298.3.299.

Nordestgaard, B. G. and Varbo, A. (2014). Triglycerides and cardiovascular disease. *The Lancet*, 384(9943), 626–635. doi: 10.1016/S0140-6736(14)61177-6.

Oberhauser, F., Schulte, D. M., Faust, M., Güdelhöfer, H., Hahn, M., Müller, N., Neumann, K., Krone, W. and Laudes, M. (2012). Weight loss due to a very low calorie diet differentially affects insulin sensitivity and interleukin-6 serum levels in nondiabetic obese human subjects. *Hormone and Metabolic Research*, 44(6), 465–470. doi: 10.1055/s-0032-1306341.

Ormazabal, V., Nair, S., Elfeky, O., Aguayo, C., Salomon, C. and Zuñiga, F. A. (2018). Association between insulin resistance and the development of cardiovascular disease. *Cardiovascular Diabetology*. BioMed Central, 17(1), 1–14. doi: 10.1186/s12933-018-0762-4.

Otvos, J. D., Jeyarajah, E. J., Bennett, D. W. and Krauss, R. M. (1992). Development of a proton nuclear magnetic resonance spectroscopic method for determining plasma lipoprotein concentrations and subspecies distributions from a single, rapid measurement. *Clinical Chemistry*, 38(9), 1632–1638. doi: 10.1093/clinchem/38.9.1632.

Otvos, J. D., Mora, S., Shalaurova, I., Greenland, P., MacKey, R. H. and Goff, D. C. (2011). Clinical implications of discordance between low-density lipoprotein cholesterol and particle number. *Journal of Clinical Lipidology*. Mosby, Inc, 5(2), 105–113. doi: 10.1016/j.jacl.2011.02.001.

Packard, C. J., Demant, T., Stewart, J. P., Bedford, D., Caslake, M. J., Schwertfeger, G., Bedynek, A., Shepherd, J. and Seidel, D. (2000). Apolipoprotein B metabolism and the distribution of VLDL and LDL subfractions. *Journal of Lipid Research*, 41(2), 305–317.

Palatini, P., Giada, F., Garavelli, G., Sinisi, F., Mario, L., Michieletto, M. and Baldo-Enzi, G. (1996). Cardiovascular effects of anabolic steroids in weight-trained subjects. *Journal of Clinical Pharmacology*, 36(12), 1132–1140. doi: 10.1002/j.1552-4604.1996.tb04167.x.

Paoli, A., Rubini, A., Volek, J. S. and Grimaldi, K. A. (2013). Beyond weight loss: a review of the therapeutic uses of very-low-carbohydrate (ketogenic) diets. *European journal of clinical nutrition*. Nature Publishing Group, 67(8), 789–96. doi: 10.1038/ejcn.2013.116.

Papandreou, C., Bulló, M., Ruiz-Canela, M., Dennis, C., Deik, A., Wang, D., Guasch-Ferré, M., Yu, E., Razquin, C., Corella, D., Estruch, R., Ros, E., Fitó, M., Fiol, M., Liang, L., Hernández-Alonso, P., Clish, C. B., Martínez-González, M. A., Hu, F. B. and Salas-Salvadó, J. (2019). Plasma metabolites predict both insulin resistance and incident type 2 diabetes: A metabolomics approach within the Prevención con Dieta Mediterránea (PREDIMED) study. *American Journal of Clinical Nutrition*. Oxford University Press, 109(3), 635–647. doi: 10.1093/ajcn/nqy262.

Park, H. S., Park, J. Y. and Yu, R. (2005). Relationship of obesity and visceral adiposity with serum concentrations of CRP, TNF-α and IL-6. *Diabetes Research and Clinical Practice*, 69(1), 29–35. doi: 10.1016/j.diabres.2004.11.007.

Parkinson, A. B. and Evans, N. A. (2006). Anabolic androgenic steroids: A survey of 500 users. *Medicine and Science in Sports and Exercise*, 38(4), 644–651. doi: 10.1249/01.mss.0000210194.56834.5d.

Parr, M. K., Müller-Schöll, A., Kristina Parr, M., Müller-Schöll, A., Parr, M. K., Müller-Schöll, A., Kristina Parr, M. and Müller-Schöll, A. (2018). Pharmacology of doping agents—mechanisms promoting muscle hypertrophy. *AIMS Molecular Science*, 5(2), 145–155. doi: 10.3934/molsci.2018.2.131.

Patel, B., Pauk, M., Amigo-Benavent, M., Nongonierma, A. B., Fitzgerald, R. J., Jakeman, P. M. and Carson, B. P. (2019). A cell-based evaluation of a non-essential amino acid formulation as a non-bioactive control for activation and stimulation of muscle protein synthesis using ex vivo human serum. *PLoS ONE*, 14(11), 1–13. doi: 10.1371/journal.pone.0220757.

Pattyn, N., Cornelissen, V. A., Eshghi, S. R. T. and Vanhees, L. (2013). The effect of exercise on the cardiovascular risk factors constituting the metabolic syndrome: A meta-analysis of controlled trials. *Sports Medicine*, 43(2), 121–133. doi: 10.1007/s40279-012-0003-z.

Petersen, K. F. and Shulman, G. I. (2002). Pathogenesis of skeletal muscle insulin resistance in type 2 diabetes mellitus. *American Journal of Cardiology*, 90(5 SUPPL.), 11–18. doi: 10.1016/S0002-9149(02)02554-7.

Petersen, M. C. and Shulman, G. I. (2018). Mechanisms of insulin action and insulin resistance. *Physiological Reviews*, 98(4), 2133–2223. doi: 10.1152/physrev.00063.2017.

Petrie, J. R., Guzik, T. J. and Touyz, R. M. (2018). Diabetes, Hypertension, and Cardiovascular Disease: Clinical Insights and Vascular Mechanisms. *Canadian Journal of Cardiology*. The Authors, 34(5), 575–584. doi: 10.1016/j.cjca.2017.12.005.

Phillips, C. M., Dillon, C., Harrington, J. M., McCarthy, V. J. C., Kearney, P. M., Fitzgerald, A. P. and Perry, I. J. (2013). Defining Metabolically Healthy Obesity: Role of Dietary and Lifestyle Factors. *PLoS ONE*, 8(10), e76188. doi: 10.1371/journal.pone.0076188.

Pirkmajer, S. and Chibalin, A. V. (2011). Serum starvation: Caveat emptor. *American Journal of Physiology - Cell Physiology*, 301(2), 272–279. doi: 10.1152/ajpcell.00091.2011.

Pope, H. G., Kanayama, G., Ionescu-Pioggia, M. and Hudson, J. I. (2004). Anabolic steroid users' attitudes towards physicians. *Addiction*, 99(9), 1189–1194. doi: 10.1111/j.1360-0443.2004.00781.x.

Pope, H. G., Wood, R. I., Rogol, A., Nyberg, F., Bowers, L. and Bhasin, S. (2014). Adverse health consequences of performance-enhancing drugs: An endocrine society scientific statement. *Endocrine Reviews*, 35(3), 341–375. doi: 10.1210/er.2013-1058.

Preis, S. R., Massaro, J. M., Robins, S. J., Hoffmann, U., Vasan, R. S., Irlbeck, T., Meigs, J. B., Sutherland, P., D'Agostino, R. B., O'Donnell, C. J. and Fox, C. S. (2010). Abdominal subcutaneous and visceral adipose tissue and insulin resistance in the framingham heart study. *Obesity*, 18(11), 2191–2198. doi: 10.1038/oby.2010.59.

Public Health England (2016). *The Eatwell Guide refreshed Nutrition Bulletin*. doi: 10.1111/nbu.12211.

Qiu, C., Zhao, X., Zhou, Q. and Zhang, Z. (2017). High-density lipoprotein cholesterol efflux capacity is inversely associated with cardiovascular risk: A systematic review and meta-analysis. *Lipids in Health and Disease*. Lipids in Health and Disease, 16(1), 1–11. doi: 10.1186/s12944-017-0604-5.

Rader, D. J. and Hovingh, G. K. (2014). HDL and cardiovascular disease. *The Lancet*. Elsevier Ltd, 384(9943), 618–625. doi: 10.1016/S0140-6736(14)61217-4.

Rahman, F. A. and Krause, M. P. (2020). PAI-1, the plasminogen system, and skeletal muscle. *International Journal of Molecular Sciences*, 21(19), 1–22. doi: 10.3390/ijms21197066.

Ramezani Tehrani, F., Amiri, M., Behboudi-Gandevani, S., Bidhendi-Yarandi, R. and Carmina, E. (2020). Cardiovascular events among reproductive and menopausal age women with polycystic ovary syndrome: a systematic review and meta-analysis. *Gynecological Endocrinology*. Taylor & Francis, 36(1), 12–23. doi: 10.1080/09513590.2019.1650337.

Rangel-Huerta, O. D., Pastor-Villaescusa, B. and Gil, A. (2019). *Are we close to defining a metabolomic signature of human obesity? A systematic review of metabolomics studies Metabolomics*. Springer US. doi: 10.1007/s11306-019-1553-y.

Rasmussen, J. J., Schou, M., Madsen, P. L., Selmer, C., Johansen, M. L., Hovind, P., Ulriksen, P. S., Faber, J., Gustafsson, F. and Kistorp, C. (2018). Increased blood pressure and aortic stiffness among abusers of anabolic androgenic steroids. *Journal of Hypertension*, 36(2), 277–285. doi: 10.1097/HJH.0000000000001546.

Rasmussen, J. J., Schou, M., Selmer, C., Johansen, M. L., Gustafsson, F., Frystyk, J., Dela, F., Faber, J. and Kistorp, C. (2017). Insulin sensitivity in relation to fat distribution and plasma adipocytokines among abusers of anabolic androgenic steroids. *Clinical Endocrinology*, 87(3), 249–256. doi: 10.1111/cen.13372.

Reilly, T., George, K., Marfell-Jones, M., Scott, M., Sutton, L. and Wallace, J. A. (2009). How well do

skinfold equations predict percent body fat in elite soccer players?. *International Journal of Sports Medicine*, 30(8), 607–613. doi: 10.1055/s-0029-1202353.

Rhee, E. J., Byrne, C. D. and Sung, K. C. (2017). The HDL cholesterol/apolipoprotein A-I ratio: An indicator of cardiovascular disease. *Current Opinion in Endocrinology, Diabetes and Obesity*, 24(2), 148–153. doi: 10.1097/MED.0000000000000315.

Riaz, H., Khan, S. U., Rahman, H., Shah, N. P., Kaluski, E., Lincoff, A. M. and Nissen, S. E. (2019). Effects of high-density lipoprotein targeting treatments on cardiovascular outcomes: A systematic review and meta-analysis. *European Journal of Preventive Cardiology*, 26(5), 533–543. doi: 10.1177/2047487318816495.

Robins, S. J., Lyass, A., Zachariah, J. P., Massaro, J. M. and Vasan, R. S. (2011). Insulin resistance and the relationship of a dyslipidemia to coronary heart disease: The framingham heart study. *Arteriosclerosis, Thrombosis, and Vascular Biology*, 31(5), 1208–1214. doi: 10.1161/ATVBAHA.110.219055.

Rodríguez-Monforte, M., Sánchez, E., Barrio, F., Costa, B. and Flores-Mateo, G. (2017). Metabolic syndrome and dietary patterns: a systematic review and meta-analysis of observational studies. *European Journal of Nutrition*. Springer Berlin Heidelberg, 56(3), 925–947. doi: 10.1007/s00394-016-1305-y.

Root-Bernstein, R., Podufaly, A. and Dillon, P. F. (2014). Estradiol binds to insulin and insulin receptor decreasing insulin binding in vitro. *Frontiers in Endocrinology*, 5(JUL), 1–13. doi: 10.3389/fendo.2014.00118.

Rossetti, M. L., Steiner, J. L. and Gordon, B. S. (2017). Androgen-mediated regulation of skeletal muscle protein balance. *Molecular and Cellular Endocrinology*, 447(3), 35–44. doi: 10.1016/j.mce.2017.02.031.

Roth, G. A., Abate, D., Abate, K. H., Abay, S. M., Abbafati, C., Abbasi, N., Abbastabar, H., Abd-Allah, F., Abdela, J., Abdelalim, A., Abdollahpour, I., Abdulkader, R. S., Abebe, H. T., Abebe, M., Abebe, Z., Abejie, A. N., Abera, S. F., Abil, O. Z., Abraha, H. N., Abrham, A. R., Abu-Raddad, L. J., Accrombessi, M. M. K., Acharya, D., Adamu, A. A., Adebayo, O. M., Adedoyin, R. A., Adekanmbi, V., Adetokunboh, O. O., Adhena, B. M., Adib, M. G., Admasie, A., Afshin, A., Agarwal, G., Agesa, K. M., Agrawal, A., Agrawal, S., Ahmadi, A., Ahmadi, M., Ahmed, M. B., Ahmed, S., Aichour, A. N., Aichour, I., Aichour, M. T. E., Akbari, M. E., Akinyemi, R. O., Akseer, N., Al-Aly, Z., Al-Eyadhy, A., Al-Raddadi, R. M., Alahdab, F., Alam, K., Alam, T., Alebel, A., Alene, K. A., Alijanzadeh, M., Alizadeh-Navaei, R., Aljunid, S. M., Alkerwi, A., Alla, F., Allebeck, P., Alonso, J., Altirkawi, K., Alvis-Guzman, N., Amare, A. T., Aminde, L. N., Amini, E., Ammar, W., Amoako, Y. A., Anber, N. H., Andrei, C. L., Androudi, S., Animut, M. D., Anjomshoa, M., Ansari, H., Ansha, M. G., Antonio, C. A. T., Anwari, P., Aremu, O., Arnlov, J., Arora, A., Arora, M., Artaman, A., Aryal, K. K., Asayesh, H., Asfaw, E. T., Ataro, Z., Atique, S., Atre, S. R., Ausloos, M., Avokpaho, E. F. G. A., Awasthi, A., Ayala Quintanilla, B. P., Ayele, Y., Ayer, R., Azzopardi, P. S., Babazadeh, A., Bacha, U., Badali, H., Badawi, A., Bali, A. G., Ballesteros, K. E., Banach, M., Banerjee,

K., Bannick, M. S., Banoub, J. A. M., Barboza, M. A., Barker-Collo, S. L., Barnighausen, T. W., Barquera, S., Barrero, L. H., Bassat, Q., Basu, S., Baune, B. T., Baynes, H. W., Bazargan-Hejazi, S., Bedi, N., Beghi, E., Behzadifar, Masoud, Behzadifar, Meysam, Bejot, Y., Bekele, B. B., Belachew, A. B., Belay, E., Belay, Y. A., Bell, M. L., Bello, A. K., Bennett, D. A., Bensenor, I. M., Berman, A. E., Bernabe, E., Bernstein, R. S., Bertolacci, G. J., Beuran, M., Beyranvand, T., Bhalla, A., Bhattarai, S., Bhaumik, S., Bhutta, Z. A., Biadgo, B., Biehl, M. H., Bijani, A., Bikbov, B., Bilano, V., Bililign, N., Bin Sayeed, M. S., Bisanzio, D., Biswas, T., Blacker, B. F., Basara, B. B., Borschmann, R., Bosetti, C., Bozorgmehr, K., Brady, O. J., Brant, L. C., Brayne, C., Brazinova, A., Breitborde, N. J. K., Brenner, H., Briant, P. S., Britton, G., Brugha, T., Busse, R., Butt, Z. A., Callender, C. S. K. H., Campos-Nonato, I. R., Campuzano Rincon, J. C., Cano, J., Car, M., Cardenas, R., Carreras, G., Carrero, J. J., Carter, A., Carvalho, F., Castaneda-Orjuela, C. A., Castillo Rivas, J., Castle, C. D., Castro, C., Castro, F., Catala-Lopez, F., Cerin, E., Chaiah, Y., Chang, J. C., Charlson, F. J., Chaturvedi, P., Chiang, P. P. C., Chimed-Ochir, O., Chisumpa, V. H., Chitheer, A., Chowdhury, R., Christensen, H., Christopher, D. J., Chung, S. C., Cicuttini, F. M., Ciobanu, L. G., Cirillo, M., Cohen, A. J., Cooper, L. T., Cortesi, P. A., Cortinovis, M., Cousin, E., Cowie, B. C., Criqui, M. H., Cromwell, E. A., Crowe, C. S., Crump, J. A., Cunningham, M., Daba, A. K., Dadi, A. F., Dandona, L., Dandona, R., Dang, A. K., Dargan, P. I., Daryani, A., Das, S. K., Gupta, Rajat Das, Neves, J. Das, Dasa, T. T., Dash, A. P., Davis, A. C., Davis Weaver, N., Davitoiu, D. V., Davletov, K., De La Hoz, F. P., De Neve, J. W., Degefa, M. G., Degenhardt, L., Degfie, T. T., Deiparine, S., Demoz, G. T., Demtsu, B. B., Denova-Gutierrez, E., Deribe, K., Dervenis, N., Des Jarlais, D. C., Dessie, G. A., Dey, S., Dharmaratne, S. D., Dicker, D., Dinberu, M. T., Ding, E. L., Dirac, M. A., Djalalinia, S., Dokova, K., Doku, D. T., Donnelly, C. A., Dorsey, E. R., Doshi, P. P., Douwes-Schultz, D., Doyle, K. E., Driscoll, T. R., Dubey, M., Dubljanin, E., Duken, E. E., Duncan, B. B., Duraes, A. R., Ebrahimi, H., Ebrahimpour, S., Edessa, D., Edvardsson, D., Eggen, A. E., El Bcheraoui, C., El Sayed Zaki, M., El-Khatib, Z., Elkout, H., Ellingsen, C. L., Endres, M., Endries, A. Y., Er, B., Erskine, H. E., Eshrati, B., Eskandarieh, S., Esmaeili, R., Esteghamati, A., Fakhar, M., Fakhim, H., Faramarzi, M., Fareed, M., Farhadi, F., Farinha, C. S. E., Faro, A., Farvid, M. S., Farzadfar, F., Farzaei, M. H., Feigin, V. L., Feigl, A. B., Fentahun, N., Fereshtehnejad, S. M., Fernandes, E., Fernandes, J. C., Ferrari, A. J., Feyissa, G. T., Filip, I., Finegold, S., Fischer, F., Fitzmaurice, C., Foigt, N. A., Foreman, K. J., Fornari, C., Frank, T. D., Fukumoto, T., Fuller, J. E., Fullman, N., Furst, T., Furtado, J. M., Futran, N. D., Gallus, S., Garcia-Basteiro, A. L., Garcia-Gordillo, M. A., Gardner, W. M., Gebre, A. K., Gebrehiwot, T. T., Gebremedhin, A. T., Gebremichael, B., Gebremichael, T. G., Gelano, T. F., Geleijnse, J. M., Genova-Maleras, R., Geramo, Y. C. D., Gething, P. W., Gezae, K. E., Ghadami, M. R., Ghadimi, R., Ghasemi Falavarjani, K., Ghasemi-Kasman, M., Ghimire, M., Gibney, K. B., Gill, P. S., Gill, T. K., Gillum, R. F., Ginawi, I. A., Giroud, M., Giussani, G., Goenka, S., Goldberg, E. M., Goli, S., Gomez-Dantes, H., Gona, P. N., Gopalani, S. V., Gorman, T. M., Goto, A., Goulart, A. C., Gnedovskaya, E. V., Grada, A., Grosso, G., Gugnani, H. C., Guimaraes, A. L. S., Guo, Y., Gupta, P. C., Gupta, Rahul, Gupta, Rajeev, Gupta, T., Gutierrez, R. A., Gyawali, B., Haagsma, J. A., Hafezi-Nejad, N., Hagos, T. B., Hailegiyorgis, T. T., Hailu, G. B., Haj-Mirzaian, Arvin, Haj-Mirzaian, Arya, Hamadeh, R. R., Hamidi, S., Handal, A. J., Hankey, G. J., Harb, H. L., Harikrishnan, S., Haro, J. M., Hasan, M., Hassankhani, H., Hassen, H. Y., Havmoeller, R., Hay, R. J., Hay, S. I., He, Y., Hedayatizadeh-Omran, A., Hegazy, M. I., Heibati, B.,

Heidari, M., Hendrie, D., Henok, A., Henry, N. J., Herteliu, C., Heydarpour, F., Heydarpour, P., Heydarpour, S., Hibstu, D. T., Hoek, H. W., Hole, M. K., Homaie Rad, E., Hoogar, P., Hosgood, H. D., Hosseini, S. M., Hosseinzadeh, M., Hostiuc, M., Hostiuc, S., Hotez, P. J., Hoy, D. G., Hsiao, T., Hu, G., Huang, J. J., Husseini, A., Hussen, M. M., Hutfless, S., Idrisov, B., Ilesanmi, O. S., Iqbal, U., Irvani, S. S. N., Irvine, C. M. S., Islam, N., Islam, S. M. S., Islami, F., Jacobsen, K. H., Jahangiry, L., Jahanmehr, N., Jain, S. K., Jakovljevic, M., Jalu, M. T., James, S. L., Javanbakht, M., Jayatilleke, A. U., Jeemon, P., Jenkins, K. J., Jha, R. P., Jha, V., Johnson, C. O., Johnson, S. C., Jonas, J. B., Joshi, A., Jozwiak, J. J., Jungari, S. B., Jurisson, M., Kabir, Z., Kadel, R., Kahsay, A., Kalani, R., Karami, M., Karami Matin, B., Karch, A., Karema, C., Karimi-Sari, H., Kasaeian, A., Kassa, D. H., Kassa, G. M., Kassa, T. D., Kassebaum, N. J., Katikireddi, S. V., Kaul, A., Kazemi, Z., Kazemi Karyani, A., Kazi, D. S., Kefale, A. T., Keiyoro, P. N., Kemp, G. R., Kengne, A. P., Keren, A., Kesavachandran, C. N., Khader, Y. S., Khafaei, B., Khafaie, M. A., Khajavi, A., Khalid, N., Khalil, I. A., Khan, E. A., Khan, M. S., Khan, M. A., Khang, Y. H., Khater, M. M., Khoja, A. T., Khosravi, A., Khosravi, M. H., Khubchandani, J., Kiadaliri, A. A., Kibret, G. D., Kidanemariam, Z. T., Kiirithio, D. N., Kim, D., Kim, Y. E., Kim, Y. J., Kimokoti, R. W., Kinfu, Y., Kisa, A., Kissimova-Skarbek, K., Kivimaki, M., Knudsen, A. K. S., Kocarnik, J. M., Kochhar, S., Kokubo, Y., Kolola, T., Kopec, J. A., Koul, P. A., Koyanagi, A., Kravchenko, M. A., Krishan, K., Kuate Defo, B., Kucuk Bicer, B., Kumar, G. A., Kumar, M., Kumar, P., Kutz, M. J., Kuzin, I., Kyu, H. H., Lad, D. P., Lad, S. D., Lafranconi, A., Lal, D. K., Lalloo, R., Lallukka, T., Lam, J. O., Lami, F. H., Lansingh, V. C., Lansky, S., Larson, H. J., Latifi, A., Lau, K. M. M., Lazarus, J. V., Lebedev, G., Lee, P. H., Leigh, J., Leili, M., Leshargie, C. T., Li, S., Li, Y., Liang, J., Lim, L. L., Lim, S. S., Limenih, M. A., Linn, S., Liu, S., Liu, Y., Lodha, R., Lonsdale, C., Lopez, A. D., Lorkowski, S., Lotufo, P. A., Lozano, R., Lunevicius, R., Ma, S., Macarayan, E. R. K., Mackay, M. T., MacLachlan, J. H., Maddison, E. R., Madotto, F., Magdy Abd El Razek, H., Magdy Abd El Razek, M., Maghavani, D. P., Majdan, M., Majdzadeh, R., Majeed, A., Malekzadeh, R., Malta, D. C., Manda, A. L., Mandarano-Filho, L. G., Manguerra, H., Mansournia, M. A., Mapoma, C. C., Marami, D., Maravilla, J. C., Marcenes, W., Marczak, L., Marks, A., Marks, G. B., Martinez, G., Martins-Melo, F. R., Martopullo, I., Marz, W., Marzan, M. B., Masci, J. R., Massenburg, B. B., Mathur, M. R., Mathur, P., Matzopoulos, R., Maulik, P. K., Mazidi, M., McAlinden, C., McGrath, J. J., McKee, M., McMahon, B. J., Mehata, S., Mehndiratta, M. M., Mehrotra, R., Mehta, K. M., Mehta, V., Mekonnen, T. C., Melese, A., Melku, M., Memiah, P. T. N., Memish, Z. A., Mendoza, W., Mengistu, D. T., Mengistu, G., Mensah, G. A., Mereta, S. T., Meretoja, A., Meretoja, T. J., Mestrovic, T., Mezgebe, H. B., Miazgowski, B., Miazgowski, T., Millear, A. I., Miller, T. R., Miller-Petrie, M. K., Mini, G. K., Mirabi, P., Mirarefin, M., Mirica, A., Mirrakhimov, E. M., Misganaw, A. T., Mitiku, H., Moazen, B., Mohammad, K. A., Mohammadi, M., Mohammadifard, N., Mohammed, M. A., Mohammed, S., Mohan, V., Mokdad, A. H., Molokhia, M., Monasta, L., Moradi, G., Moradi-Lakeh, M., Moradinazar, M., Moraga, P., Morawska, L., Velasquez, I. M., Morgado-Da-Costa, J., Morrison, S. D., Moschos, M. M., Mouodi, S., Mousavi, S. M., Muchie, K. F., Mueller, U. O., Mukhopadhyay, S., Muller, K., Mumford, J. E., Musa, J., Musa, K. I., Mustafa, G., Muthupandian, S., Nachega, J. B., Nagel, G., Naheed, A., Nahvijou, A., Naik, G., Nair, S., Najafi, F., Naldi, L., Nam, H. S., Nangia, V., Nansseu, J. R., Nascimento, B. R., Natarajan, G., Neamati, N., Negoi, I., Negoi, R. I., Neupane, S., Newton, C. R. J., Ngalesoni, F. N., Ngunjiri, J. W., Nguyen, A. Q., Nguyen, G., Nguyen, Ha Thu, Nguyen, Huong Thanh, Nguyen, L. H., Nguyen, M.,

Nguyen, T. H., Nichols, E., Ningrum, D. N. A., Nirayo, Y. L., Nixon, M. R., Nolutshungu, N., Nomura, S., Norheim, O. F., Noroozi, M., Norrving, B., Noubiap, J. J., Nouri, H. R., Shiadeh, M. N., Nowroozi, M. R., Nyasulu, P. S., Odell, C. M., Ofori-Asenso, R., Ogbo, F. A., Oh, I. H., Oladimeji, O., Olagunju, A. T., Olivares, P. R., Olsen, H. E., Olusanya, B. O., Olusanya, J. O., Ong, K. L., Ong, S. K. S., Oren, E., Orpana, H. M., Ortiz, A., Ortiz, J. R., Otstavnov, S. S., Overland, S., Owolabi, M. O., Ozdemir, R., Mahesh, P. A., Pacella, R., Pakhale, S., Pakhare, A. P., Pakpour, A. H., Pana, A., Panda-Jonas, S., Pandian, J. D., Parisi, A., Park, E. K., Parry, C. D. H., Parsian, H., Patel, S., Pati, S., Patton, G. C., Paturi, V. R., Paulson, K. R., Pereira, A., Pereira, D. M., Perico, N., Pesudovs, K., Petzold, M., Phillips, M. R., Piel, F. B., Pigott, D. M., Pillay, J. D., Pirsaheb, M., Pishgar, F., Polinder, S., Postma, M. J., Pourshams, A., Poustchi, H., Pujar, A., Prakash, S., Prasad, N., Purcell, C. A., Qorbani, M., Quintana, H., Quistberg, D. A., Rade, K. W., Anwar Rafay, A. R., Rahim, F., Rahimi, K., Rahimi-Movaghar, A., Rahman, M., Rahman, M. H. U., Rahman, M. A., Rai, R. K., Rajsic, S., Ram, U., Ranabhat, C. L., Ranjan, P., Rao, P. C., Rawaf, D. L., Rawaf, S., Razo-Garcia, C., Reddy, K. S., Reiner, R. C., Reitsma, M. B., Remuzzi, G., Renzaho, A. M. N., Resnikoff, S., Rezaei, S., Rezaeian, S., Rezai, M. S., Riahi, S. M., Ribeiro, A. L. P., Rios-Blancas, M. J., Roba, K. T., Roberts, N. L. S., Robinson, S. R., Roever, L., Ronfani, L., Roshandel, G., Rostami, A., Rothenbacher, D., Roy, A., Rubagotti, E., Sachdev, P. S., Saddik, B., Sadeghi, E., Safari, H., Safdarian, M., Safi, S., Safiri, S., Sagar, R., Sahebkar, A., Sahraian, M. A., Salam, N., Salama, J. S., Salamati, P., De Freitas Saldanha, R., Saleem, Z., Salimi, Y., Salvi, S. S., Salz, I., Sambala, E. Z., Samy, A. M., Sanabria, J., Dolores Sanchez-Nino, M., Santomauro, D. F., Santos, I. S., Santos, J. V., Santric Milicevic, M. M., Jose, B. P. S., Sarker, A. R., Sarmiento-Suarez, R., Sarrafzadegan, N., Sartorius, B., Sarvi, S., Sathian, B., Satpathy, M., Sawant, A. R., Sawhney, M., Saxena, S., Sayyah, M., Schaeffner, E., Schmidt, M. I., Schneider, I. J. C., Schottker, B., Schutte, A. E., Schwebel, D. C., Schwendicke, F., Scott, J. G., Sekerija, M., Sepanlou, S. G., Servan-Mori, E., Seyedmousavi, S., Shabaninejad, H., Shackelford, K. A., Shafieesabet, A., Shahbazi, M., Shaheen, A. A., Shaikh, M. A., Shams-Beyranvand, M., Shamsi, M., Shamsizadeh, M., Sharafi, K., Sharif, M., Sharif-Alhoseini, M., Sharma, R., She, J., Sheikh, A., Shi, P., Shiferaw, M. S., Shigematsu, M., Shiri, R., Shirkoohi, R., Shiue, I., Shokraneh, F., Shrime, M. G., Si, S., Siabani, S., Siddiqi, T. J., Sigfusdottir, I. D., Sigurvinsdottir, R., Silberberg, D. H., Santos Silva, D. A., Silva, J. P., Da Silva, N. T., Silveira, D. G. A., Singh, J. A., Singh, N. P., Singh, P. K., Singh, V., Sinha, D. N., Sliwa, K., Smith, M., Sobaih, B. H., Sobhani, S., Sobngwi, E., Soneji, S. S., Soofi, M., Sorensen, R. J. D., Soriano, J. B., Soyiri, I. N., Sposato, L. A., Sreeramareddy, C. T., Srinivasan, V., Stanaway, J. D., Starodubov, V. I., Stathopoulou, V., Stein, D. J., Steiner, C., Stewart, L. G., Stokes, M. A., Subart, M. L., Sudaryanto, A., Sufiyan, M. B., Sur, P. J., Sutradhar, I., Sykes, B. L., Sylaja, P. N., Sylte, D. O., Szoeke, C. E. I., Tabares-Seisdedos, R., Tabuchi, T., Tadakamadla, S. K., Takahashi, K., Tandon, N., Tassew, S. G., Taveira, N., Tehrani-Banihashemi, A., Tekalign, T. G., Tekle, M. G., Temsah, M. H., Temsah, O., Terkawi, A. S., Teshale, M. Y., Tessema, B., Tessema, G. A., Thankappan, K. R., Thirunavukkarasu, S., Thomas, N., Thrift, A. G., Thurston, G. D., Tilahun, B., To, Q. G., Tobe-Gai, R., Tonelli, M., Topor-Madry, R., Torre, A. E., Tortajada-Girbes, M., Touvier, M., Tovani-Palone, M. R., Tran, B. X., Tran, K. B., Tripathi, S., Troeger, C. E., Truelsen, T. C., Truong, N. T., Tsadik, A. G., Tsoi, D., Tudor Car, L., Murat Tuzcu, E., Tyrovolas, S., Ukwaja, K. N., Ullah, I., Undurraga, E. A., Updike, R. L., Usman, M. S., Uthman, O. A., Uzun, S. B.,

Vaduganathan, M., Vaezi, A., Vaidya, G., Valdez, P. R., Varavikova, E., Vasankari, T. J., Venketasubramanian, N., Villafaina, S., Violante, F. S., Vladimirov, S. K., Vlassov, V., Vollset, S. E., Vos, T., Wagner, G. R., Wagnew, F. S., Waheed, Y., Wallin, M. T., Walson, J. L., Wang, Y., Wang, Y. P., Wassie, M. M., Weiderpass, E., Weintraub, R. G., Weldegebreal, F., Weldegwergs, K. G., Werdecker, A., Werkneh, A. A., West, T. E., Westerman, R., Whiteford, H. A., Widecka, J., Wilner, L. B., Wilson, S., Winkler, A. S., Wiysonge, C. S., Wolfe, C. D. A., Wu, S., Wu, Y. C., Wyper, G. M. A., Xavier, D., Xu, G., Yadgir, S., Yadollahpour, A., Yahyazadeh Jabbari, S. H., Yakob, B., Yan, L. L., Yano, Y., Yaseri, M., Yasin, Y. J., Yentur, G. K., Yeshaneh, A., Yimer, E. M., Yip, P., Yirsaw, B. D., Yisma, E., Yonemoto, N., Yonga, G., Yoon, S. J., Yotebieng, M., Younis, M. Z., Yousefifard, M., Yu, C., Zadnik, V., Zaidi, Z., Zaman, S. Bin, Zamani, M., Zare, Z., Zeleke, A. J., Zenebe, Z. M., Zhang, A. L., Zhang, K., Zhou, M., Zodpey, S., Zuhlke, L. J., Naghavi, M. and Murray, C. J. L. (2018). Global, regional, and national age-sex-specific mortality for 282 causes of death in 195 countries and territories, 1980–2017: a systematic analysis for the Global Burden of Disease Study 2017. *The Lancet*, 392(10159), 1736–1788. doi: 10.1016/S0140-6736(18)32203-7.

Sacks, F. M., Carey, V. J., Anderson, C. A. M., Miller, E. R., Copeland, T., Charleston, J., Harshfield, B. J., Laranjo, N., McCarron, P., Swain, J., White, K., Yee, K. and Appel, L. J. (2014). Effects of high vs low glycemic index of dietary carbohydrate on cardiovascular disease risk factors and insulin sensitivity: The OmniCarb randomized clinical trial. *JAMA - Journal of the American Medical Association*, 312(23), 2531–2541. doi: 10.1001/jama.2014.16658.

Sadaf Farooqi, I. and O'Rahilly, S. (2009). Leptin: A pivotal regulator of human energy homeostasis. *American Journal of Clinical Nutrition*, 89(3), 980–984. doi: 10.3945/ajcn.2008.26788C.

Saeedi, P., Petersohn, I., Salpea, P., Malanda, B., Karuranga, S., Unwin, N., Colagiuri, S., Guariguata, L., Motala, A. A., Ogurtsova, K., Shaw, J. E., Bright, D. and Williams, R. (2019). Global and regional diabetes prevalence estimates for 2019 and projections for 2030 and 2045: Results from the International Diabetes Federation Diabetes Atlas, 9th edition. *Diabetes Research and Clinical Practice*. Elsevier Ireland Ltd, 157. doi: 10.1016/j.diabres.2019.107843.

Saeedi, R. and Frohlich, J. (2016). Lipoprotein (a), an independent cardiovascular risk marker. *Clinical Diabetes and Endocrinology*. Clinical Diabetes and Endocrinology, 2(1), 1–6. doi: 10.1186/S40842-016-0024-X.

Sagoe, D., McVeigh, J., Bjørnebekk, A., Essilfie, M. S., Andreassen, C. S. and Pallesen, S. (2015). Polypharmacy among anabolic-androgenic steroid users: A descriptive metasynthesis. *Substance Abuse: Treatment, Prevention, and Policy*, 10(1). doi: 10.1186/s13011-015-0006-5.

Sagoe, D., Molde, H., Andreassen, C. S., Torsheim, T. and Pallesen, S. (2014). The global epidemiology of anabolic-androgenic steroid use: A meta-analysis and meta-regression analysis. *Annals of Epidemiology*. Elsevier, 24(5), 383–398. doi: 10.1016/j.annepidem.2014.01.009.

Sáinz, N., Barrenetxe, J., Moreno-Aliaga, M. J. and Martínez, J. A. (2015). Leptin resistance and diet-induced obesity: Central and peripheral actions of leptin. *Metabolism: Clinical and Experimental*, 64(1),

35–46. doi: 10.1016/j.metabol.2014.10.015.

Sakamoto, K. and Holman, G. D. (2008). Emerging role for AS160/TBC1D4 and TBC1D1 in the regulation of GLUT4 traffic. *American Journal of Physiology - Endocrinology and Metabolism*, 295(1). doi: 10.1152/ajpendo.90331.2008.

Samms, R. J., Cheng, C. C., Kharitonenkov, A., Gimeno, R. E. and Adams, A. C. (2016). Overexpression of β-klotho in adipose tissue sensitizes male mice to endogenous FGF21 and provides protection from diet-induced obesity. *Endocrinology*, 157(4), 1467–1480. doi: 10.1210/en.2015-1722.

Samuel, V. T. and Shulman, G. I. (2016). The pathogenesis of insulin resistance: Integrating signaling pathways and substrate flux. *Journal of Clinical Investigation*, 126(1), 12–22. doi: 10.1172/JCI77812.

Sandesara, P. B., Virani, S. S., Fazio, S. and Shapiro, M. D. (2019). The forgotten lipids: Triglycerides, remnant cholesterol, and atherosclerotic cardiovascular disease risk. *Endocrine Reviews*, 40(2), 537–557. doi: 10.1210/er.2018-00184.

Santamarina-Fojo, S., González-Navarro, H., Freeman, L., Wagner, E. and Nong, Z. (2004). Hepatic lipase, lipoprotein metabolism, and atherogenesis. *Arteriosclerosis, Thrombosis, and Vascular Biology*, 24(10), 1750–1754. doi: 10.1161/01.ATV.0000140818.00570.2d.

Santos-Gallego, C. G., Torres, F., Badimón, J. J., Wu, M. Y., Li, C. J., Hou, M. F., Chu, P. Y., Kuipers, H., Wijnen, J. A. G., Hartgens, F., Willems, S. M. M., Khaw, K. T., Barrett-Connor, E., Jaiswal, N., Gavin, M. G., Quinn, W. J., Luongo, T. S., Gelfer, R. G., Baur, J. A., Titchenell, P. M., D'Andrea, A., Radmilovic, J., Caselli, S., Carbone, A., Scarafile, R., Sperlongano, S., Tocci, G., Formisano, T., Martone, F., Liccardo, B., D'Alto, M., Bossone, E., Galderisi, M., Golino, P., Fuchs, F. D., Whelton, P. K., White, J. P., Baltgalvis, K. A., Sato, S., Britt Wilson, L., Carson, J. A., Rasmussen, J. J., Schou, M., Madsen, P. L., Selmer, C., Johansen, M. L., Hovind, P., Ulriksen, P. S., Faber, J., Gustafsson, F., Kistorp, C., Palatini, P., Giada, F., Garavelli, G., Sinisi, F., Mario, L., Michieletto, M., Baldo-Enzi, G., Guzik, T. J., Touyz, R. M., Xu, J., Shi, G.-P., Savoia, C., Sada, L., Zezza, L., Pucci, L., Lauri, F. M., Befani, A., Alonzo, A., Volpe, M., Petrie, J. R., Guzik, T. J., Touyz, R. M., Rabiee, A., Krüger, M., Ardenkjær-Larsen, J., Kahn, C. R., Emanuelli, B., & Kotz, C. M. B. P. E. Z. A. N. H. A. A. B. C. J., Beutel, A., Bergamaschi, C. T., Campos, R. R., Preis, S. R., Massaro, J. M., Robins, S. J., Hoffmann, U., Vasan, R. S., Irlbeck, T., Meigs, J. B., Sutherland, P., D'Agostino, R. B., O'Donnell, C. J., Fox, C. S., Urban, R. J., Bhasin, S., Woodhouse, L., Casaburi, R., Singh, A. B., Bhasin, D., Berman, N., Chen, X., Yarasheski, K. E., Magliano, L., Dzekov, C., Dzekov, J., Bross, R., Phillips, J., Sinha-Hikim, I., Shen, R. R., Storer, T. W., Hartgens, F., Rietjens, G., Keizer, H. A., Kuipers, H., Wolffenbuttel, B. H. R., Storer, T. W., Basaria, S., Traustadottir, T., Harman, S. M., Pencina, K. M., Li, Z., Travison, T. G., Miciek, R., Tsitouras, P., Hally, K., Huang, G., Bhasin, S., Aguilar-Agon, K. W., Capel, A. J., Martin, N. R. W., Player, D. J., Lewis, M. P., Tamez-Pérez, H. E., Santamarina-Fojo, S., González-Navarro, H., Freeman, L., Wagner, E., Nong, Z., Bonetti, A., Tirelli, F., Catapano, A., Dazzi, D., Dei Cas, A., Solito, F., Ceda, C., Reverberi, C., Monica, C., Pipitone, S., Elia, G., Spattini, M., Magnati, G., Heine, J., Cheung, A. S., Grossmann, M., Bhasin, S., Storer, T. W., Berman, N., Callegari, C., Clevenger, B., Phillips, J., Bunnell, T. J., Tricker, R., Shirazi, A., Casaburi, R., Bennett, N. C., Gardiner, R. A., Hooper, J. D., Johnson, D. W., Gobe, G.

C., D'Elia, L., Strazzullo, P., Iacone, R., Russo, O., Galletti, F., Rizza, R. A., Frankenfeld, S. P., de Oliveira, L. P., Ignacio, D. L., Coelho, R. G., Mattos, M. N., Ferreira, A. C. F., Carvalho, D. P., Fortunato, R. S., Tam, C. S., Xie, W., Johnson, W. D., Cefalu, W. T., Redman, L. M., Ravussin, E., Godsland, I. F., Shennan, N. M., Wynn, V., Esser, N., Legrand-Poels, S., Piette, J., Scheen, A. J., Paquot, N., Pérez-Schindler, J., Handschin, C., Thompson, P. D. L., Cullinane, E. M., Sady, S. P., Chenevert, C., Saritelli, A. L., Sady, M. A., Herbert, P. N., Thieme, D., Hemmersbach, P., Fuster, V., Moreno, P. R., Fayad, Z. A., Corti, R., Badimon, J. J., Antinozzi, C., Marampon, F., Sgrò, P., Tombolini, V., Lenzi, A., Crescioli, C., Di Luigi, L., Santos-Gallego, C. G., Torres, F., Badimón, J. J., Reitzner, S. M., Hengevoss, J., Isenmann, E., Diel, P., Arazi, H., Bodine, S. C., Stitt, T. N., Gonzalez, M., Kline, W. O., Stover, G. L., Bauerlein, R., Zlotchenko, E., Scrimgeour, A., Lawrence, J. C., Glass, D. J., Yancopoulos, G. D., Seynnes, O. R., Kamandulis, S., Kairaitis, R., Helland, C., Campbel, E. L., Brazaitis, M., Skurvydas, A., Narici, M. V., Cohen, J. C., Hickman, R., Anderson, L. J., Tamayose, J. M., Garcia, J. M., Template, F., Baggish, A. L., Weiner, R. B., Kanayama, G., Hudson, J. I., Lu, M. T., Hoffmann, U., Pope, H. G., ACMD, Min, L., Simon W, R., Herbst, K. L., Amory, J. K., Brunzell, J. D., Chansky, H. A., Bremner, W. J., Santos-Gallego, C. G., Badimon, J. J., Rosenson, R. S., Daniels, T. F., Killinger, K. M., Michal, J. J., Wright, R. W., Jiang, Z., Nikolic, D., Katsiki, N., Montalto, G., Isenovic, E. R., Mikhailidis, D. P., Rizzo, M., Thongtang, N., Diffenderfer, M. R., Ooi, E. M. M., Barrett, P. H. R., Turner, S. M., Le, N. A., Brown, W. V., Schaefer, E. J., Andrews, M. A., Magee, C. D., Combest, T. M., Allard, R. J., Douglas, K. M., Sniderman, A. D., Thanassoulis, G., Glavinovic, T., Navar, A. M., Pencina, M., Catapano, A., Ference, B. A., Glazer, G., Achar, S., Rostamian, A., Narayan, S. M., Packard, C. J., Demant, T., Stewart, J. P., Bedford, D., Caslake, M. J., Schwertfeger, G., Bedynek, A., Shepherd, J., Seidel, D., Samieinasab, M. R., Shahraki, M. R., Samieinasab, F., Najafi, S., Thien, T. L. J. W. M. D. P. N. M. V. J. A. J. P. L. M. H. A. J. van't L. A., Skinner, J. W., Otzel, D. M., Bowser, A., Nargi, D., Agarwal, S., Peterson, M. D., Zou, B., Borst, S. E., Yarrow, J. F., Chen, Y., Zajac, J. D., MacLean, H. E., Traustadóttir, T., Harman, S. M., Tsitouras, P., Pencina, K. M., Li, Z., Travison, T. G., Eder, R., Miciek, R., McKinnon, J., Woodbury, E., Basaria, S., Bhasin, S., Storer, T. W., Spiegeleer, A. De, Beckwée, D., Bautmans, I., Petrovic, M., Hirschberg, A. L., Elings Knutsson, J., Helge, T., Godhe, M., Ekblom, M., Bermon, S., Ekblom, B., Saad, F., Röhrig, G., Von Haehling, S., Traish, A. M., Griggs, R. C., Kingston, W., Jozefowicz, R. F., Herr, B. E., Forbes, G., Halliday, D., Yu, J. G., Bonnerud, P., Eriksson, A., Stal, P. S., Tegner, Y., Malm, C., Lucas-Herald, A. K., Alves-Lopes, R., Montezano, A. C., Ahmed, S. F., Touyz, R. M., Holt, R. I. G., Erotokritou-Mulligan, I., Sönksen, P. H., Englund, D. A., Peck, B. D., Murach, K. A., Neal, A. C., Caldwell, H. A., McCarthy, J. J., Peterson, C. A., Dupont-Versteegden, E. E., Bodine, S. C., Baehr, L. M., Velloso, C. P., Mangan, G., Bombardier, E., Mitchell, A. S., Quadrilatero, J., Tiidus, P. M., Isenmann, E., Ambrosio, G., Joseph, J. F., Mazzarino, M., de la Torre, X., Zimmer, P., Kazlauskas, R., Goebel, C., Botrè, F., Diel, P., Parr, M. K., Xie, S. J., Li, J. H., Chen, H. F., Tan, Y. Y., Liu, S. R., Zhang, Y., Xu, H., Yang, J. H., Liu, S. R., Zheng, L. L., Huang, M. B., Guo, Y. H., Zhang, Q., Zhou, H., Qu, L. H., Morissette, M. R., Cook, S. A., Buranasombati, C., Rosenberg, M. A., Rosenzweig, A., Fink, J., Schoenfeld, B. J., Nakazato, K., Muta, Y., Tanaka, T., Hamaguchi, Y., Hamanoue, N., Motonaga, R., Tanabe, M., Nomiyama, T., Nawata, H., Yanase, T., Kovacheva, E. L., Sinha Hikim, A. P., Shen, R. R., Sinha, I., Sinha-Hikim, I., Wang, J., Harris, C., Kicman, A. T., Rahman, F., Christian, H. C., Estrada, M., Espinosa,

A., Müller, M., Jaimovich, E., Davey, R. A., Grossmann, M., Hamdi, M. M., Mutungi, G., Kristina Parr, M., Müller-Schöll, A., Antinozzi, C., Marampon, F., Corinaldesi, C., Vicini, E., Sgrò, P., Vannelli, G. B., Lenzi, A., Crescioli, C., Di Luigi, L., Sheffield-Moore, M., West, D. W. D., Phillips, S. M., Rossetti, M. L., Steiner, J. L., Gordon, B. S., Sinha-Hikim, I., Roth, S. M., Lee, M. I., Bhasin, S., Haren, M. T., Siddiqui, A. M., Armbrecht, H. J., Kevorkian, R. T., Kim, M. J., Haas, M. J., Mazza, A., Kumar, V. B., Green, M., Banks, W. A., Morley, J. E., Brown, D., Sinha Hikim, A. P., Kovacheva, E. L., Sinha-Hikim, I., Taylor, W. E., Gonzalez-Cadavid, N. F., Zheng, W., Bhasin, S., Shahidi, N. T., Thiblin, I., Garmo, H., Garle, M., Holmberg, L., Byberg, L., Michaëlsson, K., Gedeborg, R., MacKrell, J. G., Yaden, B. C., Bullock, H., Chen, K., Shetler, P., Bryant, H. U., Krishnan, V., Warburton, D. E. R., Nicol, C. W., Bredin, S. S. D., Egan, B., Zierath, J. R., Begley, E., McVeigh, J., Hope, V., Begley, E., Sagoe, D., Molde, H., Andreassen, C. S., Torsheim, T., Pallesen, S., Applegate, E. a, Baumgartner, B., Clarkson, P. M., Grivetti, L. E., Haislett, N., Mcdonald, R. B., Mills, B., Oerter, A., Suppl, M., Choi, Y. J., Choi, S. H., Kim, H. J., Han, S. J., Hwang, J. S., Chung, Y. S., Lee, K. W., Cho, H. K., Kim, D. J., Dubois, V., Laurent, M., Boonen, S., Vanderschueren, D., Claessens, F., Chappell, A. J., Simper, T., Dotson, J. L., Brown, R. T., Goldman, A. L., Pope, H. G., Bhasin, S., Kadi, F., Imperlini, E., Mancini, A., Alfieri, A., Martone, D., Caterino, M., Orrù, S., Buono, P., Kaushik, M., Sontineni, S. P., Hunter, C., Karila, T., Lippi, G., Franchini, M., Banfi, G., Muraleedharan, V., Jones, T. H., Livingstone, C., Collison, M., Pope, H. G., Wood, R. I., Rogol, A., Nyberg, F., Bowers, L., Bhasin, S., Ramirez, C. M., Goedeke, L., Rotllan, N., Yoon, J.-H., Cirera-Salinas, D., Mattison, J. A., Suarez, Y., de Cabo, R., Gorospe, M., Fernandez-Hernando, C., Quaglio, G., Fornasiero, A., Mezzelani, P., Moreschini, S., Lugoboni, F., Lechi, A., Rhee, E. J., Byrne, C. D., Sung, K. C., Rasmussen, J. J., Schou, M., Selmer, C., Johansen, M. L., Gustafsson, F., Frystyk, J., Dela, F., Faber, J., Kistorp, C., Traish, A. M., Kypreos, K. E., Hughes, D. C., Stewart, C. E., Sculthorpe, N., Dugdale, H. F., Yousefian, F., Lewis, M. P., Sharples, A. P., Hung, J., McQuillan, B. M., Thompson, P. D. L. and Beilby, J. P. (2011). The beneficial effects of HDL-C on atherosclerosis: rationale and clinical results. *Clinical Lipidology*. Elsevier Ireland Ltd, 6(2), 181–208. doi: 10.2217/clp.10.90.

Saslow, L. R., Kim, S., Daubenmier, J. J., Moskowitz, J. T., Phinney, S. D., Goldman, V., Murphy, E. J., Cox, R. M., Moran, P. and Hecht, F. M. (2014). A randomized pilot trial of a moderate carbohydrate diet compared to a very low carbohydrate diet in overweight or obese individuals with type 2 diabetes mellitus or prediabetes. *PLoS ONE*, 9(4). doi: 10.1371/journal.pone.0091027.

Sattar, N., Gill, J. M. R. and Alazawi, W. (2020). Improving prevention strategies for cardiometabolic disease. *Nature Medicine*. Springer US, 26(3), 320–325. doi: 10.1038/s41591-020-0786-7.

Saxton, R. A. and Sabatini, D. M. (2017). mTOR Signaling in Growth, Metabolism, and Disease. *Cell*, 169(2), 361–371. doi: 10.1016/j.cell.2017.03.035.

Scharner, J. and Zammit, P. S. (2011). The muscle satellite cell at 50: The formative years. *Skeletal Muscle*, 1(1), 1–13. doi: 10.1186/2044-5040-1-28.

Schellenberg, E. et al (2013). Annals of Internal Medicine Review Lifestyle Interventions for Patients With and at Risk for Type 2 Diabetes. *Annals of Internal Medicine*, 159(8), 543–551.

Schmidt, E. K., Clavarino, G., Ceppi, M. and Pierre, P. (2009). SUnSET, a nonradioactive method to monitor protein synthesis. *Nature Methods*, 6(4), 275–277. doi: 10.1038/nmeth.1314.

Schmitz-Peiffer, C., Craig, D. L. and Biden, T. J. (1999). Ceramide generation is sufficient to account for the inhibition of the insulin-stimulated PKB pathway in C2C12 skeletal muscle cells pretreated with palmitate. *Journal of Biological Chemistry*, 274(34), 24202–24210. doi: 10.1074/jbc.274.34.24202.

Schoenfeld, B. J., Ogborn, D. and Krieger, J. W. (2017). Dose-response relationship between weekly resistance training volume and increases in muscle mass: A systematic review and meta-analysis. *Journal of Sports Sciences*. Routledge, 35(11), 1073–1082. doi: 10.1080/02640414.2016.1210197.

Schwarz, J.-M., Linfoot, P., Dare, D. and Aghajanian, K. (2003). Hepatic de novo lipogenesis in normoinsulinemic and hyperinsulinemic subjects consuming high-fat, low-carbohydrate and low-fat, high-carbohydrate isoenergetic diets. *American Journal of Clinical Nutrition*, 77(1), 43–50. doi: 10.1093/ajcn/77.1.43.

Schwingshackl, L., Chaimani, A., Schwedhelm, C., Toledo, E., Pünsch, M., Hoffmann, G. and Boeing, H. (2019). Comparative effects of different dietary approaches on blood pressure in hypertensive and pre-hypertensive patients: A systematic review and network meta-analysis. *Critical Reviews in Food Science and Nutrition*. Taylor & Francis, 59(16), 2674–2687. doi: 10.1080/10408398.2018.1463967.

Schwingshackl, L. and Hoffmann, G. (2014). Comparison of the long-term effects of high-fat v. low-fat diet consumption on cardiometabolic risk factors in subjects with abnormal glucose metabolism: A systematic review and meta-analysis. *British Journal of Nutrition*, 111(12), 2047–2058. doi: 10.1017/S0007114514000464.

Scientific Advisory Committee on Nutrition (2015). Carbohydrates and Health. *TSO The Stationary Office*, (August), 1–6.

Scientific Advisory Committee on Nutrition (2019). Saturated fats and health., 203. Available at: https://www.gov.uk/government/publications/saturated-fats-and-health-sacn-report.

Sculthorpe, N., Solomon, A. M., Sinanan, A. C. M., Bouloux, P. M. G., Grace, F. and Lewis, M. P. (2012). Androgens affect myogenesis in vitro and increase local IGF-1 expression. *Medicine and Science in Sports and Exercise*, 44(4), 610–615. doi: 10.1249/MSS.0b013e318237c5c0.

Sedgwick, P. and Greenwood, N. (2015). Understanding the hawthorne effect. *BMJ (Online)*, 351(September), 1–2. doi: 10.1136/bmj.h4672.

Shah, R. V., Murthy, V. L., Abbasi, S. A., Blankstein, R., Kwong, R. Y., Goldfine, A. B., Jerosch-Herold, M., Lima, J. A. C., Ding, J. and Allison, M. A. (2014). Visceral adiposity and the risk of metabolic syndrome across body mass index: The MESA study. *JACC: Cardiovascular Imaging*, 7(12), 1221–1235. doi: 10.1016/j.jcmg.2014.07.017.

Shankaran, M., Shearer, T. W., Stimpson, S. A., Turner, S. M., King, C., Wong, P. Y. A., Shen, Y., Turnbull, P. S., Kramer, F., Clifton, L., Russell, A., Hellerstein, M. K. and Evans, W. J. (2016). Proteome-

wide muscle protein fractional synthesis rates predict muscle mass gain in response to a selective androgen receptor modulator in rats. *American Journal of Physiology - Endocrinology and Metabolism*, 310(6), E405–E417. doi: 10.1152/ajpendo.00257.2015.

Sharples, A. P., Al-Shanti, N., Lewis, M. P. and Stewart, C. E. (2011). Reduction of myoblast differentiation following multiple population doublings in mouse C 2C 12 cells: A model to investigate ageing?. *Journal of Cellular Biochemistry*, 112(12), 3773–3785. doi: 10.1002/jcb.23308.

Sharples, A. P., Al-Shanti, N. and Stewart, C. E. (2010). C2 and C2C12 murine skeletal myoblast models of atrophic and hypertrophic potential: Relevance to disease and ageing?. *Journal of Cellular Physiology*, 225(1), 240–250. doi: 10.1002/jcp.22252.

Sharrett, A. R., Ballantyne, C. M., Coady, S. A., Heiss, G., Sorlie, P. D., Catellier, D. and Patsch, W. (2001). Coronary heart disease prediction from lipoprotein cholesterol levels, triglycerides, lipoprotein(a), apolipoproteins A-I and B, and HDL density subfractions: The Atherosclerosis Risk in Communities (ARIC) Study. *Circulation*, 104(10), 1108–1113. doi: 10.1161/hc3501.095214.

Sheffield-Moore, M. (2000). Androgens and the control of skeletal muscle protein synthesis. *Annals of Medicine*, 32(3), 181–186. doi: 10.3109/07853890008998825.

Sheffield-Moore, M., Urban, R. J., Wolf, S. E., Jiang, J., Catlin, D. H., Herndon, D. N., Wolfe, R. R. and Ferrando, A. A. (1999). Short-term oxandrolone administration stimulates net muscle protein synthesis in young men. *Journal of Clinical Endocrinology and Metabolism*, 84(8), 2705–2711. doi: 10.1210/jc.84.8.2705.

Shim, J.-S., Oh, K. and Kim, H. C. (2014). Dietary assessment methods in epidemiologic studies. *Epidemiology and Health*, 7(2), e2014009. doi: 10.4178/epih/e2014009.

Shorakae, S., Ranasinha, S., Abell, S., Lambert, G., Lambert, E., de Courten, B. and Teede, H. (2018). Inter-related effects of insulin resistance, hyperandrogenism, sympathetic dysfunction and chronic inflammation in PCOS. *Clinical Endocrinology*, 89(5), 628–633. doi: 10.1111/cen.13808.

Shulman, G. I. (2000). On diabetes : insulin resistance Cellular mechanisms of insulin resistance., 106(2), 171–176. doi: 10.1172/JCI10583.On.

Shulman, G. I. (2014). Ectopic fat in insulin resistance, dyslipidemia, and cardiometabolic disease. *New England Journal of Medicine*, 371(12), 1131–1141. doi: 10.1056/NEJMra1011035.

Singh, A. B., Hsia, S., Alaupovic, P., Sinha-Hikim, I., Woodhouse, L., Buchanan, T. A., Shen, R., Bross, R., Berman, N. and Bhasin, S. (2002). The effects of varying doses of T on insulin sensitivity, plasma lipids, apolipoproteins, and C-reactive protein in healthy young men. *Journal of Clinical Endocrinology and Metabolism*, 87(1), 136–143. doi: 10.1210/jcem.87.1.8172.

Sinha-Hikim, I., Roth, S. M., Lee, M. I. and Bhasin, S. (2003). Testosterone-induced muscle hypertrophy is associated with an increase in satellite cell number in healthy, young men. *American Journal of Physiology - Endocrinology and Metabolism*, 285(1 48-1), 197–205.

Small, M., McArdle, B. M., Lowe, G. D. O., Forbes, C. D. and Prentice, C. R. M. (1982). The effect of intramuscular stanozolol on fibrinolysis and blood lipids. *Thrombosis Research*, 28(1), 27–36. doi: 10.1016/0049-3848(82)90030-5.

Sniderman, A. D., Thanassoulis, G., Glavinovic, T., Navar, A. M., Pencina, M., Catapano, A. and Ference, B. A. (2019). Apolipoprotein B Particles and Cardiovascular Disease: A Narrative Review. *JAMA Cardiology*, 4(12), 1287–1295. doi: 10.1001/jamacardio.2019.3780.

Solbach, P., Potthoff, A., Raatschen, H. J., Soudah, B., Lehmann, U., Schneider, A., Gebel, M. J., Manns, M. P. and Vogel, A. (2015). Testosterone-receptor positive hepatocellular carcinoma in a 29-year old bodybuilder with a history of anabolic androgenic steroid abuse: A case report. *BMC Gastroenterology*. BMC Gastroenterology, 15(1), 1–7. doi: 10.1186/s12876-015-0288-0.

Solinas, G. and Becattini, B. (2017). JNK at the crossroad of obesity, insulin resistance, and cell stress response. *Molecular Metabolism*. Elsevier GmbH, 6(2), 174–184. doi: 10.1016/j.molmet.2016.12.001.

Sookoian, S. and Pirola, C. J. (2011). Metabolic syndrome: From the genetics to the pathophysiology. *Current Hypertension Reports*, 13(2), 149–157. doi: 10.1007/s11906-010-0164-9.

Soto González, A., Bellido Guerrero, D., Buño Soto, M., Pértega Díaz, S., Martinez-Olmos, M. and Vidal, O. (2006). Metabolic syndrome, insulin resistance and the inflammation markers C-reactive protein and ferritin. *European Journal of Clinical Nutrition*, 60(6), 802–809. doi: 10.1038/sj.ejcn.1602384.

Sperling, L. S., Mechanick, J. I., Neeland, I. J., Herrick, C. J., Després, J. P., Ndumele, C. E., Vijayaraghavan, K., Handelsman, Y., Puckrein, G. A., Araneta, M. R. G., Blum, Q. K., Collins, K. K., Cook, S., Dhurandhar, N. V., Dixon, D. L., Egan, B. M., Ferdinand, D. P., Herman, L. M., Hessen, S. E., Jacobson, T. A., Pate, R. R., Ratner, R. E., Brinton, E. A., Forker, A. D., Ritzenthaler, L. L. and Grundy, S. M. (2015). The CardioMetabolic Health Alliance Working Toward a New Care Model for the Metabolic Syndrome. *Journal of the American College of Cardiology*, 66(9), 1050–1067. doi: 10.1016/j.jacc.2015.06.1328.

Srikanthan, P., Horwich, T. B. and Tseng, C. H. (2016). Relation of Muscle Mass and Fat Mass to Cardiovascular Disease Mortality. *American Journal of Cardiology*. Elsevier Inc, 117(8), 1355–1360. doi: 10.1016/j.amjcard.2016.01.033.

Stansfield, B. N., Brown, A. D., Stewart, C. E. and Burniston, J. G. (2020). Dynamic Profiling of Protein Mole Synthesis Rates During C2C12 Myoblast Differentiation. *Proteomics*, 2000071, 2000071. doi: 10.1002/pmic.202000071.

Stein, R., Ferrari, F. and Scolari, F. (2019). Genetics, Dyslipidemia, and Cardiovascular Disease: New Insights. *Current Cardiology Reports*. Current Cardiology Reports, 21(8). doi: 10.1007/s11886-019-1161-5.

Stergiopoulos, K., Brennan, J. J., Mathews, R., Setaro, J. F. and Kort, S. (2008). Anabolic steroids, acute myocardial infarction and polycythemia: A case report and review of the literature. *Vascular*

Health and Risk Management, 4(6), 1475–1480. doi: 10.2147/vhrm.s4261.

Storer, T. W., Basaria, S., Traustadottir, T., Harman, S. M., Pencina, K., Li, Z., Travison, T. G., Miciek, R., Tsitouras, P., Hally, K., Huang, G. and Bhasin, S. (2017). Effects of testosterone supplementation for 3 years on muscle performance and physical function in older men. *Journal of Clinical Endocrinology and Metabolism*, 102(2), 583–593. doi: 10.1210/jc.2016-2771.

Strasser, B. (2013). Physical activity in obesity and metabolic syndrome. *Annals of the New York Academy of Sciences*, 1281(1), 141–159. doi: 10.1111/j.1749-6632.2012.06785.x.

Strasser, B., Siebert, U. and Schobersberger, W. (2010). Resistance training in the treatment of the metabolic syndrome: A systematic review and meta-analysis of the effect of resistance training on metabolic clustering in patients with abnormal glucose metabolism. *Sports Medicine*, 40(5), 397–415. doi: 10.2165/11531380-000000000-00000.

Stump, C. S., Henriksen, E. J., Wei, Y. and Sowers, J. R. (2006). The metabolic syndrome: Role of skeletal muscle metabolism. *Annals of Medicine*, 38(6), 389–402. doi: 10.1080/07853890600888413.

Sun, Z. (2015). Aging, arterial stiffness, and hypertension. *Hypertension*, 65(2), 252–256. doi: 10.1161/HYPERTENSIONAHA.114.03617.

Taggart, H. M., Applebaum-Bowden, D., Haffner, S., Warnick, G. R., Cheung, M. C., Albers, J. J., Chestnut III, C. H. and Hazzard, W. R. (1982). Reduction in high density lipoproteins by anabolic steroid (stanozolol) therapy for postmenopausal osteoporosis. *Metabolism*, 31(11), 1147–1152.

Tam, C. S., Xie, W., Johnson, W. D., Cefalu, W. T., Redman, L. M. and Ravussin, E. (2012). Defining insulin resistance from hyperinsulinemic-euglycemic clamps. *Diabetes Care*, 35(7), 1605–1610. doi: 10.2337/dc11-2339.

Tangvarasittichai, S. (2015). Oxidative stress, insulin resistance, dyslipidemia and type 2 diabetes mellitus. *World Journal of Diabetes*, 6(3), 456. doi: 10.4239/wjd.v6.i3.456.

Tanti, J. F. and Jager, J. (2009). Cellular mechanisms of insulin resistance: role of stress-regulated serine kinases and insulin receptor substrates (IRS) serine phosphorylation. *Current Opinion in Pharmacology*, 9(6), 753–762. doi: 10.1016/j.coph.2009.07.004.

Tay, J., Brinkworth, G. D., Noakes, M., Keogh, J. and Clifton, P. M. (2008). Metabolic Effects of Weight Loss on a Very-Low-Carbohydrate Diet Compared With an Isocaloric High-Carbohydrate Diet in Abdominally Obese Subjects. *Journal of the American College of Cardiology*, 51(1), 59–67. doi: 10.1016/j.jacc.2007.08.050.

Tay, J., Thompson, C. H., Luscombe-Marsh, N. D., Wycherley, T. P., Noakes, M., Buckley, J. D., Wittert, G. A., Yancy, W. S. and Brinkworth, G. D. (2018). Effects of an energy-restricted low-carbohydrate, high unsaturated fat/low saturated fat diet versus a high-carbohydrate, low-fat diet in type 2 diabetes: A 2-year randomized clinical trial. *Diabetes, Obesity and Metabolism*, 20(4), 858–871. doi: 10.1111/dom.13164.

Taylor, W. E., Bhasin, S., Artaza, J., Byhower, F., Azam, M., Willard, D. H., Kull, F. C. and Gonzalez-Cadavid, N. (2001). Myostatin inhibits cell proliferation and protein synthesis in C2C12 muscle cells. *American Journal of Physiology - Endocrinology and Metabolism*, 280(2 43-2), 221–228. doi: 10.1152/ajpendo.2001.280.2.e221.

Tchernof, A. and Després, J. P. (2013). Pathophysiology of human visceral obesity: An update. *Physiological Reviews*, 93(1), 359–404. doi: 10.1152/physrev.00033.2011.

Telle-Hansen, V. H., Christensen, J. J., Formo, G. A., Holven, K. B. and Ulven, S. M. (2020). A comprehensive metabolic profiling of the metabolically healthy obesity phenotype. *Lipids in health and disease*. Lipids in Health and Disease, 19(1), 90. doi: 10.1186/s12944-020-01273-z.

Teruel, J. L., Lasuncion, M. A., Rivera, M., Aguilera, A., Ortega, H., Tato, A., Marcen, R. and Ortuño, J. (1997). Nandrolone decanoate reduces serum lipoprotein(A) concentrations in hemodialysis patients. *American Journal of Kidney Diseases*, 29(4), 569–575. doi: 10.1016/S0272-6386(97)90340-1.

Tezze, C., Romanello, V. and Sandri, M. (2019). FGF21 as modulator of metabolism in health and disease. *Frontiers in Physiology*, 10(MAR), 1–9. doi: 10.3389/fphys.2019.00419.

Thiblin, I., Garmo, H., Garle, M., Holmberg, L., Byberg, L., Michaëlsson, K. and Gedeborg, R. (2015). Anabolic steroids and cardiovascular risk: A national population-based cohort study. *Drug and Alcohol Dependence*. Elsevier Ireland Ltd, 152, 87–92. doi: 10.1016/j.drugalcdep.2015.04.013.

Thiebaud, D., Jacot, E., DeFronzo, R. A., Maeder, E., Jequier, E. and Felber, J. P. (1982). The effect of graded doses of insulin on total glucose uptake, glucose oxidation, and glucose storage in man. *Diabetes*, 31(11), 957–963. doi: 10.2337/diacare.31.11.957.

Thompson, P. D., Cullinane, E. M., Sady, S. P., Chenevert, C., Saritelli, A. L., Sady, M. A. and Herbert, P. N. (1989). Contrasting Effects of Testosterone and Stanozolol on Serum Lipoprotein Levels. *JAMA: The Journal of the American Medical Association*, 261(8), 1165–1168. doi: 10.1001/jama.1989.03420080085036.

Thompson, R. C., Allam, A. H., Lombardi, G. P., Wann, L. S., Sutherland, M. L., Sutherland, J. D., Soliman, M. A. T., Frohlich, B., Mininberg, D. T., Monge, J. M., Vallodolid, C. M., Cox, S. L., Abd El-Maksoud, G., Badr, I., Miyamoto, M. I., El-Halim Nur El-Din, A., Narula, J., Finch, C. E. and Thomas, G. S. (2013). Atherosclerosis across 4000 years of human history: The Horus study of four ancient populations. *The Lancet*. Elsevier Ltd, 381(9873), 1211–1222. doi: 10.1016/S0140-6736(13)60598-X.

Thongtang, N., Diffenderfer, M. R., Ooi, E. M. M., Barrett, P. H. R., Turner, S. M., Le, N. A., Brown, W. V. and Schaefer, E. J. (2017). Metabolism and proteomics of large and small dense LDL in combined hyperlipidemia: Effects of rosuvastatin. *Journal of Lipid Research*, 58(7), 1315–1324. doi: 10.1194/jlr.M073882.

Timmis, A., Townsend, N., Gale, C. P., Torbica, A., Lettino, M., Petersen, S. E., Mossialos, E. A., Maggioni, A. P., Kazakiewicz, D., May, H. T., De Smedt, D., Flather, M., Zuhlke, L., Beltrame, J. F., Huculeci, R., Tavazzi, L., Hindricks, G., Bax, J., Casadei, B., Achenbach, S., Wright, L., Vardas, P.,

Mimoza, L., Artan, G., Aurel, D., Chettibi, M., Hammoudi, N., Sisakian, H., Pepoyan, S., Metzler, B., Siostrzonek, P., Weidinger, F., Jahangirov, T., Aliyev, F., Rustamova, Y., Mrochak, N. M. A., Lancellotti, P., Pasquet, A., Claeys, M., Kusljugic, Z., Hudic, L. D., Smajic, E., Tokmakova, M. P., Gatzov, P. M., Milicic, D., Bergovec, M., Christou, C., Moustra, H. H., Christodoulides, T., Linhart, A., Taborsky, M., Abdelhamid, M., Shokry, K., Kampus, P., Viigimaa, M., Ryödi, E., Niemela, M., Rissanen, T. T., Le Heuzey, J. Y., Gilard, M., Aladashvili, A., Gamkrelidze, A., Kereselidze, M., Zeiher, A., Katus, H., Bestehorn, K., Tsioufis, C., Goudevenos, J., Csanádi, Z., Becker, D., Tóth, K., Hrafnkelsdóttir, P. J., Crowley, J., Kearney, P., Dalton, B., Zahger, D., Wolak, A., Gabrielli, D., Indolfi, C., Urbinati, S., Imantayeva, G., Berkinbayev, S., Bajraktari, G., Ahmeti, A., Berisha, G., Erkin, M., Saamay, A., Erglis, A., Bajare, I., Jegere, S., Mohammed, M., Sarkis, A., Saadeh, G., Zvirblyte, R., Sakalyte, G., Slapikas, R., Ellafi, K., El Ghamari, F., Banu, C., Beissel, J., Felice, T., Buttigieg, S. C., Xuereb, R. G., Popovici, M., Boskovic, A., Rabrenovic, M., Ztot, S., Abir-Khalil, S., Van Rossum, A. C., Mulder, B. J. M., Elsendoorn, M. W., Srbinovska-Kostovska, E., Kostov, J., Marjan, B., Steigen, T., Mjølstad, O. C., Ponikowski, P., Witkowski, A., Jankowski, P., Gil, V. M., Mimoso, J., Baptista, S., Vinereanu, D., Chioncel, O., Popescu, B. A., Shlyakhto, E., Oganov, R., Foscoli, M., Zavatta, M., Dikic, A. D., Beleslin, B., Radovanovic, M. R., Hlivak, P., Hatala, R., Kaliska, G., Kenda, M., Fras, Z., Anguita, M., Cequier, A., Muniz, J., James, S., Johansson, B., Platonov, P., Zellweger, M. J., Pedrazzini, G. B., Carballo, D., Shebli, H. E., Kabbani, S., Abid, L., Addad, F., Bozkurt, E., Kayikçioğlu, M., Erol, M. K., Kovalenko, V., Nesukay, E., Wragg, A., Ludman, P., Ray, S., Kurbanov, R., Boateng, D., Daval, G., De Benito Rubio, V., Sebastiao, D., De Courtelary, P. T. and Bardinet, I. (2020). European society of cardiology: Cardiovascular disease statistics 2019. *European Heart Journal*, 41(1), 12–85. doi: 10.1093/eurheartj/ehz859.

Traish, A. M. and Kypreos, K. E. (2011). Testosterone and cardiovascular disease: An old idea with modern clinical implications. *Atherosclerosis*. Elsevier Ireland Ltd, 214(2), 244–248. doi: 10.1016/j.atherosclerosis.2010.08.078.

Turban, S. and Hajduch, E. (2011). Protein kinase C isoforms: Mediators of reactive lipid metabolites in the development of insulin resistance. *FEBS Letters*. Federation of European Biochemical Societies, 585(2), 269–274. doi: 10.1016/j.febslet.2010.12.022.

Turner, M. C., Player, D. J., Martin, N. R. W., Akam, E. C. and Lewis, M. P. (2018). The effect of chronic high insulin exposure upon metabolic and myogenic markers in C2C12 skeletal muscle cells and myotubes. *Journal of Cellular Biochemistry*, 119(7), 5686–5695. doi: 10.1002/jcb.26748.

Turner, N., Cooney, G. J., Kraegen, E. W. and Bruce, C. R. (2014). Fatty acid metabolism, energy expenditure and insulin resistance in muscle. *Journal of Endocrinology*, 220(2). doi: 10.1530/JOE-13-0397.

Tyrovolas, S., Panagiotakos, D., Georgousopoulou, E., Chrysohoou, C., Tousoulis, D., Haro, J. M. and Pitsavos, C. (2020). Skeletal muscle mass in relation to 10 year cardiovascular disease incidence among middle aged and older adults: The ATTICA study. *Journal of Epidemiology and Community Health*, 74(1), 26–31. doi: 10.1136/jech-2019-212268.

U.S. Department of Health and human services (2020). *Dietary Guidelines For Americans*.

Um, S. H., D'Alessio, D. and Thomas, G. (2006). Nutrient overload, insulin resistance, and ribosomal protein S6 kinase 1, S6K1. *Cell Metabolism*, 3(6), 393–402. doi: 10.1016/j.cmet.2006.05.003.

Urhausen, A., Albers, T. and Kindermann, W. (2004). Are the cardiac effects of anabolic steroid abuse in strength athletes reversible?. *Heart*, 90(5), 496–501. doi: 10.1136/hrt.2003.015719.

Urhausen, A., Torsten, A. and Wilfried, K. (2003). Reversibility of the effects on blood cells, lipids, liver function and hormones in former anabolic-androgenic steroid abusers. *Journal of Steroid Biochemistry and Molecular Biology*, 84(2–3), 369–375. doi: 10.1016/S0960-0760(03)00105-5.

Vazquez, G., Duval, S., Jacobs, D. R. and Silventoinen, K. (2007). Comparison of body mass index, waist circumference, and waist/hip ratio in predicting incident diabetes: A meta-analysis. *Epidemiologic Reviews*, 29(1), 115–128. doi: 10.1093/epirev/mxm008.

van de Ven, K., Zahnow, R., McVeigh, J. and Winstock, A. (2020). The modes of administration of anabolic-androgenic steroid (AAS) users: are non-injecting people who use steroids overlooked?. *Drugs: Education, Prevention and Policy*. Taylor & Francis, 27(2), 131–135. doi: 10.1080/09687637.2019.1608910.

Veum, V. L., Laupsa-Borge, J., Eng, Ø., Rostrup, E., Larsen, T. H., Nordrehaug, J. E., Nygård, O. K., Sagen, J. V., Gudbrandsen, O. A., Dankel, S. N. and Mellgren, G. (2017). Visceral adiposity and metabolic syndrome after very high-fat and low-fat isocaloric diets: A randomized controlled trial. *American Journal of Clinical Nutrition*, 105(1), 85–99. doi: 10.3945/ajcn.115.123463.

Volek, J. S., Phinney, S. D., Forsythe, C. E., Quann, E. E., Wood, R. J., Puglisi, M. J., Kraemer, W. J., Bibus, D. M., Fernandez, M. L. and Feinman, R. D. (2009). Carbohydrate restriction has a more favorable impact on the metabolic syndrome than a low fat diet. *Lipids*, 44(4), 297–309. doi: 10.1007/s11745-008-3274-2.

Volk, B. M., Kunces, L. J., Freidenreich, D. J., Kupchak, B. R., Saenz, C., Artistizabal, J. C., Fernandez, M. L., Bruno, R. S., Maresh, C. M., Kraemer, W. J., Phinney, S. D. and Volek, J. S. (2014). Effects of step-wise increases in dietary carbohydrate on circulating saturated fatty acids and palmitoleic acid in adults with metabolic syndrome. *PLoS ONE*, 9(11), 1–16. doi: 10.1371/journal.pone.0113605.

Wadhera, R. K., Steen, D. L., Khan, I., Giugliano, R. P. and Foody, J. M. (2016). A review of low-density lipoprotein cholesterol, treatment strategies, and its impact on cardiovascular disease morbidity and mortality. *Journal of Clinical Lipidology*. Elsevier Inc, 10(3), 472–489. doi: 10.1016/j.jacl.2015.11.010.

Wajchenberg, B. L., Lé, B. and Wajchenberg, O. (2000). Subcutaneous and Visceral Adipose Tissue :. *Endocrine Reviews*, 21(6), 697–738.

Walker, C. L. (1983). Nutrition: The changing scene. Implementing the NACNE report. 3. The new British diet. *The Lancet*. Lancet, 322(8363), 1354–1357. doi: 10.1016/S0140-6736(83)91106-6.

Wallace, T. M., Levy, J. C. and Matthews, D. R. (2004). Use and abuse of HOMA modeling. *Diabetes*

Care, 27(6), 1487–1495. doi: 10.2337/diacare.27.6.1487.

Wang, M., Kim, S. H., Monticone, R. E. and Lakatta, E. G. (2015). Matrix metalloproteinases promote arterial remodeling in aging, hypertension, and atherosclerosis. *Hypertension*, 65(4), 698–703. doi: 10.1161/HYPERTENSIONAHA.114.03618.

Warburton, D. E. R., Nicol, C. W. and Bredin, S. S. D. (2006). Health benefits of physical activity: The evidence. *Cmaj*, 174(6), 801–809. doi: 10.1503/cmaj.051351.

Wasserman, D. H. (2009). Four grams of glucose. *American Journal of Physiology - Endocrinology and Metabolism*, 296(1), 11–21. doi: 10.1152/ajpendo.90563.2008.

Westerterp-Plantenga, M. S., Lemmens, S. G. and Westerterp, K. R. (2012). Dietary protein - Its role in satiety, energetics, weight loss and health. *British Journal of Nutrition*, 108(SUPPL. 2). doi: 10.1017/S0007114512002589.

Westlye, L. T., Kaufmann, T., Alnæs, D., Hullstein, I. R. and Bjørnebekk, A. (2017). Brain connectivity aberrations in anabolic-androgenic steroid users. *NeuroImage: Clinical*. The Authors, 13, 62–69. doi: 10.1016/j.nicl.2016.11.014.

Westman, E. C., Feinman, R. D., Mavropoulos, J. C., Vernon, M. C., Volek, J. S., Wortman, J. A., Yancy, W. S. and Phinney, S. D. (2007). Low-carbohydrate nutrition and metabolism. *American Journal of Clinical Nutrition*, (6), 276–284.

Westman, E. C., Yancy, W. S., Mavropoulos, J. C., Marquart, M. and McDuffie, J. R. (2008). The effect of a low-carbohydrate, ketogenic diet versus a low-glycemic index diet on glycemic control in type 2 diabetes mellitus. *Nutrition and Metabolism*, 5(1), 1–9. doi: 10.1186/1743-7075-5-36.

Whiteman, E. L., Cho, H. and Birnbaum, M. J. (2002). Role of Akt/protein kinase B in metabolism. *Trends in Endocrinology & Metabolism*, 13(10), 444–451. doi: 10.1016/S1043-2760(02)00662-8.

Wilcox, G. (2005). Insulin and insulin resistance. *The Clinical biochemist. Reviews / Australian Association of Clinical Biochemists*, 26(2), 19–39. doi: 10.1016/S0025-7125(03)00128-7.

Wilkenfeld, S. R., Lin, C. and Frigo, D. E. (2018). Communication between genomic and non-genomic signaling events coordinate steroid hormone actions. *Steroids*. Elsevier, 133, 2–7. doi: 10.1016/j.steroids.2017.11.005.

Williams, R., Karuranga, S., Malanda, B., Saeedi, P., Basit, A., Besançon, S., Bommer, C., Esteghamati, A., Ogurtsova, K., Zhang, P. and Colagiuri, S. (2020). Global and regional estimates and projections of diabetes-related health expenditure: Results from the International Diabetes Federation Diabetes Atlas, 9th edition. *Diabetes Research and Clinical Practice*, 162. doi: 10.1016/j.diabres.2020.108072.

Wilson, P. W. F., D'Agostino, R. B., Levy, D., Belanger, A. M., Silbershatz, H. and Kannel, W. B. (1998). Prediction of coronary heart disease using risk factor categories. *Circulation*, 97(18), 1837–1847. doi: 10.1161/01.CIR.97.18.1837.

Wilson, P. W. F., D'Agostino, R. B., Parise, H., Sullivan, L. and Meigs, J. B. (2005). Metabolic syndrome as a precursor of cardiovascular disease and type 2 diabetes mellitus. *Circulation*, 112(20), 3066–3072. doi: 10.1161/CIRCULATIONAHA.105.539528.

Winter, J. N., Jefferson, L. S. and Kimball, S. R. (2011). ERK and Akt signaling pathways function through parallel mechanisms to promote mTORC1 signaling. *American Journal of Physiology - Cell Physiology*, 300(5), 1172–1180. doi: 10.1152/ajpcell.00504.2010.

Wolfe, R. R. (2006). The underappreciated role of muscle in health and disease. *American Journal of Clinical Nutrition*, 84(3), 475–482. doi: 10.1093/ajcn/84.3.475.

Wong, C. Y., Al-Salami, H. and Dass, C. R. (2020). C2C12 cell model: its role in understanding of insulin resistance at the molecular level and pharmaceutical development at the preclinical stage. *Journal of Pharmacy and Pharmacology*, 72(12), 1667–1693. doi: 10.1111/jphp.13359.

Wood, J., Freemantle, N., King, M. and Nazareth, I. (2014). Trap of trends to statistical significance: Likelihood of near significant P value becoming more significant with extra data. *BMJ (Online)*, 348(March), 1–6. doi: 10.1136/bmj.g2215.

Woodhill, J. M., Palmer, A. J., Leelarthaepin, B., McGilchrist, C. and Blacket, R. B. (1978). Low fat, low cholesterol diet in secondary prevention of coronary heart disease. *Advances in experimental medicine and biology*. Springer, Boston, MA, 109, 317–330. doi: 10.1007/978-1-4684-0967-3_18.

Woodhouse, L. J., Gupta, N., Bhasin, M., Singh, A. B., Ross, R., Phillips, J. and Bhasin, S. (2004). Dose-Dependent Effects of Testosterone on Regional Adipose Tissue Distribution in Healthy Young Men. *Journal of Clinical Endocrinology and Metabolism*, 89(2), 718–726. doi: 10.1210/jc.2003-031492.

World Health Organization (2018a). *Healthy diet*.

World Health Organization (2018b). *The top 10 causes of death*. Available at: https://www.who.int/news-room/fact-sheets/detail/the-top-10-causes-of-death (Accessed: 1 May 2020).

World Health Organization and World Health Organization. (2019). Healthy diet., (August), 1–6. Available at: https://apps.who.int/iris/handle/10665/325828.

Wu, Z., Woodring, P. J., Bhakta, K. S., Tamura, K., Wen, F., Feramisco, J. R., Karin, M., Wang, J. Y. J. and Puri, P. L. (2000). p38 and Extracellular Signal-Regulated Kinases Regulate the Myogenic Program at Multiple Steps. *Molecular and Cellular Biology*, 20(11), 3951–3964. doi: 10.1128/mcb.20.11.3951-3964.2000.

Xu, J., Lloyd, D. J., Hale, C., Stanislaus, S., Chen, M., Sivits, G., Vonderfecht, S. N., Hecht, R., Li, L., Lindberg, R. a, Chen, J. L., Jung, D. Y., Zhang, Z., Ko, H. J., Kim, J. K. and Veniant, M. M. (2009). FGF21 Reverses Hepatic Steatosis, Increases Energy Expenditure and Improves Insulin Sensitivity in Diet-induced Obese Mice. *Diabetes*, 58(January), 250–259. doi: 10.2337/db08-0392.

Xu, J. and Shi, G.-P. (2014). Vascular wall extracellular matrix proteins and vascular diseases. *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease*, 1842(11), 2106–2119. doi:

10.1016/j.bbadis.2014.07.008.

Yamamoto, N., Ueda-Wakagi, M., Sato, T., Kawasaki, K., Sawada, K., Kawabata, K., Akagawa, M., Ashida, H., Ueda, M., Sato, T., Kawasaki, K., Sawada, K., Kawabata, K., Ashida, H., Ueda-Wakagi, M., Sato, T., Kawasaki, K., Sawada, K., Kawabata, K., Akagawa, M., Ashida, H., Ueda, M., Sato, T., Kawasaki, K., Sawada, K., Kawabata, K., Ashida, H., Ueda-Wakagi, M., Sato, T., Kawasaki, K., Sawada, K., Kawabata, K., Akagawa, M. and Ashida, H. (2015). Measurement of Glucose Uptake in Cultured Cells. *Current protocols in pharmacology / editorial board, S.J. Enna (editor-in-chief) ... [et al.]*, 71(December), 12.14.1-12.14.26. doi: 10.1002/0471141755.ph1214s71.

Yang, Q., Vijayakumar, A. and Kahn, B. B. (2018). Metabolites as regulators of insulin sensitivity and metabolism. *Nature Reviews Molecular Cell Biology*. Springer US, 19(10), 654–672. doi: 10.1038/s41580-018-0044-8.

Yao, T. and Asayama, Y. (2017). Animal-cell culture media: History, characteristics, and current issues. *Reproductive Medicine and Biology*, 16(2), 99–117. doi: 10.1002/rmb2.12024.

Yaspelkis, B. B., Davis, J. R., Saberi, M., Smith, T. L., Jazayeri, R., Singh, M., Fernandez, V., Trevino, B., Chinookoswong, N., Wang, J., Shi, Z. Q. and Levin, N. (2001). Leptin administration improves skeletal muscle insulin responsiveness in diet-induced insulin-resistant rats. *American Journal of Physiology - Endocrinology and Metabolism*, 280(1 43-1), 130–142. doi: 10.1152/ajpendo.2001.280.1.e130.

Yoon, M. S. and Choi, C. S. (2016). The role of amino acid-induced mammalian target of rapamycin complex 1(mTORC1) signaling in insulin resistance. *Experimental and Molecular Medicine*. Nature Publishing Group, 48(1). doi: 10.1038/emm.2015.93.

Yuan, H., Hu, Y., Zhu, Y., Zhang, Y., Luo, C., Li, Z., Wen, T., Zhuang, W., Zou, J., Hong, L., Zhang, X., Hisatome, I., Yamamoto, T. and Cheng, J. (2017). Metformin ameliorates high uric acid-induced insulin resistance in skeletal muscle cells. *Molecular and Cellular Endocrinology*. Elsevier Ireland Ltd, 443, 138–145. doi: 10.1016/j.mce.2016.12.025.

Yusuf, S., Joseph, P., Rangarajan, S., Islam, S., Mente, A., Hystad, P., Brauer, M., Kutty, V. R., Gupta, R., Wielgosz, A., AlHabib, K. F., Dans, A., Lopez-Jaramillo, P., Avezum, A., Lanas, F., Oguz, A., Kruger, I. M., Diaz, R., Yusoff, K., Mony, P., Chifamba, J., Yeates, K., Kelishadi, R., Yusufali, A., Khatib, R., Rahman, O., Zatonska, K., Iqbal, R., Wei, L., Bo, H., Rosengren, A., Kaur, M., Mohan, V., Lear, S. A., Teo, K. K., Leong, D., O'Donnell, M., McKee, M. and Dagenais, G. (2020). Modifiable risk factors, cardiovascular disease, and mortality in 155 722 individuals from 21 high-income, middle-income, and low-income countries (PURE): a prospective cohort study. *The Lancet*, 395(10226), 795–808. doi: 10.1016/S0140-6736(19)32008-2.

Zahnow, R., McVeigh, J., Bates, G. and Winstock, A. R. (2020). Motives and Correlates of Anabolic-Androgenic Steroid Use With Stimulant Polypharmacy. *Contemporary Drug Problems*, 47(2), 118–135. doi: 10.1177/0091450920919456.

Zang, H., Carlström, K., Arner, P. and Hirschberg, A. L. (2006). Effects of treatment with testosterone alone or in combination with estrogen on insulin sensitivity in postmenopausal women. *Fertility and Sterility*, 86(1), 136–144. doi: 10.1016/j.fertnstert.2005.12.039.

Zeng, F., Zhao, H. and Liao, J. (2017). Androgen interacts with exercise through the mTOR pathway to induce skeletal muscle hypertrophy. *Biology of Sport*, 34(4), 313–321. doi: 10.5114/biolsport.2017.69818.

Zhang, C., Rexrode, K. M., Van Dam, R. M., Li, T. Y. and Hu, F. B. (2008). Abdominal obesity and the risk of all-cause, cardiovascular, and cancer mortality: Sixteen years of follow-up in US women. *Circulation*, 117(13), 1658–1667. doi: 10.1161/CIRCULATIONAHA.107.739714.

Zhang, S., Chen, X., Huang, Z., Chen, D., Yu, B., Chen, H., Luo, J., He, J., Zheng, P. and Yu, J. (2018). Leucine promotes differentiation of porcine myoblasts through the protein kinase B (Akt)/Forkhead box O1 signalling pathway. *British Journal of Nutrition*, 119(7), 727–733. doi: 10.1017/S0007114518000181.

Zhao, L., Zhu, Z., Lou, H., Zhu, G., Huang, W., Zhang, S. and Liu, F. (2016). Polycystic ovary syndrome (PCOS) and the risk of coronary heart disease (CHD): A meta-analysis. *Oncotarget*, 7(23), 33715–33721. doi: 10.18632/oncotarget.9553.

Zurlo, F., Larson, K., Bogardus, C. and Ravussin, E. (1990). Skeletal muscle metabolism is a major determinant of resting energy expenditure. *Journal of Clinical Investigation*, 86(5), 1423–1427. doi: 10.1172/JCI114857.