Dynamic proteomic analysis of skeletal muscle adaptations to exercise training

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Declaration

I Connor Stead declare that all work presented here in this thesis was written by me and is my own. No portion of the work referred to in this thesis has been submitted in support of an application for another degree or qualification of Liverpool John Moores/ McMaster university, any other university or other institute of learning outside of the Memorandum of Understanding of the Co-Tutelle Ph.D.

Abstract

Skeletal muscle demonstrates a remarkable malleability and can adjust its metabolic and contractile properties in response to changes in environmental stimuli. The proteome represents a highly dynamic and versatile entity that coordinates the adaptive response of skeletal muscle through adjustments in individual protein abundance, modulated by changes in the synthesis and degradation rate of proteins. Previously research relating to protein turnover has been largely limited to average synthesis rates of protein mixtures e.g. from whole/ subfractions of muscle homogenates. This project utilises dynamic proteome profiling which combines deuterium oxide labelling and proteomic techniques with computational biology, to investigate muscle protein dynamics at the individual protein level. This work used 3 *in vivo* human and rodent exercise models to studying the complexity of dynamic proteome adaptation. Identifying that exercise induced adaptation can occur on the level of protein turnover, independent of classical changes in protein abundance. Which seems of particular importance in counteracting proteostatic stress (i.e. obesity and disease). Subsequently the need to investigate the relationship between protein dynamics and proteome remodelling resulted in the observation that changes in protein abundance do not follow a simple linear trajectory of adaptation. Also, protein specific synthesis rates seemingly undergo time-dependent adaptations when explaining remodelling in the context of an animal model of programmable resistance training. Finally, this thesis provides new evidence to demonstrate that human exercise training results in unique alterations in the rates of protein synthesis and degradation that is training mode and status dependent despite networks of proteins resulting in the same abundance changes. This work raises potential questions as to the extent of exercise induced adaptation explained by modulation of protein synthesis, particularly in the context of endurance training. Whereby changes in degradation are implicated as a key driver of endurance exercise induced proteome remodelling. This work represents some of the first of its kind to present novel details as to the potential roles of protein synthesis, degradation, and turnover rate in modulating differences in skeletal muscle health and function induced by exercise.

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Chapter 1. General Introduction

1.1 Muscle adaptations to exercise training

Regular physical activity and exercise training offer a profound stimulus to improve skeletal muscle function, including greater musculoskeletal health (Pedersen and Saltin, 2015) and metabolic function (Shojaee-Moradie et al., 2007). Exercise can be broadly categorized into endurance (aerobic) and resistance (strength) training, which yield distinct muscle phenotypes (Nader, 2006). Endurance training is characterized by repetitive, sub-maximal contractions and enhances muscle fatigue resistance through increases in oxidative capacity, mitochondrial density (Lundby and Jacobs, 2016), enhanced muscle ion handling (e.g. lactate transport and Ca^{2+} release) (Hostrup and Bangsbo, 2017), and oxygen/ nutrient delivery via greater muscle capillarisation (Liu et al., 2022). In contrast, resistance training involves short duration, maximal force contractions and results in increases in muscle power output and fibre cross-sectional area (CSA) through protein accretion and, in particular, expansion of the myofibrillar protein fraction (Balagopal et al., 2001, Hornberger and Farrar, 2004). Despite their dichotomy in contractile and metabolic phenotype, both endurance and resistance modes of training improve health outcomes and mitigate disease risk factors (Phillips et al., 2023), which suggests common mechanisms exist that underpin the well-established benefits of exercise (Lavin et al., 2022).

Over the course of the last several decades, targeted studies have discovered several key regulators of exercise adaptation (e.g. AMPK, PGC-1, Calcineurin, and ERK-Akt-mTOR pathways) (Egan and Zierath, 2013, Roberts et al., 2023a). However, the targeted approach may have inadvertently led to a relatively linear, 'audit-trial' in the evolution of knowledge on muscle adaptation and may have been blinkered to the full complexity of molecular adaptations to training. The completion of the human genome project and technological developments in analytical methods opened the opportunity for omics studies and a new era of 'systems biology' which approximately coincided with the turn of the century. Proteomic surveys of muscle provide a means to study the effects of exercise without prior selection of protein targets. Allowing for a description of proteome remodelling beyond the archetypal targets that have previously been associated with endurance or resistance trained phenotypes. Initially, studies in exercise proteomics used laboratory animals and investigated muscle responses to exercise using 2-D gel electrophoresis (2DGE) (reviewed in (Burniston and Hoffman, 2011)). Gel-based proteomic techniques have now been largely supplanted by liquid chromatography and online tandem mass spectrometry analyses (LC-MS/MS) (reviewed in (Hesketh et al., 2021).LC-MS/MS analyses of exercised muscle enable the abundance profile of thousands of proteins to be analysed in parallel and have provided more comprehensive insight into muscle responses to exercise training. Recent proteomic profiling of muscle from people with longstanding (>15 y) histories in either endurance, or resistance exercise or a sedentary lifestyle found differences in muscle phenotype were particularly robust between sedentary versus endurance-trained individuals. These data highlighted chronic endurance trained muscle to be associated with greater abundance of proteins associated with mitochondrial metabolism including oxidative phosphorylation subunits, TCA cycle, and fatty acid metabolism (Emanuelsson et al., 2024). However, the majority of proteomic data on the effect of exercise in skeletal muscle, utilise longitudinal study designs of training interventions to better link changes in proteome composition with specific training interventions. Early studies used experimental models of endurance activity such as chronic low-frequency stimulation of fast-twitch muscle, which results in a dramatic transition between fast- to slow-twitch phenotype. Donoghue et al (Donoghue et al., 2007) reports 60 days chronic low-frequency stimulation of rabbit tibialis anterior remodels muscle contractile machinery, ion homeostasis, vascularization, and substrate metabolism, giving insight into the potential scope of muscle adaptation in response to changes in contractile activity. Conversely, intensity-controlled treadmill running to study rat muscle responses to aerobic exercise revealed changes in the abundance of proteins associated with iron metabolism, fatty acid oxidation, and oxygen transport, as well as mitochondrial proteins (Burniston, 2008) were consistent with, but lesser in magnitude than, the muscle response to chronic low-

frequency stimulation. Similarly, Holloway et al., (Holloway et al., 2009) studied the human muscle proteome following 6 weeks of interval training, reporting adaptations in metabolic enzymes, myofibrillar proteins, heat shock proteins, and transport proteins, indicative of enhanced oxidative capacity and mitochondrial content. Analysis of proteoforms by 2-D gel electrophoresis discovered a change in creatine kinase phosphorylation after exercise training. Protein abundance profiling using isobaric tags for absolute quantitation identified increases in mitochondrial proteins involved in fatty acid beta oxidation and the electron transport chain (Holloway et al., 2009). Following numerous reports highlighting the mitochondrial protein changes to underpin endurance trained muscle, Egan et al. (Egan et al., 2013) reported high-resolution 2-DIGE of human mitochondrial proteome responses during the first 2 weeks of an endurance training programme. After 14 consecutive days of cycling (80% VO2max for 1 h per day) the whole-body VO2max in sedentary men increased by ~ 18%. Timeseries profiling found the majority of proteins that changed in abundance reached a peak after 7 days and no further gain occurred over the raimainder of the 14 d training period. There were ~1.3- to 2-fold increases in relative abundance of subunits of mitochondrial respiratory chain I and V and enzymes of the tricarboxylic acid cycle. However, no changes in mitochondrial: nuclear DNA ratio were detected, potentially indicating increases in 'qualitative' time-dependent changes of the mitochondrial proteome may precede the overall increases in mitochondrial content seen in chronic training studies. These works offered initial insight into the plasticity of the muscle proteome in response to endurance training, reproducing the understanding of protein level adaptations underpinning the endurance trained phenotype investigated using targeted biochemical analysis alongside revealing previously unexplored components associated with training.

Fewer studies have investigated the effects of resistance exercise training on muscle proteomic responses. Robinson et al (2017) compared muscle responses to 12 weeks of either resistance exercise (RT), high-intensity interval training (HIIT) or combined training (CT), which included moderate-intensity endurance exercise and resistance training, in both younger and older adults. In young adults, 265 proteins were uniquely upregulated with resistance training compared to 114 with high-intensity interval training. In older individuals, 147 were uniquely upregulated with resistance training as compared to 227 with high-intensity interval training. As such, high-intensity interval training had the strongest influence on altering mitochondrial protein abundance in older individuals. Notably, increased ribosomal proteins and other proteins significant in the translational machinery indicate an increased translational capacity in exercised muscle. Despite reports from Robinson et al., (2017) indicating robust proteome remodelling to both aerobic and resistance training, other studies report relatively few significant changes in protein abundance following chronic (> x weeks) resistance training. Deane et al., (Deane et al., 2022) assessed protein abundances from sarcoplasmic fractions of human muscle using Isobaric Tags for Relative and Absolute Quantification labelling combined with LC-MS after 20 weeks of supervised resistance training. The authors (Deane et al., 2022) report significant increases in contractile elements (e.g. alpha actin), protein chaperones and, divergent responses in metabolic enzymes (e.g. increases in fructose-bisphosphate aldolase A and decreases in pyruvate kinase) following training. Indicating the protein-specific reponses to resistance training to encompass changes across multiple functional classes of proteins. Ultimately indicating a paucity of reproducible reports information regarding how the muscle proteome is altered following chronic resistance training. As techniques develop, the depth of interrogation in the proteome has grown immensely, early studies identified <100 proteins whereas current methods are capable of studying >1000 proteins. For example, liquid chromatrography-mass spectrometry (LC-MS) based proteomics offered the comparison of >2000 proteins quantified in single fiber analysis of skeletal muscle response to 12-weeks of cycling (training 4 x per week at 75 – 90 % max heart rate) (Deshmukh et al., 2021). The authors report significant changes in the abundance of 377 proteins in response to the endurance training intervention whereby ~ 40 % and > 65 % of the significantly regulated proteome were associated with mitochondrial and metabolic processes (Deshmukh et al., 2021). Indicating proteomic analysis identified adaptation

in the mitochondrial proteome to be a hallmark of adaptation, consistent with previous targeted analysis. Whilst highlighting novel endurance-training induced adaptations associated with proteins involved in transcriptional regulation, mitochondrial metabolism, and glucose metabolism adapts to training in a fiber type- specific manner (Deshmukh et al., 2021). With deeper proteome profiling studies, the identification of changes in proteins with unknown function or relevance to adaptation is not uncommon but, nonetheless, will continue to advance our understanding of exercise influence on muscle structure and function.

Despite advancing our characterisation of skeletal muscle adaptations to training, protein abundance profiling of trained muscle offers limited insight to the mechanisms underpinning the process of adaptation. Acute exercise causes perturbations to homeostasis in skeletal muscle that occur during the onset of and recovery from contractile work including, but not limited to, greater metabolic flux (Hargreaves and Spriet, 2018), changes in mitochondrial redox state (Margaritelis et al., 2020), and mechanical loading (Tidball, 2005). The acute signalling responses to the intermittent perturbations of individual exercise bouts accumulate when regularly repeated and result in adaptations that aim to prevent future perturbations in homeostasis.

Proteomic analyses of protein post-translational modification can be used to study the co-ordinated response amongst different signalling pathways and have the power to highlight unidentified signalling responses that provide new insight into different training modes. Phosphoproteomic responses in muscle are the most well reported modification. Hoffman (Hoffman et al., 2015) conducted 2D LC-MS/MS profiling of phosphopeptides in muscle samples from 4 healthy young men before and immediately after a ~ 10 min high-intensity aerobic exercise bout. In total 928 of 1004 (92%) of phosphosites regulated by exercise were yet to be associated with upstream kinases. Potts and colleagues (Potts et al., 2017) applied similar methods of phosphoproteomic analysis of mouse tibialis anterior collected 1 h after unilateral electrical stimulation (10 sets of 6 maximal contractions) of the right limb whilst the left served as a contralateral control. Whilst the single stimulation bout did not alter the abundance of proteins, a total of 5983 phosphorylation sites (on 4,558 proteins) were identified ($n \geq 3$, per group). Including significant differences in the phosphorylation status of 621 sites (on 313 proteins). In line with the analysis by Hoffman et al (2015), most exercise responsive phospho-sites had not previously been annotated and only a few significantly regulated substrates (12 proteins) had known upstream kinases. Similarly, from static snapshots of acute signalling responses it is challenging disentangle exercise responsive signalling informing muscle adaptation from the broad, nonspecific molecular response in unaccustomed muscle exposed to the homeostatic perturbations of novel exercise induced metabolic and contractile stimuli (termed 'the first bout effect').

Phosphoproteomic studies highlight how much remains to be discovered in our quest to understand signal transduction networks. Comprehensive data on the abundance profile and site-specific post translational modifications have greatly advance the current state of knowledge of signalling networks involved in muscle adaptation. That said, the mechanistic link between acute changes in signalling networks and longer-term changes in proteome composition and muscle phenotype can only be studied using tracer techniques to investigate how changes to the synthesis and degradation of proteins resulted in the change in protein abundance (Hesketh et al., 2021).

1.2 The dynamic nature of the muscle proteome

The functional attributes of skeletal muscle are underpinned by its proteome, which encompasses the entire protein complement of the tissue. Pioneering work from Rudolph Schoenheimer established that all biological systems exist in a dynamic state. Indicating proteome composition is a result of the net balance between protein synthesis and degradation. Over the past ~90 years it has been well established that proteins undergo a continuous cycle of turnover (Ross et al., 2021). Proteins represent the critical functional units of the cell, that do not possess the

capacity for self-replication. Instead, the information required to specify individual proteins is encoded by nucleic acids, and protein abundance is regulated by a multitude of time-dependent mechanisms. Figure 1.1 illustrates the factors involved with the production of protein and the regulation of turnover.

Figure 1.1. The flow of information resulting in protein products and the regulation of protein turnover.

The production (synthesis) of a protein occurs from the flow of genetic information from DNA to RNA, to protein. A) A gene (encoded within DNA) is transcribed to RNA coding for a specific amino acid sequence (messenger RNA; mRNA). Messenger RNA then undergoes transport out of the nucleus into the cytosol where mRNA may undergo degradation or post-transcriptional events that may include alternative splicing or degradation of the mRNA that affect the rate of translation initiation of the specific mRNA. B) The resultant mRNA can then undergo synthesis into a protein product via binding of the initiation complex onto mRNA via co-operation with the eukaryotic translation initiation factor complexes. Aminoacyl-tRNA is then loaded into the 60S ribosomal subunit where peptide bonds are formed. Elongation continues until a stop codon is reached and a nascent peptide is produced. Newly synthesized proteins may undergo rapid degradation or folding via chaperones to form functional proteins. Proteins are then subjected to protein degradation by predominantly the ubiquitin proteasome system alongside the lysosomal-autophagy and caspase/calpain proteolytic systems. The abundance of a given protein depends solely on the balance between its rate of degradation versus its rate of synthesis. Proteins are the functional macromolecules producing physiological effects across the cell. If a protein has a fast synthesis rate and slow degradation rate it will increase in abundance within the cell. Conversely, proteins that have both a swift degradation and synthesis rate have the fastest turnover rates.

The central dogma of molecular biology outlines the flow of genetic information within biological systems and the transfer of biological information between DNA and RNA that ultimately results in the synthesis of new proteins (Crick, 1970). These fundamental processes are well established, and each are essential in governing the protein complement of a cell/ tissue. Muscle adaptations to training are thought to occur because of gradual changes in the protein content of muscle after repeated bouts of exercise. The adaptation is a direct reflection of the activation or suppression of specific signalling pathways that occur in response to the cellular insult inflicted by the mechanical and metabolic demand of the stimulus. Early studies on the molecular basis of exercise adaptation highlighted training induced changes in mRNA abundance. Williams and Neufer (Williams and Neufer, 1996) proposed that cumulative effects of exercise-induced changes in mRNA determine adaptive muscle responses. Temporal profiling of mRNA responses to endurance (Gustafsson et al., 1999, Pilegaard et al., 2000) and resistance exercise (Bamman et al., 2001, Willoughby and Nelson, 2002) found transient changes in gene transcription which serve as one of the fundamental mechanisms underlying exercise induced adaptation. Gene transcripts (i.e. mRNA) provide the template for protein translation but changes in transcript expression fail to reliably predict corresponding protein abundances both in model organisms (Gygi et al., 1999) and in the analysis of mammalian straited muscle (Gerdes Gyuricza et al., 2022). Overall, correlational analyses of mRNA expression and protein abundances typically suggest that transcript expression only explains ~40% of the variance in protein abundance (Vogel and Marcotte, 2012). Accordingly, changes in gene expression, particularly after resistance exercise, do not correlate with changes in muscle protein abundance in humans (Robinson et al., 2017). Some of the lack in correlation between changes in mRNA expression and protein abundance may be related to the different time-course of responses at the mRNA and protein levels. Furthermore, post-transcriptional regulation of mRNA can be responsible for further discrepancy between the mRNA expression and abundance of individual proteins. Post-translational processes were first reported in the context of resistance training, Laurent et al (Laurent et al., 1978) report that mechanical loading of chicken muscle leads to changes in protein translation rate that occur prior to RNA accumulation. Furthermore, muscle contractions induced by electrical stimulation activate translation initiation (measured via the phosphorylation of 70S6K (Baar and Esser, 1999)) and select gene transcripts are translated prior to measurable changes in the expression of the corresponding genes (Chen et al, 2001). These data question whether increases in protein synthesis always require a preceding increase in mRNA and explain why it is challenging to predict the adaptive response to training solely from transcriptome profiling data. Finally, the role of protein degradation in modulating the abundance of specific proteins has been somewhat overlooked. It was initially thought the degradation of proteins occurred via a bulk, stochastic disposal systems, but, it is now well-established that protein degradation is highly selective and precisely regulated (Goldberg, 2003). Protein degradation can occur via multiple processes within skeletal muscle, namely the autophagy-lysosomal system, caspase/calpain proteases, and the ubiquitin-proteasome system (UPS). The majority of intracellular proteins are considered to be targeted for degradation via the UPS (Pohl and Dikic, 2019). However, further mechanisms regulating organelle and compartment specific protein turnover (e.g. mitophagy and endoplasmic reticulum associated degradation) have been identified within skeletal muscle and exercise-induced adaptation.

Static data on the time course of exercise adaptation offer only a punctuate understanding of the temporal aspects of muscle adaption. Methods to monitor the dynamic processes of translation output (i.e. synthesis) and protein degradation are required to further understand proteome adaptation. For example, if a protein becomes more abundant from one time point to the next, it is not possible to know whether the increase in abundance was underpinned by greater synthesis or lesser degradation (or a combination of the 2 processes). Biosynthetic labelling of proteins *in vivo* has been used to measure protein turnover rates (technical details in Chapter 2). It is well known that resistance (Biolo et al., 1995, Yarasheski et al., 1993, Brook et al., 2015) and endurance exercise (Sheffield-Moore et al., 2004, Short et al., 2004) can increase muscle protein synthesis compared to basal rates in non-

exercise muscle. The dominant interest in muscle protein synthetic responses has resulted in mechanistic studies that focus on the regulators associated with the activation of protein synthesis. In 1999 Baar and Esser (Baar and Esser, 1999) published the first report demonstrating that the phosphorylation status of ribosomal protein S6 kinase (P70S6K) measured 6 h after training was positively associated with the degree of muscle hypertrophy quantified in rat hindlimb muscle after a 6-wk electrical stimulation model of resistance training. The prolonged phosphorylation of P70S6K was further confirmed alongside the identification of transient increases in protein kinase B (Akt) phosphorylation which is unique to hypertrophy-stimuli rather than endurance-type training (Nader and Esser, 2001). These works offered the first mechanistic data linking mechanical loading with signalling associated with increased protein synthesis which has been subsequently confirmed in the context of human resistance training (Dreyer et al., 2006). Subsequent work has built on this signalling network identifying mammalian target of rapamycin complexes 1 and 2 (mTORC1/2) as upstream kinases of P70S6K and thus the protein synthetic response to anabolic stimuli (Saxton and Sabatini, 2017). Furthermore, eukaryotic translation initiation factor 4E binding protein has subsequently been identified as a downstream target of mTORC1, involved in the activation of translation initiation (Dreyer et al., 2006). However, the focus on the synthetic arm of protein turnover has resulted in an underappreciation for protein degradation in modulating proteome remodelling in response to exercise. Protein degradation comprises a central component of the cellular protein homeostasis (proteostasis) system with a vital role in removing damaged, misfolded, and aggregated proteins (Alvarez-Castelao et al., 2012). Studies have indicated an increase in the rate of protein turnover (i.e. synthesis and degradation) in response to acute exercise (Phillips et al., 1997, Mascher et al., 2011), which is essential to the remodelling of the skeletal muscle proteome and to prevent the aggregation of damaged proteins following an exercise bout. Despite the importance of the major proteolytic systems, namely proteasome-, autophagy-, and caspase-mediated proteolysis, in maintaining cellular homeostasis, the regulatory roles of protein degradation have been sparsely studied in the context of exercise. Skeletal muscle atrophy has been the primary context of investigating degradative processes, resulting in the discovery of numerous ubiquitin E3 ligases. However, the role of these enzymes in adaptive remodelling is less well known. The muscle specific E3 ligases MuRF1 and MAFbx are transcriptionally upregulated in response to catabolic stimuli, suggesting they are regulated by similar transcription factors such as the O-type forkhead transcription factors (FOXO) (Bodine and Baehr, 2014). Subsequently, gene expression analysis of FOXO-MuRF1/MAFbx is a commonly used marker of catabolic signalling following exercise (e.g.(Mascher et al., 2008, Chen et al., 2011)), however, the role these pathways play in protein-specific degradation in response to exercise are yet to be understood. Furthermore, recent interest into E3 ligases has resulted in the identification of novel E3 ligases including the muscle specific ASB2B (Goodman et al., 2021) and UBR5 (Seaborne et al., 2019) in relation to muscle atrophy. Knockdown of UBR5 can concomitantly reduce protein synthesis (Hughes et al., 2021), which highlights the link between degradation and synthetic pathways that provides a feedback loop to maintain proteostasis. Furthermore, numerous reports have highlighted that aberrant regulation of the autophagy-lysosomal system aggravates muscle atrophy as autophagasome accumulation have been observed across myopathies (Malicdan et al., 2008). Typically, autophagy involves entire regions or organelles being engulfed by an autophagosome which is then degraded (termed macro-autophagy). The induction of autophagy is well described in response to exercise training and has been highlighted as an essential mechanism in adaptive remodelling (Wu et al., 2011, Halling and Pilegaard, 2017, Brandt et al., 2018). Whilst macro-autophagy underpins a vital quality control mechanism for the degradation of damaged proteins following cellular insult such as exercise, the regulation and contribution of selective degradation of specific proteins/ complexes via chaperone mediated autophagy to proteome remodelling is less well known. The major proteolytic systems within muscle also include cysteine aspartic proteases (caspases) which are associated with apoptotic cell death (Nicholson, 1999), Caspase-3 has been show to be a key regulator of several features of myogenesis including myoblast fusion to existing myofibers and regeneration (Fernando et al., 2002). However, the relevant caspase substrates are yet to

be well defined within this process. Furthermore, the caspase system are yet to be strongly implicated in exercise-induced protein degradation and adaptation. Despite the UPS being the main candidate for most intracellular proteolysis, each of the major degradative pathways are essential for the maintenance of protein homeostasis. Protein chaperones, including heat shock proteins, are also fundamental to proteostasis and are intimately involved at each stage of a protein's lifecycle (i.e. synthesis, folding/ re-folding and degradation) (Hartl et al., 2011). Chaperone proteins may be particularly important in skeletal muscle, which is exposed to mechanical as well as chemical stresses (Höhfeld et al., 2021).

A loss of proteostasis is one of the key hallmarks of ageing, which have emerged from studies in model organisms and experimental systems (López-Otín et al., 2023). Furthermore, losses in proteostasis have been implicated across numerous chronic diseases, including obesity and metabolic dysfunction due to chemical stresses, such as hyperinsulinemia and insulin resistance, whereby chronically activated insulin signalling, including mTORC1 activation and subsequent effects on protein turnover, may remain stimulated (Kolb et al., 2020). Furthermore, chronic inflammation and oxidative stress associated with chronic disease likely further exacerbate the impaired proteostasis resulting in increasingly toxic intramuscular environments. Therefore, disruption to intramuscular proteostasis may be a key determinant in the loss of muscle function occurring in chronic diseases. However, it is unclear as to which dynamic aspects of the proteome (e.g. synthesis, chaperoning/ protein folding, or protein degradation) may be the most responsible for declines in proteostasis induced by these chemical stresses underpinning disease. Indeed, heat shock proteins are abundant in muscle and are highly responsive to changes in contractile activity and as previously discussed the induction of UPS and autophagy-mediated degradation are key mechanisms of exercise induced protein turnover in skeletal muscle Therefore, implicating exercise training as a potentially potent stimulus for improving declines in intramuscular proteostasis. Knowledge of heat-shock responses and the involvement of select regulators of protein degradation is rapidly evolving but, as yet little is known about how synthesis, chaperoning and degradation are integrated to maintain proteostasis or achieve adaptation of the proteome to a different state.

To date, most literature has primarily reported mixed protein data on muscle protein synthesis (MPS) or breakdown (MPB) which provides an average muscle (or fraction-specific) protein synthesis/ degradation measure across all proteins within the muscle or muscle fraction. Proteomic reports indicate that these whole muscle analyses incorporate the accumulation of data from likely >10,000 proteins (Deshmukh et al., 2015). With even subcellular fractions containing > 1,000 proteins as identified within the soluble and myofibrillar fractions of muscle (Srisawat et al., 2023). Based on these bulk average data it has been challenging to quantitatively connect acute muscle responses such as cell signalling and protein synthesis with longer term muscle adaptation. Moreover, the majority of work investigating changes in muscle protein metabolism *in vivo* have used short-term (~ 3-6 h), intravenous infusions of stable isotope labelled amino acids (e.g. $^{2}H_{5}$-phenylalanine) (Witard et al., 2022). Thus, knowledge is limited to the acute elevations in protein turnover, which do not necessarily correlate with growth responses observed following chronic training (Mayhew et al., 2009, Mitchell et al., 2014). For example, Mitchell et al (2014) report no correlation between long term changes in muscle mass and acute rises in myofibrillar protein synthesis rates measured via primed constant infusion of L-[ring-$^{13}C_{6}$]-phenylalanine 1-3 h (r = 0.02) and 3-6 h (r = 0.16) after the early bouts of exercise. Damas et al., (Damas et al., 2016) demonstrated that acute measurements of exercise-induced increases in protein synthesis only correlated with muscle hypertrophy after the attenuation of muscle damage. Therefore, the initial perturbation (such as muscle damage) must be overcome before inferences on the role of adaptive mechanisms on muscle remodelling can be made. Highlighting the need for longer term/ integrated measurements across a period of exercise and recovery to limit the measurement of generic cellular perturbations associated with of the first bout effect in the understanding of muscle adaptation to exercise, However, Robinson et al., (Robinson et al., 2017) measure mixed-mitochondrial protein synthesis rate following a 3-month resistance

and endurance training intervention in humans using a 5 h infusion of [$^{13}C_6$]-phenylalanine and report a selective increase in mitochondrial protein synthesis rate in response to endurance training whilst no increase was reported in muscle in response to resistance training, despite the fact that 141 mitochondrial proteins increased in abundance following resistance training (Robinson et al., 2017). While it is obvious that measures of mixed-protein synthesis rate cannot be used to infer changes in the abundance of individual proteins, robust methods for studying the synthesis of individual proteins have only emerged in recent years.

Biosynthetic labelling of newly synthesised proteins via administration of deuterium oxide (D_2O) combined with proteomic analysis of muscle samples offers a solution to investigate longer term exercise interventions (Reviewed in chapter 2). However, most studies using deuterium oxide in humans still rely on isotope analysis of derivatised alanine extracted from muscle protein mixtures. Like the traditional application of amino acids tracers, this approach is limited to the quantification of changes in the average rate of protein synthesis for the entire protein complement of skeletal muscle or major subfractions. The assumption of this data is that training induces uniform increases in the synthesis rate of all proteins equally within skeletal muscle, but this uniform response is not consistent with protein-specific changes in abundance reported in exercised muscle (Holloway et al., 2009, Deshmukh et al., 2021b). Previous work from our lab (Hesketh et al., 2016) applied methods of biosynthetic labelling with deuterium oxide, 2DGE of muscle samples and mass spectrometry to investigate the synthesis rate of 8 proteins in rat heart, diaphragm, and fast- and slow-twitch skeletal muscle. This report indicated that the rate of synthesis of individual varied between muscles, and protein turnover rate was also not uniform within a tissue. Furthermore, during my MSc studies, we aimed to follow-up this analysis on the heterogeneity of protein turnover rates in skeletal muscle by profiling protein-specific synthesis rates in archetypal fast- and slow-twitch rat skeletal muscle (Stead et al., 2020). We identified that the long-established paradigm that protein-turnover rate is slower in fast- versus slow-twitch muscle does not hold true when data are reported at the individual protein level. Therefore, highlighting the turnover of skeletal muscle proteins is heterogenous, and a single synthesis rate cannot be used to infer data on the synthesis rate of all proteins within or across tissues. Thus, indicating the mechanistic insight that can be concluded on muscle adaptation from bulk/ average mixed-proteins synthesis rates is likely limited, particularly when investigating proteome remodelling.

The current literature indicates the need for methodologies to profile individual muscle protein abundance and synthesis responses to exercise to further our understanding on proteome adaptation. Furthermore, methods to investigate rates of protein degradation are required to understand the full extent of the dynamic processes that underpin changes in protein abundance. The development of combining contemporary proteomic techniques (i.e. protein abundance measurements) and stable isotope labelling in vivo (to measure dynamic processes) has given rise to a method termed dynamic proteome profiling (explained in detail in Chapter 2). When both protein abundance and synthesis are measured, changes in abundance that are not accounted for by changes in synthesis can be assigned to protein degradation. Likewise, changes in synthesis rate that are not accompanied by changes in abundance can be interpreted as an effect on turnover rate, which is also an important outcome in relation to proteome quality control and proteostasis. The development of dynamic proteomic techniques from our lab resulted in the first investigation into dynamic proteomic responses in human muscle in response to exercise (Camera et al., 2017). Untrained males completed a resistance training intervention consisting of 3 sessions over a 9-day period. Parallel measurement of protein-specific synthesis and abundance data for almost 100 muscle proteins enabled inferences on the role of protein degradation in muscle adaptation. Patterns of protein synthesis and abundance changes in skeletal muscle included subsets of proteins that i) increased in turnover but exhibit no change in abundance, ii) increased in abundance without a parallel rise in synthesis rate, and iii) decreased in abundance even though synthesis rate was increased. These data challenge the previously assumed linear relationship between changes in synthesis rate and protein abundance in response to exercise. Ultimately, this

opens new questions relating to how proteome remodelling occurs across a period of resistance training. However, the adaptive period studied in Camera et al., (Camera et al., 2017) was limited to just 9 days of training, which prevented meaningful study on how changes in protein synthesis rate relate to longer term changes in muscle mass. Hesketh et al (Hesketh et al., 2020) report data from independent groups of rats (n = 3 per group) that received unilateral chronic low- frequency stimulation (CLFS; 10 Hz, 24 h/d) and D_2O across a 30 d period. Dynamic proteomic analysis quantified the rates of change in abundance, synthesis, and degradation for 38 myofibrillar and 46 soluble proteins in extensor digitorum longus. Indicating changes in the degradation rate of individual proteins contributed to abundance changes equally as much as synthesis, including proteins that decreased in degradation which explained an increase abundance. Moreover, the rates of synthesis and degradation of individual proteins were different during early, mid, and late periods of the experimental intervention. Offering an insight into the capabilities of dynamic proteomic studies of exercise-induced muscle adaptation to explore both the synthetic and degradative processes underpinning protein turnover.

This thesis builds from the foundation of previous work in the Burniston laboratory and further investigates skeletal muscle proteodynamic responses to exercise. Chapter 3 reports the first analysis of proteome dynamics in the skeletal muscle of people with and without obesity at baseline and following 10-weeks of HIIT. Chapter 4 reports the first analysis of individual protein abundance and synthetic responses across a time-course of programmed resistance training in rats using unilateral stimulation *in vivo* similar to Hesketh et al (Hesketh et al., 2020). Finally, chapter 5 reports dynamic proteome responses to both acute and chronic periods of unilateral resistance or endurance exercise in human muscle.

1.3 Thesis Aims & Objectives

This thesis aims to further develop understanding of the role of individual protein dynamics in the adaptation of skeletal muscle to exercise. Muscle adaptation to exercise is a dynamic process that occurs over time in response to repeated bouts of exercise training. Currently, most knowledge on the molecular response of muscle to exercise training is from static snapshots which document the sequential processes but cannot explain how changes occurred. This thesis aims to further develop the knowledge of muscle proteome dynamics and will:

1. Expand knowledge on the complexity and heterogenous nature of protein-specific turnover rates in skeletal muscle of humans. To date, just one study (Camera et al., 2017) reports protein-specific abundance and turnover data in exercised human skeletal muscle. This initial study highlighted novel patterns of proteodynamic responses, including changes in abundance driven by protein synthesis and/or degradation but the coverage of this work (<100 proteins) was limited. It is important to verify our initial findings in separate studies and to further extend the depth (number of proteins studied) of analysis.

2. Conduct the first study of the 'intermediary' period of proteome adaptation to resistance exercise training. Currently, studies have dichotomised muscle adaptations to resistance training to either acute molecular responses or chronic functional adaptations. Proteodynamic studies of the intermediate period are required to link molecular responses to repeated bouts of training with changes in muscle mass and phenotype and develop understanding on *how* muscle adapts.

3. Use dynamic proteome profiling to discover unique and shared adaptive responses of human muscle to resistance (REX) and endurance (END) exercise. It is well established END and REX result in different muscle phenotypes yet share numerous benefits on skeletal muscle health. Protein-level data underpinning these unique and shared responses has yet to be comprehensively studied. Furthermore, dynamic processes have only been investigated using mixed-protein analysis, which lacks detail on how END and REX proteome remodelling occurs.

Chapter 2. The application of dynamic proteome profiling to quantify protein-specific abundance and synthesis rates *in vivo*.

2.1 Introduction

Dynamic processes of the proteome (i.e. protein turnover, synthesis, or degradation) can be tracked through biosynthetic labelling using isotopes of carbon (C), hydrogen (H), nitrogen (N) and oxygen (O). Stable- (^{13}C, ^{15}N, ^{19}O, and ^{2}H) isotopes of these elements are chemically and functionally indistinguishable from the primary isotope but differ in the number of neutrons (and elemental mass) and are ~2 orders of magnitude lesser abundant than the primary isotope in the natural environment (Table 2.1). The introduction of stable- or radioisotopes into a biological system (at levels higher than their natural abundance) allows for their incorporation into biomolecules to be tracked and distinguished from the primary isotope due to differences in atomic mass. Analytically tracing of heavy isotope incorporation into protein from a known amount of precursor over a set period of time allows for the estimation of dynamic processes within a system (Zilversmit, 1960).

Table 2.1. Summary of C, H, N, and O isotopes commonly used in tracer methodologies to assess protein turnover. The percent natural abundance reported in the from the 1997 report of Isotopic Abundance Measurements (Vocke, 1999).

Element	Isotopic mass	% Natural abundance
Carbon	12 13	98.93 1.07
Hydrogen	1 2	99.9885 0.0115
Nitrogen	14 15	99.632 0.368
Oxygen	16 17 18	99.757 0.038 0.205

Halliday & McKeran (Halliday and McKeran, 1975) were amongst the first to report stable isotope labelling in humans and investigated whole body and muscle specific protein turnover using demanding laboratory protocols. Including a ~12-14 h period of continuous infusion of ^{15}N labelled lysine to achieve a plateau in tracer enrichment of the precursor pool followed by serial muscle biopsies over an entire experimental period of 30 h (Halliday and McKeran, 1975). More recently applications of isotopically labelled phenylalanine have prevailed as the preferred tracer within amino acid infusion studies of protein turnover (e.g. (Phillips et al., 1999, Tang et al., 2008). The development of these methods enabled the measurement of protein fractional synthesis rate (i.e. the fraction of newly synthesised proteins) which have been extensively used in the fields of muscle protein metabolism and exercise physiology (Garlick et al., 1994).

Protein fractional synthesis rate (FSR) is derived from the precursor: product ratio, which relates to the amount of labelled amino acid tracer in the precursor pool compared to the amount incorporated into the protein product over a defined period of time (typically reported in %/h in amino acid tracer studies) (Wolfe et al., 2022). During the inception of labelling a large pre-existing pool of protein, the incorporation of label via synthesis occurs rapidly, whereas removal of labelled protein from the pool by degradation is negligible. As the experiment continues, the amount of label in the protein pool increases and, therefore, the probability that degradation will remove proteins containing labelled amino acid becomes proportionally more likely. Therefore, problems arise when methods cannot directly assess the precursor enrichment of the tRNA pool (e.g. precursor measurements inferred from

plasma or intracellular amino acids) as additional labelled amino acids may re-enter the precursor pool because of protein breakdown. Thus, amino acid tracer studies in humans tend to use short (e.g. few hours) labelling periods to study muscle protein turnover and the resulting biosynthetic labelling data largely reflect the average rate of synthesis of all new protein in a tissue. Furthermore, amino acid tracers still require intravenous infusion, which limits studies to acute windows of measurement (~ 6 h) under laboratory settings and constrain the types of interventions that can be investigated.

Improvements in sensitivity of mass spectrometers and the need for alternative biosynthetic labelling techniques that investigate longer-term muscle responses, led to renewed interest into the application of deuterium oxide (^2H_2O or D_2O) as a stable isotope (Gasier et al., 2009). Deuterium (^2H or D) is the heavy stable isotope of hydrogen (^1H) first discovered by Harold Urey, resulting in Urey's award of the Nobel prize in Chemistry in 1934. The use of D_2O in the assessment of protein synthesis is like the precursor: product whereby if the amount of stable-isotope label provided (i.e. the precursor) and the likelihood of the precursor being incorporated into protein (the number of possible exchangeable sites) is known then a rate of synthesis can be derived from the amount of stable isotope incorporated into the protein (i.e. the product). The precursor: product ratio requires the measurement of a true precursor pool, which can be challenging to measure within amino acid tracer studies (i.e. the aminoacyl-tRNA pool) but is readily achievable using D_2O wherein ^2H (or D) labelling of H-to-D exchangeable hydrogen sites comprising amino acids occurs intracellularly.

The use of D_2O as the tracer of choice also benefits from the ability to administer labelling via oral consumption, routinely used in both animals and human studies (Hesketh et al., 2016, Srisawat et al., 2023). The major strength of oral administration is the ability to label proteins with deuterium in free-living conditions, which can be sustained through the consumption of low levels of D_2O in drinking water (< 20 % of total body water) with no hazardous effect (Kushner et al., 1999). Following administration, D_2O rapidly equilibrates (< 30 mins) with body water in rat (Dufner et al., 2005) and human data indicating the presence of ingested D_2O in plasma within 5 min and complete equilibrium in ~ 100 min (Péronnet et al., 2012). Nausea and vertigo have been reported in humans in response to acute, rapid enrichment of body water to ~ 0.5 % (Gasier et al., 2010), however, side effects can be limited with adjustments to the dosing scheme. Ultimately, the combination of these factors indicates D_2O to be an ideal tool for the study of protein turnover in the context of exercise physiology.

The majority of stable isotope labelling data, including both amino acid and D_2O studies, report gross averages of the rate of synthesis for all proteins measured by determining the ratio of labelled versus unlabelled amino acids in a sample by hydrolysis, derivatization, and gas-chromatography mass spectrometry of the complete protein mixture following the use of both amino acid tracers (Phillips et al., 1997) and deuterium-labelled alanine (Brook et al., 2015). From the very earliest studies in the field, it was evident that the turnover of protein in muscle is not entirely homogeneous. Ussing (USSING, 1941) reported different rates of deuterium incorporation in whole tissue and the myosin fractions of rat muscle. Later Rooyackers et al (Rooyackers et al., 1997) reported mixed muscle fractional synthesis rate from human biopsy samples was reported to be ~ 0.05 %/h with slower rates of turnover of myosin heavy chain protein (~ 0.03%/ h) compared to that of sarcoplasmic protein (~0.045%/ h) separated by differential centrifugation followed by SDS-PAGE (Rooyackers et al., 1997, Balagopal et al., 1997). Furthermore mixed-mitochondrial protein turnover (~0.083%/ h) extracted from the soluble protein fraction was +116% faster than that of myosin heavy chain protein (Rooyackers et al., 1997). Despite the novel detail on fraction specific protein turnover, these data likely represent the average synthesis rates of potentially hundreds-thousands of proteins and limits understanding of the true heterogeneity of protein turnover in muscle. Protein separation using biochemical techniques (e.g. 1- or 2-dimensional gel electrophoresis) to extract individual proteins and assess individual protein turnover data. Hayter et al (Hayter et al., 2005) used 2-dimensional gel electrophoresis to resolve 14 subunits comprising the 20S proteasome from the skeletal muscle of Layer chicks using a dietary labelling with

[$^2H_8$]-valine. Measurement of individual protein turnover rates indicated clear variance between different subunits that comprise the same multi-protein complex. Highlighting the flaw in taking average data from across an entire compartment. Until recently technical difficulties in protein separation methods and identification were major barriers to accessing deeper analysis proteome dynamics due to biochemical separation being often unable to completely purify the protein of interest.

The re-found interest in the applications of deuterium oxide as tracer led to the development of methods to measure individual turnover rates in vivo via the combination of mass-spectrometry and D_2O labelling at low (~3%) levels of isotopic enrichment. Price et al., presented a proof-of-concept for the application of deuterium proteomics to study the turnover rate of ~100 proteins in human plasma samples (Price et al., 2012). This first report indicated that label incorporation using D_2O was sufficient for the calculation of the rate D incorporation into individual peptides and by including the likelihood of D incorporation into a peptide (i.e. the number of H-to-D exchange sites) and the level of precursor enrichment in the body water, rates of individual protein turnover can be calculated. Giving rise to a unique technique termed 'dynamic proteome profiling'.

2.2 Experimental Designs

To investigate the role of protein-specific responses in muscle adaption, experimental designs must be carefully considered: (i) A baseline sample at the start of the measurement period is required to provide a profile of the initial level of isotopic enrichment in protein. (ii) The amount of tracer provided, or rate of incorporation into peptides, must be considered to optimise accurate measurements of the isotopic profile and (iii) sampling at the end, or time-series sampling throughout, a labelling experiment must be conducted to assess deuterium incorporation into the intramuscular bound protein pool which is necessary to derive a rate of incorporation. Finally, (iv) the use of biological replication within groups is required to allow for adequate comparisons of the protein-specific responses throughout the adaptive process. Specific experimental designs for rat and human studies are reported in Chapters 4 and 5 respectively. This chapter explains the underlying principles and technical details of the sample processing and analytical methods that are common to each of the experimental chapters.

2.3 Laboratory processing

This thesis focuses entirely on muscle proteomics and the measurement of individual muscle protein synthesis rates in vivo. Skeletal muscle is a challenging substrate for proteomics and requires careful preparation as good quality proteomic data rely upon thorough and consistent extraction of protein from samples. The primary challenge is the fibrous structure of muscle, which necessitates grinding and mechanical homogenisation (Figure 2.1A). Furthermore, to improve the depth of proteomic analysis differential centrifugation is used to separate myofibrillar and soluble proteins thus reducing sample complexity (Figure 2.3B). Proteins content within each sample is quantified via Bradford assay to allow for normalisation of samples (Figure 2.1C) and improve the consistency of the digestion procedure (Figure 2.1D). Trypsin is used in the digest due to its specificity to cleave peptide bonds at the C-terminal side of lysine and arginine residues (except when followed by proline). This specificity aids protein identification inferred from subsequent peptide mass spectrometry. Digests must then be desalted and cleaned for reproducible and maximised LC separation and to limit ion suppression (Figure 2.1E). Prior to LC-MS/MS loading tests of varying peptide amounts are used to optimise the analysis (Figure 2.1F). Following LC-MS/MS inter-sample normalisation is completed during data processing to allow for comparison of protein abundances between runs. Individual peptide mass isotopomer data are then extracted and peptide fractional synthesis rate is calculated considering the number of H-to-D exchangeable sites on each peptide alongside the precursor enrichment. Protein

abundance and FSR data are then extracted and undergo statistical analysis in R with the aid of bioinformatic tools such as network analysis databases and gene ontology/ pathway analysis Figure 2.1G-I). Figure 2.1 gives a schematic overview of the processing involved within our usual workflow. Further details of sample processing and analysis used within the thesis are described below.

Figure 2.1. Schematic overview of processing workflow required for dynamic proteomic profiling.

Muscles are ground under liquid nitrogen, homogenized (A) and fractionated to myofibrillar and soluble proteins (B). Bradford assay is used to determine protein concentration (C). Protein samples are subsequently normalised and digested using the filter-aided separation process (D). Peptide mixtures are then desalted and purified (E) prior to liquid chromatography tandem-mass spectrometry (LC-MS/MS) (F). Protein abundance data is then processed to determine protein quantification and identification (G). Isotopic envelopes of each peak are analysed to determine changes in mass isotopomer distribution profile to calculate the rate of deuterium incorporation into proteins and quantify protein fractional synthesis rates (H). Subsequent protein-specific abundance and synthesis data is compared between groups and investigated using bioinformatic methods such as protein-protein interaction networks (I).

Following collection, muscle samples are snap frozen in liquid nitrogen (-196°C) to halt any enzymatic reactions and stored in -80°C pending further analysis. Samples are then pulverised into a fine powder using mortar and pestle. During grinding, sample remains frozen with liquid nitrogen, to minimise risk of enzyme activation as the sample is being processed. The relatively high content of connective and sarcomere structural tissue in muscle limit the protein yield without prior pulverisation. An accurately weighed amount of muscle powder is mechanically homogenised (using a PolyTron homogeniser) in ice-cold, weak detergent to enable muscle sample fractionation via differential centrifugation. The low salt conditions of the homogenisation buffer led to myofibrils collapsing. Therefore, differential centrifugation results in a myofibrillar protein pellet and soluble protein in the supernatant. Homogenisation buffer consisting of 1 % Triton X-100, 50 mM Tris pH 7.4 including phosphatase inhibitor and complete protease inhibitor cocktails (Roche, Indianapolis, USA) to prevent enzymatic/ protease activity during sample processing. Following homogenisation, samples are incubated on ice for 15 min and then fractionated via centrifugation (1000 x g, 4°C for 5 min). Supernatants containing soluble protein are decanted and stored on ice, and the myofibrillar pellet is resuspend (washed) in half the volume of homogenisation buffer used prior, then centrifuged again (1000 x g, 4°C for 5 min) to limit cross contamination between soluble and myofibrillar fractions. The washed myofibrillar pellet is then solubilized in 10 volumes of 7 M urea, 2 M thiourea, 4 % CHAPS, 30mM Tris, pH 8.5 (lysis buffer) and cleared by centrifugation at 12000 x g, 4 °C, 45 mins. Sample fractionation is an essential step prior to proteomic analysis to reduce sample complexity through the separation of myofibrillar and soluble fraction proteins allowing for boosting the coverage of the proteome analysis.

Soluble protein samples are then purified from the homogenisation buffer via precipitation with 5 volumes of ice-cold acetone (stored in -20°C for 1 h) resulting in the removal of salts and detergents from protein samples. Purified samples are then resuspended in lysis buffer to enable sample compatibility with the Bradford assay as Triton X-100 interferes with the Bradford reagent (Bradford, 1976). Protein concentrations of each myofibrillar and soluble protein sample are measured using the Bradford assay (Sigma-Aldrich, Poole, Dorset, United Kingdom) to allow for protein quantification and subsequent inter-sample normalization. Because our methods employ relative quantification, sample normalization is vital to standardize protein content between samples for subsequent digestion and loading prior to analysis via LC-MS/MS. The Bradford assay offers a simple yet highly sensitive technique to quantify protein after 5 min of incubation with the ability to quantify a large range of protein contents (up to 1mg/mL of protein). Within our assaying methods, the reference protein used is Bovine serum albumin (BSA) plotted on a standard curve over a range of increments in concentration: 0.000 mg/ml (Blank), 0.125 µg/µl, 0.250 µg/µl, 0.500 µg/µl, 0.750 µg/µl and 1.000 µg/µl. Standards are pipetted in triplicate into a microtitre plate and quality controlled for an R^2 of the standard curve > 0.995 with < 3% coefficient of variation of triplicate measurements. Myofibrillar samples are diluted 1:10 and proteins of the soluble fraction diluted 1: 20 to bring them in range of the standard curve. Five microliters of each sample are then pipetted in duplicate and 250 µl of Bradford reagent added to each well. The sample absorbance is read at a wavelength of 595 nm and protein concentrations are calculated by interpolation from the linear regression fitted to the BSA standards.

Following quantification, 100 ug of each myofibrillar and soluble sample are purified via precipitation with 5 volumes of ice-cold acetone and stored at -20°C overnight and then resuspended in 200 µl UA buffer (8 M urea, 100 mM tris, pH 8.5). High concentration (8 M) of urea is used within the buffer to aid protein denaturation and solubilization. Protein samples are then processed and digested for mass spectrometry analysis using the filter-aided separation process (FASP) (Wiśniewski et al., 2009). FASP has been shown to outperform in-solution digestion methods in the context of peptide extraction and minimises the number of missed lysine and arginine cleavages (Wiśniewski, 2016) which is essential for high-quality quantitative data. During FASP digestion, samples are incubated at 37 °C for 15 min in UA buffer with 100 mM dithiothreitol (DTT) to reduce the disulphide bonds between proteins and peptides. Followed by alkylation for 20 min at 4 °C in UA buffer containing 50 mM iodoacetamide (IAA) to prevent

reoxidisation of disulphide bonds. Reduction and alkylation by DTT and IAA are essential to maximise the availability of lysine and arginine sites available for digestion by trypsin. Samples are washed twice with 100 μl UA buffer and transferred to 50 mM ammonium hydrogen bicarbonate (Ambic) to prevent the loss of trypsin activity seen in high urea buffers. Sequencing grade trypsin (Promega; Madison, WI, USA) in 50 mM Ambic is added at an enzyme to protein ratio of 1:50 and the samples are digested overnight at 37 °C.

Peptides are collected following the termination of digestion by lowering the pH of the buffer to < pH 4 (inactivating trypsin) through the addition of trifluoracetic acid (TFA) to a final concentration of 0.2 % (v/v). Aliquots, containing 4 µg peptides, are desalted using C_{18} Zip-tips (Millipore, Billercia, MA, USA) and eluted in 40:60 of acetonitrile and 0.1 % TFA. Zip-Tips are a 10 µl pipette tip that contains ~0.6 µl immobilized chromatography media in the tip. The beads are immobilized in a scaffold that has been formed within the pipette tip, serving as a miniature chromatography column for desalting and concentrating peptides prior to MS analysis. Peptide solutions are dried by vacuum centrifugation for 25 min at 60 ^{o}C and peptides are resuspended in 0.1 % formic acid (FA) spiked with 10 fmol/µl yeast ADH1 (Waters Corp.) in preparation for LC-MS/MS analysis (Silva et al., 2006).

2.4 LC-MS/MS analysis

Proteomic data reported in this thesis (experimental chapters 4 and 5) were generated from LC-MS/MS analyses using an Ultimate 3000 RSLC nano liquid chromatography system (Thermo Scientific) coupled to Q-Exactive orbitrap mass spectrometer (Thermo Scientific). Peptide mixtures were analysed using an Ultimate 3000 RSLC nano liquid chromatography system (Thermo Scientific) coupled to Q-Exactive orbitrap mass spectrometer (Thermo Scientific). Samples were loaded on to the trapping column (Thermo Scientific, PepMap^{TM} 100, 5 µm C18, 300 µm X 5 mm), using ulPickUp injection, for 1 minute at a flow rate of 25 µl/min with 0.1 % (v/v) TFA and 2% (v/v) ACN. Samples were resolved on a 500 mm analytical column (Easy-Spray C18 75 µm, 2 µm column) using a gradient of 97.5 % A (0.1 % formic acid) 2.5 % B (79.9 % ACN, 20 % water, 0.1 % formic acid) to 50 % A 50 % B over 150 min at a flow rate of 300 nl/min. The data-dependent selection of the top-10 precursors selected from a mass range of m/z (mass/charge) 300-1600 was used for data acquisition consisted of a 70,000-resolution full-scan MS scan at m/z 200 (AGC set to 3^{e6} ions with a maximum fill time of 240 ms). MS/MS data were acquired using quadrupole ion selection with a 3.0 m/z window, HCD fragmentation with a normalized collision energy of 30 and in the orbitrap analyser at 17,500-resolution at m/z 200 (AGC target 5^{e4} ion with a maximum fill time of 80 ms). To avoid repeated selection of peptides for MS/MS, the program used a 30 s dynamic exclusion window.

2.4.1 Reverse phase liquid chromatography

Reversed-Phase Liquid Chromatography (RPLC) uses C18 material for the separation of peptides. In RPLC, the stationary phase, consists of beads coated in hydrophobic C18 material, which plays a pivotal role in the separation mechanism. Liquid (i.e. mobile phase) containing the peptide sample is pumped through a chromatographic column. The hydrophobic nature of the C18 stationary phase interacts with hydrophobic regions of peptides, resulting in the absorption of peptides onto the column under polar conditions facilitated by the hydrophilic aqueous mobile phase. The hydrophobicity of peptides, influenced by their length and the presence of hydrophobic residues, determines the strength of their interaction with the stationary phase. As the hydrophobicity of peptides varies, a gradient of rising organic (non-polar) solvent in the mobile phase is used causing peptides to desorb from the stationary phase, facilitating their graduated release into the mass spectrometer. This elution of peptides into the mass spectrometer occurs over time, roughly corresponding to peptides hydrophobicity. Peptides with higher hydrophilicity, interacting less strongly with the stationary phase under polar conditions, are eluted first. In contrast, more hydrophobic peptides, which naturally interact more strongly with the stationary phase, require higher

concentrations of organic solvent and elute later (Pitt, 2009). Formic acid (FA) is included in the process to maintain a consistent protonated state of peptides, ensuring optimal ionization during Electrospray Ionization (ESI). The chromatographic separation based on time simplifies the delivery of peptides to the mass spectrometer, improving coverage and reducing ion suppression and interference between peptides. The combination of RPLC preceding ESI-MS/MS forms the basis modern-day proteomic systems, allowing for the quantification of up to thousands of proteins from microgram amounts of peptide samples (Deshmukh et al., 2021b).

2.4.2 Peptide mass spectrometry

Mass spectrometry (MS) is an analytical technique that provides quantitative information on molecular mass and concentration of analytes after their conversion to ions. The most important component within MS instruments is the mass analyser which measures and separates ions based on their mass/charge ratio (m/z) which is critical for identifying and characterising analytes. The Orbitrap mass analyser is used throughout this thesis and was first made available in 2005 (Zubarev and Makarov, 2013). Orbitrap instruments include an intermediary C-trap to allow for ion accumulation which can then be selectively injected as ion packets into the Orbitrap (a small electrostatic device). Whereby the ions orbit a central electrode yielding high resolution mass spectra (Zubarev and Makarov, 2013). The ability to selectively trap ions combined with the high mass accuracy of the orbitrap offers a larger dynamic range of analyte measurement as the lower abundance ions can be accumulated to a target threshold before being passed to the analyser for quantification (Yates et al., 2006). Measurement of different ions m/z is then recorded by a computer system and the signal is recorded as ‘peaks’ to generate the first level of data (MS1). Whereby the MS1 spectra is generated based on the frequency distribution of the ions, on a similar basis to Fourier-transform ion-cyclotron resonance mass spectrometry.

MS1 data is combined with fragmentation techniques that generate a second level (MS2) of analysis. MS1 precursor ions are disassociated into smaller fragment ions by collision is gas atoms at elevated energies and commonly fragmentation occurs at the peptide bond between amino acid residues. Fragments are then passed back to mass analyser to generate structural information of the peptide ions. This second level of MS data (MS2 data) provides the essential information to identify peptides following their quantitation in the MS1 scan. The MS2 level spectra generate a ‘ladder pattern’ where the spacing between fragment ions relates to the mass of individual amino acid residues of the peptide. Therefore, fragmentation generates a complex mixture of fragment ions that represent the various combinations of different numbers of amino acid residues from the peptide (Figure 2.2.C). The collection of MS1 (quantitation) and MS2 (identification) is termed tandem-MS (MS/MS), which is essential for proteomic analysis.

Figure 2.2. Peptide quantification and identification via LC-MS/MS.

A) Peptides extracted from muscle samples are analysed via high performance reverse phase liquid chromatography-tandem mass spectrometry (HPLC-MS/MS). The measurement of different ions m/z is recorded by a computer system and the signal is recorded as 'peaks'. Whereby the greater the frequency of a specific ions signal (the abundance), the higher the peak which is plotted over time (x-axis). B) Peptide peaks can then be selected from the total ion chromatogram (TIC) and focussed on to resolve the individual peptide ion quantification peaks (MS1 data). C) Fragmentation methods can then be conducted, resulting in dissociation of precursor ions into smaller fragment ions which typically occurs at the peptide bond between amino acid residues. These resulting fragments are then analysed to generate structural information of the fragment ions where the spacing between fragment ions relates to the mass of individual amino acid residues of the peptide (MS2 data). This generates a complex mixture of fragment ions that represent the various combinations of different numbers of amino acid residues from the peptide.

Due to the high level of resolving power for the separation of ion based on mass-to-charge ratio (m/z), high mass accuracy allowing the precise determination of ion masses, and improved sensitivity allowing for low abundance ions to be detected, Orbitrap-based instruments have become one of the most common configurations in proteomics. The Q Exactive (QE) allows for the almost instantaneous filtering and selection of peptide ions for fragmentation (Michalski et al., 2011). Figure 2.3 gives a simplified schematic overview of the QE ESI-MS/MS system. ESI turns analytes in solution into droplets which enter the ion source (A). The charged droplets are then focussed into a straight beam and enter the Quadrupole mass filter which only allows for specific m/z to pass through whilst others are filtered out (B). This allows for MS1 scans to be performed at high resolution and span the expected mass range of tryptic peptides which is typically between m/z 300 – m/z 1600. The design of the QE enables accumulation of the next ion packet in parallel with the analysis of the preceding ion packet in the orbitrap analyser. Within our analysis a data dependant analysis (DDA) schedule is used whereby the top 10 most abundant peptide ions detected in the MS1 scan are selected for second level analysis (MS2), which records their fragment ion spectra. Therefore, whilst the MS1 scan (quantitation) is taking place in the orbitrap analyser, the quadruple is set so that only one peptide of interest is transmitted to the C-trap (C). The peptide ions of interest are accumulated in the C-trap and then passed to the higher-energy collision dissociation (HCD) cell for fragmentation (D), The fragment ions are then transferred back to the C-trap and delivered to the orbitrap, which records the fragment ion mass spectra (E) resulting in MS2 spectra.

Figure 2.3. Simplified representation of the key components of the Q Exactive Mass-spectrometer.

Following the separation of the sample solution over time via reverse phase HPLC, electro-spray ionization (ESI) turns analytes into droplets which desolivate and the peptides released enter the ion source (A). The charged peptides are then focused and enter the Quadrupole mass filter which only allows for specific mass-to-charge ratios (m/z) to pass through whilst others are filtered out (B). The quadruple is set so that only one peptide of interest is transmitted to the C-trap (C). The peptide ions of interest are accumulated in the C-trap and then passed to the higher-energy collision dissociation (HCD) cell for fragmentation (D). The fragment ions are then transferred back to the C-trap and delivered to the orbitrap, which record the fragment ion mass spectra (E) resulting in MS2 spectra which allows for information to be collected for peptide identification.

2.5 Data processing

2.5.1 Quantification of Protein abundance data

Bottom-up analysis of proteins using LC-MS/MS (such as in our work), involves the delivery of separated peptide mixtures to the MS by liquid chromatography. Peptides are resolved across time, resulting in a chromatogram wherein the intensity of each peptide is reported as a peak, which indicates the abundance of that peptide. Label-free quantification of our proteomics data also allows for the extraction of the isotope envelope of each peptide (Figure 2.4). Each peptide envelope consists of numerous peaks, which reflect the same peptide resolved into its different isotopic compositions (known as mass isotopomers). The monoisotopic peak (denoted as m_0) reflects the mass of the peptide with no heavy isotopes, i.e. contains only ^{12}C, ^{1}H, ^{14}N etc., (i.e. number of heavy isotopes = 0). Whereas subsequent m_1, m_2, m_3… etc, peaks indicate the mass of the peptide with the inclusion of the corresponding number of heavy isotopes (e.g. m_1 indicates a peptide with 1 heavy isotope, m_2 indicates a peptide with 2 heavy isotopes and so on). The inclusion of heavy isotopes occurs naturally because peptides are large molecules composed of many C, H, N, and O atoms, for example peptide LDPTQTSFLK (residues 278 – 287 of protein NDRG2 taken from (Stead et al., 2020)) is composed of C_{52}, H_{82}, N_{12}, O_{16}. Approximately 1 % of carbon in the natural environment is the heavy isotope (^{13}C) and ~99 % is the lighter, primary, isotope ^{12}C. Therefore, there is a 1:100 chance that a compound containing carbon will containing the heavy (^{13}C) isotope and result in a peptide quantified as m_1 mass isotopomer. Within our analysis each MS1 scan is composed of ~ thousands of copies of

each peptide, which means it is likely that some peptides will contain 1 heavy isotope and some even contain 2 or 3 heavy isotopes (visualized in Figure 2.4). Therefore, in the quantification of a peptide's abundance it is essential to include the abundance of the whole isotopic envelope to accurately measure total abundance.

Figure 2.4. Representation of the natural distribution of the isotopic envelope of peptides.

Visual representation of the natural distribution of the isotopic envelope of peptide 575.2996 m/z (LDPTQTSFLK, residues 278-287) of protein NDRG2 along with the potential elemental compositions of each mass isotopomer. The monoisotopic peak (denoted as m_0) reflects the mass of the peptide with no heavy isotope incorporated (i.e. indicating mass + 0). Whereas subsequent m_1, m_2, m_3… etc. peaks indicate the mass of the peptide with the inclusion of the corresponding number of heavy isotopes (e.g. m_1 indicates the mass + 1 heavy isotope, m_2 indicates a mass + 2, and m_3 indicates mass +3 heavy isotopes and so on). Peptide abundance is calculated as the sum of each of the heavy isotope peaks (i.e. m0, m1, m2, m3 etc)to include the quantification of peptides with naturally occurring heavy isotopes. Whereas the calculation of individual protein fractional synthesis rate requires the quantification of the individual isotopic peaks

2.5.2 Label-Free Quantitation of Protein Abundances

Within our methodologies, analysis of raw mass spectra is conducted using Progenesis Quantitative Informatics for proteomics (Water Corp, United Kingdom) as previously reported in (Burniston et al., 2014, Camera et al., 2017). The ability to combine and compare multiple LC-MS runs is vital for the analysis of protein abundance data across biological replicates and experimental conditions. However, a common feature of LC separation techniques

is the between run variation in peptide retention time. Shifts in the ambient temperatures and sample composition may lead to slight shifts (~ 2% per 1°C change in temperature) in retention time, which is the point in the run when the peptide elutes into the MS. The alignment procedures in Progenesis enable improvements in reliability of between-run comparisons in the initial processing steps (Figure 2.5). Run alignment is conducted by the identification peaks within a reference chromatogram and subsequent comparison of the retention times of those peaks with the other analytical samples. When peaks corresponding to the same ion are matched between the runs, the chromatogram is then shifted leading to the alignment of retention times. In our methods prominent ion features (>600 per chromatogram) are used as vectors to align each data set to a common reference chromatogram. An analysis window of (typically of ~350–1500 m/z) is selected, which encompasses features of peptide ions with charge states of +2 or +3. Singly charged (i.e. +1 charge state) peptides are excluded in MS settings due relatively low value of MS2 information and typical contaminants (if present) are predominantly singly charged, offering an easy method to exclude potential interference.

Following the alignment of LC-MS runs, peaks in the chromatogram (i.e. peptide ions) can be 'picked' (i.e. ion intensities are quantified) from each sample across the entire experiment. Abundance is quantified through calculating the sum of the area highlighted for each peak. Peak abundance is then log-transformed and normalized to by inter-sample abundance ratio, and relative protein abundances were calculated using nonconflicting peptides only.

Tandem MS data (MS/MS or MS2 data) collected on peptide fragments offers additional information about specific ions (termed 'fragment ion spectra'), which in addition to the accurate mass of the intact peptide is required for peptide identification. MS/MS data can sequence peptides through matching the fragments to a database of known and predicted peptide sequences. Wherein these sequences can be annotated and aligned with their full-length protein sequence. Within our processing MS/MS spectra (peak lists) are exported in Mascot generic format. Using a locally implemented Mascot server (www.matrixscience.com). The experiment and data collected thus result in an in-silico analysis whereby the experimental conditions such as species (e.g. Human/ Rat), specific enzymatic digestion protocols, potential variations in fragment characteristics due to instrumentation error are input and included in peptide fragment analysis which allow usual shifts in m/z to be considered. Based on the processing methods, enzyme specificity was set to trypsin, which allows for 1 missed cleavage. Carbamidomethyl modification of cysteine (fixed) and deamidation of asparagine and glutamine (variable modification) were selected following the reactions with iodoacetamide and DTT. Also, the oxidation of methionine (variable) is accounted for as methionine oxidation may be introduced during the proteomic processing. Also, m/z error (e.g. ± 0.3 Da) is allowed.

Figure 2.5. Representation of sample run alignment conducted using Progenesis Quantitative Informatics for proteomics (QIP; Water Corp, United Kingdom). QIP allows for all experimental runs to be imported prior to analysis. Each experimental run is then assessed for common identification peaks and a 'reference run' is selected (i.e. the run with the most peaks common to the other runs). The comparison of retention time shifts of identification peaks is then assessed relative to the reference run and a numerical score is calculated as the percentage of the 2-dimensional chromatogram deemed as well aligned to the reference run (Yellow box). Computationally generated and manually assigned vectors are added to manipulate the retention time of the peaks in each run to improve the inter-run reproducibility of peaks (i.e. alignment) according to the reference run (Red box visualizes the number of vectors added to this run resulting in >98% run alignment). The upper-right panel visualizes in detail where vectors are added (blue circles) to align the peaks in the experimental run (pink) with the reference run (green). The lower right panel offers 1 dimensional (retention time on the x-axis) visual representation of the chromatogram above showing slight deviations in the retention time of peaks between the experimental (pink) and reference (green) run.

The exported peak lists are then searched against the protein sequences in the Swiss-Prot database which is part of the UniProt knowledge base where >95 % of sequences are generated from the translation of the coding sequences which have been submitted to the public nucleic acid databases. Searching against the Swiss-Prot database specifically restricts protein sequence searches to only the high quality manually annotated and reviewed, non-redundant, protein sequences curated through experimental results, computed features, and scientific conclusions (Boutet et al., 2007). Rather than searching against the unreviewed, computationally generated annotations of sequences in the UniProtKB/TrEMBL database (Calderón-González et al., 2016). Possible protein identifications are then scored through the combined scoring of all observed MS/MS data that are matched to an amino acid sequence within a protein. Confident matches are then limited to high scoring protein identifications as well as containing queries that are unique to that protein specifically (i.e. the sequence is not present in any other protein). Subsequent Mascot search and output files (xml format) are restricted to nonhomologous protein identifications and recombined with MS profile (abundance) data. Prior to extraction of protein hits, Mascot repeats the search again but against a decoy database, that contains scrambled and reversed protein sequences, to calculate the error (false-discovery rate) of peptide matches. Only peptide with a false-discovery rate <1 % are retained for further analysis. Peptide identifications are then recombined with the matching ion abundance data then exported and analysed using statistical approaches to identify differentially abundant proteins and groups of proteins over/underrepresented across different conditions (described in 2.7). Importantly, the statistical analysis of the resultant ion abundance data is conducted through the comparison of nonconflicting peptides only.

Nonconflicting peptides consist of peptides comprising a protein that are unique only to that protein, offering the certainty that the abundance calculated relates to the allocated protein.

2.5.3 Measurement of precursor enrichment

D_2O is measured in the body water via gas chromatography-mass spectrometry. Whereby body water (extracted from saliva/ plasma) is measured against external standards that were constructed by adding ^2H_2O to phosphate buffered saline over the range from 0.0 to 5.0 % in 0.5 % increments. Deuterium enrichment of aqueous solutions is determined after exchange of ^2H (D) in water to H of acetone (McCabe et al., 2006). Samples are centrifuged at 12 000 x *g*, 4 °C for 10 min, and 20 µl of supernatant or standard is reacted overnight at room temperature with 2 µl of 10 N NaOH (high pH is required for the exchange reaction) and 4 µl of 5 % (v/v) acetone in acetonitrile. Acetone is then extracted in 500 µl chloroform and water captured in 0.5 g Na_2SO_4 before transferring a 200 µl aliquot of chloroform to an autosampler vial. Samples and standards are analysed in triplicate using an Agilent 5973N mass selective detector coupled to an Agilent 6890 gas chromatography system. A CD624- GC column (30 m x 0.25 mm x 1.40 µm) is used in all analyses. Samples (1 µl) are injected using an Agilent 7683 autosampler. The temperature program begins at 50°C and increases by 30 °C/min to 150 °C, and held for 1 min. The split ratio is 50: 1 with a helium flow of 1.5 ml/min. Acetone elutes at approximately 2.0 min. The mass spectrometer is operated in the electron impact mode (70 eV) and selective ion monitoring of *m/z* 58 and 59 is performed using a dwell time of 10 ms/ion (Figure 2.6).

Figure 2.6. Measurement of body water enrichment of D_2O via gas chromatography-mass spectrometry.

Representative images of selective ion monitoring of *m/z* 58 (H) and *m/z* 59 (^2H) following the exchanges of ^2H in body water with H of acetone at different percent enrichments of D_2O. Enrichment of D measured using an Agilent 5973N mass selective detector coupled to an Agilent 6890 gas chromatography system to quantify the ratio of ion 58 (Blue): ion 59 (Red).

2.5.4 Protein fractional synthesis rates

After the initial MS peptide abundance analysis, mass isotopomer abundance data are extracted from the MS1 spectra using Progenesis Quantitative Informatics (Nonlinear Dynamics). Like the abundance data, runs are aligned, peaks are picked, and ion quantification data is normalised. However, the abundance of the individual peaks comprising the peptide mass isotopomer envelope are quantified independently (compared to using the sum of the peaks in the abundance data). The monoisotopic peak (m_0), m_1, m_2, m_3… mass isotopomer peaks are collected over the entire chromatographic peak for each non-conflicting peptide used in label-free quantitation.

As ^2H is incorporated into peptides during provision of D_2O (in excess of deuterium's natural abundance in the environment) the distribution of the peaks within the isotopic envelope is changed (i.e. greater abundance of m_1, m_2, m_3… isotopes relative to the m_0 peak; Figure 2.9.A). Measuring the change in the distribution of the mass isotopomers enables the calculation of a synthesis rate of a protein as shifts in the distribution of the isotopic envelope indicate increases in the incorporation of ^2H-labelled amino acids (Stead et al., 2020). For each peptide a minimum of 1 unique peptide is required to calculate the molar fraction of m_0 (Equation 1 and Figure 2.7.B-C). The monoisotopic peak represents completely unlabelled protein meaning the m_0 peptide contains no deuterium. Therefore, the ratio of unlabelled (m_0): labelled (m_1, m_2, m_3 etc.) isotopes (the molar fraction of m_0; fm_0) declines as a function of deuterium incorporation into the peptide.

$$fm_0 = \frac{m_0}{m_0 + m_1 + m_2 + m_3}$$

Equation 1

The rate (k) of decay of the molar fraction of m_0 (fm_0) can be calculated from the change in m_0 from the start (t) to the end (t') of a defined period of deuterium incorporation using the formula:

$$k = \frac{1}{t' - t} \bullet -\ln\left(\frac{fm_{0_t}}{fm_{0_{t'}}}\right)$$

Equation 2

The rate of decay (k) (Figure 2.9.C) is then normalised by the number of exchangeable H positions based on the peptide sequence and the molar percentage enrichment of D in the body water compartment. Resulting in the calculation of the fraction of newly synthesised peptides over time (fractional synthesis rate; FSR) expressed as percent per day (%/d). FSR of a protein is then calculated as the median FSR of its unique peptides.

$$FSR = \frac{k}{(n \cdot p)}$$

Equation 3

Figure 2.7. D_2O incorporation into peptides results in shifts in the mass isotopomer distribution.

Example of how D_2O incorporation into muscle proteins results in shifts in the mass isotopomer distribution and the quantification of the rate of decay in the proportion of peptides comprised of 0 heavy isotopes (m0) is used for the calculation of the rate of ^2H labelling of peptides. Figure redrawn from (Stead et al., 2020). A) Incorporation of deuterium oxide in rat muscle *in vivo*. Histograms illustrate the change in the distribution of the fraction of the monoisotopic peak (fm_0) of 240 peptides quantified in soleus. Panels (left to right) represent data from control (day 0) rats that did not receive deuterium oxide (D_2O), and independent groups of rats that received D_2O for either 10 days, 20 days, or 30 days duration. The median (red line) fm_0 of peptides is reported in each panel. The incorporation of D_2O into the protein pool *in vivo* results in a decline in fm_0, evident as a time-dependent leftward shift in fm_0 distribution. B) Mass spectra from peptide $[M+2H]^{2+}$ 575.2995 *m/z* LDPTQTSFLK (residues 278-287) of protein NDRG2 from soleus taken after 0, 10, 20 or 30 days of deuterium oxide (D_2O) administration *in vivo*. The fraction of the monoisotopic peak (fm_0) declines as a function of D_2O incorporation into the protein pool over time. C) Semi-log plot of fm_0 data from peptide LDPTQTSKLK. The slope of a linear model (red line; grey shaded area = 95 % confidence interval) fitted to ln(fm_0) data was used along with equation 3 to calculate fractional synthesis rate (FSR). *n* = 3 animals at each experimental time point.

Our dynamic proteome profiling technique integrates protein specific abundance and fractional synthesis rate which can also be used to offer insights on the contribution of protein degradation to changes in abundance. A change in protein abundance is underpinned by changes in the synthesis and/or degradation rate of the protein. By plotting changes in abundance alongside changes in synthesis rate, it is possible to visualise and infer different patterns of response to an intervention. For example, if a protein exhibits relatively similar direction and magnitude of change in both protein abundance and synthesis rate, it can be considered that the change in synthesis is primarily responsible. Whereas if there is a disconnect, or opposing pattern, of changes in abundance and synthesis then a change in degradation may be responsible for the abundance change (Represented in Figure 2.8).

Figure 2.8. The integration of abundance and synthesis measurements.

Measurement of individual protein abundance and synthesis responses can be presented on quadrant plots to visualize the relationship between changes in abundance and synthesis rate of individual proteins. Proteins residing in the top right/ bottom left panel indicate changes in synthesis to explain changes in abundance. Whereas proteins in the top left/ bottom right indicate an inverse relationship between changes in synthesis and abundance indicating changes in protein degradation must be driving the abundance change.

2.6 Data handling and analysis

2.6.1 Data handling and quality control

Dynamic proteome profiling is unique in its ability to quantify protein-specific data of abundance alongside insights into different components of protein turnover. This offers further understanding of an intervention that induces 'change' or adaptation within a tissue as proteome responses can be further understood through considering if changes in the synthesis or degradation of individual proteins underpin changes in abundance. Previous work from our lab has shown that the analysis of protein abundances and fractional synthesis rates are reproducible (Srisawat et al., 2020). Srisawat et al., (2020) investigated the reproducibility of protein abundance and FSR data following LC-MS/MS analysis of muscle samples collected from healthy, recreationally active males (n = 4). The median coefficient of variation (%CV) of abundance data analysed from technical replicates was 3.6% (1st quartile = 1.7 %, 3rd quartile = 6.7 % Whereas the median %CV of FSR data of 54 proteins was 10.1% (interquartile range = 13%). These measurements of repeatability indicate the % CV of dynamic proteome profiling to compare well against the assessment of repeatability of other proteomic analyses which include a technical % CV of protein abundances from rat gastrocnemius of 7.3% reported previously by our group (Burniston et al., 2014) alongside 12% measured in yeast (O'Connell et al., 2018).

Statistical analyses are only conducted on proteins that are void of any missing values across all biological replicates and time-points in all experimental groups, without the use of any prior data imputation to computationally enhance the number of proteins quantified.

2.6.2 Statistical analysis

Where appropriate, complete multi-factorial analysis is applied to include all information derived from the full experimental design of a study (e.g. Two-way analysis of variance) to investigate the true interaction/main effects between experimental condition and time on dependant dynamic proteomic variables. Furthermore, the probability of type I error in multiple testing scenarios is controlled through the calculation of false-discovery rates (q-values; (Storey and Tibshirani, 2003)). Whereby, the q-value is the proportion that significant features (i.e. those identified as $p < 0.05$) are estimated to be false-positive results.

2.6.3 Bioinformatic analysis

To identify important and/or unique responses within the data, multiple downstream analyses are conducted to give a biological perspective to the data. The most used analysis for -omics data, and throughout this thesis, is the application of gene ontology (GO) enrichment tools. GO classification typically assigns protein inputs in to three different classes: Biological processes, cellular component, and molecular functions. Similarly, the Kyoto Encyclopaedia of Genes and Genomes (KEGG) database can be used to group proteins based on biological systems and pathways they are involved in (Kanehisa and Goto, 2000), offering a potentially powerful tool to simplify the interpretation of omics data. Correlation networks are also used throughout this thesis to give a further perspective on proteins that are different between experimental groups. Our tool of choice is the 'Search Tool for the Retrieval of Interacting Genes/Proteins' (STRING) database of known and predicted protein-protein interactions (Szklarczyk et al., 2019). STRING uses information from curated databases (such as GO classes and KEGG), experimental determined interactors, computationally predicted models and text mining of proteins co-occurring within literature to build protein network. User-defined proteins can be input into STRING to create a network of proteins that are connected based on the aforementioned parameters, allowing for the visualisation of protein groups that may then aid manual biological interpretation of the data. Within this thesis, proteins identified as significantly different between groups are investigated using STRING (Version 12.0) to aid in the biological

interpretation of our proteomic analysis. Where possible, significantly enriched GO terms are reported to offer the most untargeted view of what comprises the proteins of interest. However, qualitative assessment of the interaction networks and further literature mining of individual proteins is used to gain a comprehensive understanding of the biological data. Statistical analysis can then be conducted using statistical tests to calculate *P* values of 'enrichment' for different GO terms. A significant enrichment of $p < 0.05$, would therefore estimate that there is a less than 5 % probability that the overlap between the lists is due to randomness. Because the test investigates multiple overlapping groups of proteins simultaneously, *P* values are corrected for multiple testing using the Benjamini-Hochberg procedure to compute a false discovery rate (fdr) (Benjamini and Hochberg, 1995). An enrichment of a network is deemed significant where fdr <5 %. A previously underappreciated aspect of the downstream analysis is the considerable susceptibility to bias. All -omics technologies, particularly LC-MS/MS-based proteomics, has an inherent biased for detecting and measuring certain proteins. For example, LC-MS analysis of the muscle proteome is bias towards the quantification of the most abundant proteins which in muscle comprise GO terms associated with muscle contraction and glycolysis/glycolytic processes. Therefore, these multiple sources of bias must be adjusted for through the inclusion of a user defined background list which includes all proteins that were accepted for statistical analysis (Timmons et al., 2015). Networks were visualised using Cytoscape version 3.9.1 (Shannon et al., 2003). Furthermore, proteins were included in mitochondrial proteome analysis of abundance or FSR if annotated to the Gene ontology cellular component term "mitochondrion". Whereby, total mitochondrial protein abundance was calculated as the sum of all mitochondrial protein abundances. Mixed protein/ total mitochondrial FSR was assessed by calculating the abundance normalised average FSR from individual proteins (median FSR weighted by abundance) quantified in all samples.

In Chapters 4 and 5 temporal analyses are conducted on proteins exhibiting significant main effects of time and interactions in abundance and/or synthesis rate using soft clustering analysis using the fuzzy c-means clustering algorithm with the Mfuzz R package (Kumar and Futschik, 2007). To allow for plotting of temporal profiles data are collapsed by fold-difference between condition at each time point (e.g. mean of condition *x* / mean condition *y*) and $\log_2$ transformed. The optimal number of clusters is decided through inspection of scree plots and qualitative iterative assessment of cluster numbers. The minimum membership value for inclusion into a cluster was set at 0.3. Proteins comprising each temporal cluster of abundance/ synthesis rates were further investigated using the bioinformatic methods previously mentioned.

Chapter 3. People with obesity exhibit losses in muscle proteostasis that are partly improved by exercise.

Note: Portions of this chapter have been published previously in:

SRISAWAT, K.*, **STEAD, C. A**.*, HESKETH, K., POGSON, M., STRAUSS, J. A., COCKS, M., SIEKMANN, I., PHILLIPS, S. M., LISBOA, P. J., SHEPHERD, S. & BURNISTON, J. G. 2023. People with obesity exhibit losses in muscle proteostasis that are partly improved by exercise training. *Proteomics*, e2300395. *Indicates joint 1st authorship.

3.1 Introduction

This PhD commenced in February 2021 amid corona virus restrictions in the UK. Therefore, the first component of this PhD was in silico analysis of pre-existing data. This chapter reports bioinformatic analysis of new data generated by high-resolution mass spectrometry of samples collected during the PhD studies of Kanchana Srisawat. The PhD thesis of Dr Srisawat reports separate analysis of lower resolution proteomic data from the same samples. I gratefully acknowledge the provision of data from these samples by Dr Srisawat and her supervisory team. The data, including bioinformatic analysis and new biological interpretation, reported in this chapter have been published (Srisawat et al., 2023) and the published version of the complete experiment is appended (Appendix 1.1). This chapter includes brief details of the rationale for the study and a summary of the experimental design followed by more detailed presentation of the bioinformatic analyses and biological interpretation.

Skeletal muscle dysfunction contributes to the metabolic abnormalities in people with obesity, but it is uncertain whether the turnover of muscle proteins is increased or decreased. Traditional amino acid tracer methods to investigate mixed-protein turnover have been unable to resolve whether protein synthesis is elevated or suppressed in the muscle of obese insulin-resistant individuals. Chronically elevated insulin levels suppress translocation of glucose transporters to the cell membrane but other aspects of the insulin receptor signalling cascade, including mTORC1 signalling and subsequent effects on protein turnover, may remain stimulated (Kolb et al., 2020). Indeed, kinase activity profiling in muscle from healthy lean vs obese insulin-resistant (OIR) individuals (Qi et al., 2020) found hyperactivation of JNK stress kinase signalling and hypo-activation of negative regulators of the mTOR pathway in OIR muscle.

It is well established that protein turnover is essential to protein homeostasis (proteostasis) but under fasting conditions (Tran et al., 2018, Tran et al., 2016) or in the post-absorptive state (Guillet et al., 2009), the turnover of mixed protein may be lower in the muscle of people with obesity. When proteins of the myofibrillar fraction are studied, no differences in protein turnover are evident between individuals with or without obesity, but healthy-weight individuals have a greater rise in myofibrillar protein fractional synthesis rate (FSR) after a protein-rich meal (Beals et al., 2016). The average FSR of proteins in the muscle mitochondrial fraction is also less in people with obesity (Guillet et al., 2009, Tran et al., 2018), but the protein synthetic response to amino acids may be either impaired (Guillet et al., 2009) or enhanced (Tran et al., 2018). The lack of consensus regarding the effects of obesity on muscle protein turnover could arise due to the lack of protein specific detail and the acute (< 12 h) measurement period used in stable isotope labelled amino acid methods.

Overall, the above observations implicate dysregulation of muscle protein turnover in the context of obesity and question whether protein-specific responses may occur rather than changes *en masse* to the turnover of muscle proteins.

3.2 Methods

3.2.1 Study design

This experiment examined if a loss in muscle proteostasis occurs in people with obesity and whether endurance exercise positively influences either the abundance profile or turnover rate of proteins in this population. Normal-weight men (n = 4) and men with obesity (n = 3) between the ages of 30-45 years were recruited. Each participant gave their informed consent to the experimental procedures approved (16/WM/0296) by the Black Country NHS Research Ethics Committee (West Midlands, UK) and conformed with the Declaration of Helsinki, except

registration in a clinical trials database. Anthropological and physiological data were collected at least 3 d prior to commencing the first 14-day period of D_2O consumption (i.e. baseline investigation period). Throughout baseline measurements, saliva and blood samples were collected, and muscle samples were obtained before D_2O administration (0 day) and after 4, 9, and 14 days of D_2O consumption. Normal-weight participants completed the baseline investigation only, whereas the men with obesity also undertook a 10-week HIIT intervention. During the final 2 weeks of the HIIT intervention (weeks 8 to 10), participants consumed D_2O and samples were collected consistent with the baseline investigation (Figure 3.1). Physiological measurements were repeated in men with obesity at least 72 h after completing the 10-week HIIT intervention.

Figure 3.1 – Experimental design and deuterium incorporation

A two-week deuterium oxide (D_2O) labelling experiment was conducted with normal-weight healthy control (NWC; n = 4) and obese insulin resistant (OIR; n = 3) participants to collect baseline data (weeks -2 – 0). Saliva sampling and D_2O administration (4 x 50 ml) were conducted daily, and venous blood samples were collected every other day. Percutaneous biopsies of vastus lateralis muscle were conducted on days 0, 4, 9 and 14. The OIR group then completed a 10-week high-intensity interval training (HIIT) program. During the last 2 weeks of the training intervention, a repeat of the labelling experiment was conducted to investigate the effects of exercise on the OIR muscle proteome. Physiological data, including peak aerobic power (W_{max}), maximum oxygen uptake (VO_2max) and oral-glucose tolerance (OGT), were measured 48 h prior to the collection of baseline biological samples or 48 h after completion of the 10-week HIIT intervention.

3.2.2 Statistical and bioinformatic Analysis

Statistical analysis was performed in R (Version 3.6.2). Baseline protein abundances were calculated in each participant from the median abundance of each protein across the time-series, whereas post-exercise protein abundances were quantified from the final biopsy only.

Baseline comparisons of participant physiological data, protein abundances, and protein turnover rates between the NWC and OIR groups were analysed using between-subjects ANOVA. Whereas within-subjects ANOVA was used to assess differences between baseline and post-exercise measures in OIR participants. Significance was identified as $P < 0.05$. False-discovery rates (q-values) were calculated for all protein data to test for false positives. Bioinformatic analyses were conducted using the methods described in Chapter 2. All supplementary dynamic proteomic data is appended (Appendix 1.2 – 1.5)

3.3 Results

3.3.1. Participant health and exercise characteristics

Normal-weight participants and those with obesity had a BMI of 24.2 ± 2.4 kg.m^{-2} and 34.0 ± 5.8 kg.m^{-2}, respectfully. Participants with obesity had a significantly (P < 0.01) lower VO_2peak and W_{max} compared to normal-weight individuals. Fasting insulin concentrations tended (P = 0.07) to be greater in people with obesity, but fasting blood glucose levels were not different between the groups. In response to the OGTT, the area under the curve (AUC) of plasma glucose was similar between groups, whereas for the AUC of insulin tended to be greater in people with obesity. Participants with obesity were classified as insulin-resistant based on Matsuda Index (MI; 1.7 ± 0.6 mmol), which was significantly (P < 0.01) less than normal-weight participants (5.7 ± 1.4 mmol). Based on the above characteristics, the abbreviations obese insulin resistant (OIR) and normal-weight controls (NWC) are used throughout the manuscript when referring to the groups of people with and without obesity.

After 10-weeks of HIIT, VO_2peak of OIR participants increased by 9 % and W_{max} increased 14 % (Table 3.1). There was no change in fat mass or body fat percentage and HIIT did not significantly alter fasting concentrations of glucose or insulin. The mean glucose and insulin AUC were less in response to OGTT after the 10-week exercise programme, but these improvements did not reach statistical significance. Similarly, the Matsuda index of OIR participants (2.0 ± 1.0) was 18 % greater than baseline (1.7 ± 0.6) after the HIIT intervention, improvement in insulin sensitivity also did not reach statistical significance.

Table 3.1 - Participant characteristics and physiological data

	NWC (n = 4)	OIR (Baseline) (n = 3)	OIR (post-exercise) (n = 3)
Age (Years)	38 ± 8	38 ± 6	
Height (cm)	177.1 ± 5.9	179.7 ± 4.1	
Weight (kg)	75.6 ± 5.1	113 ± 25	112 ± 27
BMI (kg.m^2)	24.2 ± 2.4*	34.0 ± 5.8	34.8 ± 6.1
Fat mass (kg)	13.5 ± 3.9*	32.9 ± 14.11	32.4 ± 14.0
Fat free mass (kg)	55.9 ± 3.0**	70.3 ± 5.7	70.6 ± 7.5
Percent body fat (%)	18.3 ± 4.3*	30.2 ± 7.5	30.1 ± 6.4
VO_{2peak} (ml.kg FFM^{-1}.min^{-1})	61.30 ± 9.2**	38.8 ± 2.0	41.8 ± 7.6
Watt$_{max}$ (W.kg FFM^{-1})	4.65 ± 0.87**	2.47 ± 0.27	2.89 ± 0.56
Fasting glucose (mmol.l^{-1})	5.0 ± 0.3	5.0 ± 1.6	5.4 ± 0.4
Glucose AUC (mmol.l^{-1})	703.3 ± 81.5	849.3 ± 441.4	739.0 ± 298.8
Fasting insulin (uIU.ml^{-1})	8.6 ± 2.9$^{\#}$	36.8 ± 28.6	31.5 ± 26.9
Insulin AUC (uIU.ml^{-1})	5422 ± 2627$^{\#}$	9292 ± 172	15058 ± 8023
Matsuda Index	5.7 ± 1.4*	1.9 ± 0.7	2.1 ± 1

Physical and physiological characteristics measured during pre- and post-experimental testing of the normal-weight healthy control (NWC) and obese insulin resistant (OIR) individuals at baseline and after 10-weeks HIIT exercise. Data presented as mean ± standard deviation. *P < 0.05; **P < 0.01; $^{\#}$P < 0.1 vs OIR baseline.

3.3.2. Dynamic proteome profiling of human muscle.

Deuterium enrichment of body water rose at a rate of 0.25 ± 0.06 %/d to a maximum of 3.54 ± 0.5 % during the first 14-day measurement period. After 8 weeks without deuterium oxide consumption (i.e., day 0 of the second measurement period), body-water enrichment of deuterium had returned to 0.08 %. During the second 14-day measurement period, deuterium enrichment of body-water rose at a rate of 0.22 ± 0.07 %/d to a maximum of 3.11 ± 0.5 %. There were no significant differences in the rate of body-water enrichment calculated from measurements made using equivalent plasma or saliva samples. Proteomic analysis encompassed 40 muscle samples, including day 0, 4, 9 and 14 time points at baseline (n=4 NWC and n=3 OIR) and day 0, 4, 9 and 14 time points during weeks 8-10 of the HIIT intervention (n=3, OIR only). Overall, 1,422 proteins were confidently identified and after filtering to exclude missing values amongst biological replicates, the abundance of 858 proteins was measured across all sampling times in at least n = 3 participants per group. Protein abundances were stable (Figure 3.2A) in each participant across each time-series (day 0, 4, 9 and 14) of samples used to investigate the biosynthetic labelling of proteins with deuterium. Between group ANOVA highlighted 352 significant ($P < 0.05$, $q < 0.05$) differences in protein abundance, including 289 proteins that were more abundant in OIR and 63 that were more abundant in NWC muscle at baseline (Figure 3.2C). In addition, within-subject ANOVA of day 0 baseline samples and samples taken after 10 weeks HIIT in OIR participants highlighted 53 statistically significant ($P < 0.05$) changes in protein abundance, including 33 proteins that increased and 20 proteins that decreased in response to the HIIT intervention (Figure 2F).

Protein turnover rates were measured for 301 proteins that had high-quality peptide mass isotopomer data matched between $n = 3$ participants in the OIR and NWC groups at baseline or 386 proteins matched within the n=3 OIR participants at baseline and after the HIIT intervention. Protein-specific turnover values were aggregated to derive the average rate of turnover (%/d) of mixed protein, which tended ($P = 0.061$) to be greater in NWC (1.95 ± 0.68) than OIR (0.91 ± 0.32) muscle at baseline (Figure 3.2D) and increased 1.5-fold ($P = 0.12$) to 1.59 ± 0.63 %/d in OIR muscle during weeks 8-10 of HIIT (Figure 3.2G).

Nineteen individual proteins exhibited differences ($p < 0.05$) in turnover rate at baseline (Figure 3.2E), including 11 greater in NWC and 8 greater in OIR. Seven proteins were significantly more abundant in OIR muscle, but their turnover rate was significantly less than in NWC muscle. In addition, 10 proteins exhibited significant differences in the turnover rate but were not different in abundance between OIR and NWC groups (Figure 3.2H). There were significant ($P < 0.05$) changes in the turnover of 22 individual proteins between baseline and the final 2-weeks of training. Twenty-one proteins changed in turnover independent of changes in protein abundance (19 increasing, 2 decreasing in turnover rate) (Figure 3.2D). Whereas only 1 protein, 14-3-3 protein Epsilon (14-3-3E), increased in abundance and turnover rate in response to the HIIT.

Proteins that exhibited significant differences between NWC and OIR baseline or changes in OIR from pre- to post-HIIT were enriched for KEGG pathways relating to energy metabolism (Figure 3), proteasome and cell stress (Figure 4).

A
OIR 2 Abundance Data (Baseline)
D0
D4
D9
D14
1.00
0.98
1.00
0.96
0.93
1.00
0.98
0.97
0.94
1.00
B
OIR 1
OIR 2
OIR 3
1.00
0.56
1.00
0.59
0.36
1.00
C
Principal Component 2 (20.52 %)
Principal Component 1 (46.54 %)
Group
NWC
OIR
D
PC2 (29.90 %)
PC1 (37.04 %)
Group
NWC
OIR
E
PC2 (23.21%)
PC1 (51.34 %)
Group
Baseline
HIIT
F
PC2 (20.98 %)
PC1 (43.19 %)
Group
Baseline
HIIT
G
P Value (-Log 10)
OIR/NWC (Log 2 Fold Diff)
H
p = 0.06
Average Turnover Rate (%/day)
OIR
NWC
I
OIR/NWC FTR (Log 2 Fold Diff)
OIR/NWC Abundance (Log 2 Fold Diff)
J
P Value (-Log 10)
HIIT/Baseline (Log 2 Fold Diff)
K
p = 0.12
Average Turnover Rate (%/day)
Baseline
wk 8-10
L
HIIT/Baseline FTR (Log 2 Fold Diff)
HIIT/Baseline Abundance (Log 2 Fold Diff)

Figure 3.2 – Dynamic proteome profiling of human muscle

(A) Representative correlation matrix illustrating the technical reproducibility of muscle protein abundance data (n = 880 proteins quantified) from a participant sampled at days 0, 4, 9 and 14 of the baseline experimental period. (B) Representative correlation matrix illustrating the biological variation of protein fractional turnover rates (n = 301 proteins) quantified in n = 3 OIR participants during baseline experimental period. (C - D) Principal component analysis illustrating differentiation in OIR and NWC participants based on protein abundance profile and fractional turnover rates, respectively. (E - F) Principal component analysis illustrating differentiation in OIR at baseline and after 10-weeks of high intensity interval training (HIIT) participants based on protein abundance profile and fractional turnover rates, respectively. (G) Volcano plot illustrating Log_2 fold-difference in protein abundance between OIR and NWC muscle at baseline (day 0). Statistically significant ($p < 0.05$) data with a false discovery rate (FDR) < 5 % are highlighted in red. (H) Average turnover rate of proteins (n = 301) quantified in OIR (n = 3) and NWC (n = 4) participants during the baseline measurement period. (I) Scatter plot of co-occurring differences (Log_2 transformed data) in protein abundance (x-axis) and turnover rate (y-axis) in OIR compared to NWC participants. (J) Volcano plot illustrating Log_2 fold-change in protein abundance in OIR participants between baseline (day 0) and the end (day 14) of the HIIT intervention. Statistically significant ($p < 0.05$) data are highlighted in red, the FDR threshold for this data is >40 %. (K) Average turnover rate of proteins (n = 301) quantified in OIR (n = 3) during the baseline measurement period and final two-weeks of the HIIT intervention. (H) Scatter plot of co-occurring changes (Log_2 transformed data) in protein abundance (x-axis) and turnover rate (y-axis) in OIR participants after the HIIT intervention.

3.3.3. Proteodynamic analysis of proteins associated with muscle energy metabolism pathways.

In total, 13 proteins associated with glycolysis/gluconeogenesis (of 24 quantified) were significantly different in abundance between OIR and NWC groups. The majority (11 proteins) of glycolytic proteins were more abundant in OIR muscle. Turnover rates were measured for 17 proteins associated with glycolysis/gluconeogenesis and the turnover of each protein tended to be less in OIR than NWC muscle (Figure 3A). In response to HIIT, 2 proteins associated with glycolytic metabolism (glycerol-3-phosphate phosphatase; PGP) and glycogen phosphorylase; PYGM) increased ~1.2-fold in abundance (P = 0.038 and 0.030, respectively). Similarly, peroxisomal multifunctional enzyme 2 (DHB4) exhibited a robust increase (~5-fold; P = 0.036) in abundance after HIIT (Pre = 4.478 ± 1.408, Post = 23.338 ± 5.469 fmol/µg). The turnover rate of triosephosphate isomerase increased (P = 0.046) from 0.26 ± 0.25 %/d at baseline to 0.45 ± 0.31 %/d in trained OIR muscle and the turnover of phosphoglucomutase-1 (PGM1) increased ~3-fold (P = 0.011) between baseline (0.34 ± 0.36 %/d) and the final 2-weeks of exercise (0.92 ± 0.46 %/d). In each case, the increases in protein turnover rate were not associated with differences in the abundance of these proteins between baseline and HIIT conditions (Figure 3B).

Significant differences were detected amongst 6 enzymes (of 20 quantified) involved in fatty acid oxidation (FAO) and 9 enzymes (of 21 quantified) of the tricarboxylic acid (TCA) cycle. Most enzymes associated with FAO and TCA cycle were more abundant in OIR muscle (5/6 and 8/9, respectively), whereas dihydrolipoamide dehydrogenase (DLD) was significantly greater in abundance in NWC muscle. Like the pattern exhibited by enzymes of glycolytic metabolism, the turnover of the FAO enzymes was generally lower in OIR muscle (Figure 3A). For example, the turnover of enoyl-CoA hydratase (ECHM), was 2.4-fold slower (P = 0.044) in OIR (0.60 ± 0.42 %/d) compared to NWC (1.41 ± 0.23 %/d) and ECHM abundance was 1.8-fold greater (P = 0.01, q = 0.02) in OIR muscle. The rate limiting enzyme of vitamin B6 metabolism, pyridoxine-5'-phosphate oxidase (PNPO), was 2.5-fold more abundant but had a 16-fold lower turnover rate in OIR (0.24 ± 0.41 %/d) compared to NWC (4.04 ± 2.07 %/d) muscle. The redox enzyme, dehydrogenase/ reductase SDR family member 7 (DHRS7), was 4-fold more abundant (P = 0.005, q = 0.01) but had a 2.3-fold (P = 0.015) slower turnover rate in OIR compared to NWC muscle at baseline. Exercise led to changes in the abundance or turnover rate of several proteins associated with

metabolic pathways (Figure 3B). Long-chain fatty acid CoA ligase-1 (ACSL1) and delta(3,5)-delta(2,4)-dienyol-CoA isomerase, mitochondrial (ECH1) increased in abundance ~1.6 fold (P = 0.03 and P = 0.27, respectively). Whilst short-chain specific acyl-CoA dehydrogenase (ACADS) did not change in abundance, ACADS increased (P = 0.024) 2.4-fold in turnover rate in response to exercise training.

3.3.4. Proteodynamic analysis of respiratory chain subunits.

Overall, 24 proteins (of 61 quantified) belonging to the KEGG pathway "oxidative phosphorylation" (OXPHOS) exhibited significant differences in abundance between OIR and NWC groups (Figure 3A). NADH dehydrogenase (Complex I) exhibited the greatest number of differences and 10 subunits (of 28 quantified) exhibited significant differences in abundance between OIR and NWC groups. Six proteins (NDUB5, NDUS3, NDUCR, NDUA2, NDUA5, and NDUBA) were less abundant in OIR muscle. However, 4 subunits were more abundant in OIR muscle (NDUB9, NDUS6, NDUS5, and ACPM). Subunits A and B of succinate dehydrogenase (SDH; Complex II) were significantly (p < 0.05, FDR < 5 %) more abundant in OIR muscle compared to NWC, but there was no difference in abundance of the 2 membrane anchoring SDH subunits, C and D. Eight subunits of Complex III (Cytochrome c reductase) were quantified and 5 exhibited significant differences between OIR and NWC participants. Cytochrome c (CY1) and 2 other core subunits of the cytochrome b-c1 complex (QCR1 and QCR2) were significantly more abundant in OIR as was subunit 6 (QCR6) which is associated with the low molecular weight component of Complex III. However, subunit 8 (QCR8), which is also associated with the low molecular weight sub-complex, was significantly less abundant OIR muscle. Cytochrome c oxidase subunit 5A (COX5A) was the only subunit of 7 quantified from Complex IV that was significantly less abundant (P = 0.03, q = 0.04) in OIR muscle. Eleven subunits of ATP synthase (Complex V) were analysed, and 3 exhibited significant differences in abundance between NWC and OIR muscle. ATP synthase subunits f (ATPK) and the endogenous inhibitor (ATIF1) were more abundant (8.4-fold and 2.9-fold, respectively), in OIR muscle. Conversely, ATP5I was more abundant in NWC muscle and (1.8-fold). Alongside protein abundance profiling our analysis quantified the turnover rates of 15 OXPHOS subunits. Generally, the turnover data indicated a theme of lower mean turnover rate in OIR muscle (Figure 3) and the rate of turnover of 1 protein, Cytochrome b-c1 complex subunit Rieske (UCRI), was statistically (P = 0.025) greater in NWC (2.78 ± 1.20 %/d) compared to OIR (0.37 ± 0.55 %/d) muscle.

Two subunits of respiratory Complex I, including alpha-subcomplex 12 (NDUAC) and beta subcomplex subunit 10 (NDUBA) that were significantly less abundant in OIR compared to NWC at baseline, increased in abundance by 2.4-fold (P = 0.018) and 7.8-fold (P = 0.045), respectively after HIIT (Figure 3B). ATP synthase subunit beta (ATPB) increased (1.3-fold, P = 0.04) from 32.73 ± 9.94 fmol/µg at baseline to 42.27 fmol/µg post-HIIT. Two other ATP synthase subunits (G; ATP5L and A; ATPA) exhibited greater turnover rates after 10 weeks HIIT but their abundance was unaffected. ATP5L exhibited a robust increase (~4-fold, P = 0.007) in turnover rate from 0.21 ± 0.34 %/d at baseline to 0.82 ± 0.36 %/d across the final 2-weeks of exercise. ATPA increased >2-fold (P = 0.045) in turnover rate in response to training in OIR muscle.

Figure 3.3 – Dynamic proteome profiling of muscle energy metabolism pathways

Nodes represent proteins organized to their principal energy metabolism pathway in muscle and are annotated by their UniProt knowledgebase identifier. **(A)** Node fill colour represents Log_2 fold-difference in abundance and node boarder colour represents Log_2 fold-difference in fractional turnover rate (FSR) between obese-insulin resistant (OIR) and normal-weight healthy control (NWC) participants at baseline. **(B)** Node fill colour represents Log_2 fold-change in abundance and node boarder colour represents Log_2 fold-chance in fractional synthesis rate in obese-insulin resistant (OIR) after the 10-week high-intensity interval training (HIIT) intervention. Grey borders indicate missing FSR data. CI – CV represent mitochondrial respiratory chain complexes.

3.3.5. Proteodynamic analysis of proteins associated with the proteasome, ubiquitination, or cellular stress response.

In addition to metabolic enzymes, proteins belonging to the KEGG "Proteasome" pathway were highly enriched (q = $5.3e^{-4}$) amongst the significant differences between OIR and NWC muscle. Several enzymes involved in protein ubiquitination exhibited significantly greater abundance in OIR muscle (Figure 4), including the E1 enzyme, ubiquitin-like modifier-activating enzyme 1 (UBA1), E2 ubiquitin-conjugating enzymes UBE2N, UBE2K, UB2V2 and the E3 ligases, UBAC1 and TRI72. There were some exceptions to this pattern, for example variant 1 of the E2 enzyme, UB2V1, was ~2-fold greater (p = 0.003, q = 0.009) in NWC muscle and the abundance of the E3 ligase RNF123, was ~3-fold greater (P = 0.002, q = 0.008) in NWC. Three subunits of the COP9 signalosome (a deactivator of Cullin-RING ubiquitin ligases) were more abundant in OIR muscle, and a protein involved in mitophagy, FUN14 domain-containing protein 2, was ~9-fold greater in abundance in OIR (0.3407 ± 0.0920 fmol/µg) than NWC (0.0374 ± 0.0424 fmol/µg).

Our analysis encompassed 24 of the 43 known subunits of the 26S proteasome, including all 7 alpha (PSMA) and 7 beta (PSMB) subunits that make up the 20S catalytic core, 4 subunits of the 19S base region (PSMC), 5 subunits of the 19S lid region (PSMD), and one subunit of the 11S proteasome activator (PSME). Non-ATPase regulatory subunit 3 (PSMD3) and proteasome regulatory subunit 6A (PRS6A) were significantly more abundant in OIR muscle, alongside greater abundances of alpha subunits 3, 4, 5, and 6, and beta subunits 1 and 5 of the core particle (Figure 4C). Conversely beta subunits 2, 3, and 7, and the proteasome activator complex subunit 1 (PSME1) were significantly more abundant in NWC muscle. Protein turnover rates were quantified for 6 proteasome subunits but no statistically significant differences in turnover were identified between OIR and NWC groups.

Fifteen proteins associated with response to stress and chaperone function differed in abundance between OIR and NWC muscle, including 4 isoforms of peroxiredoxin (PRDX), each more abundant in OIR than NWC muscle (Figure 4E). PRDX1 exhibited the greatest difference and was ~2.5-fold greater (P = 0.008, q = 0.02) in OIR muscle. Similarly, PRDX3, PRDX5, and PRDX6 were each ~1.5-fold more abundant in OIR muscle. Parkinson disease protein 7 (PARK7) was 2.5-fold greater (P < 0.001, q = 0.001) in abundance in OIR muscle (26.89 ± 0.22 fmol/µg) than NWC (10.54 ± 1.80 fmol/µg), whereas the mitochondrial superoxide scavenging enzyme, superoxide dismutase (SODM) was greater (P < 0.001, q = 0.003) in abundance in NWC (1.965 ± 0.300 fmol/µg) than OIR muscle (0.3132 ± 0.1259 fmol/µg).

Several proteins associated with maintaining proteostasis were more abundant but had a slower turnover rate in OIR muscle. Heat-shock 70 kDA protein 1 (HSP72) exhibited a > 5-fold slower turnover (p = 0.01) in OIR (0.74 ± 0.56 %d) compared to NWC muscle (4.11 ± 1.14 %/d) whilst being ~1.5-fold more abundant (P = 0.017, q = 0.028) in OIR. Similarly, the detoxifying enzyme (aldo-keto reductase, mitochondrial; AK1A1) exhibited a >3-fold greater abundance in OIR muscle but turned over at a rate of 1.11 ± 0.31 %/d in OIR and 8.50 ± 1.18 %/d in NWC muscle (7.66-fold slower in OIR; p < 0.001). Mitochondrial aldehyde dehydrogenase 2 (ALDH2) is the second major enzyme associated with alcohol metabolism and protects against oxidative stress. ALDH2 also had a significantly greater turnover rate in endurance trained muscle (4.12 ± 1.14 %/d) compared to OIR (1.44 ± 1.18 %/d), however no difference in abundance was identified. Whereas the plasma protein Hemopexin (HEMO), which protects against heme-mediated oxidative stress was 3-fold greater in abundance (P = 0.009, q = 0.19) and 2-fold greater in FSR (p = 0.011) in OIR than NWC muscle at baseline.

Following the HIIT intervention, 4 proteasome subunits that were more abundant in OIR muscle than NWC under baseline conditions became significantly less abundant after exercise (Figure 4D). Ten weeks of HIIT also led to robust changes in the abundance and/or turnover rates of proteins associated with chaperone functions (Figure

4F). The abundance of the mitochondrial heat shock protein, HSP 75 kDa (TRAP1), increased 1.5-fold (P = 0.021) from 0.096 ± 0.030 fmol/µg at baseline to 0.143 ± 0.026 fmol/µg after 10 weeks HIIT. Similarly, chaperonin 60 (CH60) exhibited a 1.6-fold increase (P = 0.041) in abundance after the HIIT intervention from 2.790 ± 0.190 fmol/µg at baseline to 4.431 ± 0.665 fmol/µg post-training. The adapter protein 14-3-3E, which may positively regulate the heat shock response increased in abundance from 8.178 ± 1.891 fmol/µg to 9.029 ± 2.218 fmol/µg post-exercise (p = 0.046). Notably, the abundance of the chaperone, heat shock cognate 71 kDa (HSP7C) increased 1.4-fold (p = 0.042) after HIIT specifically in the myofibrillar fraction. The abundance of HSP7C within the soluble fraction remained stable between pre- and post-exercise in OIR muscle, whereas HSP7C turnover increased 2.7-fold (p = 0.004). The chaperone HS90-beta also significantly increased in turnover rate (P = 0.046) between baseline (4.46 ± 1.07 %/d) and in response to exercise (5.74 ± 1.23 %/d). In addition, exercise training increased the turnover rates of PRDX2 and ALDH2, which exhibited a significantly greater FSR (P = 0.050 and 0.037, respectively) in trained OIR muscle (0.58 ± 0.25 and 5.58 ± 1.87 %/d, respectively) in comparison to baseline (0.43 ± 0.26 and 1.44 ± 1.18 %/d) (Figure 4F).

Figure 3.4 – Dynamic proteome profiling of the muscle proteostasis network

Nodes represent proteins annotated by their UniProt knowledgebase identifier and organized to their principal proteostasis network components, including ubiquitin ligase (A and B), proteasome (C and D) or heat shock protein and antioxidant system (E and F). (A, C and E) Node fill colour represents Log_2 fold-difference in abundance and node boarder colour represents Log_2 fold-difference in fractional synthesis rate (FSR) between obese-insulin resistant (OIR) and normal-weight healthy control (NWC) participants at baseline. (B, D and F) Node fill colour represents Log_2 fold-change in abundance and node boarder colour represents Log_2 fold-chance in fractional turnover rate in obese-insulin resistant (OIR) after the 10-week high-intensity interval training (HIIT) intervention. Grey borders indicate missing FSR data.

3.4. Discussion

Proteostasis describes the maintenance of proteome quality and encompasses the processes of protein turnover and the actions of chaperone systems that safeguard proteins during discrete stages of their lifecycle. Our current findings point to dysregulation of proteostasis in OIR individuals, while longitudinal analysis of OIR muscle after a 10-week programme of HIIT revealed some restoration of muscle proteostasis. Proteins accumulate damage through irreversible spontaneous modifications (e.g. deamination) in a time-dependent manner (Robinson and Robinson, 2001). Deamination caused by nucleophilic attack may particularly occur during the catalytic cycle of enzymes (Hipkiss, 2011) and turnover is required to maintain the quality of the enzyme pool. Glycolytic enzymes were more abundant but had slower rates of turnover in OIR muscle at baseline (Figure 3), which may indicate a poorer protein quality compared to NWC muscle. In model systems, the catalytic activity of metabolic enzymes is positively correlated with their turnover rate (Garcia-Aguilar et al., 2019). Therefore, the lesser turnover of glycolytic enzymes in OIR muscle agrees with the lower rates of glucose utilisation reported (Kelley et al., 1992) in the muscle of people with obesity. However, this accumulation of glycolytic enzymes with suboptimal rates of turnover may burden protein quality control systems responsible for the removal of damaged proteins. Indeed, the fundamental components of the proteostasis network, including the ubiquitin proteasome system (UPS) and heat-shock protein (HSP) chaperones, featured prominently amongst the differences between OIR and NWC muscle proteomes.

This investigationg discovered differences in the abundance and turnover rate of UPS components, including the 20S core proteasome, 19S regulatory particle, 11S proteasome activator, ubiquitin (E1, E2 and E3) ligases and components of super-complexes (e.g. Cop9 signalosome) that regulate protein ubiquitination and degradation. E3 ubiquitin ligases underpin the selectivity of UPS-mediated protein degradation and have been a focus of previous mechanistic studies. The E3 ubiquitin ligase, tripartite motif-containing protein 72 (TRIM72), was more abundant and had a lower turnover rate in OIR muscle. TRIM72 ubiquitinates the insulin receptor and insulin receptor substrate-1 (IRS1) and negatively affects muscle insulin signalling (Song et al., 2013). Consistent with our findings, muscle TRIM72 abundance is greater in models of obesity and insulin resistance, whereas knock-down of TRIM72 protects against muscle insulin resistance induced by a high-fat diet (Hu and Xiao, 2018). HIIT did not alter TRIM72 abundance but did significantly increase TRIM72 turnover, which may be an early indication of a beneficial effect of exercise training. OIR muscle also had a greater abundance of the E3 ubiquitin ligase RNF123, which is the catalytic subunit of the Kip1 ubiquitin promoting complex (KPC). The KPC is responsible for the degradation of Kip1 (cyclin dependent kinase inhibitor 1B) (Kamura et al., 2004) and is an acknowledged regulator of the cell cycle that may also protect against stress-induced apoptosis in striated muscle (Yuan et al., 2019).

OIR muscle also exhibited differences in the Cop9 signalosome, which regulates the large family of cullin-RING ubiquitin E3 ligases (CRL) by removing the NEDD8 ubiquitin-like modifier from cullin subunits. Only NEDD8-modified CRL complexes are catalytically active and 2 subunits of the COP9 signalosome (CSN3 and CSN7A) were more abundant in OIR muscle at baseline (Figure 3.4), which may indicate lesser activation of CRL enzymes. Cullin-associated NEDD8-dissociated protein 2 (CAND2) is also specific to striated muscle and suppresses the activity of SCF (Skp1-Cullin1-F-box protein)-like ubiquitin E3 ligase complexes by binding culin1 to prevent neddylation (Shiraishi et al., 2007). No difference in CAND2 abundance was detected, but the turnover of CAND2 was lesser in OIR compared to NWC at baseline and increased in OIR muscle after exercise training. In addition, the deubiquitinating enzyme, ubiquitin carboxyl-terminal hydrolase isozyme L3 (UCHL3), which hydrolyses the peptide bond of both ubiquitin and nedd8 modifications (Wada et al., 1998), was significantly more abundant in OIR muscle. Together these findings indicate disruption to NEDD8 post-translational modifications that regulate the activity of key E3 ligase families in muscle.

Ubiquitin E2 enzymes (UBE2- N, K, V1 and V2) also exhibited different abundances between OIR and NWC

muscle, particularly those associated with the regulation of K^{63}-polyubiquitination. Ubiquitin-conjugating enzyme E2 N (UBE2N) forms heterodimers with either UBE2 variant 1 (UBE2V1) or UBE2 variant 2 (UBE2V2) and regulates the assembly of K^{63}-polyubiquitin chains, whereas UBE2K is responsible for generating branched chains containing both k^{48}- and k^{63}-linked ubiquitin's. Polyubiquitin chains joined at ubiquitin K^{48} are an acknowledged degradative signal, whereas the inclusion of K^{63} linkages may counter the signal for proteasomal degradation (Yang et al., 2014). The UBE2N/V2 heterodimer (each more abundant in OIR muscle) is associated with protection against DNA damage (Andersen et al., 2005) whereas only UBE2V1 isoform was enriched in NWC muscle. UBE2V1 modulates ubiquitin proteasome responses to proteotoxic stress (Xu et al., 2020) and the greater likelihood of UBE2N/UBE2V1 heterodimers in NWC muscle may be associated with better proteostasis. Conversely, UBE2V2 can be modified by reactive electrophiles and may lead to hyperactivation of UBE2N to promote K^{63}-polyubiquitination and genome protection (Zhao et al., 2018). UBE2K was also significantly more abundant in OIR muscle and is responsible for the formation of branched polyubiquitin chains that contain K^{48}- as well as K^{63}-linkages (Pluska et al., 2021). UBE2K may enhance proteasomal degradation of proteins carrying K^{63}-polyubiquitin chains (Ohtake et al., 2018). These findings highlight a complex interplay between E2 ligases and suggest the distribution of K^{48}- and K^{63}-linked polyubiquitin chains was altered in the muscle of OIR participants.

The catalytically active subunits of the core proteasome (beta 1, 2 and 5) differed in abundance between OIR and NWC muscle. The beta-1 and beta-5 subunits were significantly more abundant in OIR muscle, whereas the beta-2 subunit was significantly less abundant compared to NWC muscle. Previous studies (Hwang et al., 2010, Vanderboom et al., 2022) similarly report some but not all proteasome subunits exhibit differences amongst the muscle of people with and without obesity. Currently, it is uncertain whether the abundance of individual proteasome subunits measured in muscle homogenates reflects the activity of the proteasome (Jenkins et al., 2020). Proteasome activity is also modified by changes to regulatory subunits, including the 11S proteasome activator (PA28α; PSME1), which increases specifically in the muscle of people with obesity after acute exercise (Vanderboom et al., 2022). Similarly, PSME1 was more abundant in OIR muscle after 10-weeks HIIT (Figure 4D) and our earlier analysis of rat heart responses to exercise (Burniston, 2009) also demonstrated that endurance training increases the abundance of the PSME1 subunit. Overexpression of PSME1 is associated with increased degradation of oxidatively damaged proteins in rat neonatal ventricular myocytes (Li et al., 2011). Whereas streptozotocin-induced insulin-dependent diabetes is associated with reduced muscle PSME1 content and loss of proteasome activity (Merforth et al., 2003). Furthermore, PA28-null mice exhibit hepatic steatosis, decreased hepatic insulin signalling, and increased hepatic glucose production (Otoda et al., 2013). Therefore, despite ambiguous differences in catalytically active subunits of the core proteasome, 10-weeks HIIT likely improved the capacity for proteasomal degradation in OIR muscle via 11S proteasome activation.

Heat shock proteins (HSP) are major constituents of the proteostasis network and are categorized into major families (e.g. 90 kDa-, 70 kDa- and small (< 45 kDa) heat-shock proteins) by their molecular weight. Small heat shock proteins (sHSP) exhibited differences in abundance between OIR and LHC muscle that were consistent with previous literature. For example, HSP27 (HSPB1) was significantly more abundant in OIR and is also more abundant in the muscle of Goto-Kakizaki rats (Mullen et al., 2011) and in myoblasts generated from the muscle of type 2 diabetic patients (Al-khalili et al., 2013). HSPB1 and HSPB6 (HSP20) are well studied in the context of skeletal muscle responses to exercise and each of these proteins exhibited higher rates of turnover after 10-weeks HIIT (Figure 4F). Small HSP (sHSP) function in homo- or hetero-oligomers of various sizes and complexity and the observed differences across several sHSP (Figure 4E) may indicate changes to the size or composition of sHSP oligomers. sHSP bind efficiently with misfolded proteins but lack ATPase activity and cannot (re-) fold substrate proteins directly (Haslbeck et al., 2019), so work cooperatively with other chaperone complexes, e.g. by preparing proteins for refolding by HSP70 (Goncalves et al., 2021). The inducible HSP72 and the constitutively expressed

heat shock cognate (HSP7C) were each more abundant in OIR than NWC muscle. Chronic elevation of HSP72 in OIR muscle may be evidence of sustained stress and an elevated requirement for refolding damaged proteins (Gupta et al., 2010). Indeed, muscle-specific overexpression of HSP72 can protect against the development of insulin resistance induced by a high-fat diet in mice (Chung et al., 2008). In the current work, HIIT did not affect HSP72 but did significantly increase the turnover rate of HSP7C, which may support an improvement in proteome quality by enhancing the capability of HSP7C to orchestrate chaperone-mediated degradative process (Fernández-Fernández and Valpuesta, 2018). HSP70 complexes may, in turn, pass client proteins to HSP90 complexes, including HSP90-alpha (HS90A) and HSP90-beta (HS90B), which are abundant cytosolic proteins that (re-) fold newly synthesized or incorrectly folded protein clients. Pharmacological inhibition (Lee et al., 2013) or knockdown (Jing et al., 2018) of HSP90 improves insulin sensitivity in rodent models of diabetes or diet-induced obesity. Consistent with findings in patients with type 2 diabetes (Venojärvi et al., 2014), HSP90B was more abundant in OIR muscle (Figure 4E). HIIT did not alter the abundance of either HSP90 isoform but the turnover rate HS90B increased significantly in OIR muscle after 10-weeks of training (Figure 4F). HSP90 function is regulated by post-translational modifications, including oxidation (Backe et al., 2020), and a greater turnover of HSP90 in trained muscle may equate to a greater proportion of non-modified HSP90 proteins, which have preserved functional capacity (Beck et al., 2012). In addition, the mitochondrially targeted homolog of HSP90, TRAP1, was more abundant in OIR muscle after HIIT, and TRAP1 may offer greater protection against mitochondrial apoptosis induced by reactive oxygen species (Montesano Gesualdi et al., 2007).

Redox signalling contributes to the muscle response to exercise, and oxidative stress is a proposed mechanism of muscle dysfunction associated with obesity. PARK7 (DJ-1) and peroxiredoxins (PRDX) were generally more abundant in OIR than NWC muscle. PARK7 is a redox-sensitive chaperone that may reverse methylglyoxal and glyoxal-glycated protein modifications (Richarme et al., 2015) that can be elevated in the muscle of people with obesity or type 2 diabetes (Mey et al., 2018). PRDX enzymes are the primary scavengers of cellular H_2O_2 and may underpin the hormesis response of muscle to exercise-induced oxidative stress (Xia et al., 2023). Our findings (Figure 4E and F) add to reports that PRDX2 and PRDX6 are more abundant in the skeletal muscle of patients with type 2 diabetes (Brinkmann et al., 2012), and that PRDX5 in more abundant in myoblasts derived from muscle biopsies of type 2 diabetic patients (Al-khalili et al., 2013). No change in PRDX abundances occurred after the 10-week HIIT intervention, whereas (Brinkmann et al., 2012) reported muscle PRDX5 abundance increases in type 2 diabetic patients after exercise training. PRDX enzymes undergo reversible redox modifications, for example, PRDX3 becomes more oxidized in human muscle during HIIT (Pugh et al., 2021). This analysis reports the turnover rate of PRDX3 was relatively low in OIR compared to LHC muscle and further declined after the 10-week HIIT intervention, which may be associated with changes to the modification state and dimerization of PRDX3 in exercised muscle.

Our protein-by-protein analysis of abundance and turnover responses has yielded new insight into losses in muscle proteostasis associated with obesity. Our findings are based on highly stringent data handling, including critical analysis of the alignment of LC-MS peptide retention patterns and the exclusion, rather than imputation, of peptides with missing data. During each measurement period, a time series of n = 4 samples was collected from each participant to determine the turnover rate and abundance of each protein in each experimental condition. Our statistical analyses of protein-level data were conducted using only those proteins that are detected in every participant at every timepoint, which is crucial in within-subject repeated measures designs. However, it must be acknowledged that more extensive studies are required to pursue this line of enquiry in the future and previous power analyses (Srisawat et al., 2019) suggest n >12 may be required in order to limit the effect of type 2 error (false negatives) particularly associated with the reporting of no change in protein abundance and therefore inferring changes in protein turnover to be a key outcome in trained muscle in obese individuals. Where data exist

in the previous literature, our current findings align well with existing knowledge regarding the effects of obesity on skeletal muscle. In agreement with meta-analysis of protein abundance profiling literature (Srisawat et al., 2017), glycolytic enzymes were enriched in OIR muscle and mitochondrial Complex I emerged as a point of convergence between the effects of metabolic disease and exercise training. However, differences in protein-specific turnover rates were the more prominent feature observed across metabolic enzymes between OIR and LHC at baseline (Figure 3.3A) or in OIR muscle before and after the 10-week HIIT intervention (Figure 3.3B), which add new information on the effects of obesity in human muscle. Our data align with the limited data on protein-specific synthesis rates. For instance, Tran et al., (2019) used targeted analysis of ATP synthase beta (ATPB) in $^{2}H_{10}$-leucince labelled samples to report a lesser turnover of ATPB in the muscle of people with obesity. Similarly, this analysis indicated the protein-specific turnover rate of ATPB was less in OIR muscle, and our non-targeted analysis, which encompassed a further 10 subunits of Complex V, highlighted that subunits AT5F1, ATP5L and ATPO also exhibited lesser rates of turnover in OIR muscle (Figure 3A). When our protein-specific turnover data are aggregated, the average turnover rate of mixed protein tended to be less in OIR than NWC individuals (Figure 2H). However, this pattern was not uniform at the protein-specific level and the turnover of proteins, including kelch-like protein 41 (KLH41), HSPB3 and 2 subunits (PSA6 and PSB3) of the core proteasome tended to be greater in OIR than LHC muscle (Figure 3.4E). Furthermore, this data indicated a trend towards a greater average protein turnover in OIR muscle undergoing HIIT (Figure 3.2K) but this response pattern was, again, not uniform at the protein-specific level. In particular, some subunits of the core proteasome, heat shock proteins and peroxiredoxins exhibited lesser turnover rates after HIIT in OIR muscle (Figure 3.4D and F). Therefore, dynamic proteome profiling adds protein-specific detail to trends observed in mixed protein data and highlights proteins that exhibit responses that are inverse to the overall trend in average turnover of the protein mixture.

3.5. Conclusion

In conclusion, the muscle proteome of obese insulin-resistant humans exhibits widespread evidence of losses in proteostasis, and elevated proteome stress characterised by differences in the abundance and turnover rate of heat shock proteins and perturbations to the ubiquitin proteasome system. Ten-weeks of HIIT tended to improve the quality of the proteome by altering proteasome composition and enhancing the turnover rates of metabolic enzymes. Furthermore, changes in the turnover rate of energy metabolism enzymes without exercise-induced changes in protein abundance were observed; therefore, this proteodynamic analysis offers new knowledge into muscle exercise responses. Changes to the turnover rate of individual proteins is an underappreciated aspect of the cytoprotective effects of exercise.

Chapter 4. Temporal profiling of skeletal muscle protein-specific abundance and synthesis over 30 days of programmed resistance training.

4.1 Introduction

Molecular studies on resistance training adaptations have focussed on muscle protein synthetic responses to acute exercise or longer-term functional end points (e.g. hypertrophy) after weeks-months of training, and there is currently a gap in knowledge regarding the intermediate period where processes associated with repeated bouts of exercise result in phenotypic adaptation. Unilateral models of skeletal muscle mechanical loading in rodents serve as a valuable resource in the investigation of mechanisms regulating skeletal muscle hypertrophy. Unilateral models offer the use of within animal analyses as the contralateral limb within an animal serves as a non-exercised control. Also, unilateral models are typically applied using electrical stimulation of motor units, allowing for a training 'programme' to be elicited with control of the range of repetitions, sets, and load applied to the target muscles. Furthermore, mechanistic studies of hypertrophy in rodents allows for the entire muscle to be extracted for accurate analysis of muscle size, aiding in biological interpretations. Wong and Booth (Wong and Booth, 1988) were some of the first to introduce a model of electrical stimulation and external mechanical loading of the plantar flexors in anesthetized rats that increased gastrocnemius wet weight by ~15 % after 16 weeks of twice weekly training. Unexpectedly, growth (~30 %) also occurred in the tibialis anterior of the stimulated limb as a consequence of co-activation and subsequent eccentric contraction of the tibialis anterior during each concentric contraction of the plantar flexors (Wong and Booth, 1990). Stimulation of the sciatic nerve to simultaneously contract the innervated hind limb muscles without an external mechanical load were later developed on the basis that maximal stimulation of the plantarflexors (gastrocnemius, soleus, and plantaris) produce more force than the dorsiflexors (tibialis anterior and extensor digitorum longus) resulting in plantar flexion of the ankle joint through concentric contraction of the plantarflexors and maximal eccentric contraction of the dorsiflexors (Baar and Esser, 1999). Twice weekly training for 6 weeks increased wet weight of the dorsiflexors ~14% alongside no changes in plantarflexors muscle weights (Baar and Esser, 1999). Electrical stimulation models overcome concerns regarding non-physiological stimulus of compensatory overload (e.g. (Goldberg et al., 1975)) and can be applied unilaterally to avoid between animal comparisons necessary in voluntary training models such as ladder climbing as used previously (Hornberger and Farrar, 2004). However, the need for repeated rounds of anaesthesia alongside the training protocol may lead to physiological and immunological stress responses which could confound the interpretation of the response to exercise training (Layton et al., 2023). Schmoll and colleagues (Schmoll et al., 2018) report a model of unilateral, co-contraction of the plantar- and dorsi-flexors by implantation of electrical stimulators in vivo, enabling programmable muscle contractions without the need for repeated bouts of anaesthesia. This co-contraction model in vivo increases the wet weight of the tibialis anterior ~15 % after 4 weeks of training (Schmoll et al., 2018, Viggars et al., 2023) and is used in this chapter to investigate the time course of dynamic proteome responses.

Radioisotope infusions used alongside electrical stimulation models reported significant increases in muscle protein synthesis rates of 47 % after 17-hours and 57 % after 41-hours compared to non-exercise control limbs (Wong and Booth, 1990). Furthermore, studies on cell signalling found the phosphorylation status of 70 kDa S6 protein kinase (70S6K) 6 hours after electrically stimulated eccentric contractions was positively correlated with the extent of hypertrophy in rat hindlimb muscles following 6 weeks of training (Baar and Esser, 1999). Resulting in the identification of molecular signalling events specific to hypertrophy-inducing stimuli (Nader and Esser, 2001) linked to the later transient increases in muscle protein synthesis which are thought to underpin gains in muscle mass. However, it has been challenging to quantitatively connect acute muscle responses such as cell signalling and protein synthesis with longer term muscle growth due to the reliance on short-term intravenous infusions of stable isotope labelled amino acids. Limiting the knowledge to only acute elevations in protein turnover quantified after a single bout of exercise.

Ultimately a change in abundance offers a direct reflection of the net-balance of synthesis and degradation on the

individual protein level. Isfort et al., (Isfort et al., 2002) was amongst the first work that used proteomic techniques in skeletal muscle and investigated muscle hypertrophy induced by a return to normal loading conditions after a period of hindlimb-suspension in rats. Muscle homogenates were separated by two-dimensional gel electrophoresis (2-DE) and differential analysis of gel images highlighted significant increases in the abundance of 15 proteins following re-loading of the soleus. The observations indicated that metabolic (e.g. beta-enolase, lactate dehydrogenase B, and carbonic anhydrase), contractile and chaperone proteins were selectively altered during hypertrophy and argue against uniform increases in the abundance of all proteins assumed in the interpretation of mixed-protein FSR data. The significant changes in the abundance of 15 proteins of ~50 that could be confidently matched between the different 2-DE samples reflects 30% of the total number of proteins that could be successfully mapped indicating a potentially considerable proteome remodelling if greater coverage could be achieved. Also, the specificity of response indicates a much more complex molecular coordination of the adaptive process to chronic training than that previously suggested by short term amino acid infusion studies. A more comprehensive understanding of the events that connect the molecular responses to acute exercise with the chronic improvements in skeletal muscle function will enhance the precision of exercise training prescription for health and performance gains.

The combination of stable isotope labelling *in vivo* and proteomic techniques utilising peptide mass spectrometry allow for the quantification of individual protein synthesis rates, which offer the potential to investigate protein-specific remodelling on a protein-by-protein basis. Shankaran et al., (Shankaran et al., 2016b) combined D_2O labelling with LC-MS/MS analysis of peptides to quantify individual protein fractional synthesis rates in rats that received an anabolic selective androgen receptor modulator (SARM). Skeletal muscle samples were collected after 10 or 28 days of SARM treatment at 0.3 mg/kg and 1.0 mg/kg body weight which resulted in ~ 10 % greater muscle weight following 28 days of SARM treatment. The fractional synthesis rate of 94 proteins was studied and muscle growth observed after 28 days SARM treatment was preceded by greater fractional synthesis rate of 11 myofibrillar proteins, including actin (+1.41-fold), myosin heavy chain (+1.2-fold), and myosin regulatory light chain 2 (+1.8-fold) quantified during the first 10 days of treatment. However, this study lacked quantification of protein abundances, meaning the effect of changes in synthesis resulting in the accretion of specific proteins could not be investigated. Therefore, the application of dynamic proteome profiling as used previously in our lab (Camera et al., 2017) enables inferences of all dynamic processes regulating protein abundance (i.e. synthesis, turnover, and degradation) to be considered. Currently it is not known how resistance exercise modulates protein-specific changes in abundance across a typical time-course of training (i.e. weeks-to-months), and little is known about how chronic training regulates the synthetic and degradative characteristics of muscle. This work aimed to utilise our dynamic proteomic methodologies to understand the complete time-course of muscle adaptation to programmed resistance training (PRT). Aiming to add new insights on the short-term dynamic adaptation of the proteome to training, and how that links with the remodelling observed following chronic exercise adaptation. Herein, a combination of D_2O labelling *in vivo* and peptide mass-spectrometry were applied to conduct dynamic proteomic analysis across 4 weeks of daily PRT, known to elicit unilateral muscle hypertrophy of rat tibialis anterior via spillover electrical stimulation allowing the contralateral control limb to act as a within-animal control (Schmoll et al., 2018, Viggars et al., 2023). Our analysis included temporal profiling of protein-specific abundance and synthesis rates after 2, 10, 20, and 30 days of PRT. It is hypothesised that electrical stimulation induced hypertrophy would induce global, time dependant changes in the dynamic muscle proteome that have been overlooked by previous studies.

4.2 Methods

4.2.1 Experimental Design

The electrical stimulation model, animal husbandry, surgery, and administration of the training protocol (Figure 1A-C) was completed by Prof. Jonathan Jarvis and his team alongside the animal facility staff at Liverpool John Moores University. Proteomic analyses reported in this chapter are unique to this thesis whereas the experimental design and transcriptomic profiling of the muscles analysed in this chapter has been published previously (Viggars et al., 2023). The animal experiments were conducted under the provisions of the Animals (Scientific Procedures) Act 1986 and approved by the British Home Office (PA6930221). Male Wistar rats were group-housed with 2–3 per cage maintaining an alternating 12h light 12h dark cycle. In total 15 animals underwent proteomic analysis (n = 3 in each group), the mean age when euthanized was 23 ± 2 weeks. Mean pre- and post-body masses were as follows for sham surgery (430 ± 33 g to 390 ± 34 g), 2 days (498 ± 57 g to 494 ± 44 g), 10 days (497 ± 13 g to 497 ± 10 g), 20 days (575 ± 60 g to 595 ± 74 g), and 30 days of training (558 ± 44 g to 605 ± 45 g).

4.2.2 Resistance training protocol

Animals received 1 session per day of SpillOver resistance training in the left hindlimb via stimulation from an implanted pulse generator (IPG) as previously described, (Schmoll et al., 2018, Viggars et al., 2023) for 2, 10, 20, or 30 days or underwent sham surgery. Seven days were allowed for recovery from surgery before the start of the training protocol. Training was conducted within the first 2 h of the relatively inactive light cycle of the rat. Briefly, for high load (SpillOver) exercise to elicit slight stretch under load, the dorsiflexor muscles, tibialis anterior (TA), and extensor digitorum longus (EDL), received supramaximal activation via a cathode placed underneath the common peroneal nerve (CPN), while the anode was positioned underneath the tibial nerve and the stimulation current was adjusted if necessary by remote programming, to recruit enough of the gastrocnemius, plantaris, and soleus (plantarflexors muscles) to provide resistance against the contraction of the dorsiflexors. Daily training was delivered automatically by the IPG (surgical implantation and configuration of the stimulators are explained in detail in (Viggars et al., 2023)). Training consisted of an initial 10 seconds of preparatory stimulation at a low frequency (F = 4 Hz, phase width = 258 µs, current = approximately 1 mA), followed by 5 sets of 10 tetanic contractions at 100Hz. Each contraction lasted for 2 s with 2 s rest between contractions and 2.5 min of rest between sets. The stimulation was delivered only in the left hindlimb, so muscles of the right hindlimb could be used as unstimulated contralateral controls. Stimulation with these settings and the amplitude chosen to balance dorsiflexion and plantarflexion described above was well-tolerated by all animals without further anaesthesia or sedation. Regular observations during daily training across the time course revealed no adverse behavioural signs.

4.2.3 Deuterium oxide administration

D_2O labelling of rats was initiated by a bolus intraperitoneal injection of 10 µl/g of 99 % ^2H_2O-saline. Due to the smaller size of the animals enough D_2O can be provided in the initial bolus injection resulting in a rapid rise in precursor (i.e. D_2O loading). Following the precursor loading a maintenance design of D_2O provided in the drinking water result in steady-state maintenance of precursor enrichment which results in much simpler calculations of individual peptide FSR values.

4.2.4 Muscle collection and processing

Muscles were received and processed for proteomic analysis following collection at day 0 (sham-operated animals) and 1 hour after training on days 2, 10, 20, and 30 of stimulation. Animals were euthanized in a rising concentration of CO_2 followed by cervical dislocation. Tibialis anterior from the left stimulated limb (Stim) and the right non-stimulated limb (Ctrl) were isolated. TA wet weight was measured immediately after sample collection. Each

muscle was cleaned of fat and connective tissue then weighed before being frozen in liquid nitrogen and stored at −80°C pending the proteomic analysis outlined in Chapter 2.

4.2.5 Statistical and Bioinformatic Analysis

All data processing and statistical analyses were conducted in *R* (version 3.6.2). Two-way analysis of variance (ANOVA) was applied to investigate the interaction between experimental condition (Stim vs Ctrl limbs) and time (0, 2, 10, 20, and 30 days) from muscle wet weight and protein content. Where appropriate (non-omics data) significant ($p < 0.05$) interactions were followed up by Tukey post-hoc analysis.

Prior to statistical analysis, protein abundance and synthesis data were filtered so that only values quantified in all muscles (stimulated and control) across all sampling timepoints in n = 3 animals per group were analysed. This data handling practice avoids the use data imputation to replace missing values and enables robust statistical analysis by 2-way ANOVA to investigate the interaction between experimental condition (Stim vs Ctrl; within-animal factor) and time (muscles harvested from individual groups of rats across the time-course; between-group factor). Statistical significance was set at $p < 0.05$ and where appropriate assessed for multiple comparisons by calculating false-discovery rates (q-values; (Storey and Tibshirani, 2003)). Protein-specific abundance and/or FSR identified as exhibiting a significant ($p < 0.05$, $q < 0.1$) interaction (condition x time) were used for bioinformatic analysis to understand the effect of time on the muscle dynamic proteome response. Temporal profiles ($\log_2$ transformed Stim/Ctrl fold-difference across time) of proteins exhibiting significant interactions in abundance and/or synthesis rate underwent soft clustering analysis as described in chapter 2. All supplementary dynamic proteomic data is appended (Appendix 2.1 – 2.2).

Correlations between gene expression and proteomic data was conducted through calculation of the Pearson correlation coefficient of the relationship between the mean $\log_2$ fold-difference (Stim/Ctrl) in protein abundance and DEseq expression of each transcript from each sampling time point (n = 3 biological replicates).

4.3 Results

4.3.1 Daily training leads to progressive increases in muscle wet weight and protein content

Two-way ANOVA indicated a significant interaction (time x condition) in TA wet weight ($p = 0.001$) and protein content ($p = 0.002$) (Figure 4.1D and 4.1E). Post-hoc analysis indicated no significant difference in wet weight (Figure 4.1D) or protein content (Figure 4.1E) between the left (sham-operated) and right (contra-lateral control) TA at the onset of the experiment (Day 0) ($p = 0.997$ and 0.993, respectively). There was no significant change in TA wet weight or protein content of unstimulated, Ctrl TA after 10-day ($p = 1.00$ and 0.75, respectively), 20 day ($p = 0.995$ and 0.985, respectively) and 30-day ($p = 0.925$ and 0.999, respectively) time points compared to day 0 Ctrl (Figure 4.1D and 4.1E, respectively). However, significant increases in wet weight (13 ± 5 %; Figure 4.1D) and protein content (42 ± 15 %; Figure 4.1E) were identified in the Stim TA versus Ctrl after 10 days ($p = 0.021$ and 0.003, respectively), 20 days ($p = 0.006$ and 0.0001, respectively), and 30 days ($p = 0.0003$ and 0.0024, respectively) of programmed resistance training (PRT) (Figure 1D and E).

Figure 4.1. Daily stimulation results in progressive increases in muscle mass and protein content.

A) Programmed resistance training (PRT) in vivo was achieved using implantable pulse generators placed in the abdomen with electrodes routed subcutaneously to the left limb. The anode was placed under the tibial nerve and the cathode under the common peroneal nerve to produce programmed resistance training. Stimulation was optimised to fully activate the dorsiflexor muscle (tibialis anterior) and partially activate the larger antagonistic plantarflexors. B) Schematic of the within-animal unilateral PRT model, encompassing loaded contractions of the tibialis anterior (resisted by the plantarflexors) in the left hindlimb whilst the right remains unstimulated. C) Schematic representation of the time-course of training. Rats (n = 3, per sampling point) were stimulated daily during the early phase of the light period. Prior to the initiation of deuterium oxide labelling on day 0, control and sham-operated control TA muscles were collected (red arrow). Deuterium oxide (^2H_2O) labelling was initiated at day 0 and maintained by administration of ^2H_2O in the drinking water available *ad libitum*. Stimulated and control limb muscles were collected 1 h post training after 2, 10, 20, and 30 days. PRT consisted of 5 sets of 10 repetitions of 2s on 2s off, tetanic contractions at 100 Hz with 2.5 min of rest between sets. D) Mass (TA wet weight; milligrams) of the stimulated and control limbs across the experimental time-course. E) TA protein content (milligrams) measured by Bradford assay. Data are presented as Mean ± Standard deviation and analysed using a two-way ANOVA. Significant interactions (Condition x Time) were investigated using Tukey post-hoc analysis. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$.

4.3.2 Time-series analysis of protein abundance across 30 days of muscle adaptation to PRT.

Proteomic analysis of myofibrillar and soluble fractions quantified a total of 942 proteins in rat TA and the abundance of 889 proteins (679 from the soluble fraction and 210 from the myofibrillar fraction; Figure 4.2A) was successfully quantified in all Stim and Ctrl muscles across the experimental time course (i.e. n = 3 per group at days 0, 2, 10, 20, and 30) and were accepted for statistical analysis. No significant differences were observed between the Ctrl and sham-operated stim TA at day 0, the Pearson correlation coefficient (r) for protein abundance in left (sham) and right (Ctrl) muscles were r = 0.93 (Figure 4.2B). Mitochondrial content was estimated by extracting abundance of mitochondrially annotated proteins and was significantly ($p = 0.0106$) lower in Stim muscle across the time course, irrespective of time (main effect of condition). Two-way analysis of protein abundance data identified 269 proteins exhibited significant ($p < 0.05$, $q < 0.05$) interactions (Time x Condition) across the experimental time-series (Supplementary Figure S4.1). Gene-ontology (GO) analysis found significant enrichment of GO terms related to mitochondrial cellular components were over-represented in the network of proteins with significant interactions between Time and Condition (Figure 4.2C). Soft clustering analysis of the time series data separated protein abundance data displaying a significant interaction into 5 temporal clusters (Figure 4.2D).

Figure 4.2. Temporal profiling of muscle protein abundance across 30 days of daily PRT

A) Venn diagram of proteins successfully identified in rat tibialis anterior muscle by bottom-up proteomics using liquid-chromatography tandem mass-spectrometry (LC-MS/MS) and quantified by label free quantification. Values represent 889 protein abundances quantified across all time points in all Stim and Ctrl biological replicates. B) Pearson correlation coefficient (*r*) of 889 protein abundances (log_2) in TA of n = 3 sham-operated and contralateral control (Ctrl) limbs representing the beginning (Day 0) of the experimental period. Grey data points represent proteins not-significantly different ($q > 0.1$) in abundance between the day 0 muscles. Data points represent mean log_2 abundance between n = 3 biological replicates per condition. Day 0 protein abundance data analysed via paired t-test of protein abundance data between the stim and ctrl muscle. C) Gene ontology (GO) analysis of cellular components for all proteins exhibited a significant ($p < 0.05$, $q < 0.1$) interaction of protein abundance across the experimental period. GO terms plotted by -log10 Benjamini-Hochberg adjusted p-value (BH-adjusted p-value). The red vertical line indicates threshold for statistically significant enrichment of a term (BH-adjusted $p < 0.05$). D) Temporal profiles (log_2 transformed Stim/Ctrl fold-difference at each time point) of proteins exhibiting significant interactions in abundance underwent soft clustering analysis using the fuzzy c-means clustering algorithm with the Mfuzz R package. The optimal number of clusters applied was decided through inspection of the elbow plot (top left panel) as well as qualitative assessment of temporal clusters. The minimum membership value for inclusion into a cluster was set at 0.4. The redder the time-series trace, the greater the membership value to that cluster.

Bioinformatic analysis of each cluster indicated that proteins with similar biological characteristics (function/ compartment) had shared temporal responses to training. Proteins in cluster 1 (n = 83 proteins) and 4 (45 proteins) declined in abundance in Stim muscle vs. Ctrl after 2- and 10-days of PRT (Figure 4.2D). Cluster 1 proteins (C1; Figure 4.3A) were characterised by a distinct decrease after 2 days of PRT which was later restored in Stim muscle at days 20 and 30 of the intervention.

Cluster 1 was enriched with enzymes associated with lipid metabolism and the tricarboxylic acid (TCA) cycle (Figure 4.3B). STRING analysis further highlighted a network of ATP synthase subunits (8 proteins; ATP-A, -B, -D, -G, -O, -MK, 5I, and AT5F1) were included in cluster 1 (Figure 4.3C). Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PRGC1; more commonly known as PGC-1α) was also included in cluster 1 (membership value = 0.46) and exhibited a highly significant interaction ($p = 1.65e^{-05}$, $q = 0.0007$) between condition and time. PGC-1α abundance decreased ($\log_2$ FD = -0.25) at day 2 and then increased in Stim muscle after 20 days ($\log_2$ FD = 0.56) and 30 days ($\log_2$ FD = 0.74) of PRT.

Cluster 4 can be uniquely identified by a distinct pattern of decreased protein abundance in Stim TA occurring after 10 days of PRT. Cluster 4 was specifically enriched in biological pathways associated with respiratory chain structure and function (Figure 4.3E and F), including NADH Dehydrogenase (respiratory complex I) core (NU1M, NDUS1, NDUS2 and NDUV2) and accessory subunits (NDUS4, NDUS6, NDUA5, NDUA9, NDUAA, NDUAB) (Figure G). Alongside subunits of respiratory complex III (CYB, QCR1, QCR2, and UCRI) and IV (COX2, CX6C2, COX5A, COX5B, and COX41) (Figure 4.3E). Overall, both cluster 1 and cluster 4 contained mitochondrial proteins and had similar trends of decreased abundance during the 'early' (≤ day 10 days) phase of the PRT intervention.

Clusters 1 and 4 also included proteins associated with mitochondrial quality control. Two isoforms of prohibitin (PHB and PHB2) were less abundant (log2 FD = -0.31 and -0.25, respectively) after 2 days PRT (Cluster 1). Cluster 1 also included the mitochondrial chaperonin (60kDa heat-shock protein), CH60, and co-chaperonin CH10. Whilst cluster 4 contained 2 subunits of the mitochondrial contact site and cristae organising system (MIC-10 and 60) and the sorting and assembly machinery component 50 (SAM50). All 3 voltage-dependant anion-selective channel protein isoforms (VDAC1, 2, and 3) were included in cluster 4, as well as the mitochondrial apoptosis inducing factor 1 (AIFM1). Indicating there were decreases in abundance across a selection mitochondrial chaperones and proteins associated with mitochondrial quality during the first 10 days of PRT. Proteins associated with ribosome formation followed a similar pattern of response to that of the mitochondrial chaperones. Including the nucleolar protein, nucleolin, and 40S (RS-3, 4X, and 8) and 60S (RL18) ribosomal subunits, which may indicate a potential link between ribosomal translation and changes to mitochondrial protein chaperones.

Cluster 2 (32 proteins) was characterised by an inverse temporal pattern to that of clusters 1 and 4 (Figure 2) and contained proteins associated with cytosolic chaperone function and protein translation. Including the 90- and 70-kDa heat shock-proteins (HSP90A and HS71C, respectively), small heat shock protein B8 (HSPB8), and prefoldin subunit 2. Therefore, the time-courses differed between the response of mitochondrial (cluster 1 and 4) and cytosolic chaperones (cluster 2).

A)
Cluster 1
B)
Biological Process
Small molecule metabolic process
Fatty acid metabolic process
Fatty acid catabolic process
Carboxylic acid metabolic process
Carboxylic acid catabolic process
Term
BH-adjusted p-value (-log10)
KEGG Pathway
Fatty acid degradation
Fatty acid metabolism
TCA Cycle
Peroxisome
Carbon metabolism
C)
KEGG: TCA Cylce
KEGG: FA Metabolism
ATP synthase
FD abundance (log2; Stim/Ctrl)
Time (days)
Uniprot ID
D)
PGC-1a
Abundance (log2)
Time (Days)
E)
Cluster 4
F)
Biological Process
Respiratory electron transport chain
Mitochondrial Respiratory chain complex I assembly
Mitochondrial Respiratory chain complex assembly
Aerobic electron transport chain
ATP synthesis coupled electron transport
BH-adjusted p-value (-log10)
G)
Respiratory Complex I
Respiratory Complex III
Respiratory Complex IV

Figure 4.3. Temporal regulation of mitochondrial protein abundance across 30 days of PRT.

A) STRING protein interaction network for proteins displaying significant ($p < 0.05$, $q < 0.05$) interaction (Time x Condition) in abundance data across the time-course of PRT belonging to cluster 1 reported in Figure 2. Coloured nodes represent proteins in cluster 1 annotated to the KEGG pathways "TCA Cycle" (Green), "Fatty acid metabolism" (Blue), and a network of ATP synthase subunits (Red). B) Gene ontology (GO) analysis of biological process (upper panel) and KEGG pathway analysis (lower panel) of proteins included in cluster 1. GO terms plotted by -log10 Benjamini-Hochberg adjusted p-value (BH-adjusted p-value). The red vertical line indicates the threshold BH-adjusted $p < 0.05$ for statistically significant enrichment of a term. C) Line graphs displaying the protein-specific abundance change (units/ scale??) across the time-course of PRT. Panels represent proteins annotated to the KEGG pathway "TCA Cycle" (Left), "Fatty-acid metabolism" (Middle), and the network of ATP synthase subunits (Right). D) Boxplot displaying $\log_2$ relative abundance of PRGC1 (a.k.a PGC-1α) from the Stim (blue) and Ctrl (black) muscle ($n = 3$, per group) across the 30 days of PRT. Two-way ANOVA identified a significant ($p < 0.05$, $q < 0.1$) interaction (Time x Condition) for PGC-1a in response to PRT. E) STRING protein interaction network for proteins displaying significant ($p < 0.05$, $q < 0.05$) interaction (Time x Condition) in abundance data across the time-course of PRT which comprise temporal cluster 4. Coloured nodes represent subunits belonging to mitochondrial respiratory chain complex I (Orange), complex III (Yellow), and complex IV (Pink). F) Gene ontology (GO) analysis of biological process of proteins included in cluster 4. GO terms plotted by -log10 Benjamini-Hochberg adjusted p-value (BH-adjusted p-value). The red vertical line indicates threshold (BH-adjusted $p < 0.05$) for statistically significant enrichment of a term. G) Line graphs displaying the respiratory chain abundance change across the time-course of PRT. Panels represent subunits of respiratory complex I (Left), III (Middle), and IV (Right). STRING interaction networks generated using a minimum interaction score of 0.4. Data points in C and G represent mean ± standard deviation of $\log_2$ fold-difference (FD) in abundance (Stim/Ctrl) at each sampling point. Red horizontal line indicated a log2 FD = 0 (i.e. positive values = more abundant in Stim and negative = more abundant in Ctrl).

Cluster 3 (64 proteins) exhibited a continuous downregulation of protein abundances in the Stim TA across the 30 days of PRT. GO analysis indicated cluster 3 consisted of enzymes annotated to the KEGG pathway "Glycolysis/ Gluconeogenesis" (10 proteins, BH-adjusted $p = 0.0443$). A network of contractile proteins was also identified in cluster 3, including myosin light chain -1, -3, myosin light chain kinase 2, Troponin T (fast skeletal muscle isoform), and Troponin I (slow skeletal muscle isoform inhibitor). Alterations in voltage dependant calcium channel proteins were also highlighted in cluster 3 (CACB1, CAC1S, CA2D1, and CCG1). The downregulation of enzymes associated with glycolysis, sarcomere structure, and calcium handling proteins co-occurred with reciprocal increases in mitochondrial proteins suggesting changes in energy metabolism indicating clear alterations in muscle proteome composition suggestive of changes in phenotype of the TA induced by 30 days of PRT.

Cluster 5 consisted of 42 proteins that progressively increased in abundance across the period of PRT. Whilst cluster 5 was not enriched for any GO terms, 12 proteins typically associated with blood and heme-stress formed a dominant protein-protein interaction network.

4.3.3 Temporal profiling of protein-specific synthesis rates across 30 days of PRT.

In total, our analysis quantified the fractional synthesis rates (FSR) of 659 proteins. After filtering for missing values, 340 proteins-specific synthesis rates were quantified successfully across the entire experimental period (n = 30, total number of independent samples) (Figure 4.4A). Both mixed-protein and mitochondrial-protein FSR was significantly (main effect of condition; $p = 0.00243$ and 0.023, respectively) greater in Stim compared to Ctrl muscle (Figure 4.4B and Supplementary Figure 4.1B).

Analysis of protein-specific synthesis rates identified the FSR of 97 proteins exhibited a significant ($p < 0.05$, $q < 0.1$) interaction (condition x time). Temporal clustering (Figure 4.4C) identified 4 unique clusters of protein synthesis rate responses to PRT. GO analysis of FSR data found no significantly enriched GO terms/ pathways amongst proteins within each cluster. However, STRING analysis highlighted biological networks of proteins within each cluster.

Cluster 2 was characterised by rises in protein synthesis rate in Stim muscle after 2 days of PRT which later plateaued during the remainder of the experiment period. Cluster 2 consisted of proteins associated with chaperone functions and proteostasis, including mitochondrial superoxide dismutase (SODM), the calcium binding chaperone (Calreticulin), and 4 heat-shock related chaperones (HS71A, HSP7C, BiP, and F10A1). Indicating an upregulation of turnover rates of quality control proteins in Stim muscle in response to training (Figure 4.4D).

Sarcomere proteins were included in both cluster 1 (MYH8, LMNA, DESM, COF1, PROF2, FHL1, SMPX, and PDLI5) and cluster 3 (MYLK2, MYL11, CASQ1, ACTB, WDR1, and TPM2), which exhibited different temporal patterns. Proteins in cluster 1 displayed a greater FSR in Stim after the 2 and 10 days of PRT, which then decreased in FSR after 20 days. Whereas cluster 3 proteins were considerably lower in FSR after the first 2 days of PRT and then their FSR increased and plateaued after 10 days. These findings indicate protein-specific responses in synthesis rate across different structural components of skeletal muscle sarcomere during the 30-day training period.

Figure 4.4. Temporal profiling of muscle protein synthesis across 30 days of daily PRT.

A) Venn diagram of number of protein-specific fractional synthesis rates (FSR) successfully quantified during each experimental period. In total the FSR of 659 protein was quantified, including 408 during 0-2, 576 during 2-10, 601 during 10-20, and 480 during 20-30. B) Mixed-protein FSR responses to PRT based on the weighted median FSR (%/d weighted by protein abundance) of 340 proteins quantified in all samples. Data are presented as mean ± sd of n = 3 biological replicates and were analysed using a two-way ANOVA. C) Temporal profiles (log_2 transformed Stim/Ctrl fold-difference at each time point) of proteins exhibiting significant ($p < 0.05$, $q < 0.1$) interactions (Time x Condition) in synthesis (assessed by 2-way ANOVA) underwent soft clustering analysis using the fuzzy c-means clustering algorithm with the Mfuzz R package. The optimal number of clusters applied was decided through inspection of the elbow plot (top left panel) as well as qualitative assessment of temporal clusters. The minimum membership value for inclusion into a cluster was set at 0.4. The bluer the time-series trace, the greater the membership value to that cluster. D-E) Line graphs displaying change in FSR of chaperones grouped in cluster 2 (D) and enzymes associated with glycolysis/gluconeogenesis in cluster 4 (E) FSR the across the time-course of PRT. Data points in D and E represent mean ± standard deviation of log_2 fold-difference (FD) in abundance (Stim/Ctrl) at each sampling point. Red horizontal line indicated a log2 FD = 0 (i.e. positive values = more abundant in Stim and negative = more abundant in Ctrl).

Cluster 4 represents proteins that displayed a progressive increase in synthesis rate across the 30 days of PRT. Network analysis found these proteins generated a highly inter-connected network of enzymes associated with energy metabolism. Namely, muscle and mitochondrial creatine kinase as well as 9 proteins involved in glycolysis/ gluconeogenesis (ENOA, ENOB, ENOG, ALDOA, TPIS, PGM1, PYGB, and PGAM2) (Figure 4.4E).

Thirteen subunits of multiprotein complexes comprising oxidative phosphorylation pathways were temporally grouped in cluster 3 (NDUV2, NDUAB, NDUS1, CYC, COX41, QCR6, ATPD and ATPO) and cluster 4 (NDUA5, QCR2, ATPK, ATP5L, ATP5J) which exhibit a lower rate of synthesis in Stim muscle over the first 2 days of PRT and then increased over the remainder of the experimental period.

Figure 4.5 summarizes fold-differences (Stim/Ctrl) in protein abundance plotted against fold-differences (Stim/Ctrl) in protein synthesis rate across the time course of PRT. Proteins in the upper left quadrant were less abundant in Stim but exhibit a greater rate of synthesis versus the Ctrl muscle. Whereas proteins in the lower right exhibit greater abundance but a lower rate of synthesis in the Stim versus Ctrl muscle. Integration of the 'early' response to PRT (Figure 4.5B) indicated the decreases in abundance of enzymes associated with oxidative phosphorylation was primarily due to an increase in the rate of breakdown (i.e. abundance was less but FSR was slightly greater, indicating the rate of degradation increased to a greater extent than that of synthesis).

The increases in the abundance of respiratory chain and ATP synthase subunits identified after 20- and 30-days PRT was likely due to increases in the rate of synthesis to a greater extent than changes in degradation. The clearest pattern of response was identified in glycolytic enzymes. Whereby, 10 proteins associated with glycolysis/gluconeogenesis were clustered and displayed a continuous decrease in abundance in Stim muscle across the time course of PRT (Figure 4.2D; cluster 3), whereas the FSR of these proteins exhibited a continuous increase in response to PRT (Figure 4.4E). Parallel analysis of the abundance and synthesis change of these proteins indicated these proteins were grouped in the top left panel after 20 and 30 days of PRT (Figure 4.5C and 4.5D), which indicates the synthesis rate of these enzymes was greater in Stim, but there was a relatively greater increase in the degradation of these proteins that resulted in the decrease in protein abundance.

Figure 4.5. Dynamic proteome response of muscle proteins to PRT.

Dynamic proteome profiling of proteins associated with glycolysis/ gluconeogenesis (purple), ATP synthase subunits (red), and respiratory chain subunits (complex I = orange, complex III = yellow, and complex IV = pink) after 2 days (A), 10 days (B), 20 days (C), and 30 days (D) of PRT. Proteins that a greater in abundance and synthesis rate in Stim muscle after PRT (upper right quadrant). Proteins that are lesser in abundance and synthesis rate in Stim muscle after PRT (lower left quadrant). Proteins that are more abundance but lower in synthesis rate in Stim muscle after PRT (lower right quadrant). Proteins that are less abundant but greater in synthesis rate in Stim muscle after PRT (upper left quadrant). Data points represent mean $\log_2$ fold-difference (Stim/Ctrl) in abundance and FSR (n = 3, per group).

4.4 Discussion

Long-term resistance training is associated with beneficial mitochondrial adaptations but knowledge of the role of mitochondrial remodelling during resistance training is currently incomplete (Parry et al., 2020). Our temporal analysis of protein abundance profiles highlighted 2 clusters of mitochondrial proteins (abundance cluster 1 and 4) (Figure 4.2 and 4.3), which were characterized by a decrease in protein abundance across the first 10 days of PRT followed by a later increase after 20 days PRT. Cluster 1 mainly consisted of proteins associated with TCA cycle, fatty-acid metabolism, and oxidative phosphorylation (OXPHOS), whereas cluster 4 was comprised of mitochondrial electron transport proteins including subunits on respiratory complex I, III, and IV (Figure 4.3A). The observed changes in the abundance of specific OXPHOS subunits alongside similar decrements in the total abundance of mitochondrial proteins (Supplementary figure 4.1), suggest an overall decrease in mitochondrial content after 2 and 10 days of PRT. However, the average FSR of mitochondrial annotated proteins was not different after 2 days PRT and only slightly increased after 10 days PRT. Implying degradation of mitochondrial proteins was elevated during the first 10 days of PRT. Our proteomic analysis encompassed >800 proteins, including 10 core (4 alpha and 6 beta), 9 regulatory proteasome subunits and 9 proteins associated with ubiquitination (e.g. E3 ligases RN123 and RD23B and E2 enzymes UB-2D2, -2V2, and UBE2N) but no significant differences in proteins involved in degradation during the first 10 days of PRT were identified. Similarly, recent transcriptomic analysis (Viggars et al., 2023) revealed no significant change in mRNAs annotated to the GO term 'Ubiquitin mediated proteolysis' during the first 10 days of PRT. However, significant increased expression of genes associated with 'Proteasome' and 'foxO signalling' were identified after 2 and 10 days respectively. Potentially indicating increased in signalling pathways associated with degradation despite no changes in the abundance of proteins responsible for proteolysis.

The mitochondrial inner membrane protein prohibitin 2 (PHB2) was amongst the proteins in cluster 1 (Figure 4.3A) and was less abundant after 2 days of PRT (Supplementary figure 4.1C). PHB2 can act as a receptor for the induction mitophagy in mammals if it becomes exposed to the cytosol (Wei et al., 2017). From our data it is unclear whether the PHB2 alongside other mitochondria proteins indicates the involvement of mitophagy. Ogborn et al, (Ogborn et al., 2015) reported a similar loss of mitochondrial protein in human muscle after unaccustomed resistance exercise, but targeted markers of mitophagic processes, including gene expression of the mitophagy receptor, NIX, was not altered in exercised muscle (Ogborn et al., 2015). Similar data from older human muscle indicated no increases in mitophagy 24 hr following a resistance exercise bout (Mesquita et al., 2020). However, the protein content of PHB2 (and Pink1/Parkin measured in previous reports) represent proxy markers of mitophagy. The critical regulator of mitophagy, Pink1, is a serine/threonine-protein kinase which is regulated through autophosphorylation at Ser^{228} and Ser^{402} (Okatsu et al., 2012). Therefore, future phosphoproteomic analysis may bring a more complete understanding of whether mitophagy is involved in the muscle response to resistance training.

The synthesis of 6 proteins associated with the term "response to stress" increased after 2 days PRT (Figure 4.4D), including key chaperones and folding sensors, BiP (Binding immunoglobulin protein) and CALR (Calreticulin) which act as regulators of the endoplasmic reticulum (ER) unfolded protein response (UPR) (Hetz, 2012). Superoxide dismutase (SODM) also had a greater synthesis rate after 2 days of PRT. SODM metabolises mitochondrial reactive oxygen species (ROS) and determines the rate that superoxide anions are converted to H_2O_2 (Pani et al., 2009). The greater synthesis of SODM potentially indicates elevated cellular stress (e.g. ROS). Both ROS and ER signalling via ER chaperones (e.g. BiP and CALR) lead to the activation of the unfolded protein response to limit the impact of metabolic and cellular stress (Ron and Walter, 2007) which is linked to the mitochondrial UPR.

Previous investigations have identified the activation of the UPR and significant increases in the abundance of BiP and expression of ATF6 following a single bout of resistance exercise in human muscle (Ogborn et al., 2014). In yeast, the activation of the mitochondrial unfolded protein response (UPR^{mt}) is increased in response to mistargeted mitochondrial proteins and greater cell stress (Bragoszewski et al., 2013). However, the presence of mitochondrial degradation via UPS induced by UPR is less clear in higher organisms. Defects in protein quality control and mitochondrial protein import in C.Elegans have been shown to lead to proteasome activation via UPR^{mt} (Wu et al., 2018, Sladowska et al., 2021). Activation of the UPR can inhibit protein synthesis, for example by eIF2a kinases and this may explain the unexpected decrease in mixed protein FSR during the first 2 days of PRT (Figure 4.4B). eIF2a appears to play a key role in regulating translation initiation in response to cellular stress (Starck and Shastri, 2016) with phosphorylation of eIF2a at serine^{51} resulting in reduced eIF2 complex formation and inhibition of translation initiation (Merrick and Pavitt, 2018). Further highlighting phosphoproteomic analysis as follow-up to potentially explain changes in protein turnover induced via PRT through regulation of protein phosphorylation status.

In the longer-term (days 20 – 30) PRT resulted in a greater abundance of mitochondrial proteins and enzymes of the TCA cycle and fatty-acid metabolism (Figure 4.3), which are more traditionally associated with endurance adaptations (Furrer et al., 2023). Early studies reported resistance training leads to an increase in myofibrillar content the relative density of mitochondria decreases in human triceps after a 6-month programme of resistance training (MacDougall 1979). Subsequently, Luthi and colleagues (Lüthi et al., 1986) clarified that while resistance training decreases the relative volume of muscle mitochondria, the absolute mitochondrial volume remains constant when the data are adjusted for increase in muscle mass. However, more recent studies (Groennebaek and Vissing, 2017) report significant gains in the synthesis of mitochondrial protein and respiratory function of human muscle after 6 weeks of high-load resistance exercise training, and there continues to be debate in the literature on whether resistance training leads to the expansion of muscle mitochondrial content (Parry et al., 2020).

Mesquita and colleagues (2020) reported gains in mitochondrial abundance based on targeted analysis of electron transport chain components in the muscle of older humans after a 10-week period of resistance exercise but there were no co-occurring changes in markers of mitochondrial biogenesis (e.g. PGC-1α protein content). Here, in rat muscle, a relatively robust increase in PGC-1α was detected that was temporally aligned with the enzymes of fatty acid metabolism and the TCA cycle in cluster 1 (Figure 4.3A and D). PGC-1α is widely regarded as a regulator of mitochondrial biogenesis and improved metabolic capacity in the context of endurance exercise (Furrer et al., 2023). However, Ruas et al., (Ruas et al., 2012) reports a specific isoform (PGC-1α4), characterised by alternative N-terminal amino acid sequences, is preferentially expressed in mouse and human muscle in response to resistance exercise. Our LC-MS/MS analysis did not include peptides from the N-terminal region of PGC-1α so it is not possible to unambiguously identify the isoform quantified in our analysis. Nevertheless, the corresponding increases in abundance of numerous enzymes comprising fatty-acid and TCA cycle metabolic pathways (Figure 4.3) indicates the typical 'endurance' isoform (PGC-1α1) was the most likely candidate linked to the adaptive phenotype in our data. However, it is currently unclear if a true endurance trained muscle phenotype (such as improved oxidative capacity and mitochondrial content) is induced as a result of PRT or rather a broad spectrum adaptive programme occurs encompassing mitochondrial and metabolic improvements alongside muscle growth following the onset of relative high levels of contractile activity in an archetypical fast twitch muscle comprised of almost entirely type IIX and B fibers.

Despite the greater abundance of PGC-1α, greater synthesis rate of mitochondrial proteins and increase in some mitochondrial proteins in the later response to PRT, the total abundance of mitochondrial annotated protein did not completely recover in trained muscle to the levels in untrained muscle at 30 days (Supplementary figure 4.1A and B). Thus, PRT may not have resulted in greater mitochondrial density. However, data previously published from a

separate investigation of the samples analysed in this study indicated greater mitochondrial respiratory complex I activity in response to PRT (Viggars et al., 2023). Furthermore, in humans following resistance training, ex vivo measurements of mitochondrial respiration significantly increased alongside increases in complex I abundance despite no-change in transcripts involved in mitochondrial biogenesis (Porter et al., 2015). Taken together this data provide strong evidence for resistance training induced increases in protein-specific abundance of oxphos subunits alongside greater capacity for oxidative phosphorylation. The apparent lack of mitochondrial biogenesis indicated resistance exercise to induce protein-specific remodelling leading to greater mitochondrial quality rather than the bulk increases in mitochondrial content.

Our abundance and FSR analyses indicated an inverse relationship between the change in abundance of enzymes associated with glycolysis/ glycolytic processes and their fractional synthesis rates. The abundance of these proteins progressively decreased in Stim muscle (Figure 4.2D), whereas FSR of 9 of these proteins (ENOA, ENOB, ENOG, ALDOA, TPIS, PGM1, PYGB, and PGAM2) progressively increased (Figure 4.4E) across the 30-day PRT intervention. This phenomenon of greater synthesis yet decreased abundance of glycolytic enzymes (Figure 4.5C and D) agrees with dynamic proteomic analysis of human muscle following resistance exercise (Camera et al., 2017). Together these findings point to a selective increase in the degradation of glycolytic enzymes that outpaces their rate of synthesis and leads to a loss in protein abundance. Inferences on the role of protein degradation can only be interpreted from simultaneous measurements of the synthesis and abundance of individual proteins. However, studies that focused only measuring protein synthetic responses to hypertrophic stimuli also found a similar pattern of response in enzymes associated with glycolytic processes. Measurement of individual protein turnover rates in humans during 2 weeks of 3x weekly resistance training indicated the turnover rate of 4 glycolytic enzymes significantly increased compared to non-exercising muscle (Murphy et al., 2018). Similarly, pharmacologically induced hypertrophy via administration of selective androgen receptor modulators reported increases in the turnover rates of 9 enzymes associated with glycolytic processes, with the increase in turnover proportional to the magnitude of muscle hypertrophy (Shankaran et al., 2016b). Collectively these data indicate a greater rate of turnover of proteins associated with glycolytic processes/ glycolysis seem to be a conserved response to hypertrophic stimuli. Indicating the turnover rates of these enzymes being associated with the expected increases in enzymatic/ metabolic activity in response to the fuel demand of resistance exercise and/or to aid in energy provision to facilitate muscle growth. Transcriptome analysis of PRT revealed no change in gene expression associated with glycolysis/ gluconeogenesis (Viggars et al., 2023). Indicating no change in upstream signalling at the mRNA level driving the protein abundance change. Therefore, the decreased protein abundance may be the result of increased catalytic activity of these glycolytic enzymes which results in a greater rate of turnover due to the accumulation of damage. However, the rate of protein synthesis is outweighed by the need to increase degradation to maintain proteostasis, resulting in a net negative protein abundance. Thus, potentially providing early insights into the role of metabolic/ enzyme activity in regulating protein turnover on an individual protein basis. Welle and Nair (Welle and Nair, 1990) identified relatively strong, positive correlations ($r = \sim 0.83$) between metabolic rate and indices of protein turnover (measured via leucine flux following 4 hr infusion of L[1-^{13}C]-Leucine). However, to date, little work is available assessing metabolite-enzyme relationships in the regulation of protein-specific turnover.

The key strength of our dynamic proteome profiling techniques is the ability to infer the contribution of synthesis and degradation on changes in protein abundance. Our data and that of Camera et al (Camera et al., 2017) clearly highlight the role of protein degradation in regulating the adaptive response to different biological processes. Whereby the key theme emerging seems to be the decreased abundance of glycolytic enzymes is due to an increase in their rate of degradation that is greater than the increase in protein synthesis, resulting in the net loss of protein abundance. Our group have also reported dynamic proteome profiling of rat muscle in response to

chronic-low frequency stimulation (CLFS, i.e. endurance-type exercise). CLFS similarly resulted in decreased abundance of glycolytic enzymes (Hesketh et al., 2020) suggesting lower abundance of these proteins may be a consequence of endurance and resistance training in untrained muscle. However, the incorporation of synthesis data to explain the change in abundance of these protein indicated that the decrease in abundance was a result of a decrease in protein synthesis rate following CLFS (Hesketh et al., 2020) rather than a result of increase in degradation rate in response to resistance training. Highlighting a key difference in the regulatory mechanism of proteome remodelling in response to different modes of exercise. Consolidating the need for data on the role of protein-specific degradation to completely understand the adaptive responses to exercise.

The relationship between the acute transcriptional responses and subsequent proteome remodelling are currently not well understood. Transcriptomic analysis of the PRT muscle samples within this chapter have been previously published (Viggars et al., 2023). RNA-sequencing methods indicated adaptation in the transcriptional response to training measured 1 h post-exercise across the time-course of hypertrophy. The accumulation of transcriptomic and dynamic proteomic analysis highlights the extreme complexity underpinning muscle adaptation. Viggars et al (2023) report mitochondrial encoded genes to follow a similar temporal response to that of our proteomic analysis whereby Complex I – V mRNA profiles exhibit decreases in expression after 2 and 10 days of PRT. However, the expression of these mitochondrially encoded genes were identified to be greater following 30 days of PRT. Similarly, GO terms associated with oxidative phosphorylation and fatty-acid metabolism were significantly lower in Stim muscle after 2 and 10 days of PRT. Whereas GO terms ‘processing by the endoplasmic reticulum’ and ‘proteasome’ (potentially indicative of UPR/ ER stress and greater degradation) were significantly enriched after 2 days of PRT. Furthermore, markers of mitochondrial adaptation (PPAR signalling and oxidative phosphorylation) were significantly upregulated after 20 and 30 days of PRT (Viggars et al., 2023). Taken together the biological interpretation of the response using GO/ network analysis indicates a considerable amount of overlap between the transcriptome and clustering of the dynamic proteomic data reported here. The unexpectedly high degree of similarity in the biological interpretation of the transcriptome and our proteomic data indicates the adaptive signalling response aligns well between the different data sets. Despite that changes in mRNA do not necessarily explain changes in protein abundance. Overall, these data indicate temporal alterations in the transcriptional, translational, and protein abundance response to hypertrophic stimuli. Suggesting a unique signalling and adaptive response occurs on the level of transcription and translation across a period of remodelling offering further evidence as to why acute markers and measurement of adaptation may poorly explain the long-term adaptive phenotype.

The implementation of programmed resistance training (PRT) to induce hypertrophy of the rat tibialis anterior was first developed by Schmoll et al., (Schmoll et al., 2018). This method employs a unilateral programmable electrical stimulation model in vivo that allows training to be elicited without the need for repeated anaesthesia. This programmed resistance training results in resisted/ loaded eccentric contractions of the dorsiflexors. Each bout of contractions induced by stimulation has been reported to lead to significant increases in the maximal tetanic force output by the tibialis anterior (+124%) compared to unloaded controls. Daily loading for ~ 3 weeks resulted in significant increases in the wet weight of the TA (+13.9%) and EDL (+10%) compared to contralateral controls. Alongside ~ +19% greater mean myofiber cross sectional area (CSA) in the TA, however, the smallest fibres (first quartile of CSA) experienced the greatest magnitude of hypertrophy (>50%) (Schmoll et al., 2018). The extent of muscle hypertrophy aligns favourably with the unilateral stimulation model used by Baar and Esser (1999) which resulted in increases (+ 14 %) in TA wet weight following twice weekly training for 6 weeks. Further applications of the novel unilateral stimulation model applied daily PRT across a 30 day time-course experiment to a larger cohort of animals (n = 47, in total), of which the animal samples reported in our work originated (Viggars et al., 2023). Daily training resulted in 13.1 % increases in TA wet weight after 10 days of PRT progressing to + 17.1 % after 30 days. Similarly, fibre CSA progressively increased after 10 (+ 3.5%), 20 (+ 8.8%), and 30 days (+18.7 %)

compared to contralateral control animals (Viggars et al., 2023). Fibre-type specific profiling showed that Type I and Type IIA fibre CSA only was significantly increased after 30 days. A subset (n = 3, per group) was then analysed for transcriptomics reported in Viggars et al., and dynamic proteomics reported within this chapter. Alongside the previous data generated from the application of this PRT model, this study provided evidence for wet weight and CSA increases to be accompanied with increases in total protein content of the muscle. Further consolidating the strength of the model to induce robust hypertrophy rather than being limited to confounding variables associated with dramatic surgeries or repeated anaesthesia as used in previous models.

The development of D_2O labelling with LC-MS/MS proteomics has enabled the reporting of fractional synthesis rates on a protein-by-protein basis in skeletal muscle. Proteome-wide analysis of FSRs demonstrate that the measurement of mixed-muscle FSR offers limited understanding of full extent of the dynamic responses of muscle to training. Previous work from our lab (Camera et al., 2017) and this data report that changes in protein abundance in response to hypertrophic stimuli are underpinned by complex and time/ training status-dependant regulation of either synthetic or degradative processes. Whilst this concept is not new, the reporting of empirical data measuring individual protein abundance and rates of synthesis in response to chronic training is sparse. Dynamic proteomic methodologies can provide novel data on the synthesis, abundance, and degradation of individual proteins. Indicating the use of these methods in future studies are of vital importance to advance the knowledge of the dynamic processing involved in exercise adaptation. Our current dynamic proteomic methods do not allow for complete consideration for a protein's cellular localisation. Which ultimately is a vital piece of biological information needed for the interpretation of molecular responses to exercise. Furthermore, our proteomic analysis uses proteolytic digest of proteins into peptides with a predictable sequence which can be measured via LC-MS/MS to infer information about the proteins within a sample. Whilst this sequence information is enough to assign peptides to its parent protein, bottom-up proteomics miss important information pertaining to post-translational modification and the resulting proteoforms that can arise from the same protein isoform. The analysis of different proteoforms, via top-down proteomics, allows for a greater understand of the signalling response due to the information provided on peptide modifications which can have vital implications on the structure and function of proteins. However, top-down approaches are often limited by the need of extensive fractionation or biochemical separation techniques (e.g. 2-dimensional gel-electrophoresis). This can lead to extensive sample processing and instrument run time whilst also inhibiting the depth of the proteome that can be quantified compared to that of current bottom-up proteomic analysis.

4.5 Conclusion

In conclusion, this is the first data to integrate the profiling of abundance and synthesis rates on a protein-by-protein basis across the time-course of training induced muscle hypertrophy. This data highlighted daily, programmed resistance training induced chronic (i.e. 20 and 30 days) remodelling of the proteome like that of endurance-type adaptation. Resulting in greater abundance of proteins associated with the mitochondrial inner membrane proteins and improve metabolic capacity (e.g. TCA cycle and fatty acid oxidation). Proteodynamic analyses provided unique insights suggesting this adaptive phenotype is preceded by potential losses in mitochondrial proteostasis and decreases in the abundance of mitochondrial inner membrane proteins in response to acute (2 and 10-days) PRT, induced by greater rates of degradation. Suggesting that adaptation of the proteome may be underpinned by non-linear changes in protein abundance and synthesis rate. Our work highlights the importance of integrating the measurement of the acute and chronic responses to training to understand the complexity of the adaptive process. Similarly, acute (2 day) alterations in protein FSR do not accurately reflect changes in individual synthesis rates observed throughout the entirety of muscle adaptation. Further highlighting the inability to extrapolate short-term changes in muscle protein synthesis to explain resistance training induced adaptation.

Chapter 5. The dynamic proteomic response to endurance and resistance training in human skeletal muscle.

5.1 Introduction

To date, studies have largely focused on protein metabolism responses to different exercise modes and have reported mixed protein synthesis rates. It is understood that mixed-protein synthesis is stimulated by resistance (Chesley et al., 1992; Biolo et al., 1995) and endurance (Carraro et al., 1990) training in humans, alongside concomitant increases in rates of mixed-protein breakdown (Phillips et al., 1997, Sheffield-Moore et al., 2004). However, the co-occurring changes in the rates of synthesis and breakdown of protein mixtures does not give insight to the nature of the responses of different classes of proteins that result in changes in net protein abundance associated with endurance or resistance training. Wilkinson et al., (Wilkinson et al., 2008) addressed this question by measuring mixed-myofibrillar and -mitochondrial protein synthesis at rest, following an acute bout of resistance (REX) or endurance (END) exercise, and following 10 weeks of training in young men. REX significantly increased myofibrillar (+67 %) and mitochondrial (+69 %) protein synthesis following a single bout of exercise in untrained men. However, after chronic training only myofibrillar protein synthesis responded (+36 %) to REX. Conversely, END specifically increased the rate of mitochondrial protein synthesis in both the untrained (+154 %) and trained (+105 %) state but did not affect myofibrillar protein synthesis rate. Therefore, the synthetic response of muscle subfractions differs between training modes and only resistance exercise training is associated with gains in myofibrillar protein synthesis. However, these data did not allow for inferences to be made on how changes in mixed-protein FSR underpin proteome remodelling. Ultimately, it has been challenging to connect the acute muscle responses such as cell signalling to protein synthesis and longer-term adaptation. The use of traditional stable isotope methodologies and proxy markers of translation initiation (e.g. eIFs and p70S6K) only allow inferences to be made on the transient changes in bulk/ mixed-protein synthesis rates following a single bout of training. Robinson et al, (Robinson et al., 2017), reports mixed mitochondrial protein synthesis rates, and investigated chronic human muscle responses to 3 exercise modes, including high-intensity interval exercise (HIIT; cycling at >90% VO2max 5 x / week), resistance exercise (REX; 4 sets of 8–12 repetitions, 4 x / week) or combined training (CT) encompassing cycling and moderate resistance exercise 5 days and 4 days a week, respectively for 3-months. These data indicate a ~ 25 % increase in the synthesis rate of mixed mitochondrial protein that was specific to endurance exercise and absent from the human muscle response to resistance exercise training. However, the supplementary proteomic analysis found increases in the abundance of 141 mitochondrial proteins following resistance training (Robinson et al., 2017). Therefore, short duration measurements of average synthesis rate poorly reflect changes in the abundance of individual proteins.

This work used dynamic profiling methods to explore detailed protein-specific responses integrated over 5 days of exercise during the first week and 10th week of training. Previously, Shankaran et al. (Shankaran et al., 2016a) reported the first data on protein-specific synthesis rates in sedentary and endurance-trained human muscle. In total the FSR of 139 proteins in the muscle of at least 3 sedentary and 3 exercised participants and found a significant increase in the synthesis rate of 20 proteins involved in glucose metabolism, muscle contraction, and cellular respiration in exercised muscle. Furthermore, Murphy et al. (Murphy et al., 2018) reports the FSR responses of 190 proteins (n ≥ 2 participants per group) to resistance exercise in overweight males during a 2-week period of energy restriction and noted that 175/ 190 proteins exhibited a significant increase in synthesis rate in response to exercise, including increases in mitochondrial and sarcoplasmic proteins. Taken together these data indicate that individual proteins in human muscle exhibit a range of different individual responses and highlight the potential oversight of reporting mixed protein FSR in the interpretation of training adaptations. However, both these reports (Shankaran et al., 2016a, Murphy et al., 2018) lack data on individual protein abundance to complement the analysis of protein synthesis rates. In the context of exercise-induced proteome remodelling, this leaves questions as to whether the reported changes in synthesis rate represent changes in protein turnover or underpin changes in protein abundance.

Currently, it is not known how different training stimuli modulate protein-specific changes in abundance across an acute and/ or chronic period of training in human skeletal muscle. Furthermore, no reports have incorporated the analysis of proteomic responses to resistance and endurance training within the same group of individuals to limit the effect of inter-individual variation responsiveness. Previous work investigating the differential response to endurance and resistance exercise utilized a model of unilateral exercise training in humans, in which one leg completed resistance-training, consisting of knee extension exercise, while the contralateral leg completed an endurance training of one-legged cycling (Wilkinson et al., 2008). Unilateral training resulted in significant increases in knee extensor force output in the resistance trained leg only. Whereas the contralateral cycling limb resulted in significant increases in Vo_{2peak} and markers of mitochondrial content, quantified via citrate synthase activity that were unique to the endurance trained limb. Indicating the model as a valid method of within-subject endurance and resistance training, without any interference effect on physiological outcomes between limbs.

In an effort to elucidate the shared and unique adaptive response underpinning the endurance and resistance trained phenotype, this work optimized methods previously used in within our laboratory (Camera et al., 2017) to profile the individual protein abundance and synthesis response to endurance and resistance training. By applying the same unilateral model of endurance and resistance exercise as used in Wilkinson et al., (2008) combined with stable isotope labelling with deuterium oxide to quantify protein-specific abundance and synthesis rates at rest and across the acute response (1 week) and chronic period (week 10) of both endurance and resistance training within each subject. The primary aim of this study was to profile the human muscle protein-specific responses in synthesis rate and abundance changes that underpin the acute and chronic responses to endurance and resistance exercise. The integrated analysis of protein-specific abundance and synthesis rate offers the possibility to resolve novel mechanisms to aid in our understanding of how different training modes result in divergent response of skeletal muscle ultimately leading to distinct differences in muscle phenotype.

5.2 Methods

5.2.1 Experimental Design

Using a within-subject repeated measures design of unilateral exercise training, 14 participants (n = 8 females, n = 6 males) were recruited to participate in this study. Participants were healthy, recreationally active, and between the ages of 18-30. Before participation, participants gave their informed consent to the experimental procedures approved by the Hamilton Integrated Research Ethics Board (HIREB #15317) and conformed to the standards for the use of human subjects in research as outlined by the Canadian Tri-Council Policy (TCPS 2 2022) on the ethical use of human subjects in research and the declaration of Helsinki.

Participants' legs were randomly assigned to resistance (REX) or endurance (END) exercise group. Vastus lateralis biopsies (Bx) were collected at the start (unilateral Bx on day 0) and end (bilateral Bx on day 4) of the baseline period. Participants were told to refrain from moderate-vigorous exercise across the baseline period. Immediately following the end of the baseline period, participants began a 10-week unilateral exercise intervention. Training 3 x per week unilateral lower-limb resistance and endurance exercise. Dynamic proteomic responses to training were quantified over the first 5 days of training (acute study period; bilateral biopsies on day 8) and over the final 5 days of training (chronic study period) with bilateral biopsies collected at the start (day 0) and end (day 4) of week 10. Prior to the final biopsies of the acute and chronic study periods, participants laid supine and bilateral biopsies were collected 3 h following the final exercise bout. Biosynthetic labelling of proteins with D_2O was initiated via 2 days of loading doses (days -2 and -1 of baseline and chronic study period) and 5 days of labelling was conducted throughout the baseline, acute, and chronic study periods via administration of maintenance doses (described below). Throughout D_2O labelling periods, saliva samples were collected daily (Figure 5.1). Anthropological and

physiological testing was complete > 72 hours before the initiation of D_2O labelling in the baseline, free living period and the chronic study period. As well as in replace of a training session in week 5 to adjust exercise prescription if required. During each measurement period participants were provided with a standardised diet (>1.4 $g.kg^{-1}.d^{-1}$ protein).

Figure 5.1 Experimental design and physiological responses across 10-weeks of unilateral resistance and endurance training.

A) Participants (n = 14; 8F/6M) were recruited and legs were randomised to either resistance (REX) or endurance (END) exercise. Once randomised body composition measurements and unilateral exercise tests were completed pre-training. B) Seventy-two hours after exercise testing participants underwent a unilateral vastus lateralis biopsy (day 0) and began D_2O consumption. Participants refrained from exercise until after the bi-lateral biopsy on day 4 (baseline measurements) then performed unilateral REX and End training on days 4, 6, and 8. Three hours after completing the training on day 8, bilateral biopsies were collected (acute response measurements). D_2O consumption was ceased during weeks 2-8 of training. C) Participants then repeated 6 days of D_2O consumption and bilateral biopsies across the final week of exercise in line with the acute measurement period.

5.2.2 Physiological assessments

Body composition and leg lean mass was assessed using whole body dual-energy X-ray absorptiometry (DEXA) scan (QDR-4500A, software version 12.31; Hologic, Bedford, MA). Subjects were fasted (~10 h) and had not completed any exercise during the 48 h before the scan taken pre-training prior to the initiation of the baseline-acute and chronic measurement period (Figure 5.1). The DEXA was calibrated prior to each participants arrival using a three compartment Universal Whole BodyDEXA Phantom (Orthometrix, Naples, Florida). All subjects were centrally aligned prior to the scan. Following DEXA, participants performed 1-repetition maximum unilateral (REX limb only) strength tests on quadriceps press and extension. Participants were instructed on proper lifting technique

and carried out a warm up set of 10 repetitions at ~ 50% 1RM. Participants provided feedback on the difficulty of each set using an adapted Borg Scale (CR-10) (Buckley and Borg, 2011) and the load was progressively increased by ~ 5-20% for each repetition until a true 1RM was achieved, as previously described (McGlory et al., 2014). Three min rest was given between each attempt. A successful attempt required the participant to move the load with the correct form throughout the full range of motion. Participants also competed a unilateral (END limb only) incremental exercise test to exhaustion on a cycle ergometer (Velotron, RacerMate; Seattle, WA) as previously described (Thomas et al., 2022). Heart rate was monitored continuously with a heart rate monitor (Polar A3, Lake Success, NY). The test began with a 2-min warm-up at 50 watts (W), followed by incremental increases in resistance (1 W every 4 s) until volitional exhaustion or the point at which cycling cadence fell below 60 rpm. Maximal workload (W_{max}) was recorded as the highest power output achieved during the last 30 s of the test.

5.2.3 Unilateral training protocol

The training period consisted of participants visiting the lab 3 times per week to complete supervised exercise sessions. On each visit, subjects completed unilateral endurance and resistance training in a randomised order. Resistance exercise consisted of 3 sets × 10-12 reps 80% 1RM leg press followed by 3 sets × 10-12 reps 70-80% 1RM leg extension, with 2 min recovery between sets and exercises. Endurance exercise consisted of 2.5 min warmup at 40% $watt_{max}$ followed by 4 bouts of 5 min cycling at 65% $watt_{max}$ with 2.5 min rest (40% $watt_{max}$) between sets, followed by 2.5 cooldown at 40% $watt_{max}$. Throughout each training session participants were periodically monitored for heart rate (Polar A3, Lake Success, NY) and RPE (Borg CR10).

5.2.4 Stable isotope labelling in vivo

Within this work, a similar experimental design as used in rats in chapter 4, consisting of a 'loading phase' and a 'maintenance phase' of D_2O provision was applied. However, due to the greater body weights of humans orally administered loading doses were given over the course of 2 days. Enrichment of D_2O was then maintained via oral administration of smaller 'maintenance' doses of D_2O. Labelling was initiated on day -2 of each measurement period. D_2O loading (days -2 and -1) consisted of participants consuming 10 doses of 0.66ml.kg body $mass^{-1}$ of 99.8 atom % deuterium oxide (D_2O; Sigma-Aldrich) approximately 1 hour apart each day (equating to D_2O loading of 13.2 ml.kg body $mass^{-1}$). D_2O enrichment was then maintained by consumption of 1 dose of 0.66ml.kg body $mass^{-1}$ D_2O daily during each measurement period. Body water D_2O was measured in saliva samples by cavity ring-down spectroscopy using a Liquid Water Isotope Analyzer with automated injection system (Los Gatos Research, Mountain View, CA). The water phase of the saliva was injected six times, and the average of the last three measurements was used for data analysis (McKendry et al., 2019).

5.2.5 Muscle collection and processing

Muscle biopsies were obtained from the vastus lateralis (VL) under local anaesthesia (1% xylocaine) using the Bergström needle technique (Bergstrom, 1975) that was adapted for manual suction. Muscle tissue was quickly blotted to remove any visible fat and connective tissue before being frozen in liquid nitrogen and then stored at −80° C pending further analysis. Muscle samples from a subset of participants (n = 5, 1M, 4F) processed for dynamic proteomic analysis according to Chapter 2.

5.2.6 Calculation of individual protein fractional synthesis rates

The fractional synthesis rate of each protein was calculated across the baseline (unilateral; BL - Pre) period and the acute (REX/ END limb; day 4 - 8) and chronic (REX/ END limb; day 0 – 4) training periods using equations (1-3) presented in Chapter 2.

5.2.7 Statistical and Bioinformatic Analysis

One-way analysis of variance was applied to investigate the physiological responses to training across the training period. Where possible 2-way ANOVA was used to investigate differences between exercise mode on physiological data (e.g. leg lean mass). Where appropriate significant ($p < 0.05$) differences in physiological data were followed up by Tukey post-hoc analysis.

Prior to statistical analysis, protein abundance (n = 5 participants) and synthesis (n ≥ 3 participants) data were filtered so that only values quantified in all sampling timepoints (BL, Pre-, Acute-, Wk9-, and Post-) and conditions (END and REX). Due to the unilateral BL biopsy, within our analysis of protein abundance data (expressed as femtomole.μg protein^{-1}; fmol/μg) the BL sample was excluded from analysis to enable a complete 2 factor within-subject analysis of the main effects of time (Pre-, Acute-, Wk9-, and Post-), condition (END and REX), and the interaction between the abundance responses to exercise (time x condition).

The labelling periods within our design allow for the quantification of baseline protein fractional synthesis rates (unilaterally). As well as the protein specific FSR response to 1 week (acute) and 10 weeks (chronic) resistance and endurance training. Mixed-protein FSR was assessed by calculating the abundance normalised average FSR from individual proteins (median FSR weighted by abundance) quantified in all time-points in n ≥ 3 participants. Paired t-tests were applied to compare individual synthesis rates after acute and chronic REX and END to baseline turnover rates. Statistical significance was set at $p < 0.05$ and where appropriate assessed for multiple comparisons by calculating false-discovery rates (q-values; (Storey and Tibshirani, 2003)). Dynamic proteomics data (protein-specific abundance and FSR) identified as exhibiting a significant response to training were used for bioinformatic analysis to understand the effect of time (i.e. conserved responses to END and REX) or interactions (i.e. differential response of END and REX over time) on the muscle dynamic proteome response. All supplementary dynamic proteomic data is appended (Appendix 3.1 – 3.2).

5.3 Results

5.3.1 Physiological response to unilateral training

Fourteen (8F/6M) young (20 ± 1 years), healthy recreationally active individuals were recruited and completed a 10-week (3 sessions per week) unilateral endurance (END) and resistance (REX) exercise protocol. Dynamic proteomic analysis of 5 participants from the n = 14 cohort are reported within this analysis. Participants' physiological data are reported in Table 5.1. All participants completed ≥ 90 % of the programmed training sessions. Within the 5 participants, 10 weeks unilateral REX significantly increased quadriceps press 1RM ($p = 9.66e^{-07}$; mean diff = + 65 ± 10 kg) and quadriceps extension 1RM ($p = 4.26e^{-05}$; mean diff = + 16 ± 5 kg) (Figure 2A and B), whereas END significantly ($p = 0.0253$) increased unilateral Wmax (+ 21 ± 17 W) (Figure 5.2C). Quadriceps press/ extension 1RM was not performed in the END trained leg nor Wmax test was not performed in REX trained leg). Overall, 10 weeks of training resulted in a slight, yet significant ($p = 0.045$) decrease (mean diff = -0.76 ± 0.59 kg) in body mass (Figure 5.2D) and significant ($p = 0.0125$) increases in leg lean mass in both the END and REX limb (Figure 5.2E).

Table 5.1 - Participant characteristics and physiological data

Variable	Baseline	Week 5	Post
n = 5 (1M/4F)			
Age	20 ± 2		
Height (cm)	166 ± 7		
Weight (kg)	69.98 ± 21.28	-	69.22 ± 21.13*
Quadriceps press 1RM (kg)	55 ± 32	109 ± 30**	119 ± 32**
Quadriceps Extension 1RM (kg)	21 ± 7	30 ± 7**	37 ± 8***$
Peak power output (Wmax)	125 ± 22	132 ± 23	146 ± 31*
Max Heart Rate (bpm)	168 ± 18	167 ± 12	168 ± 22
Leg Lean Mass (Kg)			
Endurance Limb	7.9 ± 2.0	-	8.2 ± 2.1 #
Resistance Limb	7.9 ± 2.0	-	8.5 ± 2.4 #

Physical and physiological characteristics measured at baseline, after 5 weeks, and after 10-weeks of unilateral exercise. Data presented as mean ± standard deviation. *P < 0.05; **P < 0.005; ***P < 0.0005 vs Pre. $p < 0.05 vs week 5. #main effect of time p < 0.05.

Figure 5.2. Physiological response to unilateral training

A-D) Comparisons of physiological measurements pre-exercise (red), after 5 weeks (yellow), and after 10 weeks of unilateral training (green). Data analysed using 1-way repeated measures ANOVA. E) Comparison of leg lean mass measured via DEXA in the endurance (END) and resistance (REX) trained limb pre-(red) and post-(green) 10 weeks of unilateral exercise. Measurement times labelled with different letters indicate significant ($p < 0.05$) differences in response to unilateral training. # main effect of time $p < 0.05$. Circles represent male participants (n = 1) and triangles represent females participants (n = 4).

5.3.2 Proteomic analysis

Overall, 2883 proteins were confidently identified with ≥1 unique peptides at a false-discovery rate of identification ≤ 1%. After data were filtered to remove missing values amongst biological replicates, the abundance of 2012 proteins were measured in all muscle samples (n = 9) within each individual (n = 5). The dynamic range of the abundance (fmol.μg of protein^{-1}; fmol.μg) of the proteins quantified spanned 6 orders of magnitude from 0.00098 ± 0.00082 fmol.μg (Aldehyde dehydrogenase family 1 member A3) to 3171.0810 ± 3549.6032 (Actin, alpha skeletal muscle) (Figure 5.2A). The top 10 most abundant proteins included type I (MYH7; 784.2223 ± 982.4508 fmol.μg), type IIa (MYH2; 1527.9694 ± 2473.8860 fmol.μg) and type IIx (MYH1; 352.7275 ± 623.9851 fmol.μg) myosin heavy chain (MYH). Analysis of relative abundance of MYH at baseline indicated MYH7, 2, and 1 account for ~99.85 % of MYH proteins (from 10 isoforms quantified in total). MYH7 accounted for 46.85 ± 31.28 % of MYH profile within baseline biopsies across our participants. Whereas MYH2 (IIa) and MYH1 (IIx) accounted for 43.67 ± 25.25 % and 9.33 ± 6.48 %, respectively (Figure 5.2B).

Deuterium oxide consumption resulted in an average body water enrichment of 1.88 ± 0.10 % across the baseline-acute period (days 0 – 8) and 1.85 ± 0.16 % across the chronic measurement period (Figure 2C-D). Dynamic proteome profiling quantified the fractional synthesis rate (FSR; %/d) of 1465 proteins (in ≥ 1 participant). Protein FSR data was accepted for statistical analysis if quantified in ≥ 60% of participants (n = 3-5) across the baseline and first week of training (acute response) or across the baseline and final week of training (chronic response). Analysis of 1173 muscle protein turnover rates at baseline indicated our individual protein FSR data spanned from 0.1526 %/d (Ferrochelatase, mitochondrial) to 63.5479 %/d (Thrombospondin-4), median FSR of individual muscle proteins was 3.0462 %/d (Q1 = 1.6524, Q3 = 5.3136) (Figure 5.2E).

Figure 5.3. Dynamic proteome profiling of human skeletal muscle.

A) Distribution plot of abundance data (log_{10}) from 2012 proteins successfully quantified in human vastus lateralis at baseline by bottom-up proteomics using liquid-chromatography tandem mass-spectrometry (LC-MS/MS) and quantified by label free quantification. Values represent 2012 protein abundances quantified across all time points in all muscle samples (n = 5 participants). B) Stacked bar plot of the relative proportion (%) of the typical muscle specific myosin heavy chain isoforms. Furthest left bar ("mean") represents the average MYH distribution across all 5 participants at baseline. C) Precursor enrichment of deuterium measured by cavity ring-down spectroscopy in the body water compartment collected via daily saliva sampling across the baseline (0 – 4 d), acute (4 – 8 d) experimental period and the (D) chronic experimental period (0 – 4 d). E) Distribution plot of fractional synthesis rates (log_{10} %/d) of 1173 proteins FSR successfully quantified in n = 3-5 human vastus lateralis at baseline by dynamic proteome profiling.

5.3.3 Proteome adaptation common to endurance and resistance training

Two-way analysis of protein abundance data over time (pre, acute, 9-wks, and chronic) in both the endurance (END) and resistance (REX) trained limb identified 284 proteins that significantly changed in abundance irrespective of exercise mode (main effect of time; $p < 0.05$, q value < 0.13). The conserved response to both REX and END exhibited no significant enrichment of gene ontology (GO) terms or KEGG pathways. Soft-clustering analysis of the significant main effects of time across pre, acute, and chronic training separated the conserved training responses into 3 distinct clusters (minimum membership value = 0.3) (Figure 5.3A-D).

Cluster 1 consisted of 132 proteins and was characterised by an increase in abundance after 1-week of training followed by decreased abundance/return to baseline in chronically trained muscle (Figure 3B). GO analysis indicated no significant enrichment in GO terms/ pathways. STRING analysis revealed a strong network (confidence = 0.9) of 10 proteins associated with cytoplasmic translation (Figure 5.3E). This network included 4 eukaryotic initiation factor 4 (eIF4) subunits (-B, -H, -A2, and -G1) (Figure 5.3F). Alongside 3 proteins associated with the 40S component of the ribosome (RS21, RSSA, RACK1) and eIF3B, 2A, and 4. Cluster 1 also included 21 proteins associated with proteolysis which exhibited patterns of decreased abundance in response to chronic training. Including the E3 ubiquitin ligases; E3A, RNF114, and Cullin-5, and the deubiquitinase Ubiquitin thioesterase OTUB1 (Figure 5.3G) and proteasome alpha core complex subunits (PSA1, PSA5, and PSA7) (Figure 5.3H).

A
Min. centroid distance
B
Cluster 1
Expression Change
Pre
AC
CR
Membership value
C
Cluster 2
D
Cluster 3
E
RL14
RSSA
RACK1
RS21
EIF2S1
EIF4G1
EIF4B
EIF3B
EIF4A2
EIF4H
EIF5
RNF114
OTUB1
E3A
CUL5
PSMA1
PSMA7
PSMA5
F
eIF4
IF4A2
IF4B
IF4G1
IF4H
Abundance (fmol.ug)
Pre AC CR Pre AC CR
END
REX
G
E3 Ligases
CUL5
OTUB1
UBE3A
RNF114
H
Proteasome alpha subunits
PSA1
PSA5
PSA7

Figure 5.4. Proteome adaptation common to endurance and resistance training exhibit acute increases in protein abundances associated with protein turnover.

A-D) Temporal profiles (log_2 transformed acute/baseline and chronic/ baseline fold-difference) of proteins that exhibited significant main effects of time in abundance analysed by soft clustering using the fuzzy c-means algorithm (Mfuzz). The optimal number of clusters applied was decided through inspection of the elbow plot (top left panel) as well as qualitative assessment of temporal clusters. The minimum membership value for inclusion into a cluster was set at 0.4. E) STRING protein interaction network for proteins displaying significant ($p < 0.05$) main effects of time in abundance data across the time-course belonging to cluster 1. Coloured nodes represent proteins in cluster 1 annotated to the biological function "Cytosolic translation" (Green and Blue) and "Proteolysis" (Orange and Red). F-H) Boxplots displaying the protein-specific abundance data (fmol/ per microgram of protein) of proteins extracted from cluster 1 across pre-exercise, 1-week of training (Acute; AC), and 10-weeks of training (Chronic; CR) of unilateral exercise. Panels represent proteins associated with the eIF4F complex (F), E3 Ubiquitin-ligases (G), and Proteasome subunits (H).

Cluster 2 encompassed 96 proteins that exhibited an increased abundance after chronic training irrespective of training mode (Figure 5.4C). Cluster 2 was significantly enriched (FDR = 0.0034) for the GO cellular component 'mitochondrion' (48 proteins). Twenty-eight proteins (Figure 4A) were annotated to the cellular component 'mitochondrial inner membrane' (FDR = 0.00028). Including 4 mitochondrial respiratory complex 1 accessory subunits (NDU-A5,-A8, -B4, and ACPM), the complex 1 assembly factor (ACAD9) (Figure 5.5B) and 3 subunits comprising respiratory complex III (QCR1, QCR2, and QCR6). The mitochondrial regulatory proteins: Dynamin-like 120 kDa protein (OPA1), the m-AAA-protease interacting protein (MAIP1), and AFG3-like protein 2 (AFG32) as well as 3 proteins associated with mitochondrial organisation and cristae formation (MIC26, GHITM, and CHC10) each increased in abundance after 10 weeks of training in both END and REX muscle.

In contrast, proteins grouped into cluster 3 (54 proteins) exhibited consistent decreases in abundance in both REX and END muscle (Figure 5.4D). Network analysis indicated no significant GO/ pathway enrichment, however interaction networks with high confidence (0.7) were identified between 4 proteins associated with proteasomal degradation including the 26S regulatory subunits 6A (PRS6A) and 10B (PRS10) alongside the multiubiquitin chain receptor RD23B (UV excision repair protein RAD23 homolog B). The E1 enzyme (UBA1) and Lys^{48}-ubiquitin chain cleaving protein, UBP5, were also included in cluster 2. The small ribosomal precursor (RS5) and the RNA binding component of eIF3 (EIF3A) and the eIF2 subunit (IF2G) were included in cluster 2. Indicating different components of translation initiation and the ubiquitin proteasome system were decreased in both REX and END training after either 1 week (cluster 2) or 10 weeks of training (cluster 1).

Figure 5.5. Both endurance and resistance exercise increase the abundance of mitochondrial accessory subunits

A) STRING protein interaction network for proteins displaying significant ($p < 0.05$) main effects of time in abundance across the time-course that belonged to cluster 2 and were annotated to the GO cellular component "Mitochondrion". Nodes with a blue border indicate proteins annotated to the cellular component "Inner mitochondrial membrane". Coloured nodes represent proteins associated with respiratory complex I (Green) and III (Orange) B) Boxplots displaying protein-specific abundance data (fmol/ per microgram of protein) of proteins extracted from the network in panel A across pre-exercise, 1-week of training (Acute; AC), and 10-weeks of training (Chronic; CR) of unilateral exercise. Panels represent Complex I accessory (non-catalytic) subunits.

5.3.4 Training mode-specific regulation of protein abundance

In total, 123 protein abundances exhibited a statistical ($p < 0.05$) interaction between time and training mode. GO analysis of all significant interactions indicated significant enrichment of proteins annotated to the cellular component 'mitochondrion' (73 proteins, fdr = $4.53e^{-13}$). More specifically interactions were significantly enriched for 34 proteins annotated to the KEGG pathway 'oxidative phosphorylation' (fdr = $2.09e^{-14}$) and 20 of those proteins annotated as 'Mitochondrial respiratory chain complex 1' (fdr = $6.05e^{-09}$). Soft-clustering analysis of the time-course of the interaction between training modes (log_2 END/REX) revealed 3 differential responses between REX and END (minimum membership value = 0.3) (Figure 5.6A-D). Cluster 1 (Figure 5.6B) included 21 proteins grouped by a decrease in abundance in END (Figure 5.6E) and increased in REX trained muscle after 1-week of training (Figure 5.6F) whereby the differences is conserved into the chronic period of training (Figure 5.6H). Despite the relatively small number of proteins, the cluster network was enriched for proteins annotated to the Uniprot key words 'Cell membrane' (10 proteins, fdr = 0.0353) and 'Inflammatory response' (4 proteins, fdr = 0.0408) (Figure 5.6E-K). The inflammatory response proteins included the calcium- and zinc-binding proteins S100-A8 and -A9 which were 74 % and 87 % more abundant in REX trained muscle after 1-week of training. Monocyte differentiation

antigen CD14 is associated with the inflammatory response and was 122 % and 82 % more abundant in REX trained muscle compared to END after 1-week and 10-weeks of training. Also, the scavenger receptor cysteine-rich protein M130 (a.k.a CD163) exhibited the greatest increase in abundance in REX (135 % and 461 %) compared to END after 1-week and 10-weeks of training, respectively. Signal transducer and activator of transcription 3 (STAT3) exhibited a 30 % greater abundance in REX muscle after 1-week of training but then returned to similar levels in END and REX after the chronic training period. The immunoglobulin E binding protein, Gelectin-3 (LEG3) was included in the inflammatory network and exhibited a similar pattern of response as STAT3. The ATP-dependant RNA helicase**,** Eukaryotic initiation factor 4A-I (IF4A1), exhibited a significant interaction (time x condition; $p = 0.04$, $q = 0.73$) and was 23 % more abundant after 1-week of resistance training compared to END and then returned to similar abundance between the training modes after chronic training.

Significant interactions in protein abundances grouped in clusters 2 and 3 include proteins that were greater in abundance after 1-week (cluster 3) or 10-weeks (cluster 2) of endurance training (Figure 5E-K).

Cluster 3 contained 58 proteins that uniquely increased in abundance in END muscle after 1-week of training. GO/ KEGG pathway analysis found significant enrichment of the cellular component 'mitochondrial envelope' (25 proteins, fdr = $1.40e^{-05}$) and the KEGG pathway 'Oxidative phosphorylation' (10 proteins, fdr = 0.031). Proteins annotated to the oxidative phosphorylation pathway (OXPHOS) formed a high confidence network (confidence = 0.7) (Figure 5.6G) consisting of mitochondrially encoded Complex I core subunit (MT-ND6) and the Complex I alpha subcomplex accessory subunits (NDUA3, NDUA7, and NDUAD). The OXPHOS network in cluster 3 also included cytochrome c oxidase (COX; Complex IV) subunits 5A, 7A2, the assembly chaperone, COX17, and ATP synthase subunits ATP8, ATPO, and ATP5L. Cluster 3 contained 13 proteins associated with chaperone-function and proteome quality control. Including 3 small heat shock proteins (HSPB1, 2, and 3) and mitochondrial stress-70 protein (GRP75). The mitochondrial co-chaperone TIM14 and assembly factors TMM70 and LYRM7 were also amongst the proteins that exhibited differences during acute adaptive period between END and REX muscle.

A
Cluster number
B
Cluster 1
Pre
AC
CR
Time
C
Cluster 2
D
Cluster 3
Membership value
E
Cluster 1
F
Cluster 2
G
Cluster 3
H
1-wk END
Cluster 1
Cluster 2
Cluster 3
I
1-wk REX
J
10-wk END
K
10-wk REX
P Value (−Log10)
END Abundance Fold Change (Log2 AC/Pre)
REX Abundance Fold Change (Log2 AC/Pre)
END Abundance Fold Change (Log2 CR/Pre)
REX Abundance Fold Change (Log2 CR/Pre)

Figure 5.6. Unique proteomic adaptations in response to acute and chronic endurance and resistance training.

A-D) Temporal profiles (log_2 transformed AC/Pre and CR/Pre fold-difference) of proteins exhibiting significant ($p < 0.05$) interactions (time x condition) in abundance underwent soft clustering analysis using the fuzzy c-means clustering algorithm with the Mfuzz R package. The optimal number of clusters applied was decided through inspection of the elbow plot (top left panel) as well as qualitative assessment of temporal clusters. The minimum membership value for inclusion into a cluster was set at 0.4. E - G) STRING protein interaction network for proteins displaying significant ($p < 0.05$) main effects of time in abundance data across the time-course belonging to cluster 1 (E), 2 (F), and 3 (G). Coloured nodes represent proteins in associated with muscle inflammatory responses (Yellow) and mitochondrial respiratory Complex I (Light blue), II (Orange), IV (Green), and ATP synthase (Dark blue) as well as heat-shock proteins (Red). H – K) Volcano plots of fold change (log2 AC/Pre and CR/Pre) in response to 1-week (H – I) and 10-weeks (J – K) of endurance (Left panels) and resistance (Right panels) training. Plotted against -log10 p value of the interaction. Coloured points represent proteins comprising cluster 1 (Blue), 2 (Red), and 3 (Yellow).

Cluster 2 describes significant interactions (time x condition) which were significantly increased in abundance in endurance trained muscle compared to resistance trained after chronic-training (Figure 5.6G-H). The 42 proteins comprising cluster 2 were significantly enriched for the biological process 'Cellular respiration' (27 proteins, fdr = $1.24e^{-17}$). The network of proteins associated with cellular respiration forms a strong (confidence = 0.9) highly inter-connected network (198 edges) (Figure. The network includes 16 subunits of respiratory complex I (NADH dehydrogenase), the 2 core subunits of complex II (Succinate dehydrogenase), 2 subunits of complex IV (Cytochrome c oxidase) and 3 complex V subunits (ATP synthase). Also citrate synthase was included in cluster 2 and exhibited a greater abundance ($p = 0.04$, $q = 0.60$) in endurance (24 %) compared to resistance trained muscle after chronic training. Overall, clusters 2 and 3 highlight interactions in mitochondrial and OXPHOS abundances as key differentiators between the END and REX response to acute and chronic training.

5.3.5 Training-induced changes in protein turnover rates

Calculation of mixed-protein FSR was derived from 306 individual protein FSRs (measured on n = 3-5 individuals) which incorporated the synthesis rates of the top 90 % of the most abundant proteins within our analysis. No significant differences in mixed-protein FSR ($p > 0.05$) were observed across END and REX conditions. However, on average mixed-protein FSR was ~34 % greater after 1-week of REX (2.12 ± 0.34 %/d) compared to baseline (1.58 ± 0.19 %/d). Ten-weeks of training resulted in an ~15 % increase in mixed-protein FSR in both REX (1.84 ± 0.41) and END (1.82 ± 0.19) trained muscle compared to baseline (Figure 5.7A)

Paired t-tests were used to compare individual protein synthesis rates at baseline to the acute and chronic response to REX and END. From a total of 399 proteins measured across the acute response period, 25 and 27 proteins exhibited significant ($p < 0.05$, $q < 0.60$) changes in synthesis rate in response to REX (18 up, 7 down) and END (14 up, 13 down), respectively (Figure 5.7B-C). Only 4 (out of 48) proteins (APOA1, COQ8A, HPT, VTDB) were commonly regulated (decreased) in synthesis rate between END and REX during the acute response to exercise (Figure 5.7D).

Proteins that were significantly increased in synthesis rate were unique to END and REX exercise (Figure 5.7D). Twenty-two of 25 proteins significantly changed in FSR after 1-week of REX but did not significantly change in

abundance during this experimental period, i.e. training primarily altered the turnover rate of these proteins. STRING analysis indicated no significant enrichment of GO terms/ KEGG pathways amongst proteins that increased in FSR in response to 1-week of REX but several including, VINC, CAC1S, TITIN, ANKR2, XIRP1 and CRYAB, were associated with the myofibrillar apparatus (Figure 5.7C). Musculoskeletal embryonic nuclear protein 1 (MUSTN1) exhibited the greatest increase ($p = 0.009$, $q = 0.55$) in FSR after 1-week of REX. Also, turnover of the transcriptional cofactor, LIM and cysteine-rich domains protein 1 (LMCD1) was log2 FC = 1.91 greater ($p = 0.022$, $q = 0.55$) after acute REX. Superoxide dismutase (copper-zinc isoform; SODC) significantly ($p = 0.03$, $q = 0.55$) increased in turnover rate (log2 FC = 0.74) in response to acute REX. Four metabolic enzymes significantly increased in turnover rate after 1-week of REX, including lactate dehydrogenase A (log2 FC = 1.49), the mitochondrial isoform of malate dehydrogenase (log2 FC = 0.44), and 2 enzymes (ECI2 and ECHB) associated with acetyl-CoA metabolism (log2 FC = 1.72 and 0.80, respectively). Also, the turnover rate of elongation factor 1D, was significantly ($p = 0.036$, $q = 0.55$) greater after 1-week REX compared to baseline. Also, the autophagy associated Toll-interacting protein (TOLIP) significantly ($p = 0.035$, $q = 0.55$) decreased in turnover rate uniquely in response to 1-week of REX.

In contrast to REX, the majority (13/27) of proteins significantly ($p < 0.05$) decreased in FSR after 1-week of END (Figure 5.7B). Network analysis revealed a high confidence network (confidence ≥ 0.7) of 8 proteins enriched (fdr = 0.00063) for the UniProt annotated keyword “signal” which relates to a domain sequence common to proteins destined for secretion or incorporation into membranes. These proteins were also enriched for the term “Glycoprotein’ (fdr = 0.0056). Network analysis of the proteins that increased in FSR in response to 1-week of END revealed no statistically significant enrichment, but the network included 7 proteins associated with the carbohydrate metabolic process. Five out of these 7 proteins formed a high confidence network (confidence ≥ 0.7) consisting of the glycolytic enzymes; Fructose-bisphosphate aldolase A (ALDOA), ATP-dependant 6-phosphofructose kinase, and beta-enolase. As well as the glycogen regulating enzymes phosphoglucomutase 1 and UTP--glucose-1-phosphate uridylyltransferase. The rapamycin binding protein (FKBP3) significantly ($p = 0.039$, $q = 0.526$) increased in turnover rate after 1-week of END compared to baseline. Also, the non-ATPase regulatory proteasome subunit (PSD13) significantly ($p = 0.029$, $q = 0.526$) increased in turnover rate (log2 FC = 0.327) after 1-week of END training.

Figure 5.7. Changes in protein fractional synthesis rate in response to 1-week of training

A-D) Mixed-protein fractional synthesis rates (% / d) was derived from 306 individual protein FSRs (measured in n = 3-5 individuals) which incorporated the synthesis rates of 9/10 of the most abundant proteins within our analysis alongside numerous highly abundant muscle proteins. No significant changes in mixed-protein FSR ($p > 0.05$) were observed across all conditions. On average mixed-muscle FSR was ~34 % greater after 1-week of REX compared to baseline. Ten-weeks of training resulted in an ~ 15% increase in mixed-muscle FSR in both REX and END trained muscle compared to baseline. B - C) Volcano plots of fold change (log2 AC/BL) in FSR to 1-week of endurance (B) and resistance (C) training plotted against -log10 p value. Coloured points represent proteins identified as significantly different compared to baseline. Significant differences are labelled with their respective uniport identifier. Data points represent fold change of the mean value in AC and BL (n = 3-5 individuals). D) Venn diagram of the overlap between protein-specific FSR increased (Left) and decreased (Right) between END and REX after 1-week of training.

In total 596 protein-specific FSR were measured across the chronic response period (Figure 5.7A-C). Thirty-one proteins significantly altered in their synthesis rate in response to REX (24 up, 7 down) and END (25 up, 6 down), respectively. Only 5 proteins were commonly regulated ($p < 0.05$) between the chronic REX and END response (Figure 5.8C). Four proteins significantly increased after chronic training in both REX and END (CRYAB, PSB3, RAB1A, and YBOX1). Proteasome subunit beta-3 (PSB3) exhibited the largest significant ($p < 0.05$) increase in turnover rate in both chronically trained END (log2 FC = 2.71) and REX (log2 FC = 2.50). Only basigin (BSG a.k.a CD147) commonly decreased ($p < 0.05$) in FSR in response to chronic REX (log2 FC = -0.35) and END (log2 FC = -0.56) (Figure 5.8A-B).

No clear networks could be drawn from proteins significantly increasing/ decreasing in FSR in response to chronic END or REX. However, numerous proteins associated with post-transcriptional regulation were amongst those significantly regulated in response to REX and END training. The mitochondrial elongation factor Tu and complement component 1 Q subcomponent-binding protein, are each implicated in the regulation of mitochondrial protein synthesis and exhibited significant decreases in turnover rate in response to 10-weeks of endurance training. The pre-mRNA processing enzyme Heterogeneous nuclear ribonucleoprotein H significantly increased in FSR after chronic END. Eukaryotic translation initiation factors (eIFs) seemed to exhibit subunit and training mode specific regulation in FSR after chronic exercise. Ten-weeks of END resulted a significant ($p = 0.0077$, $q = 0.66$) increase (log2 FC = 0.68) in turnover rate of eukaryotic translation initiation factor 5A-1 (IF5A1). Whereas chronic REX resulted in a significant ($p = 0.025$, $q = 0.64$) increase (log2 FC = 1.21) in turnover rate of Eukaryotic translation initiation factor 5 (IF5). Also, eIF4G1 synthesis increased in response to chronic END whereas eIF4B synthesis decreased in response to chronic REX. The synthesis of eIF4H tended to decrease in response to both chronic REX and END, however REX also exhibited trends for increases in the FSR of IF4A1 and IF2S1.

Overall, 5 proteins that significantly increased in FSR in response to REX were consistent between the acute and chronic training response (CRYAB, HEMO, LDHA, TITIN, and XIRP1) and no proteins exhibited a consistent downregulation in FSR across both acute and chronic REX. Similarly, only ALDOA was common amongst proteins that were significantly regulated in response to acute and chronic END. Therefore, of 105 protein-specific synthesis rates significantly regulated in response to training (i.e. acute or chronic and REX or END) only ~5.7 % (6/105) of proteins were commonly regulated in the acute and chronic training response to either training mode.

Figure 5.8. Changes in protein fractional synthesis rate in response to 10-weeks of training.

A - B) Volcano plots of fold change (log2 CR/BL) in FSR after 10-week of endurance (A) and resistance (B) training plotted against -log10 p value. Coloured points represent proteins identified as significantly different compared to baseline (p <0.05). Significant differences are labelled with their respective uniport identifier. Data points represent fold change of the mean value in CR and BL (n = 3-5 individuals). C) Venn diagram of the overlap between protein-specific FSR increased (Left) and decreased (Right) between END and REX after 10-weeks of training.

Inspection of the relationship between the change between individual protein fractional synthesis rates (log2 fold change) between the different training modes (i.e. END versus REX) indicated moderate positive linear relationships between changes in protein FSR after 1-week (r = 0.56, 95 % CI = 0.490 –0.62) and 10-weeks (r = 0.58, 95% CI = 0.52 – 0.63) of training, respectively (Figure 5.9A and B). Generally, this linear relationship was maintained between the change in FSR of proteins related to mitochondrial respiratory complex I, complex V, eIF proteins, and proteasome subunits (Figure 5.9A and B). However, no relationship and -0.672) was observed between changes in the relative abundance of proteins between END and REX after 1-week (r = 0.19, 95% CI = 0.10 – 0.29) and 10-weeks (r = -0.07, 95% CI = - 0.15 – 0.01) of training, respectively (Figure 5.9C and D).

Figure 5.9. Dynamic proteome response of muscle proteins to training.

Correlational analysis between changes in protein-specific synthesis rate (A and B) and abundance (C and D) between endurance and resistance training after 1-week (A and C) and 10-weeks (B and D) of training. Red and black dots represent mitochondrial respiratory complex I and V, respectively, which were key abundance networks differentially regulated between END and REX. Blue and green dots represent eIF proteins and proteosome subunits which were previously identified to be typically conserved in their abundance response between END and REX. Data points represent mean $\log_2$ fold-difference (Post/Pre) in abundance (n = 5) and FSR (n = 3-5).

5.4 Discussion

The benefits of resistance and endurance training on skeletal muscle health are acknowledged but, currently, little is known about the shared vs unique muscle proteome responses to these training modes. Our proteome-wide analysis highlighted chronic increases in the abundance of mitochondrial accessory subunits alongside an acute increase in abundance of proteins associated with the regulation of protein synthesis (e.g. eukaryotic elongation initiation factors and ribosomal subunits) and degradation (e.g. proteasome subunits and enzymes associated with the ubiquitin proteasome system) characterised the conserved adaptive response of muscle to each training mode. However, the integration of individual abundance and synthesis rate changes implicate protein degradation as a key feature differentiating proteome remodelling between endurance and resistance trained muscle (Figure 5.9).

Networks of proteins associated with mitochondrial respiratory complex I and V segregated the proteome responses to chronic endurance and resistance training. There was a moderate, positive relationship between changes in protein-specific FSR in END and REX trained muscle (Figure 5.9) but no relationship in abundance changes between the training modes (Figure 5.9). Therefore protein-specific degradation may represent a key difference in proteome remodelling between endurance and resistance trained muscle. The ubiquitin-proteasome system is the most well described selective degradation pathway in skeletal muscle. However, no clear differences in the abundance of E3 ligases that may explain differences in protein degradation were identified. From a total of 6 E3 ligases identified in our analysis (CHIP, RN123, RN114, HUWE1, UBE3A, and ARI1), only RN114 and UBE3A significantly changed in abundance, which were similar between END and REX trained muscle (Figure 5.4). Parker et al., (2020) proposed that the ubiquitin-like modification, NEDDylation, was a regulator of endurance-training induced changes in UPS-mediated protein degradation. This work detected no training mode-specific differences in the abundance of NEDD8 or subunits of the Cullin-E3 ligase (5/6 subunits quantified). Moreover, the decrease in proteasome subunits detected after 10-weeks of either training mode (Figure 5.4), aligns with a recent cross-sectional analysis of the muscle proteome of individuals that chronically (>15 y) trained in either resistance or endurance exercise (Emanuelsson et al., 2024), which also found a lesser abundance of UPS components compared to untrained individuals. Using dynamic proteomics, a robust increase (> 5-fold) was identified in the turnover rate of the beta-3 core subunit of the 20S proteasome after 10-weeks of either endurance or resistance training (Figure 5.8). PSB3 is a non-catalytic core subunit that has a structural role in proteasome assembly (Tanaka, 2009). Also a greater turnover of PSB3 in the muscle of individuals with obesity compared to lean healthy controls was identified in Chapter 3. This commonality in UPS response between both END and REX (and across two separate non-targeted studies) may highlight a key change in protein quality control mechanisms. The proteasome is tightly regulated through post-translational modification, in particular phosphorylation via cAMP/Protein kinase A pathway, which is relevant in exercise-induced activation of the proteasome (Lokireddy et al., 2015). This work quantified the abundance of 3 of the 4 subunits of cAMP and detected no differences between the training modes. Future phosphoproteomic analysis may be useful but PSB3, in particular, is more commonly modified by ubiquitination than phosphorylation and di-glycine remnant profiling may also be necessary to decipher differences in proteasome activity.

Our dynamic proteomic profiling revealed a significant increase in the turnover rate of eIF5A specifically in endurance trained muscle after 10-weeks of training (Figure 5.8). eIF5A1 is the only known protein in humans to undergo hypusination of Lys^{50} as a result of modification by deoxyhypusine synthase and deoxyhypusine hydroxylase (Park and Wolff, 2018), which is critical for eIF5A function (Cano et al., 2008). Increased abundance (~ 70-fold) and hypusination of eIF5A has been previously identified in skeletal muscle after 10 days of bed rest (Murgia et al., 2022). However, none of the 7 peptide sequences quantified in our MS/MS data covered the Lys^{50} residue. Hypusination of eIF5A has been shown to promote the maintenance of TCA cycle and oxidative phosphorylation (OXPHOS) in macrophage populations (Puleston et al., 2019), aligning well with our finding of

greater abundances of 35 OXPHOS subunits and enzymes comprising the TCA cycle (e.g. citrate synthase) specifically in endurance trained muscle (Figure 5.6). Knockdown of eIF5A results in downregulation of mitochondrial biogenesis and metabolic switching from oxidative to glycolytic metabolism (Pereira et al., 2021). Furthermore, eIF5A1 is essential for the translation of transcription factor EB (TFEB) (Zhang et al., 2019). TFEB may regulate mitochondrial biogenesis independent of PGC1a following endurance exercise (Mansueto et al., 2017), which implicates the eIF5A-hypusine axis as a potentially unique post-transcriptional mechanism underpinning endurance training specific adaptation of human skeletal muscle.

TFEB is often considered the master transcription factor of autophagy, with eIF5A also required for the translation of ATG3, involved in the lipidation of LC3B and autophagosome formation (Barba-Aliaga and Alepuz, 2022). Therefore, the endurance training specific adaptation may be regulated via autophagic pathways. It is well known that exercise training results in the stimulation of autophagy (Halling and Pilegaard, 2017) and some data suggest autophagy may be required for endurance training adaptation (Lira et al., 2013, Ju et al., 2016). However, how autophagy may regulate the specificity of proteome remodelling is unclear. The targeting of substrates for autophagosome engulfment is regulated via different receptor proteins (Stolz et al., 2014). Within our data the autophagy receptor, TOLIP, exhibited a significant ~ 50 % decrease in turnover rate (FSR decreased with no change in abundance) after 1-week RE specifically (Figure 5.7). Conversely, the abundance of optineurin (OPTN) was specifically up regulated after 10-weeks of END (Figure 5.6). Both TOLIP and OPTN have been reported to exhibit clear differences in selective autophagy in vitro (Zellner et al., 2021) and OPTN is required for successful autophagosome assembly around ubiquitinated mitochondria (Wong and Holzbaur, 2015). Significant decreased abundance of the mitochondrial Tu elongation factor, which has a non-canonical role in the regulation of mitophagy involving PINK1 dependent phosphorylation at serine^{222} (Lin et al., 2020) was also identified. Currently it is unclear if these changes in autophagy may result in modulation of protein abundance or greater turnover of mitochondria as an adaptive feature to maintain mitochondrial health. However, this data does implicate changes in protein-specific degradation via autophagy/ mitophagy as potential candidates for endurance training induced mitochondrial adaptation.

Increases in protein synthesis after resistance training have been previously associated with enhanced translation initiation via phosphorylation of eIF4E-binding protein 1 and subsequent binding to the 5' end of mRNA to form the active eIF4F complex (Saxton and Sabatini, 2017). Also, the phosphorylation of ribosomal-S6 kinase leads to the phosphorylation of the 40S ribosomal S6 subunit (RS6) and eIF4B (Marabita et al., 2016), which activates protein synthesis. Unfortunately, eIF4E/ 4E- BP1 were not included in our current proteome profiling and the phosphorylation status of proteins was not studied. However, the FSR of eIF4B tended ($p = 0.08$) to increase after 1-week of REX only ($p = 0.50$ after 1-week of END). Furthermore, eIF4B was significantly decreased in synthesis rate after 10-weeks of resistance training. Indicating potential time-dependant changes in eIF4B turnover specific to resistance training. The activation of protein synthesis via eIF4F occurs in response to phosphorylation of eIF4B^{Ser422} by p70S6K (Raught et al., 2004) greater activation of p70S6K is a well described response to resistance training-type stimulus in animal models (Baar and Esser, 1999). However, no change was identified in eIF4B phosphorylation either immediately or 6 h after lower body resistance exercise in human muscle (Witard et al., 2009). Within this work (Witard et al., 2009) phosphorylation of S6K1^{Thr389} was also not identified, indicating that most likely eIF4B phosphorylation was missed in the time course analysis. Greater activation of eIF4F (measured via 4E-BP1 phosphorylation) aligns with the enhanced myofibrillar protein synthesis specifically in response to resistance training (Wilkinson et al., 2008). Furthermore, ribosomal profiling in model organisms have indicated eIF4B is required for the maintenance of translational efficiency of longer mRNAs (Sen et al., 2016). Therefore, REX-induced changes in eIF4B synthesis combined with the significant increase in synthesis rate of 6 proteins associated with the myofibrillar apparatus (Figure 5.6C) may suggest a role for changes in eIF4B dynamics in

regulating myofibrillar protein translation. Potentially illustrating the complexity of cellular translational pathways and the need to investigate the role of changes in post-transcriptional mechanisms affecting protein specific synthesis rates. The combination of dynamic proteome profiling combined with gain- and loss-of-function models targeted towards specific components of the translation initiation pathways in muscle cells may offer a necessary next step to understand how training results in unique differences in protein translation.

Currently there is limited data on which components of exercise-induced muscle remodelling underpin the shared health benefits associated with both endurance and resistance training. Previously, restrictions imposed by the necessary measurement of bulk/ mixed-protein FSR led to a discourse that segregated endurance and resistance training responses to increases in either mitochondrial or myofibrillar fractions, respectively(Wilkinson et al., 2008). However, these methods crucially do not provide details on protein-specific turnover and in particular mask the responses of lower abundance proteins, whose turnover rate is less likely to affect the average mixed-protein FSR measurement. The moderate correlation in protein-specific synthesis response (Figure 5.9) suggests both training modes resulted in a pattern of greater turnover of 276 proteins. More specifically, 57 proteins associated with the KEGG term 'metabolic pathways', including enzymes comprising TCA cycle (5 proteins) and fatty-acid oxidation (6 proteins) had greater FSR after 10-weeks of END and REX (i.e. Figure 5.9B top right quadrant). These patterns of increased turnover of proteins associated with metabolic pathways may represent a key feature of the exercise induced improvements in skeletal muscle health. Therapeutic targets to improve proteostasis are highly sought-after in mechanistic investigations of declines in cellular function, such as ageing (Taylor and Dillin, 2011) whereby increased cellular stress and protein damage may result in impaired protein degradation and increases in cytotoxic protein aggregates (Balch et al., 2008). Dynamic proteomic analysis resolved protein-specific increases in turnover (i.e. increased synthesis rate independent of changes in abundance) induced by both training modes. Therefore, exercise induced increases in protein turnover rates may represent a critical benefit of exercise on skeletal muscle in the context of improving intracellular proteostasis. Ultimately, these improvements in proteome quality likely contribute to the improvements in tissue function and thus overall health benefits that are associated with both endurance and resistance training, previously unresolved by traditional static measurements. However, a more work is required to distinguish the functional relevance of enhanced turnover rates on protein functioning.

Forty-eight mitochondrial proteins (main effect cluster 2) were commonly increased in abundance after both training modes (Figure 5.4). Network analysis revealed proteins associated with mitochondrial quality including the mitochondrial Complex I assembly factor (ACAD9) and 4 Complex I accessory subunits. Accessory subunits are often considered as 'supernumerary' subunits that are not essential for Complex I function (Sharma et al., 2009). However, their role in regulating Complex I structure, and function is not yet known. Like most details on individual OXPHOS subunits, there is little-to-no data on the role of ACAD9 in muscle and in response to exercise. However, ACAD9 has been identified in the Complex I intermediate assembly complex which is required for assembly of functional Complex I units (Formosa et al., 2020). Knockdown of ACAD9 in HEK293 cells resulted in decreased Complex I activity and abundance without effecting the abundance of other OXPHOS complexes (Nouws et al., 2010). Indicating ACAD9 may be an essential Complex I assembly factor that is increased after both REX and END. Suggesting qualitative improvements in mitochondria common to both training modes. This qualitative improvement in the mitochondrial proteome aligns with previous investigations into the short-term (14 d) response to endurance training (Egan et al., 2013), highlighting potential changes in individual proteins comprising the mitochondrial proteome prior to bulk increases in mitochondrial content/ mitochondrial biogenesis. Therefore, the shared protective properties of both REX and END training on musculoskeletal health and function may be underpinned by selective remodelling of proteins associated with mitochondrial quality, rather than mitochondrial biogenesis per se.

An essential difference in the regulation of Complex I protein abundance within our data was the ability of END

training to result in robust increases in the abundance numerous Complex I subunits (20 out of 45 known) including 4 Complex I core subunits. The core catalytic subunits of Complex I contain sequence motifs required for the binding of the redox centres of Complex I and are highly conserved across species (Friedrich and Weiss, 1997). Indicating an essential role in the maintenance of Complex I function as it is assumed that core subunits alone are sufficient for energy transduction (Sharma et al., 2009). Also 16 accessory subunits were uniquely regulated in response to endurance training. Thus, our data suggests potential remodelling of Complex 1 structure in response to END only. Furthermore, endurance training specifically increased the abundance of the 2 succinate dehydrogenase catalytic subunits (SDHA and B). Alongside increases in the abundance of the ATP synthase subunits ATP8 and ATPMK as well as 2 subunits (ATP-F1 and -F2) that are required for the formation of the F1 portion (catalytic) of the ATP synthase complex (Pícková et al., 2005). Recent proteomic analysis further indicated enrichment of proteins associated with oxidative phosphorylation as the most significantly upregulated in chronically (> 15 years) endurance trained muscle (Emanuelsson et al., 2024). Furthermore, network analysis of proteomic abundance profiles (Emanuelsson et al., 2024) indicated a network of Complex I and V proteins strongly correlate with citrate synthase activity (a commonly used marker of mitochondrial density). Indicating the specific increases in Complex I and V proteins within the endurance trained response likely also reflect an increase in total mitochondrial density, that is specific to endurance trained muscle.

Our preliminary analysis highlights mitochondrial proteome remodelling as a key feature of the beneficial effects of chronic exercise training. Furthermore, changes in protein degradation are inferred as important components of training-mode specific proteome remodelling. On the individual protein level, complex roles for eIFs were shown in response to END and REX both on the level of protein abundance and turnover rate. However, post-translational modification is key to protein function, particularly in the context of eIFs and UPS/ degradative activity, therefore, phosphoproteomics would be an obvious next step to further investigate the molecular responses to END and REX. Proteomic techniques such as titanium dioxide enrichment (Yu and Veenstra, 2013) would provide an untargeted insight into proteome-level changes in phosphorylation status. However, this would lack details on individual proteoforms that are more clearly resolved using Top-down proteomic analysis (i.e. 2-DIGE) to allow for a greater variety of modifications to be identified. However, the resolution of eIF or UPS proteins is challenging using traditional 2-DIGE techniques. Therefore, biochemical enrichment/ extensive fractionation would be required to specifically target these components such as those used in Hayter et al., (2005) to resolve individual components of the proteasome in skeletal muscle. Ultimately, differential PTM mapping could provide the evidence needed to allow for clearer mechanistic questions/ targeted analysis (e.g. loss- or gain-of-function studies) of individual proteome components that may underpin the specificity of proteome remodelling.

Furthermore, it must be noted that our current work investigated differences in the relative abundance of proteins and is best suited to highlighting qualitative changes to the muscle proteome (i.e. that align with change in muscle phenotype) rather than absolute changes in muscle mass associated with protein accretion. Consistent with previous abundance profiling studies on human muscle (Deane et al., 2022, Roberts et al., 2023b, Emanuelsson et al., 2024) which indicate very few changes in relative protein abundance in response to resistance training. These data suggest limited changes in the muscle phenotype rather than proteome adaptation being absent from resistance training adaptations. Therefore, developments in the absolute quantification of proteomics data may be required to adequately demonstrate muscle proteome adaptation associated with protein accretion that underlie changes in muscle mass induced by resistance training. However, this would require accurate determination of changes in muscle volume to calculate total protein content. Whilst currently no clear differences were resolved in muscle mass between END and REX (Figure 5.2), previous work using similar resistance training regiments have shown significant increases (+ ~8 %) in VL CSA after chronic training (3 sets of 8-12 repetitions leg press and extension at 80 % 1RM training 3x / wk for 10 weeks) (Stokes et al., 2020). Therefore, it may be inferred that

resistance training induces expansion in the total size proteome which likely underpin the improvement in strength/ force output rather than reorganisation of the relative abundance of proteins (i.e. changes in phenotype) as more predominantly seen in endurance trained muscle. However, our data along with previous supplementary proteomic analysis by Robinson et al., (2017) do indicate qualitative changes in the muscle mitochondrial proteome that may, at least in part, underpin the resistance training induced improvements in skeletal muscle health.

5.5 Conclusion

In conclusion, this work provides the first data to integrate the profiling of abundance and synthesis rates on a protein-by-protein basis after both resistance and endurance training, in parallel. To date, this work likely represents the largest analysis of protein-specific fractional synthesis rates in human muscle in vivo. Allowing for the quantification and comparison of 399 and 596 protein synthesis rates after 1- and 10-weeks of training, respectively, to complement the analysis of >2000 protein abundances. Our data indicate increases in networks of proteins comprising complex I and V were identified as specifically increases to endurance training, likely underpinning the robust increase in aerobic capacity. However, both resistance and endurance training seemingly benefit from increases in mitochondrial complex I accessory subunit abundance and greater turnover of metabolic enzymes, representing both training modes to result in improved mitochondrial and proteome quality. Also, changes in UPS components were identified as prominent adaptive features comprising the shared response to endurance and resistance training. However, the lack of training mode specific changes in UPS suggest common improvements in muscle proteostasis. Co-analysis of changes in abundance and synthesis rates allow for inferences on protein degradation and suggest the selective degradation of mitochondrial proteins (potentially by autophagic processes) may be a key mechanism of training-mode specific adaptation. More specifically, selective autophagic degradation induced by the eIF5A hypusination pathway is highlightedas a potential regulator of training-modes specific proteome remodelling.

Chapter 6. General Discussion

6.1 General discussion

Skeletal muscle is a diverse tissue that exhibits a remarkable ability to adapt and remodel its functional constituents in response to repeated metabolic and mechanical stimuli. Exercise induced changes in muscle function occur via selective alteration of individual protein abundances. The central dogma of molecular biology offers a vital framework to understand how proteins are synthesised from mRNA templates and preceding DNA. Currently most literature on protein synthetic responses to exercise report the aggregation/ averaging of all muscle proteins or fractionated samples. Mixed protein FSR is a common target in the investigation of molecular responses to training. Across our studies we reported typical patterns of stimulation in mixed-protein FSR (calculated from the aggregation of individual protein data) both in response to resistance exercise in rats (Figure 4.4) and humans (Figure 5.7). However, this work (Chapter 4 and 5) indentified heterogeneity in the synthetic response to exercise when assessing FSR on a protein-by-protein basis. In Chapter 4 differential patterns of FSR response for numerous proteins that comprise the same subcellular fraction were clearly identified. For example, temporal clustering revealed a network or myofibrillar proteins (MYH8, LMNA, DESM, COF1, PROF2, FHL1, SMPX, and PDLI5) that had a greater FSR in Stim after 2 and 10 days of PRT, which then decreased after 20 days. Whereas a separate cluster of myofibrillar proteins (MYLK2, MYL11, CASQ1, ACTB, WDR1, and TPM2) exhibited considerably decreased FSR after the first 2 days of PRT and then FSR increased and plateaued after 10 days. Furthermore, 9 proteins involved with glycolysis/gluconeogenesis exhibited a progressive increase in synthesis rate. However, 13 oxidative phosphorylation subunits exhibited a more complex pattern of response whereby synthesis rate was lower in stimulated muscle over the first 2 days of PRT and then increased over the remainder of the experimental period. These findings clearly highlight protein-specific synthesis rates are required to understand the changes in dynamic responses across a time course of training.

Heterogeneity of protein specific FSR responses were also clearly evident in human muscle following 1-week (Chapter 5) and 10-weeks of training in both skeletal muscle of young healthy (Chapter 5) and obese individuals (Chapter 3). The incorporation of individual-protein synthesis and abundance data ais required to assess exercise induced changes in protein turnover, as without knowledge on the abundance change inferences on the biological role of changes in FSR cannot be made. This thesis further consolidates previous work from our lab indicating that changes in protein synthesis rate independent of abundance (i.e. turnover rate of proteins) represents an important adaptive feature of exercise. Both resistance and endurance exercise in young, health muscle (Chapter 5) and HIIT in obese muscle (Chapter 3) were found toelicit increases in the protein-specific turnover rate of metabolic enzymes comprising the TCA cycle and fatty-acid oxidation, which was also conserved in animal models of resistance training (Chapter 4). These increases in protein turnover likely occur in response to the greater metabolic load associated with exercise training (de Freitas et al., 2017). Whereby protein turnover may be increased to prevent the accumulation of irreversible protein modification/ damage because of greater catalytic function of enzymes. Similar improvements in proteostasis represent a key target of therapeutic interventions to counteract the cellular decline associated with ageing and chronic disease (Balch et al., 2008). A loss of proteostasis, characterised by alterations in degradative components and patterns of slower rates of individual protein turnover in metabolically impaired skeletal muscle (Chapter 3) was also highlighted using dynamic proteomic profiling, which is consistent with our work reporting impaired protein turnover of mitochondrial proteins as a potential pathological component of muscular dystrophy (Nishimura et al., 2023). Ultimately, the consistent evidence for the improved turnover of individual proteins, irrespective of the context or mode of exercise investigated, likely highlights an essential yet underappreciated aspect of of regular training inimproving skeletal muscle proteostasis. Improvements in proteome quality likely contribute to the improvements in muscle function contributing towards the overall health benefits associated with regular training. Critically, such data on proteome dynamics cannot be resolved using static abundance measurements and therefore, the use of dynamic proteomic techniques offers

novel insight into the therapeutic effects of exercise and underappreciated details on the heterogeneity of proteodynamic responses to training.A vital strength to non-targeted analysis is the ability to generate hypotheses in an unbiased or less biased manner than traditional deductive processes. Strikingly, significant changes in the abundance of proteins comprising oxidative phosphorylation and more specifically mitochondrial complex I were identified throughout each of our separate analyses. Chapter 3 highlighted dysregulation to the abundance of complex I subunits in the muscle of people with obesity. Whereby six proteins (NDUB5, NDUS3, NDUCR, NDUA2, NDUA5, and NDUBA) were less abundant in the muscle of obese individuals. Two Complex I accessory subunits (NDUAC and NDUBA) which were less abundant at baseline significantly increased in the muscle of individuals with obesity after 10-weeks of HIIT. Furthermore, both Chapters 4 and 5 identified increases in protein abundance of complex I subunits after exercise training. In chapter 5, increases in the abundance of 4 accessory subunits was conserved between endurance and resistance training. Whereas chronic (> 20 days) programmed resistance training of rat TA and endurance training in healthy humans (10-weeks) specifically increased the abundance of numerous (10 and 20 proteins, respectively) Complex I accessory and catalytic subunits (Chapter 4 and 5). The measured increase in the abundance of just 2 Complex I subunits after 10-weeks of HIIT in obese individuals compared to >20 proteins in young, healthy adults (Chapter 5), may represent an adaptive component of training that is blunted in muscle of metabolically impaired individuals. However, the Complex I accessory subunit, NADH:Ubiquinone Oxidoreductase Subunit S4 (NDUS4), was significantly increased in abundance following endurance training in both obese (Chapter 3) and young individuals (Chapter 5) as well as programmed resistance training in rat fast twitch muscle (Chapter 4). Respiratory Complex I (NADH Dehydrogenase) represents the largest and most complicated component of the respiratory chain and is the major entry point for electrons to the electron transport chain and ultimately a rate limiting step in aerobic respiration (Wirth et al., 2016). Whilst mitochondrial dysfunction has been implicated in various human chronic diseases, isolated deficiency of Complex I function is the most frequent across mitochondrial disorders (Sharma et al., 2009). Indicting the maintenance of Complex I structure and function is at the centre of energy provision and metabolic health within muscle. A systematic review of muscle proteomics literature highlighted Complex I abundance as a main feature of exercise induced adaptation particularly in the context of type 2 diabetes and obesity (Srisawat et al., 2017). Whilst improvements in the efficiency of oxidative phosphorylation and markers of Complex I are well reported in targeted biochemical analyses of exercise adaptation, the prevalence of Complex I proteins as central components to the training response in unbiased proteomic analysis reinforces these proteins as a key exercise responsive target in skeletal muscle. Currently the functional significance of all subunits is not well known but the findings point to a general qualitative improvement in Complex I structure and function that may underpin the improved metabolic function and associated health benefits common to both endurance and resistance training. However, throughout the work changes in the turnover rate of individual Complex I subunits were detected. It is currently challenging to infer what the changes in turnover rates have on assembly of the Complex I multiprotein complex. Increases in protein turnover rates may possibly indicate greater assembly/ disassembly of Complex I subunits as a result of damage accumulated from greater activity after training. Whereas the increased abundance may aid in the expansion in the number of respirasome supercomplexes in endurance trained muscle. Previous work has reported exercise induced changes in the stoichiometry and formation of respirasomes within skeletal muscle following 16-weeks of endurance training (Greggio et al., 2017). However, recent work has suggested the functional relevance of multiprotein complex stoichiometry and supercomplex formation is not clearly understood and may serve as a more structural component of the mitochondrial inner membrane (Brischigliaro et al., 2023). Furthermore, stable isotope labelling methods in HeLa cells indicated the assembly of Complex I is much less efficient compared to that of Complex V (Bogenhagen and Haley, 2020). Whereby, complex I proteins are often ‘over synthesised’ and rapidly degraded generally within 3 hours. This could have potential implications for the disparity in Complex I protein abundance response in humans between Chapter 3 and Chapter 5. Our work in Chapter 5 collected biopsies 3 h

following the final exercise bout in week 1 and week 10. Therefore, the dramatic upregulation in Complex I abundance (i.e. > 20 subunits) compared to the increase in 2 subunits in Chapter 3 (samples collected 72 h post training) may be a result of the excess synthesis of Complex I subunits. However, recent cross-sectional analysis of endurance and resistance training in humans with > 15 years of either training modality, reported similar increases in the abundance of Complex I were a prominent characteristic of endurance trained muscle (biopsies collect > 72 h post training) (Emanuelsson et al., 2024). Questions remain on the cellular location or incorporation of these individual mitochondrial proteins. Despite their confident association with mitochondria, the majority of these proteins require active transport to the mitochondria via their mitochondrial targeting sequence (Bolender et al., 2008). Therefore, to understand the complete biological role of increased abundance of Complex I subunits, structural insights into cellular location and supercomplex formation are necessary. However, based on our current data Complex I protein abundance is a key target for the development of therapeutic interventions to improve health and metabolic function of skeletal muscle.

Currently, an over focus on Crick's model (e.g. mRNA transcription and protein synthesis) in molecular biology and technical challenges in the measurement of protein degradation, have meant relatively few investigations focus on the role of protein degradation in proteome remodelling. Protein degradation is potentially as influential and equally sophisticated and a tightly regulated system as synthesis with the potential to alter the abundance of individual proteins. Alongside previous reports from our laboratory (Camera et al., 2017, Hesketh et al., 2020), throughout this thesis the combination of changes in protein specific-abundance and synthesis rate were applied to resolve inferences on the role of protein degradation in adaptive remodelling of skeletal muscle. Data reported in Chapter 4 suggests potential changes in mitochondrial degradation underpin early (< 10 day) responses to daily programmed resistance training of rat tibialis anterior. Also, our analysis in Chapter 5 implicates changes in the degradation of mitochondrial complex I and V proteins underpin the endurance training specific increases in proteins comprising oxidative phosphorylation. The proteasome represents the core complex responsible for proteolytic degradation of muscle proteins following ubiquitin conjugation by E2 ligases, transferred to substrate proteins via E3 ligases (Kitajima et al., 2020). The ubiquitin proteasome system represents the most well described degradative pathways in skeletal muscle, and there has been a focus on E3 ligases involved in the aberrant degradation of muscle proteins (Peris-Moreno et al., 2021). Throughout this thesis, the protein abundance profiling data generally had good coverage of proteasome subunits as well as the abundance of E2 enzymes and E3 ligases. However, significant changes in UPS components in response to PRT or that clearly segregated the endurance and resistance training responses were not resolved. Therefore, UPS-dependant processes may not be the main mechanism of protein degradation observed throughout our training adaptations in healthy muscle. In contrast, alterations in the abundance of proteasome subunits were a key characteristic of the training response in obese muscle, further implying changes in UPS as a potential proteostatic mechanism. Moreover, this workhighlights increases in the mitophagy receptor, PHB2, as a potentially important candidate responsible for regulating the degradation of mitochondrial proteins across the first 10 days of training in Chapter 4. Similarly, numerous autophagy receptors, including OPTN and TOLIP, were differentially regulated in their abundance following endurance and resistance training. Also a complex regulation of eIF5A1 turnover, specific to endurance trained human muscle was identified. eIF5A1 is essential for the synthesis of the autophagy master regulator transcription factor EB. Overall, these data suggest that selective autophagy of proteins may represent a key exercise responsive mechanism resulting in changes in proteome remodelling in response to different training modes. Currently, it is known that autophagy is upregulated in response to different exercise interventions (Halling and Pilegaard, 2017) however, this is typically discussed in the context of macro-autophagy. Studies *in vitro* highlight different proteostatic perturbations result from changes in the cargo proteins selectively engulfed by the autophagosome via recognition by autophagy receptors (Stolz et al., 2014). This highlights potential future lines of

enquiry on how different training modes changes the targeting of individual proteins by autophagic degradation using autophagosome content profiling (Zellner et al., 2021) to define the role of different autophagy receptor proteins on cargo selectivity.

Overall, this work further reinforces the utility of our dynamic proteome profiling technique for discovering novel details on how protein abundance is regulated in response to exercise. Dynamic proteomic methods is a potentially powerful tool for studying how signalling processes may relate to subsequent changes in protein abundance. Integration with profiling of site-specific post-translational modification such as phosphorylation and ubiquitination (Hoffman et al., 2015, Parker et al., 2020)) are an obvious line to follow up the current work in this thesis. The parallel profiling of changes in protein abundance and turnover alongside changes in the post-translational modification of proteins could delineate how different signals regulate adaptation (i.e. abundance) through the downstream manipulation of protein synthesis/ degradation. However, systematic analysis of different modifications (including phosphorylation, acetylation, and ubiquitination) found no uniform link between individual post-translational modifications and the degradation or synthesis rate of a the protein (Zecha et al., 2022). Therefore, future analysis may need to integrate data across several different types of modification on different residues of a protein to understand the link between signal transduction pathways and subsequent changes in synthesis, degradation, and abundance of proteins. Furthermore, it well acknowledged that mRNA fail to reliably predict corresponding protein abundances (Gygi et al., 1999, Gerdes Gyuricza et al., 2022). Co-measurement of gene expression, synthesis, abundance and degradation on an individual protein level (Martin-Perez and Villén, 2017) can improve the predictive models that attempt to link mRNA expression with changes in protein abundance. Therefore, it appears crucial that the variable roles of synthesis, degradation, and overall turnover rates are considered on a protein-by-protein basis in the development of models to accurately represent subsequent proteome remodelling in response to exercise.

6.2 Limitations

Whilst this thesis adds crucial data on the importance of dynamic proteome profiling in developing the understand of skeletal muscle adaptation, our analysis of proteome dynamics does not come without limitations. Based on the data on protein synthesis and abundance changes presented in this thesis, changes in degradation are inferred to be a key feature of exercise-induced adaption. However, it must be acknowledged that protein degradation was not measured directly in these methods. Net changes in protein abundance are a result of changes in synthesis and degradation (i.e. abundance = synthesis – degradation). Therefore, through measurement of abundance and synthesis rate a role for protein degradation can be inferred, i.e. when changes in abundance are not easily explained by synthesis. Pulse-chase experiments (Elgendy, 2017) using deuterium oxide could offer more direct measurement of both protein synthesis and degradation. However, these investigations over longer period of times (i.e. days) in human participants are unlikely to be successful because of the slow rate of decay of deuterium in the body water compartment.

All calculations on dynamic protein measurements rely on mathematical assumptions. Throughout this thesis relatively simple equations are applied to study the rate of incorporation of deuterium into muscle proteins. The rate of synthesis is calculated using the product: precursor ratio, which assumes steady states in abundance to calculate the fraction of protein synthesised over time (i.e. newly synthesised protein / total protein). However, sometimes these biological systems are dynamic and include changes in protein abundance. Furthermore, whilst numerous proteins exhibit no change in their relative abundance (i.e. the steady state assumptions are met), currently it cannot be confirmed that the assumption that muscle size has not changed and may have affected the absolute abundance of proteins. The calculations that attempt to correct for changes in protein abundance (Zak et al., 1979), suggest that relatively large (i.e. several fold) changes in abundance are required before significant

errors in the synthesis rate calculation arise. However, such calculations have not been applied here as they again rely on further assumptions (e.g. change in synthesis/ pool size is constant) which cannot fully account for changes in abundance in such complex systems, particularly in the context of proteome-wide data. For example, the formula for the calculation of turnover rate assumes turnover is constant and that changes in turnover (or synthesis/ degradation) occur instantly and lead to linear changes in abundance resulting in a new equilibrium being reached. Particularly, in the context of muscle adaptation and exercise whereby stimuli are intermittent not result in linear changes in abundance. However, these assumptions for the calculation of protein dynamics have been currently accepted in the field. These mathematical assumptions may be true if the period of assessment is very short, however, the application of D_2O method is best suited for longer periods of investigation. In Chapter 4 muscle responses to programmed resistance training across different time intervals do not follow a linear pattern of response. However, currently it is unknown if the 3 x 10-day periods (i.e. 10, 20, and 30 days) were too large to provide the most accurate biological information. For example, the key changes in protein abundance to mitochondrial proteins seem to have occurred between day 10 and 20 indicating an adaptive response likely occurred at some point between these times. Similarly, an increase in the turnover rate of chaperones and proteins associated with the response to stress are highlighted as key components of the first 2 days of training. However, it is unclear if this was too fine of a resolution or too early in the adaptive response to provide biologically meaningful information on muscle adaptation rather than generic cellular stress.

Skeletal muscle is an extremely challenging substrate for proteomic analysis due to its large dynamic range. Nevertheless, contemporary proteomic methods can compare > 2000 proteins in human muscle from a relatively simple fractionation of muscle samples into myofibrillar and soluble proteins. However, in all proteomic experiments the number of proteins that can be identified will be largely greater than the number that can be quantified due to the common issue with missing data between groups and biological replicates. (Emanuelsson et al., 2024) identified ~6000 muscle proteins using TMT-labelling combined with isoelectric fractionation prior to LC-MS/MS analysis, but the number of proteins that could be matched across 90 % of samples was ~ 2900. The confident identification of a protein can be made from just one sample but quantitative analyses require that peptides unique to that protein are clearly resolved in each biological replicate. Often this issue is circumvented by accepting a less than full number of biological replicates for statistical testing and this has tended to inflate the perceived number of participants reported in some studies compared to the true n used in protein quantification and statistical analysis. Despite the continued development of imputation techniques for artificially complete missing values, this should not be considered best practice. Particularly in the context of typical exercise physiology studies that often use repeated measures designs of relatively small sample sizes. In this scenario, the handling of missing data must take account of the entire intervention within each individual. Our dynamic proteome profiling requires capturing high quality mass isotopomer distribution profiles for each peptide during their entire chromatographic profile, in each sample to calculate a synthesis rate. Therefore, some peptides may be excluded because co-eluting peptides with similar or overlapping mass isotopomer envelopes contaminate each other and so the number of proteins submitted to statistical analyses is further reduced. Because of the above-mentioned quality control processes, the analysis of some proteins is based on data from single peptides, whereas other proteins may have numerous protein-specific peptides that are well resolved. Profiling of the same peptide across different experimental groups is robust but it is not yet certain how representative one peptide is of the abundance or synthesis rate of the entire protein.

Despite these potential technical limitations, our data offers novel insights into the regulation of protein abundance within skeletal muscle. However, there are a number of caveats that must be considered in the interpretation of this data. The lack of control groups, particularly in Chapter 3, hinders the ability to discern whether observed changes in turnover rates and minimal changes in protein abundance are attributable to the specific characteristics

of the studied group (i.e., individuals who are obese and insulin-resistant) or if the training protocol elicited few abundance changes due to its relatively low intensity. Furthermore, while the use of two modes of unilateral exercise within the same individual permits a within-subject comparison of resistance and endurance training during the same period, it does not eliminate concerns regarding the potential influence of systemic factors such as alterations in circulating cytokines/myokines, and hormones. For instance, exercise-induced myokines such as interleukin-6 (IL-6) can exhibit both pro-inflammatory and anti-inflammatory effects, depending on the exercise context (Pedersen & Febbraio, 2012). These circulating factors may therefore result in a 'cross-talk' that ultimately may influence the molecular response to exercise. Therefore, the dual-mode approach, although beneficial for within-subject comparisons, does not fully account for the independent systemic effects of each training modality or their potential interactions.

Throughout this thesis, it must be acknowledged that relatively low sample sizes and biological replicates were used to investigate dynamic proteomic responses to exercise. The potential effects of type I error (reporting of false positives) are routinely controlled through the calculation of false discovery rates. Also, these protein abundance profiling studies demonstrate good reproducibility of findings with previous targeted analyses of exercise interventions. However, it is important to note the novel reporting of changes in protein turnover rates (i.e. non-significant alterations in protein abundance, alongside significant increases in protein synthesis rates) should be recognised as a result of analysis from a discovery cohort of samples. Whilst these works align with changes in turnover rates measured across different experimental studies from our group (Camera et al., 2017, Hesketh et al., 2020), it is necessary for follow-up studies with larger cohorts to more rigorously test the hypothesis that changes in protein-specific turnover rates can occur independently of changes in abundance. Such follow-up is crucial to validate these preliminary findings and to enhance knowledge on exercise induced protein turnover as an adaptive feature of exercise trained muscle. Similarly, the results of Chapter 5 were collected from a predominantly female cohort (n = 4 females, 1 male) who were not controlled for differences in menstrual cycle phase. The protein abundance profiling does align well with previous reports on proteome differences collected from a larger cohort of male and female participants (Emanuelson et al., 2023). However, it must be acknowledged that changes in protein dynamics and the regulation of protein-specific turnover represent the outcome of the signalling responses to each training bout. Therefore, measurements of dynamic process such as protein turnover may exhibit sex-specific differences across a training intervention despite the physiological responses being similar between males and female. Therefore, the interpretation of the findings in this thesis should consider these potential sex-specific molecular responses, which necessitates further investigation in larger mixed-sex cohorts to fully understand the implications of training on muscle proteome dynamics with the potential to highlight sex-specific changes in the regulation of abundance, synthesis, degradation, and turnover of proteins to further understand differences un the molecular responses to training between males and females.

The selection of sampling time points must also be taken into consideration to interpret any potential effect of the last bout of exercise on the skeletal muscle responses to training. Chapter 2 and 3 samples were collected 1 h and 3 h following the cessation of the training, respectively. Therefore, by collecting samples during the hours of recovery following the final exercise bout we cannot rule out the potential influence of the final training bout leading to changes in protein abundances. However, previous phosphoproteomic analysis utilising sample collection in the hours following a bout of exercise highlighted minimal effects of a single bout of training on protein abundance data despite robust changes in the cellular signalling responses (Hoffman et al., 2015, Potts et al., 2017). Furthermore, utilising the benefit of deuterium oxide provision across days/ weeks allows for the measurement of protein synthesis rates to be integrated across the labelling period, therefore, limiting the potential impact of the acute post-exercise response on these data. To limit the potential influence of the last bout effects, sampling times ≥ 72 h post the cessation of exercise, like those used in Chapter 1, should be used. However, 72 h post training samples are

not optimised for the future study integrating cellular signalling responses alongside changes in the synthesis, abundance, and degradation of proteins to understand how proteome adaptation occurs. Also, it must be noted that the current work consists of the analysis of mixed-fiber samples in humans in Chapter 3 and 5. Similarly, Chapter 4 utilises a novel animal model of programmed resistance training targeted to the archetypal fast-twitch tibialis anterior. Therefore, the use of more rigorous fiber typing procedures of biopsy samples or the study of fiber-type specific responses through single fibre analysis ,similar those to used previously Deshmukh et al., 2021, Murgia et al., 2021), may place the interpretation of the dynamic proteome profiling in a greater context as to the muscle samples that have been investigated. Therefore, limiting the inter-individual variability of training responses due to differences in fiber profile analysed between individuals. Whilst also potentially highlighting fiber-type specific responses in proteome dynamics which may offer further insight into the heterogeneity of muscle adaptation between different muscle fiber types.

Ultimately, our findings highlight the extent of the complexity of protein metabolic processes that modulate proteome remodelling and subsequent changes in function. Moreover, the collection of dynamic proteomic data across this thesis and published previously, provides clear evidence that the use of mixed protein synthesis rates gives an inadequate reflection of the dynamic processes regulating protein abundances. This work has provided new evidence for selective synthesis and degradation of individual proteins at varying rates alongside offering some of the first insights indicating that changes in protein synthesis rate and abundance do not necessarily follow a uniform pattern across a period of adaptation (e.g. early changes in synthesis do not reflect the chronic changes in synthesis rate). Raising further questions on how repeated bouts of exercise produce the well reported chronic phenotype, particularly in the context of how proteins are selectively synthesized and degraded differently across a period of adaptation.

6.3 Conclusions

Studies at an individual-protein resolution are required to understand specifically which proteins undergo alterations in their synthesis, degradation, or turnover rate during the adaptation to exercise. Chapters 3 and 5 represent only the 2nd and 3rd dynamic proteomic studies of exercise adaptation in skeletal muscle of humans. Consistent with Camera et al., (2017) each of these analyses highlighted large degrees of heterogeneity in protein-specific FSR responses to training. Chapter 5 highlights the significant increases in protein FSR are largely unique between acute and chronic exercise. Whilst in Chapter 4, time-series analysis across a 30-day period of adaptation highlighted a more complex relationship of time-dependant changes in the synthesis rates of proteins associated with different biological networks.

Furthermore, throughout our investigation of dynamic proteome responses to training changes in the turnover rate of proteins independent of changes in abundance were identified, which provides new knowledge on muscle adaptation. Across all chapters of this thesis, a general pattern of increases in the turnover rate of metabolic enzymes was obsereved independent of training mode. These increases in protein turnover likely represent an essential health-centric response to repeated exercise training associated with improvements in proteostasis and muscle proteome quality in response to exercise. Furthermore, this work provides the first study to show loss of proteostasis in young obese individuals which has predominantly been associated with the effect of ageing on skeletal muscle (Paez et al., 2023).

Through the integration of protein abundance and synthesis changes within this these,selective changes in the degradation rate of individual proteins may underpin important aspects of proteome remodelling in response to training. Chapter 5 demonstrates that despite a relatively linear relationship between synthetic responses, protein

abundance changes do not correlate between endurance and resistance training. Furthermore, Chapter 4 highlights changes in mitochondrial protein synthesis rate do not completely explain changes in abundance. Therefore, indicating that changes in the degradation rates of proteins, particularly mitochondrial proteins, and oxidative phosphorylation subunits, may represent key mechanisms resulting in proteome remodelling. However, throughout this data changes in proteins associated with UPS did not exhibit selective changes across training conditions. Rather we propose changes in autophagy/ mitophagy to represent a key adaptive mechanism in exercise induced proteome remodelling.

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