Investigating the relationship between skeletal muscle inflammation, protein synthesis and mass in humans, using eccentric exercise, limb immobilisation, critical care, and resistance training models.

Submitted by Thomas Stanley Osborne Jameson to the University of Exeter
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I certify that all material in this thesis which is not my own work has been identified and that no material has previously been submitted and approved for the award of a degree by this or any other University.

Signature: ……..

Abstract

Inflammation and muscle protein turnover are necessary processes that underpin the plasticity, mass and function of skeletal muscle during health and disease.

The principle aim of this thesis was to test the hypothesis that inflammation regulates myofibrillar protein synthesis rates to determine changes in muscle function and mass in healthy and critically ill humans. Firstly, a nutrition intervention approach was used to investigate the time course of free-living myofibrillar protein synthesis rates and transcriptional inflammatory NF-κB and proteolytic signalling with respect to the recovery of muscle function after muscle-damaging eccentric contractions. Eccentric contractions were then combined with a unilateral limb immobilisation model to investigate the regulation of myofibrillar protein synthesis rates, muscle atrophy and muscle function by inflammation under disuse conditions. Using critically ill patients as a model of pathophysiological inflammation and muscle protein turnover, the effect of an exercise intervention administered in the intensive care unit on gene expression associated with inflammation and protein turnover was then investigated with respect to prospective functional outcomes in survivors. Finally, this thesis aimed to determine if early gains in muscle function in response to an eccentric biased resistance exercise training programme can be expedited nutritionally.

The studies presented in this thesis demonstrate for the first time that an increase in myofibrillar protein synthesis rates are likely to be directly related to the decline in muscle function after myofibrillar injury. A primary novel finding of this thesis is that using nutrition or immobilisation to manipulate muscle function occurs independently of changes in myofibrillar protein synthesis rates. Additionally, this thesis presents novel data to show that inflammatory NF-κB signalling does not regulate myofibrillar proteins synthesis rates in healthy individuals and likely does not regulate changes in muscle function. We present preliminary data suggesting that muscle protein breakdown may instead be important. A final novel and pertinent conclusion of this thesis is that pathophysiological inflammation in the critically ill patient is associated with failed skeletal muscle remodelling in response to muscle contraction, and this is associated with poor functional outcomes in survivors. These findings could be of major relevance for athletic,

general and clinical populations where muscle mass and function underpin athletic performance, quality of life and life itself.

Declaration

All of the data collected, and procedures presented throughout this thesis, including blood and skeletal muscle sample preparation and biochemical and molecular biological analysis, have been performed by myself, with the following exceptions:

Dr Francis Stephens, Dr Ben Wall, Dr Marlou Dirks and Dr Sarah Jackman took all of the muscle biopsy samples in **Chapters 3**, **4**, **5** and **7**. Dr Sean Kilroe performed data collection of the "Control" condition in **Chapter 5**. Dr Frantisek Duska and colleagues at Charles University provided muscle samples in **Chapter 6**. Doaa Reda Abdelrahman performed mass spectrometry in **Chapters 3**, **4**, **5** and **7** at The University of Texas Medical Branch. Dr Jonathan Fulford performed magnetic resonance imaging in **Chapters 5** and **7** with image analysis performed by me. **Chapters 3**, **4** and **7** formed part of larger investigations, and data collection was performed by George Pavis and me.

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Publications

Abstracts

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Papers

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Chapter 1

General introduction

1.1 Overview

Skeletal muscle plays a leading role in maintaining metabolic health and physical function. The redistribution of amino acids from the skeletal muscle reservoir also supports immune function and wound healing. Consequently, disturbances to skeletal muscle structure, function and mass can have profound negative implications for physical and physiological functioning during both health and disease. To mitigate this, skeletal muscle possesses remarkable plasticity. This allows it to remodel and adapt to both acute injury and chronic alterations in the demand for contractile work in physiological and pathophysiological states. The degeneration and regeneration of skeletal muscle is characterised by alterations in inflammatory processes and the turnover of skeletal muscle proteins. However, our understanding of how these two processes interact to regulate muscle mass and muscle function during health and disease remains incomplete.

1.2 Muscle protein turnover

1.2.1 Regulation of muscle protein synthesis and breakdown

To maintain a constant mass of skeletal muscle over time, there must be equilibrium between the rates of muscle protein synthesis and muscle protein breakdown. Muscle protein synthesis and breakdown rates are dynamically regulated such that there is a constant fluctuation in the synthesis and breakdown of muscle protein throughout the course of a day and night. The difference between protein synthesis and protein breakdown represents the net protein balance and ultimately changes in muscle mass. Muscle protein is gained in a positive protein balance (muscle protein synthesis > breakdown) and lost in a negative protein balance (muscle protein synthesis < breakdown). In healthy untrained individuals, skeletal muscle mass is constant and displays turnover rates of ~1.2% per day. There are many factors which affect the synthesis and breakdown of skeletal muscle, thereby leading to acute or chronic perturbations in muscle protein balance. Muscle contraction (or a reduction thereof) (1–3), and nutrition are the primary regulatory factors. Other factors include inflammation (4) critical illness (5), myofibrillar injury (6), pharmaceuticals (7), hormones (8, 9), and age (10, 11).

Our mechanistic understanding of the acute regulation of muscle protein turnover is based on studies using intravenous infusions of isotopically labelled amino acids (such as [^{13}C]leucine or [^{13}C]-, [^{15}N]-, or [^{2}H]phenylalanine) (12). These amino acids are essentially identical to their endogenous counterparts but can be distinguished by their mass difference using mass spectrometry techniques. The principal is that the labelled amino acid mixes with the endogenous pool and becomes incorporated into muscle protein over time. By performing serial muscle biopsy sampling during constant infusion protocols, the rate of incorporation of these amino acids into individual muscle fractions (i.e., myofibrillar) or the mixed muscle pool can be measured to determine the synthetic rate of that protein fraction. The synthesis rate of the mixed muscle protein pool is influenced by the synthetic rate of its composite fractions and indeed the synthesis rate of individual fractions is a reflection of the average synthesis rate of the individual proteins within that fraction. The magnitude and duration of alterations in the synthetic rate of individual protein fractions is regulated differently by the type of contraction (i.e., resistive vs endurance) and nutrition (1, 13, 14). When investigating changes in skeletal muscle function, it is therefore important to directly assess changes in the myofibrillar protein fraction given its primary role in force production.

A central limitation with isotopically labelled amino acid infusion protocols is the inability to obtain measurements of protein synthesis rates over an extended period (i.e., ~ >12 hours) and under free-living (i.e., not laboratory-restricted) conditions. More recently, the re-emergence of deuterated water ($^{2}H_2O$) to measure the turnover of muscle protein has been widely adopted to overcome these limitations. Hans Ussing first described the use of deuterated water to measure protein turnover in 1941 (15). Upon oral ingestion, deuterated water equilibrates with the body water pool within ~2 hours in humans (16). ^{2}H-labelling of carbon-bound hydrogens of amino acids then occurs intracellularly via transamination reactions, and the ^{2}H labelled amino acid is incorporated into muscle proteins. Importantly, steady body water deuterium enrichments can safely be maintained for weeks in humans. This integrates important lifestyle factors that may contribute to chronically altered protein synthesis rates (e.g., repeated mixed meal ingestion, physical (in)activity, repeated postprandial, post-absorptive and overnight periods, hormonal and diurnal fluctuations etc.).

Measuring muscle protein breakdown is methodologically more challenging, and our understanding of the regulation of muscle protein breakdown is more immature as a consequence. Several methods can provide an estimation of muscle protein breakdown. However, a primary requirement of these methods is that the muscle is in a physiological steady state (17). Consequently, perturbations in protein metabolism caused by exercise and nutrition are not compatible with the techniques currently used to directly assess muscle protein breakdown. An indirect method to calculate muscle protein breakdown involves measuring urinary excretion of 3-methylhistidine, which is derived mainly from the breakdown of the protein actin. However, the breakdown of cardiac and smooth muscle can contribute to the appearance of 3-methylhistidine in urine in addition to dietary sources (i.e., meat). Appropriate caution is therefore required when interpreting these data (18, 19).

The preponderance of studies that wish to investigate changes in muscle protein breakdown typically measure changes in the muscle mRNA expression of candidate proteins that comprise the molecular pathways of muscle protein breakdown. There are four primary proteolytic systems in human skeletal muscle responsible for the majority of cellular protein degradation: caspase (20), cathepsin (21), calcium-dependent calpain (22) and the ubiquitin-proteasome (UPS) (23, 24) pathways. The UPS is considered to be of particular importance for the degradation of myofibrillar protein. However, intact myofibers must first be disrupted either mechanically (i.e., contraction) or via an alternative proteolytic system (25). For protein degradation to occur, target proteins must first be tagged by ubiquitin which is a process achieved predominantly by the action of the E3 ubiquitin ligases MuRF1 and MAFbx. Ubiquitinated proteins are then recognised by the 26S proteasome, where they undergo degradation (26). The finding that MuRF1 and MAFbx expression is increased in multiple animal models of skeletal muscle atrophy has led to them being considered primary markers of muscle protein breakdown (23, 24, 27). Autophagy is an additional degradation pathway which removes damaged protein and organelles in myofibers by forming autophagosomes which fuse with the lysosome. Degraded end products (e.g., amino acids) are released into the cells which in turn can participate in molecular signalling pathways. Autophagy in skeletal muscle is modulated by contraction (28), disuse (29) and critical illness (30), among others.

At a molecular level, muscle protein synthesis is primarily regulated at the level of translation initiation (31). The mechanistic target of rapamycin complex 1 (mTORC1) intracellular signalling pathway is arguably the most well studied in this regard, central to which is the serine/threonine protein kinase, mTORC1. Activation of mTORC1 signalling occurs primarily in response to growth factors such as insulin (32), amino acids (33) and muscular contraction (34), but also to inflammatory signalling (35). Underlining its importance, inhibition of mTORC1 signalling in human skeletal muscle by rapamycin abolishes the increase in mixed muscle protein synthesis rates caused by contraction (36) and amino acids (37). Consequently, the protein and phosphorylated protein expression of mTOR is routinely measured to provide a molecular basis for changes in muscle protein synthesis.

1.2.2 Acute skeletal muscle contraction

In 1992, Chesley and colleagues provided the first direct evidence that resistance exercise increases the synthesis rate of mixed muscle proteins (38). Using a unilateral exercise model in combination with an L-[1-^{13}C]leucine tracer, they reported that mixed muscle protein synthesis rates increased from 0.067%·h^{-1} in the rested arm to 0.101%·h^{-1} in the exercised arm immediately post-exercise. In a separate participant cohort, they also demonstrated that mixed muscle protein synthesis rates increase from 0.045%·h^{-1} in the rested arm to 0.094%·h^{-1} in the exercised arm 20-24 h post-exercise. Biolo and colleagues provided the first evidence that resistance exercise increases muscle protein breakdown in 1995 (39). The arterial-venous balance method was used to demonstrate that protein breakdown increased by 51% during a 3 h post exercise period, concomitant with a 108% increase in mixed muscle protein synthesis rates. Consequently, the post-exercise protein balance remained negative. Shortly afterwards, Phillips and colleagues provided a more temporally resolved picture of the change in mixed muscle protein synthesis and breakdown rates after resistance exercise (19). Their data demonstrated an increase in mixed muscle protein synthesis rates from rest (~0.06%·h^{-1}) after three hours (~0.12%·h^{-1}) which remained elevated 24 (~0.10%·h^{-1}) and 48 (~0.08%·h^{-1}) h later. Protein breakdown was also elevated from ~0.11%·h^{-1} at rest, to ~0.15%·h^{-1} 3 h post-exercise, which was measured using the tracer dilution approach. Protein breakdown measured using

urinary excretion of 3-methylhistidine was also reported to be elevated 24 h (~0.14%·h^{-1}), but not 48 h (~0.12%·h^{-1}) post-exercise.

The study by Phillips and colleagues was the first study that attempted to delineate the effect of contraction mode (eccentric vs concentric) on mixed muscle protein synthesis and breakdown rates (19). This was based on the principle that eccentric contractions had been shown to cause more myofibrillar disruption (using electron microscopy) than concentric contractions (discussed in **1.4.1**). Accordingly, it was hypothesised that myofibrillar injury would increase muscle protein breakdown rates (40). However, no difference in muscle protein synthesis or breakdown rates were reported between concentric and eccentric contractions which the authors concluded, possibly in haste, was due to an underpowered study. An alternative explanation could be that their eccentric contraction protocol which was performed at 80% of concentric one repetition maximum, did not necessitate enough eccentric force to cause myofibrillar injury (41). It is unsurprising, therefore, that no difference in protein synthesis or breakdown rates was reported between contraction modalities. Additionally, by measuring the synthetic rates of mixed muscle proteins, meaningful changes in myofibrillar protein synthesis rates may have been masked by variation in the synthetic rate of other protein fractions. Indeed, collagen protein shows a similar post-exercise increase in synthesis rate regardless of contraction modality (6). Subsequent studies comparing mixed muscle and myofibrillar protein synthesis rates between eccentric and concentric contraction modalities performed at the same absolute intensity have also demonstrated no difference in post-exercise muscle protein synthesis rates between contraction modalities (42, 43).

The first study to demonstrate a difference in myofibrillar proteins synthesis rates between eccentric and concentric contraction modalities was performed by Moore and colleagues in 2005 (6). They used a unilateral model whereby one leg performed a maximal bout of eccentric contractions, and the contralateral leg performed maximal concentric contractions until an equivalent amount of work was completed. The same relative (maximal) intensity between contraction modes was achieved as a consequence. This unilateral approach, whereby the response between contraction modalities is compared within the same individual markedly reduces inter-individual variation and affords a more robust study

design (44). Using a [1,2-^{13}C]leucine tracer, Moore and colleagues reported that relative to at rest (~0.07%·h^{-1}), post-exercise myofibrillar proteins synthesis rates increased to a greater degree in the eccentrically contracted leg (to ~0.110%·h^{-1}) compared to the concentrically contracted leg (~0.090%·h^{-1}). Moreover, myofibrillar disruption (z-band steaming) was reported only in the eccentrically contracted leg. Moore and colleagues did not attempt to correlate the individual increase in myofibrillar protein synthesis rates with the individual increase in myofibrillar disruption. Still, this study nonetheless provided the first evidence that these two processes may be associated.

Since this study, there has been little attempt to investigate this further. Consequently, the significance of the greater increase in myofibrillar protein synthesis rates after eccentric compared to concentric contractions is still to be determined. At the time, Moore and colleagues concluded that the larger amount of work performed when exercising concentrically caused a delay in the rise in myofibrillar protein synthesis rates, suggesting that they did not think myofibrillar injury was relevant (6). In mice, the recovery of muscle function 48-120 h following electrically stimulated eccentric contractions is associated with an increase in mixed muscle protein synthesis and breakdown rates for at least five days (45). Furthermore, inhibiting mTOR signalling with rapamycin treatment attenuates the recovery of muscle strength by 20% after 7 and 14 days (46). It would therefore be reasonable to suggest that the greater increase in myofibrillar protein synthesis rates after eccentric contractions is in part due to the requirement synthesise new myofibrillar protein. This new protein would then be used to repair and replace myofibrillar protein damaged by eccentric contraction. Indeed, myofibrillar injury causes a decline in muscle function that can persist for up to 7 days and longer in some individuals (discussed in **1.4.1**) (41). Consequently, the recovery of muscle function, which has been directly correlated with myofibrillar injury (47), could be underpinned by the capacity to upregulate myofibrillar protein synthesis rates after eccentric contractions. There are two primary lines of evidence that have not been investigated and are required to test this hypothesis. Firstly, a measure of myofibrillar protein synthesis rates over multiple days is required; a single ‘snapshot’ measure obtained over a few hours is unlikely to represent the dynamic remodelling of myofibrillar injury

accurately. Secondly, a time course of muscle function needs to be measured concomitantly with myofibrillar protein synthesis rates.

Gasier and colleagues in 2012 were the first to apply deuterated water to demonstrate that resistance exercise increases myofibrillar protein synthesis during a 16 hour period of free-living, reporting rates of 0.94%·h^{-1} in the exercised leg relative to 0.75%·h^{-1} in the rested leg (48). This shows good agreement with rates of ~0.13 and 0.10%·h^{-1} measured 3 h and 24 h post-resistance exercise, respective using a [$^{2}H_{5}$]phenylalanine labelled amino acid tracer under laboratory-restricted conditions (19). A primary benefit of using deuterated water compared to traditional stable isotope infusion methodologies is the ability to measure cumulative changes in myofibrillar protein synthesis rates over multiple days. Wilkinson and colleagues demonstrated that relative to the contralateral rested leg (1.35%·d^{-1}), resistance exercise performed every other day for eight days increased daily myofibrillar protein synthesis rates to 1.79%·d^{-1} averaged over these eight days (49). By sampling muscle tissue at various time points (i.e., days 0-2, 2-4 and 4-8), this study also demonstrated the utility of deuterated water to investigate time-course changes in myofibrillar protein synthesis rates. In agreement with these data, Holwerda and colleagues have recently shown that unilateral resistance exercise performed on three consecutive days increases myofibrillar protein synthesis rates from 1.642%·d^{-1} in the control leg to 1.984%·d^{-1} in the exercise leg (50).

Collectively these early data demonstrate novel opportunities afforded by deuterated water to investigate chronic changes in myofibrillar protein synthesis rates. This is particularly relevant to intervention studies where chronic alterations in myofibrillar protein synthesis rates are expected, such as in response to myofibrillar injury or during a period of limb immobilisation.

1.2.3 Chronic skeletal muscle contraction

Repeatedly performing resistance exercise during a period of resistance exercise training facilitates the accumulation of time in which the muscle is in a positive protein balance. This is considered to underpin the accretion of muscle proteins that leads to skeletal muscle hypertrophy and can be measured at the level of individual fibre cross-sectional areas (CSA) or the whole muscle level using

computerised tomography (CT) scanning or gold standard magnetic resonance imaging (MRI). In untrained individuals, quadriceps hypertrophy of ~0.7%/week is typically reported in during the first 8-12 weeks of resistance exercise training (51–55), and this is accompanied by a ~1.5%/week increase in quadriceps strength (52, 54–56).

A causative role of muscle protein synthesis rates in underpinning hypertrophic adaptations to resistance exercise training is not clear. Studies using stable isotope labelled amino acid infusions have failed to correlate the acute post-exercise increase in muscle protein synthesis rates after the first training session with ensuing hypertrophy after a period of resistance exercise training (57, 58). This can likely be attributed to a variety of factors. These include the considerable interindividual variation in myofibrillar protein synthesis rates and hypertrophy, especially when a bilateral training model is used (59), and the “snapshot” approach whereby muscle protein synthesis is measured over just several hours using intravenous infusions of labelled amino acids combined with a single measurement of hypertrophy performed at a pre-determined time point.

Brook and colleagues have recently applied deuterated water to investigate changes in muscle protein synthesis rates over an integrated period of resistance exercise training (60). They addressed this using a unilateral model whereby chronic alterations in protein synthesis rates and hypertrophy can be corrected to non-training induced alterations in the untrained contralateral leg. It was demonstrated that mixed muscle protein synthesis rates were greatest in the first three weeks of training (1.60%·d^{-1}) compared to the subsequent three weeks of training (1.29%·d^{-1}). Furthermore, they demonstrated that vastus lateralis hypertrophy was greatest during the first three weeks of training with no subsequent increase after that. Although the authors concluded that this showed hypertrophy to be most active during the initial phase of a resistance exercise training programme, this is open to interpretation. Firstly, measuring hypertrophy after just three weeks of resistance exercise training in previously untrained individuals is highly likely to be confounded by oedematous swelling (61). Protein accretion after three weeks of training is expected to be small and hard to sensitively detect, especially with the ultrasound technique they employed (relative to a gold standard MRI approach). It seems that at least six weeks of

training is required to detect true hypertrophy reliably (59). It is known that in untrained individuals, unaccustomed exercise causes myofibrillar injury (40). As discussed previously, higher myofibrillar protein synthesis rates occur concomitantly with myofibrillar injury (6). Consequently, greater rates of myofibrillar protein synthesis rates during the first three weeks of training are likely to be a response to myofibrillar injury and are unlikely to be directed at hypertrophic remodelling.

This hypothesis has recently been tested by Damas and colleagues (51). They used deuterated water to measure myofibrillar protein synthesis rates over a 48 h post-exercise period after the first, fifth (3 weeks) and 19th (10 weeks) training session concomitant with a direct measurement of myofibrillar injury (Z line streaming). They report that myofibrillar protein synthesis rates and myofibrillar injury were both greatest after the first training session. When myofibrillar protein synthesis rates were corrected to the magnitude of myofibrillar injury, there was no difference in the increase in myofibrillar protein synthesis rates between the three training periods. This corroborates the hypothesis presented in **1.2.2** that myofibrillar protein synthesis rates increase in response to myofibrillar injury likely in order to repair damage. Furthermore, Damas and colleagues demonstrated that the increase in myofibrillar protein synthesis rates only after the fifth and 19th training sessions (when there was minimal myofibrillar injury) correlated with skeletal muscle hypertrophy. This illustrates that remodelling of injury is prioritised over hypertrophic remodelling after unaccustomed resistance exercise which causes myofibrillar injury. Nonetheless, there are currently no intervention studies that have investigated this. For example, it is unknown if attenuating myofibrillar injury and the associated inflammatory response at the onset of resistance exercise training can accelerate the refinement of myofibrillar protein synthesis rates leading to more rapid hypertrophic adaptations.

1.2.4 Removal of muscle contraction

Musculoskeletal injuries often necessitate a short-term period of muscle disuse typically administered using limb immobilisation. The removal of muscle contraction also has profound effects on net protein balance and muscle mass. Experimental models of muscle disuse began in the 1940s when a bed rest and

leg immobilisation model was shown to induce a negative nitrogen balance and muscle atrophy of the legs in otherwise healthy, young men after just two weeks (62). In the last decade, unilateral leg immobilisation models of experimental disuse have been favoured. The short-term immobilisation model induces rapid muscle atrophy due to imbalances in muscle protein turnover, making it a valuable empirical tool to elucidate the *in vivo* mechanisms that regulate muscle mass and function. Moreover, using a unilateral model allows the effect of removing contraction *per se* to be delineated by comparing to the contralateral ambulant leg. A concerted effort has been made to identify the mechanisms regulating skeletal muscle atrophy during short term periods of disuse (i.e., <7 days), primarily because most periods of injury and illness that require home-based recovery or reduced physical activity generally last less than one week. In light of this, however, a caveat of the studies presented in the following section is that they investigate the response of young, healthy individuals to limb immobilisation. Therefore, the application of this data to a clinical population is not clear.

Quadriceps muscle atrophy of 0.78%/day is reported to occur after 3-7 days of unilateral limb immobilisation (3, 63–65). Underlining the rapid and substantial decline in muscle mass that occurs with disuse, to gain the equivalent amount of muscle mass lost after seven days of leg immobilisation would take approximately eight weeks of resistance exercise training. Mechanistically, muscle atrophy results from a persistent imbalance between muscle protein synthesis and breakdown rates. Wall and colleagues in 2015 were the first to investigate myofibrillar protein synthesis rates after short-term (five days) leg immobilisation using intravenously infused labelled phenylalanine and leucine tracers (3). In the postabsorptive state, immobilisation resulted in a decline in myofibrillar protein synthesis rates from $0.051\%\cdot h^{-1}$ to $0.026\%\cdot h^{-1}$. Furthermore, postprandial myofibrillar protein synthesis rates after ingesting 25 g of protein also declined after immobilisation to from $0.062\%\cdot h^{-1}$ to $0.032\%\cdot h^{-1}$.

More recently, our group have measured integrative myofibrillar protein synthesis rates during seven days of leg immobilisation using deuterated water, thus incorporating both the postabsorptive and postprandial decline in myofibrillar protein synthesis rates, that presumably occur for 24 hours per day, into an

integrative measure. Myofibrillar protein synthesis rates in the immobilised leg declined to 0.81%·d^{-1} relative to rates of 1.26%·d^{-1} in the control leg after 7 days of immobilisation, and a numerical decline was evident after just 2 days (1.11%·d^{-1}) (63). Corroborating the robust decrease in myofibrillar protein synthesis rates with short-term immobilisation, our group later showed a ~28% decrease in myofibrillar protein synthesis after three days of leg immobilisation in a separate participant cohort (64). Underlining the role the decline in myofibrillar protein synthesis rates plays in governing muscle mass, the individual decline in quadriceps volume observed after seven days of immobilisation was strongly positively correlated with the decline in integrative myofibrillar protein synthesis rates (63). However, an interesting discussion point from this study was that the decline in integrative myofibrillar protein synthesis rates was calculated to only explain ~47% of the loss in muscle mass after seven days of immobilisation, and only 25% after two days. This would suggest that a large and rapid increase in muscle protein breakdown could also be driving the decline in muscle mass with short term disuse.

Direct measurement of muscle protein breakdown has not yet been performed in humans during short term immobilisation. Therefore, most of our understating stems from examining changes in mRNA expression of candidate proteins that comprise the molecular pathways of muscle protein breakdown. Jones and colleagues provided the first human evidence of increased skeletal muscle MAFbx and MuRF1 mRNA expression in addition to calpain-3 after two weeks of leg immobilisation (66). More recently, an increase in MuRF1 and MAFbx expression has been reported after three (67), five (68) and seven (69) days of immobilisation. Urso and colleagues have applied a microarray and gene ontology approach to investigate transcriptional perturbations induced by 48 h of leg immobilisation (70). An interesting and relevant observation from this study was that 11% of all differentially regulated genes were associated with the ubiquitin-proteasome system. However, when using RT-PCR to investigate fold changes at the individual gene level, the expression of MuRF1 and MAFbx expression did not increase. This is congruent with studies showing an increase in MuRF1 and MAFbx expression after seven, but not two days of immobilisation (69). Nonetheless, this study demonstrates the power of a gene array approach when coupled with gene ontology analysis to identify potentially important

changes in whole and potentially novel pathways which may be missed when examining changes at the individual gene level.

1.2.5 Satellite cells

Satellite cells are the primary source of myonuclei in postnatal skeletal muscle. Satellite cells remain in quiescence until stimulation by mechanical load or injury whereupon they proliferate. A proportion of activated satellite cells will differentiate into myoblasts and fuse together to form new myofibers or fuse to existing myofibers. The remaining activated satellite cells return to a quiescent state to replenish the resident satellite cell pool. Consequently, satellite cells are considered important for skeletal muscle remodelling. The progression of satellite cells through sequential activation, proliferation and differentiation phases is orchestrated by a series of myogenic regulatory factors (MRFs) including paired box transcription factor 7 (Pax7) and myoblast determination protein 1 (MyoD) which are expressed in proliferating satellite cells followed by the increased expression of myogenin, alongside sustained MyoD expression, to promote terminal differentiation of satellite cells. Satellite cells can also become senescent due to injury and ageing (71). Senescence is associated with an increase in the secretion of growth factors and pro-inflammatory cytokines termed the senescence-associated secretory phenotype which is linked to impaired muscle regeneration and muscle atrophy.

Satellite cells are considered essential for muscle fibre repair as muscle regeneration in satellite cell depleted animals is severely impaired (72). A single bout of eccentric muscle contractions which cause myofibrillar injury increases satellite cell number in human skeletal muscle between 24-72 h post-exercise (73–76). Indeed, skeletal muscle mRNA expression of MyoD, Myf5 and myogenin is increased 48 h after a bout of eccentric contractions, and the mRNA expression of whole muscle homogenates accurately reflects MRF mRNA expression observed specifically in isolated satellite cells (77). Human muscle fibre hypertrophy is also accompanied by an increase in satellite cell or myonuclei number, and satellite cell number positively correlates with muscle fibre size after a prolonged period of resistance exercise training (78–80). Whether satellite cells are *required* for muscle fibre hypertrophy in humans, however, is not clear.

Satellite cell activation, proliferation and differentiation is considered to be regulated by a variety of factors during skeletal muscle repair and remodelling. These include inflammatory cytokines such as IL-6 (74) and TNF related weak inducer of apoptosis (TWEAK) (81), growth factors such as insulin-like growth factor 1 (IGF-1) (75) and myostatin (82–84), and transcription factors such as nuclear factor-kappa B (NF-κB) (85). The regulation of satellite cells by inflammatory signalling is particularly intriguing given inflammation is hallmark of myofibrillar injury (discussed in **1.3**). In particular, classical NF-κB signalling which is rapidly activated after myofibrillar injury (86) (discussed in **1.3**) has been demonstrated to promote satellite cell proliferation, but inhibit differentiation in regenerating skeletal muscle in part by degrading the MyoD protein (85). More recently, soluble factors secreted by infiltrating macrophages in injured skeletal muscle have been identified as regulators of satellite cell fate. Seven days following electrically stimulated eccentric contractions (which cause a greater extent of myofibrillar injury than eccentric contractions performed voluntarily), pro-inflammatory M1 macrophages which *in vitro* secrete TNF-α and IL-1β, localise with regions of injured skeletal muscle containing proliferating satellite cells. Conversely, anti-inflammatory M2 macrophages, which *in vitro* secrete TGFβ to promote satellite cell differentiation, localise with regenerating areas of skeletal muscle which contain differentiating satellite cells (87). Consequently, inflammation is a likely key regulator of satellite cell homeostasis to coordinate regeneration and remodelling of skeletal muscle and consequently contractile function.

1.2.6 Protein nutrition

In the late 1990s, the effect of protein nutrition on post-exercise mixed muscle protein synthesis rates was being investigated for the first time. Combing a stable isotope tracer approach with an additional continuous amino acid infusion (approximately 11 g of mixed amino acids per hour) was shown to increase post-exercise mixed muscle protein synthesis rates by 75% during a 3-h post-exercise period (from 0.0657 to 0.1442%·h^{-1}) (88). Muscle protein breakdown did not change (the post-exercise increase was suppressed by amino acid infusion), providing the first direct evidence in humans that resistance exercise and amino

acids synergistically promote a positive protein balance in skeletal muscle, at least during the 3 h post-exercise period. Following up on this, the same group demonstrated that a similar increase in post-exercise (4.5 h) mixed muscle protein synthesis rates could be achieved with the oral ingestion of 40 g of a mixed and essential amino acid drink (89).

Since these initial amino acid infusion and feeding intervention studies, subsequent studies have attempted to elucidate the optimal dose and timing of protein ingestion to maximally potentiate post-exercise myofibrillar protein synthesis rates. At a molecular level, amino acids synergistically and dose-dependently increase the post-exercise phosphorylation of upstream (protein kinase B (Akt)) and downstream (mTOR and ribosomal protein S6 kinase (p70S6K)) mTOR signalling proteins (90–94). Indeed, inhibition of mTOR signalling by rapamycin abolishes the 60% increase in resting mixed muscle protein synthesis rates induced by the ingestion of 10 g of essential amino acids (37). Under overnight fasted conditions, Moore and colleagues demonstrated a dose-response between whole egg protein and post-exercise mixed muscle protein synthesis rates, showing a progressive increase in muscle protein synthesis rates with 5, 10 and 20 g of protein, with no further increase with 40 g of protein (95). Following on from this, Witard and colleagues provided evidence of a dose-response relationship between whey protein ingestion and post-exercise *myofibrillar* protein synthesis rates (96). Using a [$^{13}C_6$]phenylalanine tracer and a unilateral resistance exercise approach, myofibrillar protein synthesis rates were measured for 4 h in response to 0, 10, 20 and 40 g of whey protein. Relative to 0 g (0.041%·h^{-1}), 20 (0.061%·h^{-1}) and 40 (0.064%·h^{-1}) g of whey protein increased myofibrillar protein synthesis rates with no difference between 20 and 40 g. Moreover, this was performed 3 h after a high-protein breakfast was consumed as opposed to overnight fasted conditions, thereby increasing the practical applications of these data. When resistance exercise training is combined with protein ingestion, protein potentiates the gains in fat-free mass (by 27%), mid-femur CSA (by 14%), type I (by 45%) and type II (by 54%) muscle fibre CSA and one repetition maximum strength (by 9 - 20%) compared to no supplementary protein ingestion (97, 98).

Muscle protein synthesis rates decline during postabsorptive or fasting conditions, such as during overnight sleep (99). As a consequence, combining resistance exercise with both post-exercise *and* pre-bed protein ingestion to support overnight muscle remodelling has been investigated. This was first demonstrated by Res and colleagues (100). In their study, participants performed evening leg resistance exercise and ingested 20 g of post-exercise whey protein. Immediately before sleep, participants consumed an additional 40 g of casein protein, and mixed muscle protein synthesis rates were measured overnight using a continuous [$^{2}H_{5}$]phenylalanine infusion whilst participants slept in the laboratory. Relative to a non-caloric placebo (0.048%·h^{-1}), overnight mixed muscle protein synthesis rates increased with 40 g of pre-sleep casein protein ingestion (to 0.059%·h^{-1}). A similar synthetic response of the myofibrillar protein fraction has since been demonstrated in elderly individuals (101). These data illustrate that the addition of pre-sleep casein protein is an effective means of maximising post-exercise protein synthesis rates.

As discussed in **1.2.1,** there are methodological difficulties that make measuring muscle protein breakdown in response to resistance exercise and nutrition challenging. Nonetheless, moderate elevations of plasma insulin achievable by consuming a mixed-meal or protein beverage have been shown to reduce muscle protein breakdown rates by ~50% at rest (102–104), and likely attenuate the increase in muscle protein breakdown rates when ingested following a bout of resistance exercise (105).

There are a limited number of studies applying deuterated water to determine if supplementary protein ingestion increases cumulative myofibrillar protein synthesis rates when exercise is performed across several days. In a series of studies, Davies and colleagues applied deuterated water for the first time to demonstrate that ~25g of whey protein in combination with a session of bilateral resistance exercise increases myofibrillar protein synthesis rates from rest (~0.065 %·h^{-1}) over a 5 h post-exercise period (to ~0.085 %·h^{-1}) (106). However, when translating this into a cumulative increase in myofibrillar protein synthesis rates over five days (involving three resistance exercise sessions performed on non-consecutive days), they reported no increase in myofibrillar protein synthesis rates by exercise *per se*. Additionally, no effect of daily ~25g whey protein

ingestion on cumulative myofibrillar protein synthesis rates was reported (107). By combining a bilateral exercise intervention with deuterated water to measure myofibrillar protein synthesis rates over multiple days, interindividual variation will be compound as differences in ecological factors between individuals such as dietary patterns and habitual physical activity will cumulatively influence myofibrillar proteins synthesis rates over the incorporation period. This can easily be addressed by using a unilateral exercise approach and correcting to the non-exercised contralateral control leg.

1.3 Skeletal muscle inflammation

1.3.1 Regulation of muscle protein synthesis by inflammation

Inflammation is an essential cellular process that plays a crucial regulatory role in the degeneration and regeneration of skeletal muscle under physiological and pathophysiological conditions. There are several signalling pathways in skeletal muscle which can act independently and in unison to promote inflammatory events. The p38 mitogen activated protein kinase (MAPK) is stimulated by cellular stress and pro-inflammatory cytokines and acts to increase gene expression associated with inflammation and regulate myogenesis in skeletal muscle (108). Additionally, the janus kinase (JAK)/signal transducer and activator of transcription (STAT) signalling pathway is also implicated in regulating transcription of inflammatory mediators in skeletal muscle upon activation by cytokine binding, particularly interleukin (IL) -6 (109). Finally, the most putatively important inflammatory signalling pathway in skeletal muscle is the NF-κB signalling pathway. Classical NF-κB signalling is primarily activated by the pro-inflammatory cytokines tumor necrosis factor-α (TNF-α) and IL-1β which initiate a signalling cascade leading to nuclear translocation of NF-κB and initiation of transcription (110). Activation of NF-κB signalling regulates the expression of a vast array of genes, associated with a diverse range of biological processes including both myogenic (85) and atrophic (111) pathways in skeletal muscle. Inflammatory NF-κB signalling, therefore, represents an intriguing contender as a regulator of muscle function and muscle mass in humans.

Our current mechanistic understanding of the effect of inflammatory signalling on muscle protein synthesis rates is still predominantly informed by cell and animal studies. Using the cytokine TWEAK to induce NF-κB signalling in cultured myotubes inhibits the activity of the phosphoinositide-3 kinase PI3K/Akt signalling pathway which was shown to reduce downstream mTOR and p70S6K phosphorylation (112). Additionally, cultured myotubes exposed to the acute phase C-reactive protein (CRP) demonstrated decreased Akt and p70S6K phosphorylation and a decrease in protein synthesis rates (113). However, TWEAK and CRP signalling increases in humans after resistance exercise, and as discussed resistance exercise increases mTOR phosphorylation and muscle protein synthesis (114, 115). In C2C12 myoblasts, TNF-α and IL-1β have been shown to inhibit differentiation into myotubes through the activation of NF-κB signalling (116) and human macrophages displaying a pro-inflammatory M1 phenotype have been shown to secrete TNF-α and IL-1β to decrease myotube formation *in vitro* (87), suggesting that inflammation impairs myogenesis. Indeed, inhibition of NF-κB increases the diameter of regenerating myofibers five days after cardiotoxin damage in mice (117). In humans, seven days after electrically stimulated eccentric contractions, M1 macrophages localise with proliferating satellite cells whereas anti-inflammatory M2 macrophages localise with differentiating satellite cells (87). This demonstrates a spatial and temporal association between inflammation and skeletal muscle anabolism after myofibrillar injury, but the *in vivo* significance of this in humans is not known.

Observationally, elevated systemic CRP concentrations are associated with a loss of lean body mass and the age-related loss of muscle mass (118–120). Moreover, it has been shown that NF-κB protein concentration in elderly individuals is four-fold higher than in young individuals and elderly individuals demonstrate an impaired ability to increase muscle protein synthesis rates in response to nutrition or resistance exercise (121, 122). To establish a causative link between inflammation and muscle protein synthesis in healthy humans, studies have used acute cytokine and endotoxin infusions to induce a systemic inflammatory response which is associated with the activation of the NF-κB signalling pathway. Declines in resting mixed muscle protein synthesis rates of ~36% and ~58% have been reported after infusion of endotoxin and IL-6, respectively. In contrast, infusion of TNF-α does not affect mixed muscle protein

synthesis rates (123–125). The decline in muscle protein synthesis rates with IL-6 infusion was attributed to a reduction in arterial amino acid availability due to increased amino acid uptake by other tissues, rather than an impairment in protein translation in skeletal muscle. Indeed, an amino acid and endotoxin co-infusion has been shown to increase mTOR phosphorylation compared to endotoxin infusion alone, suggesting the sensitivity of the mTOR signalling pathway to amino acid availability in skeletal muscle is not blunted under an inflammatory burden in young individuals (126).

The results from human studies using a pharmacological approach to dampen inflammation are also unclear. Ibuprofen and acetaminophen ingestion has been shown not to affect resting mixed muscle protein synthesis rates (7). After a bout of unliteral eccentric contractions which induce an inflammatory response in skeletal muscle (discussed in **1.4.2**), both ibuprofen and acetaminophen have been shown to prevent the increase in mixed muscle protein synthesis rates 24 h post-exercise (7). This would suggest the local inflammatory response in skeletal muscle induced by exercise promotes muscle protein synthesis; however, it is unknown how this affects muscle function. This is intriguing given the earlier discussion that an increase in protein synthesis could underpin the remodelling of myofibrillar injury and therefore, the recovery of muscle function. However, subsequent studies have found no change in mixed muscle protein synthesis rates after submaximal resistance exercise and ibuprofen ingestion in patients with knee osteoarthritis (127) or after eccentric contractions and a local indomethacin infusion in young individuals (128).

1.3.2 Regulation of muscle protein breakdown and atrophy by inflammation

The regulation of muscle protein breakdown and muscle atrophy by inflammation appears to be more robust. Cai and colleagues in 2004 were the first to demonstrate in mice that activation of NF-κB promoted skeletal muscle atrophy and identified using a gene microarray that this was primarily driven by a 3.3-fold upregulation in skeletal muscle MuRF1 mRNA expression (knockout of MuRF1 reversed ~50% of skeletal muscle atrophy) which was identified as a direct target of NF-κB (129). In denervated skeletal muscle, inhibiting NF-κB signalling has

been shown to prevent disuse atrophy and maintain muscle function, which was paralleled by a reduction in MAFbx and MuRF1 mRNA expression (117).

There is a lack of data concerning inflammation and muscle atrophy during short term disuse. Indeed, our current understanding is derived from studies investigating muscle atrophy in healthy individuals. In contrast, short-term limb immobilisation or reductions in physical activity most commonly occur consequent with injury or illness when systemic and local inflammation is elevated. In healthy individuals, the skeletal muscle mRNA expression of the NF-κB p50 subunit has been shown to increase seven days after unilateral leg immobilisation in young, healthy males. This occurred concomitant with an increase in MAFbx and MuRF1 mRNA expression, a 7% decline in quadriceps volume and a 36% decline in myofibrillar protein synthesis rates (69). These data suggest that immobilisation *per se* induces NF-κB signalling, but any causative role of inflammation on muscle protein turnover cannot be established without an intervention approach. Supplementation with omega-3 fatty acids, which can reduce inflammation (130), has been shown to result in an 8% decline in quadriceps volume, relative to a larger 14% decline in a placebo condition in young, healthy females (67). Interestingly, the decline in myofibrillar protein synthesis rates during immobilisation in the omega-3 condition (from ~1.55%·d^{-1} to ~1.35%·d^{-1}) was not different to the decline in the placebo condition (from ~1.55%·d^{-1} to ~1.25%·d^{-1}). Skeletal muscle MuRF1 mRNA expression was increased ~3 fold after three days of immobilisation in the placebo condition only thereby identifying a possible role for inflammation in driving an increase in muscle protein breakdown rather than a decline in myofibrillar protein synthesis rates during short term disuse.

In light of the absence of studies that empirically manipulate inflammation to delineate the effect of inflammation on skeletal muscle protein turnover, comparing disuse atrophy between healthy and clinical populations can provide some further insight. The decline in rectus femoris CSA in critically ill patients resident in an intensive care unit (ICU) area has been shown to decline by 13% after seven days (131). In contrast, a decrease of just 4.5% is reported after seven days of uninjured limb immobilisation in healthy individuals (132). Furthermore, the decline in vastus lateralis volume has been shown to decrease

by 29% after 60 days of immobilisation following an anterior cruciate ligament tear (133) whereas with strict bedrest of healthy volunteers, combined vastus lateralis and medialis volume has been shown to decrease by just 16% in an equivalent amount of time (134).

1.3.3 Critical illness and skeletal muscle inflammation

A specific instance where unrestrained muscle atrophy can have serious implications for long term functional outcomes is in critically ill patients in the intensive care unit setting. Critical illness is characterised by rapid skeletal muscle wasting in patients at the onset of intensive care unit (ICU) residency (131) and is associated with an increased length of ICU stay and mortality (135). Furthermore, muscle atrophy during ICU residency contributes to ICU acquired weakness (ICUAW). ICUAW is characterised by fatiguability and decreased aerobic performance which can persist for multiple years after discharge from hospital and is the key contributor to failed functional outcomes in survivors of critical illness (136, 137). Moreover, inflammation in critically ill patients is markedly elevated and is in itself a cause of prolonged ICU stays, low-grade organ dysfunction (138) and ICUAW (139). Thus, an advanced understanding of the aetiology of muscle wasting and inflammation in critically ill patients is crucial in developing interventions to improve survival and functional outcomes in survivors.

Relative to healthy controls, mixed muscle protein synthesis rates have been shown to decline (day one in ICU) (131), increase (median day four in ICU) (4) or not change (median day five and day seven in ICU) (131, 140) in patients during critical illness. This contrasts with the repeatable and robust decline in muscle protein synthesis rates in healthy individuals undergoing short-term limb immobilisation (3, 63). At the molecular level, the gene expression and protein phosphorylation of Akt, mTOR and p70S6K is increased in critically ill patients relative to healthy controls (median day 7.5 and day 3 in ICU) (141, 142).

In contrast to muscle protein synthesis, critically ill patients demonstrate a more consistent increase in muscle protein breakdown (5, 140). Indeed, the negative protein balance observed in critically ill patients that results in substantial skeletal muscle atrophy is primarily driven by an increase in muscle protein breakdown

as opposed to changes in muscle protein synthesis (5, 131, 140). Accordingly, increased skeletal muscle MuRF1 and MAFbx protein and mRNA expression is a consistent observation in critically ill patients (131, 140). It is therefore interesting to speculate if the large increase in muscle protein breakdown consequent with a critical illness (possibly mediated to a degree by inflammatory signalling) is in part an adaptive mechanism to increase amino acid availability in order to support immune function and wound healing. Such an increase in amino acid availability could consequently stimulate an increase in muscle protein synthesis which would explain elevated mTOR signalling and the tendency for muscle protein synthesis rates to remain unchanged, despite prolonged bed rest and elevated inflammation, in critically ill patients. This process has been described in burns patients where amino acids are redistributed from skeletal muscle to superficial tissues to support healing (143–145). Still, it does not appear to have been discussed in the context of critical illness or indeed exercise-induced myofibrillar injury.

There are likely several factors in addition to bed rest that are driving the profound muscle wasting that is observed in critically ill patients, including hypoxia (146), glucocorticoid administration (147) and inflammation. C-reactive protein has been shown to positively correlate with the loss of muscle volume in critically ill patients, and myofibre necrosis and macrophage infiltration has been identified in ~50% of critically ill patients, illustrating the likely role of inflammation-mediated muscle protein breakdown in critically ill patients (131). Experimental models demonstrate that NF-κB activity is increased within four h of the induction of sepsis (148), a common presentation in critically ill patients (149). This may be compounded by comorbidities such as diabetes which can independently regulate NF-κB signalling (150, 151). Furthermore, treatment of septic rats with curcumin dose-dependently reduces and indeed abolishes muscle protein breakdown in parallel with a marked reduction in NF-κB DNA binding but without changes in MuRF1 or MAFbx expression (152). However, there are no intervention studies that have investigated the relationship between inflammation and muscle protein turnover in critically ill patients and how this may improve functional outcomes in survivors.

Muscle contraction is associated with a reduction in inflammation and all-cause mortality in healthy individuals and patients with chronic disease (151, 153, 154). In type II diabetics, eight weeks of endurance exercise training has been demonstrated to increase resting protein expression of the NF-κB p50 subunit (considered a repressor of NF-κB-mediated translation) and the NF-κB inhibitors IκBα and IκBβ, restoring them to comparable levels to those seen at baseline in healthy controls (151). In turn, TNF-α protein content, which is a potent inducer of proteolysis (155), was concomitantly decreased in people with type II diabetes following training. It has also been shown that seven days of neuromuscular electrical stimulation of skeletal muscle in critically ill patients increases mTOR phosphorylation and prevents the 24% decrease in type II myofibre CSA observed in the contralateral control leg (142). However, how muscle contraction during ICU residency affects acute skeletal muscle inflammation is not known. Crucially there are no prospective studies that have investigated the effect of exercise performed during ICU residency on persistent skeletal muscle inflammation and ICUAW in survivors of critical illness.

In summary, although not a consistent observation, there are data to suggest that inflammation may impair protein synthesis rates in healthy individuals during ambulation. There are, however, no empirical data concerning the effect of inflammation on protein synthesis rates during short-term disuse. Inflammation is also associated with an increase in muscle protein breakdown in clinical populations. Although this is also likely to occur in healthy individuals, there is currently not enough direct evidence to conclude this. A schematic which summarises our current understanding of the relationship between inflammation and muscle protein turnover is presented in **Figure 1.1**. There is a need for controlled intervention studies in healthy and clinical populations that manipulate skeletal muscle inflammation and determine the consequent changes in myofibrillar protein synthesis and breakdown. Importantly, the functional implications of this with regards to changes in muscle volume and muscle function require investigation.

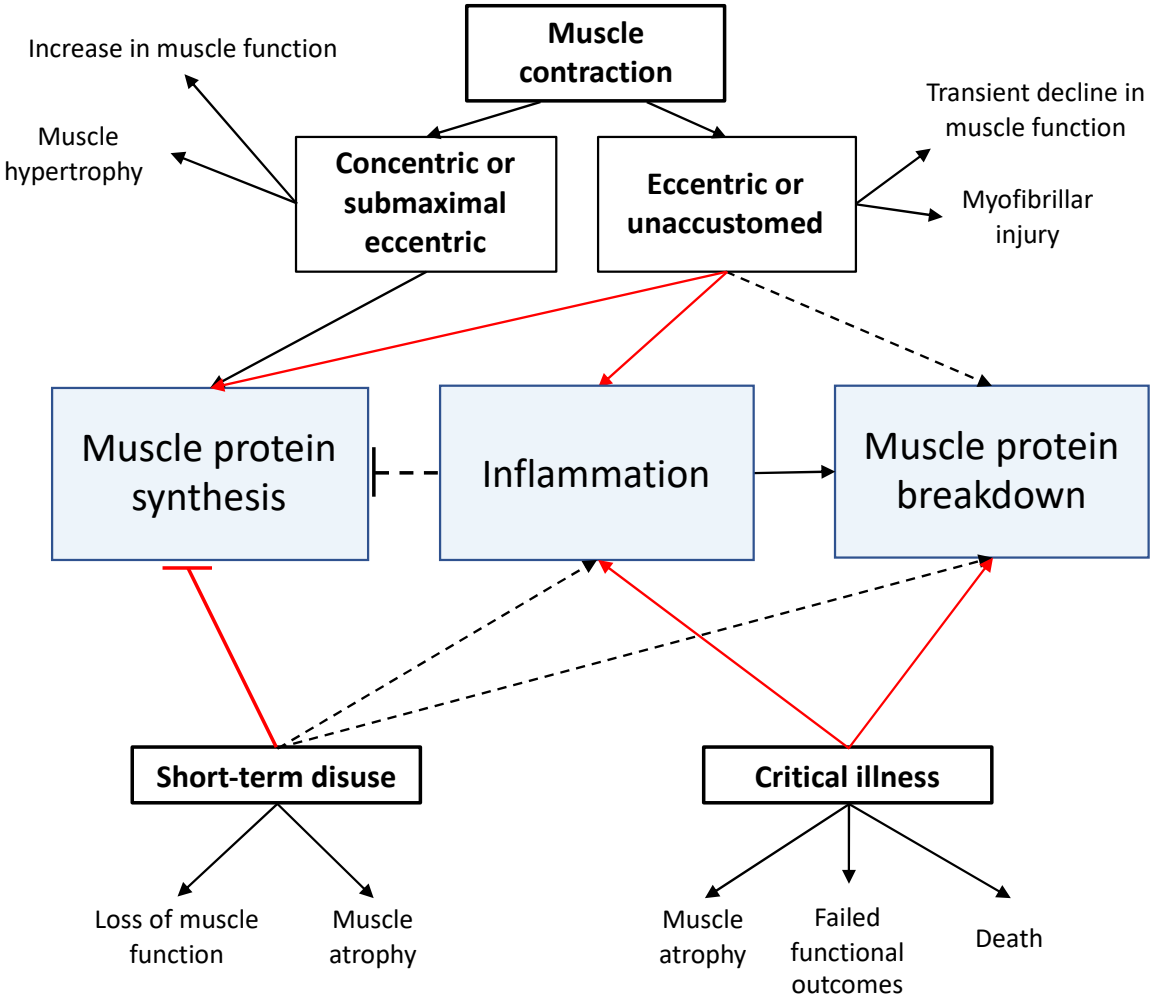

Figure 1.1. A schematic to illustrate the proposed relationship between muscle protein synthesis, muscle protein breakdown and inflammation in skeletal muscle and how this is regulated by contraction, disuse and critical illness. Dashed lines represent a theoretical relationship which has not yet been investigated in humans. Red lines have been used to illustrate relationships for which there is strong empirical evidence in humans. Arrows ending in a flat line represent a downregulation.

1.4 Eccentric contractions as a model to induce muscle inflammation

1.4.1 Eccentric contractions cause muscle injury

Eccentric contractions occur when the muscle is lengthened under load, which can lead to damage. It is largely agreed that the initial phase of eccentric contraction-induced muscle damage results from the uncontrollable lengthening of weak sarcomeres on the descending limb of the length-tension curve where sarcomeres inhomogeneities develop. At this point, tension is then absorbed by passive non-contractile proteins such as Z-lines which become deformed (156). When the muscle relaxes, not all overstretched sarcomeres re-interdigitate leading to damage of the sarcolemma, transverse tubules and sarcoplasmic reticulum (157, 158).

The magnitude of unaccustomed experimental muscle damage and the subsequent inflammatory response is dependent on the eccentric contraction protocol used. Downhill or downstairs running and resistance exercise with equal concentric and eccentric loading phases (i.e., traditional isotonic weight stack resistance exercise) cause mild muscle damage (<20% decline in muscle function) which is typically insufficient to induce an inflammatory response. Conversely, single-joint, maximal eccentric contraction protocols have repeatedly been shown to cause moderate to severe muscle damage (20 – 50% to >50% decline in muscle function, respectively) and leukocyte accumulation (41). Indeed, 300 maximal eccentric knee extension contractions are commonly used to damage the quadriceps muscle group (47, 114, 159–165). Using this protocol, myofilament disorganisation and loss of Z disk integrity are reported immediately post-exercise (47), with the largest decrement in muscle function occurring after 48 h. Full recovery of muscle function typically occurs within seven days (41). Notably, single joint eccentric contraction protocols can be performed unilaterally. This allows the contralateral leg to act as an internal control and changes in the “injured” leg can be corrected to the contralateral leg to reduce interindividual variation, in addition to intraindividual variation between repeated measurements.

1.4.2 Eccentric contractions cause inflammation

Eccentric contractions induce a robust degenerative and regenerative inflammatory response in skeletal muscle. Jones and colleagues (166) and Round and colleagues (166) first reported mononuclear cell infiltration in human skeletal muscle 5 – 14 days after eccentric elbow flexor exercise. Since, radiolabelled leukocytes, primarily neutrophils, have been shown to accumulate in micro vessels 1 – 24 h (167, 168), and in the extracellular space 24 and 48 h following eccentric contractions (159, 163, 169). Macrophages typically accumulate after 24 h and remain present for up to seven days or longer in extreme cases (159, 165, 170, 171). Evidence, predominantly from mice using eccentric contraction protocols, has shown macrophages displaying an M1 phenotype infiltrate tissue first (12 – 48 h) and participate in mounting an inflammatory response, degradation and phagocytosis of damaged myofibres and the stimulation of satellite cell proliferation by the delivery of cytokines and growth factors (IL-1, IL-6, TNF-α (at high levels) and vascular endothelial growth factor (VEGF)). Macrophages then transition to an M2 phenotype (3 – 7 days) to resolve inflammation and stimulate satellite cell entry into terminal myogenesis by producing transforming growth factor-beta (TGF-β), TNF-α (at low levels) and insulin-like growth factor 1 (IGF-1) (87, 172). Thus, the correct temporal regulation of macrophage phenotype is considered necessary for successful regeneration of skeletal muscle following damage. Additionally, this illustrates the large number of effectors that regulate the inflammatory response in skeletal muscle and highlights the importance of a widespread approach to investigating changes in the mRNA or protein expression of inflammatory pathways.

Hyldahl and colleagues in 2011 provided the first evidence that eccentric contractions induce NF-κB signalling in skeletal muscle (86). This was achieved using a microarray and gene ontology approach, which identified NF-κB signalling to be the most enriched pathway in skeletal muscle three h after eccentric contractions. From a methodological perspective, this study illustrates the power of a gene array approach coupled with ontological analysis in identifying novel targets that regulate skeletal muscle protein turnover and function under conditions of elevated inflammation. Using this approach, Hyldahl and colleagues identified a panel of genes associated with up and downstream signalling of NF-κB. As a result, this gene panel can be used as a measurement

of NF-κB mediated transcription and inflammatory signalling in skeletal muscle. Indeed, Hyldahl and colleagues only investigated NF-κB signalling three hours after eccentric contractions. Consequently, the temporal expression of these genes and how they relate to changes in protein turnover, muscle function and muscle mass is mostly unknown. More recently, it has been demonstrated that NF-κB phosphorylation is increased 2 and 8 days following 300 eccentric contractions (164, 165), illustrating prolonged NF-κB signalling after eccentric contractions. Furthermore, NF-κB signalling induced by eccentric contractions appears to be associated with muscle function given that peak NF-κB phosphorylation two days after eccentric contractions has been shown to coincide with the peak decline in muscle function (164, 165).

1.4.3 Role of nutrition in accelerating recovery from injury

Combining measurements of muscle protein synthesis and inflammation with a nutritional intervention approach targeted at manipulating the recovery of muscle function can provide novel insight into the mechanistic regulation of muscle function.

The efficacy of protein ingestion to accelerate the recovery of muscle function after eccentric contractions is mixed, with studies demonstrating positive (164, 173–175) or no (173, 176, 177) effects. However, only one study has investigated protein ingestion after a high-volume, single joint eccentric contraction protocol. In this study, Draginidis and colleagues report that daily ingestion of 20 g of milk protein (80 g on the day of eccentric contractions) accelerated the recovery of muscle function to baseline within three days of eccentric contractions. In contrast, muscle function was impaired for five days with an isocaloric carbohydrate placebo (164). Interestingly, protein ingestion attenuated NF-κB phosphorylation after 48 h concomitant with the peak decline in muscle function, suggesting that inflammation may exacerbate the impairment in muscle function after injury. Muscle protein synthesis was not measured, so it is unknown how this was regulated by NF-κB signalling.

Polyphenols are also gaining a reputation for their role in accelerating the recovery of muscle function following eccentric contractions. Polyphenols are ubiquitous within plants where they offer resistance to pathogens and

environmental stressors (178). *In vitro*, fruit-derived polyphenols have been shown to dose-dependently attenuate endotoxin-induced NF-κB mRNA expression and p65 phosphorylation (179, 180), although this is yet to be investigated in skeletal muscle. In humans, fruit-derived polyphenol ingestion reduces systemic cytokine concentrations associated with NF-κB signalling (181). Under inflammatory conditions after eccentric contractions, polyphenol ingestion has been shown to accelerate recovery of muscle function with extracts of cherry (182) and pomegranate (183–185) among the most efficacious. Once again, however, it is not known if this is related to changes in inflammation or muscle protein turnover.

Ingestion of protein and polyphenols in combination may synergistically accelerate recovery of muscle function following muscle-damaging eccentric contractions, possibly more rapidly than with the ingestion of protein or polyphenol in isolation. Indeed, a recent publication demonstrated the decrement in muscle function with ingestion of a combined protein and polyphenol drink was numerically less than with ingestion of protein alone (186). Although this was not significant, only the initial 24 h period was measured. Therefore, it is interesting to speculate if this difference would be more pronounced after 48h when the largest loss of muscle function occurs.

1.5 Summary and thesis aims

Eccentric contractions induce myofibrillar injury, an increase in myofibrillar protein synthesis rates and a robust inflammatory response and NF-κB signalling in human skeletal muscle. This unilateral eccentric contraction model can be applied to investigate changes in muscle protein turnover and how they relate to changes in muscle function under controlled inflammatory conditions. Combining the eccentric contraction model with a time course investigation using nutritional and short-term disuse interventions will provide novel insight into the regulation of myofibrillar protein turnover by inflammation, and importantly the significance of this with respect to functional endpoints including muscle contractile function and muscle mass. Additionally, critical illness can be used to investigate the role of pathophysiological inflammation and muscle protein turnover to delineate the regulation of muscle function further.

In **Chapter 3** this thesis first tested the hypothesis that myofibrillar protein synthesis rates and transcriptional NF-κB signalling are associated with the recovery of muscle function after eccentric contractions. This was achieved by applying deuterated water to measure myofibrillar protein synthesis rates and semi-targeted RT-PCR gene array with ontological analysis in combination with a protein-polyphenol nutrition approach to accelerate the recovery of muscle function. **Chapter 4** developed the findings of **Chapter 3** by testing the hypothesis that the loss of muscle function after eccentric contractions is accompanied by an increase in skeletal muscle mRNA expression associated with muscle protein breakdown. **Chapter 5** tested the hypothesis that inflammation induced by eccentric contractions would exacerbate skeletal muscle disuse atrophy during seven days of leg immobilisation in healthy individuals by promoting a further decline in free-living myofibrillar protein synthesis rates and increasing skeletal muscle mRNA expression associated with muscle protein breakdown. **Chapter 6** tested the hypothesis that an exercise intervention administered to critically ill patients during ICU residency would reduce inflammatory NF-κB signalling, and be associated with improved functional outcomes in survivors six months after ICU admission. Finally, **Chapter 7** tested the hypothesis that myofibrillar injury induced by unaccustomed resistance exercise at the onset of a 30 session resistance exercise training programme delays the time course of gains in muscle function. Consequently, it was hypothesised that a protein-polyphenol

intervention would accelerate the early remodelling of myofibrillar injury resulting in more rapid gains in muscle function and a greater post-training increase in skeletal muscle hypertrophy. This thesis is concluded with a general discussion in **Chapter 8**.

Chapter 2

General methods

2.1 Human volunteers

Healthy males and females were recruited by advertisement and word of mouth from the student, and general population of Exeter to participate in studies presented in **Chapters 3**, **4**, **5** and **7** of this thesis. These studies were approved by The University of Exeter's Sport and Health Sciences Research Ethics Committee in accordance with the Declaration of Helsinki (187). Following a thorough written and verbal explanation of the study purpose, procedures and potential risks, participants gave their written consent to participate. Participants were made aware they could withdraw from the study at any time. Participants were recreationally active, defined as participating in sporting activities for more than two h per week but had not engaged in structured exercise training in the six months preceding participation. Before commencing participation in a study, participants underwent a routine screening visit and completed a general health questionnaire to determine eligibility for the study (i.e., absence of any musculoskeletal injury, medication use, diagnosed metabolic or cardiovascular impairment, or motor disorders). Individuals routinely consuming nutritional supplements and non-steroidal anti-inflammatory drugs or who had a habitual protein intake of <0.8 $g \cdot kgBM^{-1} \cdot d^{-1}$ were excluded from participation.

Participants in **Chapter 6** were recruited from 4 intensive care units (ICUs) at Královské Vinohrady University Hospital (FNKV), Prague, Czech Republic. This study was approved by the Královské Vinohrady University Hospital Research Ethics Board in accordance with the Declaration of Helsinki (187). All patients admitted to participating ICUs were screened daily by research nurses, and investigators approached all eligible patients or their representatives within 72 hours of ICU admission.

2.2 Functional testing procedures

Tests of muscle function and muscle soreness were performed in **Chapters 3**, **4**, **5** and **7**.

In **Chapters 3**, **4** and **7,** exercise was performed on an isokinetic dynamometer (Biodex Medical Systems, Shirley, New York, USA). Participants were seated with 85^{o} of hip flexion with the lateral epicondyle of the femur aligned with the axis

of rotation of the dynamometer and with a Velcro strap securing the ankle to the dynamometer attachment. The length of the dynamometer attachment was the same for the right and left legs to standardise the moment arm. Extraneous movement was restrained using shoulder, hip and thigh straps. The Biodex configuration was recorded during the familiarisation, and the Biodex configuration remained the same for each participant for sequential tests. Torque data were sampled at 2000 Hz using an analogue-to-digital converter (Power1401-3A, Cambridge Electronic Design Ltd., Cambridge, UK **Chapters 3** & **4**; PowerLab 4/26, AD Instruments, Sydney, Australia; **Chapter 7**) connected to a computer running Spike2 software (Cambridge Electronic Design Ltd., Cambridge, UK; **Chapters 3** & **4**) or LabChart (AD Instruments, Sydney, Australia; version 8; **Chapter 7**) to render a torque-time curve, which was then low-pass filtered at 100 Hz during off-line analysis.

2.2.1 Eccentric contraction protocol

Studies in **Chapters 3**, **4** and **5** used a voluntary maximal, unilateral knee extensor eccentric contraction protocol to induce myofibrillar injury and initiate an inflammatory response in the knee extensor muscles. Participants performed a total of 300 eccentric contractions (10 sets of 30 contractions); a number of eccentric contractions previously shown to induce ultrastructural sarcomere injury and leukocyte infiltration as determined by electron microscopy and immunohistochemistry, respectively (47, 159). Each contraction was performed at $60^{o}\cdot sec^{-1}$ over an 80^{o} range of motion which ended at full voluntary knee flexion to induce maximal muscle injury (188). Each set was separated by 2 minutes of passive rest. Participants were instructed to resist the movement maximally throughout the full range of motion in every repetition and were provided with verbal encouragement to ensure maximal effort.

2.2.2 Knee extensor isometric torque

Knee extensor isometric torque was measured in **Chapters 3**, **4** and **7**. Isometric torque was measured unilaterally at 75^{o} of knee flexion (where 0^{o} is equal to maximal voluntary knee extension). Participants first performed an incremental warm-up of four submaximal, three-second contractions at 50%, 50%, 75% and

90% of their perceived maximum, with 30 seconds of rest between contractions. Subjects then performed three maximal, three-second knee extensor contractions, with 60 seconds of rest between contractions. Subjects were provided with verbal encouragement to ensure a maximal effort. The peak instantaneous isometric torque achieved after three maximal contractions was used to represent isometric torque.

2.2.3 Knee extensor isokinetic torque

Knee extensor isokinetic torque was measured in **Chapter 7**. Isokinetic knee extensor contractions were performed unilaterally at $75^{o} \cdot sec^{-1}$ through an 80^{o} range of motion equidistant from voluntary maximal knee extension and flexion. Participants first completed a five-repetition submaximal warm-up followed by three maximal contractions. Subjects were provided with verbal encouragement to ensure a maximal effort. The peak instantaneous isokinetic torque achieved after three maximal contractions was used to represent isometric torque.

2.2.4 Knee extensor isokinetic work

Knee extensor isokinetic work was measured in **Chapters 3**, **4** and **7**. Isokinetic knee extensor contractions were performed unilaterally at $75^{o} \cdot sec^{-1}$ through an 80^{o} range of motion equidistant from voluntary maximal knee extension and flexion. Participants first completed a five-repetition submaximal warm-up followed by 30 maximal contractions. Subjects were provided with verbal encouragement to ensure a maximal effort. The area under the torque-time curve, which represents total work performed, was used to represent isokinetic work.

2.2.5 Knee extensor 1-RM testing

Unilateral knee extensor one repetition maximum (1-RM) testing was performed in **Chapter 5** on a standard leg extensor machine using an incremental multiple repetition testing procedure. After two warm-up sets of eight and four repetitions at self-determined 25% and 50% of 1-RM, respectively, single repetitions at 1-RM were attempted. The weight was increased incrementally until no additional weight could be lifted, with each attempt separated by a 2-min rest. The final 1-RM lift was taken as the heaviest repetition that was completed with the correct

technique where a full range of motion was achieved. All exercise was performed unilaterally first by the immobilised leg then by the control leg. Verbal encouragement was provided throughout all exercise to ensure maximal effort.

2.2.6 Visual analogue scale

Muscle soreness was measured in **Chapter 3** using a visual analogue scale (VAS). VAS are frequently used to assess exercise-induced muscle soreness and have been reported to show good reliability (189, 190). Muscle soreness was measured by asking participants to stand from a seated position and mark the sensation of pain induced by this movement on a 100 mm visual analogue scale anchored by “no pain” at 0 mm and “worst possible pain” at 100 mm. Muscle soreness was quantified as the distance from 0 mm using a ruler.

2.2.7 Pressure pain threshold

Pressure pain threshold (PPT) of the vastus medialis, vastus lateralis and rectus femoris muscles was measured in **Chapter 3** using algometry. The PPT method has been reported to show good reliability when measuring the pain threshold of skeletal muscle (191, 192). PPT was measured by applying a continually increasing pressure of 10 $nm \cdot s^{-1}$ through the 1 cm diameter probe of a pressure algometer (Wagner Instruments, Riverside, USA) to the mid-belly of the vastus medialis and rectus femoris, and distal half of the vastus lateralis muscles. Participants were instructed to indicate when the sensation of pressure transitioned to a sensation of pain at which point the corresponding force was recorded. Algometry was performed on the muscle of both legs sequentially, and the average of three repeats for each muscle was taken.

2.3 Dietary analysis and control

2.3.1 Habitual diet analysis

In **Chapters 3**, **4**, **5** and **7**, all participants completed a three-day diet diary (two weekdays and one weekend day) before the beginning of the study periods to determine eligibility and habitual energy and macronutrient intake. All dietary

intake data was analysed using online software (Nutritics, Swords, Co. Dublin, Ireland).

2.3.2 Dietary control

In **Chapters 3**, **4** and **7**, participants were provided with a fully controlled diet during all of (**Chapters 3** and **4**) or part of (**Chapter 7**) the study period. Diets were weighed out and prepared in a metabolic kitchen. Participants received all food products in individual packets and received step-by-step recipes. All meals and snacks were provided, whereas water and non-caloric drinks were allowed ad libitum. Caffeinated drinks were permitted before midday only. Energy requirements were calculated as the basal metabolic rate (193) multiplied by an activity factor of 1.6. Daily protein intake was standardised at 1.2 $g \cdot kgBM^{-1} \cdot d^{-1}$ (~17% of energy intake) with the remaining calories being contributed by fat (~33% of energy intake) and carbohydrate (~50% of energy intake). Compliance with the nutritional intervention was assessed via completed food diaries, returned food containers, and daily communication with the participants.

2.4 Deuterated water ingestion

In **Chapters 3, 4, 5** and **7** orally consumed deuterated water (D_2O; 70 atom percent; Cambridge Isotope Laboratories Inc, Tewksbury, Massachusetts, USA) was used to measure free-living myofibrillar protein synthesis rates. Upon oral ingestion, D_2O equilibrates with the body water pool within ~two hours in humans (16), allowing ^2H-labelling of carbon-bound hydrogens of amino acids to occur intracellularly via transamination reactions. All amino acids can become ^2H-labelled at their α-carbon position, but alanine is turned over rapidly (194) and has 4 potential ^2H-labelling sites (both α- and β- hydrogens) (195) and is therefore relatively easy to detect using pyrolysis-isotope ratio mass spectrometry (IRMS). The total ^2H hydrogen labelling of alanine is ~3.7 times that of ^2H-labelled body water (196), and this difference in ^2H labelling of the precursor (body water) and product (myofibrillar protein) is accounted for when using the precursor-product equation to calculate myofibrillar protein synthesis rates (**2.10 Calculations**).

The deuterated water dosing protocol consisted of 1 loading day of 70% D_2O to label the body water pool to ~0.6%, followed by consecutive maintenance days to maintain steady 0.6% body water ^2H labelling for the duration that measurements of myofibrillar protein synthesis rates are required. In **Chapters 3, 4** and **7** this was achieved by dosing deuterated water relative to body mass as total body water (i.e. the precursor pool) is primarily influenced by body mass (197). Based on the assumption that the body water pool contributes 60-70% body mass in healthy lean individuals (197), participants ingested a total of 6 ml·kgBM^{-1} of 70% D_2O during an 8 hour loading period. To minimise the risk of participants experiencing symptoms of vertigo or nausea, the total volume was separated into eight doses and ingested 1.5 hrs apart (196). Participants attended the laboratory following an overnight fast at ~0800 h and ingested the first dose shortly after arriving. Breakfast was provided after the first dose was consumed and participants remained in the laboratory until the fourth dose was consumed (i.e., 1230 h), at which point they returned home and were instructed to consume the remaining four doses every 1.5 hrs apart, with the last dose consumed at 1830 h. To maintain body water ^2H labelling at 0.6%, a daily maintenance dose of 0.54 ml·kgBM^{-1} of 70% D_2O was consumed by the participant at home upon waking. This dose was calculated based on the assumption that body water turns over at 7 – 10 %·d^{-1} (198).

In **Chapter 5**, deuterated was not dosed relative to body mass to maintain methodological consistency with a data set collected by our research group, which was used as the control in this study. In this instance, the dosing protocol was the same as discussed above, but participants ingested an absolute 400 mL of 70% D2O on the loading day (i.e., 8 x 50 mL doses), and 50 mL on the maintenance days.

2.5 Venous blood sampling

Chapters 3, 4, 5 and **7** involved venous blood sampling during the experimental visit(s). Participants rested semi-supine in a bed whilst a butterfly needle was inserted into a superficial antecubital vein, and approximately 10 mL of venous blood was drawn into a 10 mL vacutainer. For the collection of blood plasma, blood was drawn into lithium heparin-coated vacutainers and, after immediate

centrifugation (2850 x*g* at 4°C for 10 min), the plasma fraction was aliquoted into individual microcentrifuge tubes and stored at -80 °C until further analysis.

2.6 Blood and saliva analysis

2.6.1 Body water deuterium enrichment

In **Chapters 3, 4** and **7**, the measurement of the ^{2}H enrichment of body water was performed by Elemtex Ltd (Gunnislake, UK). Plasma was initially centrifuged to remove any suspended material, and 500 µL was aliquoted into glass HPLC vials. Hydrogen isotope ratios ($^{2}H/^{1}H$) of plasma were determined in triplicate by injecting samples into a high-temperature conversion elemental analyser (TCEA Flash 2000, ThermoFisher Scientific, Waltham, MA, USA) coupled to an IRMS (Delta V, ThermoFisher Scientific). Raw isotope ratio values were normalised with in house reference materials calibrated to Vienna Standard Mean Ocean Water (VSMOW). In **Chapter 5***,* body water deuterium enrichment was measured in saliva samples on an automated on-line gas preparation system. A ThermoFisher Delta V Advantage Isotope Ratio mass spectrometer (IRMS) (Bremen, Germany) equipped with a Finnigan GasBench II (Thermo Fisher Scientific, Waltham, MA, USA), was used for stable hydrogen isotope ratio measurements. After uncapping a 12 mL Exetainer (Labco Limited, Lampeter, UK), 5 mg of activated charcoal (Thermo Fisher Scientific) and 200 mg of copper powder (Thermo Fisher Scientific) were introduced into the Exetainer followed with a platinum catalytic rod (Thermo Fisher Scientific). The activated charcoal and copper powder were added to remove any potential contaminants in the samples that might poison the platinum catalyst. After putting 200 uL of saliva into the Exetainer, the Exetainer was recapped and placed into the GasBench II and flushed with a Helium/Hydrogen gas mixture. The samples were allowed to equilibrate for 4 hours before the gas from the vial headspace was sampled by the automated analyser and introduced in to the isotope ratio mass spectrometer for analysis. A standard calibration curve was prepared using 99.9% deuterium-enriched water (Sigma-Aldrich, St. Louis, MO, USA), and the deuterium (2H) enrichment in duplicate saliva samples was determined (199).

2.6.2 Plasma creatine kinase

In **Chapters 3** and **4**, creatine kinase activity was analysed in blood plasma by Clinical Chemistry at the Royal Devon and Exeter Hospital using a photometric assay and measured using a Cobas 8000 automated analyser (702 module) (Roche Diagnostics, USA).

2.6.3 Plasma C-reactive protein

In **Chapters 3** and **4**, C-reactive protein concentration was analysed in blood plasma by Clinical Chemistry at the Royal Devon and Exeter Hospital using an immunoturbidimetric assay and measured using a Cobas 8000 automated analyser (702 module) (Roche Diagnostics, USA).

2.6.4 Plasma cytokine concentrations

In **Chapter 3**, the protein expression of IL-1β, IL-1ra, IL-6, IL-8, IL-10, IL-33, CCL2 and TNF-α was measured in blood plasma using a multiplex assay according to the manufacturer's instructions and using manufacturer supplied reagents and antibodies (U-PLEX, Meso Scale Diagnostics LLC (MSD), Rockville, Maryland, USA). This assay uses biotinylated antibodies coupled to U-PLEX Linkers, which self-assemble onto unique spots on the U-PLEX plate. After analytes in the sample bind to the antibodies, detection antibodies conjugated with electro chemiluminescent labels (MSD GOLD SULFO-TAG) bind to the analytes to complete the sandwich immunoassay. The plate is placed into an MSD instrument where the amount of analyte present in the sample is measured against a standard curve.

Briefly, 200 uL of antibody was added to 300 uL of linker, vortexed and incubated at room temperature for 30 minutes. Following incubation, 200 uL of stop solution was added, the solution was vortexed and centrifuged for a further 30 minutes. Next, 600 uL of each solution was combined into a single tube, and the total volume was made up to 6 mL with stop solution. Fifty uL of the solution was added to each well of the 96 well U-PLEX plate, and the plate was sealed and shaken at room temperature for 1 h (750 rpm). The plate was then washed 3 times with wash buffer. The following day, 250 uL of a diluent supplied by the manufacturer was added to a standard and incubated for 30 minutes. Eight standards were

then prepared using a serial dilution. Plasma samples were then defrosted, vortexed and centrifuged (1000 x *g* at 4ºC for 5 min) and then diluted in an equal volume of diluent. Then, 25 uL of the standards and samples were added in duplicate to the U-PLEX plate, incubated for 1 hour with shaking (750 rpm), and washed 3 times as described above. Sixty uL of each detection antibody was then combined and made up to a total volume of 6 mL with diluent, and 50 uL of this solution was added to each well, and the plate was incubated for 1 h with shaking (750 rpm). The plate was then washed three times, and 150 uL of read buffer was added to each well, and the plate was placed into the MSD instrument to measure the concentration of each cytokine against a standard curve.

2.7 Skeletal muscle sampling

All studies (**Chapters 3, 4, 5, 6** and **7**) involved skeletal muscle sampling during the experimental visit(s). Muscle samples were obtained from the vastus lateralis using the percutaneous needle biopsy technique (200) modified with suction whilst participants rested in a supine position. Briefly, the skin within a 10 cm perimeter of the area to be sampled was shaved (if necessary) and sterilised using an iodine solution. After that, an incision <1 cm was made in the skin and fascia under a local anaesthetic (2% lidocaine hydrochloride, B. Braun, Melsungen, Germany) and a 5 mm gauge Bergström biopsy needle was used to sample ~100 mg wet weight muscle tissue. The muscle sample was frozen immediately in liquid nitrogen-cooled isopentane (Sigma-Aldrich Company Ltd., Gillingham, UK) and then the isopentane was rapidly blotted off, and the muscle sample was stored at -80 °C until further analysis. Consecutive biopsies obtained from the same muscle were performed a minimum of 2.5 cm proximally or medially from a previous biopsy, and the needle was angled so as not to sample any tissue in proximity to previous biopsies. Approximately 50 mg of wet muscle tissue was used for the determination of myofibrillar bound [^{2}H]alanine (**Chapters 3, 4, 5** and **7**) and total and phosphorylated mammalian target of rapamycin (mTOR) protein (**Chapter 4**) and ~20 mg was used for the determination of mRNA expression (**Chapters 3, 4, 5** and **6**).

2.8 Muscle analysis

2.8.1 Myofibrillar amino acid extraction

In **Chapters 3, 4, 5** and **7**, the myofibrillar fraction of skeletal muscle tissue samples was extracted to measure the enrichment of myofibrillar protein-bound [^{2}H]alanine. To do this, ~50 mg of whole frozen muscle was mechanically homogenised in 7.5 volumes of ice-cold homogenisation buffer (Tris-HCL (pH 7.4) 50 mM, EDTA 1 mM, EGTA 1 mM, β-glycerophosphate 10 mM, NaF 50 mM, activated sodium orthovanadate 0.5 mM and 1 complete mini protease inhibitor cocktail tablet per 50 mL of buffer [Roche Holding AG, Basel, Switzerland]). Homogenised samples were centrifuged (2200 x *g* at 4^{o}C for 10 min) and the supernatant containing the sarcoplasmic protein fraction was removed and stored at -80^{o}C for western blotting analysis. The remaining pellet was washed in 500 µL ice-cold homogenisation buffer, centrifuged (700 x *g* at 4^{o}C for 10 min), and solubilised (750 µL 0.3 M sodium hydroxide at 50^{o}C for 30 min). After centrifugation (10,000 x *g* at 4^{o}C for 10 min), myofibrillar proteins were precipitated from the supernatant by adding 500 µL of 1 M perchloric acid and vortexing for 30 seconds and pelleted by centrifugation (700 x *g* at 4^{o}C for 10 min). The pellet was washed twice in 70% ethanol and amino acids were hydrolysed in 2 mL of 6 M hydrochloric acid at 110^{o}C for 24 hours. The samples were subsequently dried under a vacuum (Savant^{TM} SpeedVac^{TM}, ThermoFisher Scientific), reconstituted in 3 mL 25% acetic acid, passed over cation exchange resin columns (100 – 200 mesh; H^+ form; Dowex 50WX8; Sigma-Aldrich Company Ltd., Gillingham, UK) and eluted with 6 M NH_4OH, before being dried again under vacuum. Samples were resuspended in 1 mL distilled water and 1 mL 0.1% formic acid in acetonitrile, centrifuged (10,000 x *g* at 4°C for 3 min), and the supernatant was aliquoted, dried under a vacuum and stored at -20°C.

2.8.2 Myofibrillar protein-bound [^{2}H]alanine quantification

Amino acids were derivatised by adding 50 µL N-tert-Butyldimethylsilyl-N-methyltrifluoroacetamide (MTBSTFA) + 1% tert-butyl-dimethylchlorosilane and 50 µL acetonitrile and vortexed and heated at 95°C for 40 min. The samples were then transferred to a gas chromatography vial. D4-alanine enrichment was analysed using a ThermoFisher Delta V Advantage Isotope Ratio mass

spectrometer (IRMS) (Bremen, Germany) fitted with a Trace 1310 gas chromatograph with an on-line high-temperature thermal conversion oven (HTC) at 1420°C. The sample (1µL) was injected in splitless mode at an injection port temperature of 250°C. The peaks were resolved on a 30 m × 0.25 mm ID × 0.25 µm film Agilent Technologies DB-5 capillary column (temperature program: 110°C for 1 min; 10°C·min^{-1} ramp to 180°C; 5°C·min^{-1} ramp to 220°C; 20°C·min^{-1} ramp to 300°C; hold for 2 min) before pyrolysis. Helium was used as the carrier gas with a constant flow of 1 mL/min. Any amino acid eluting from the gas chromatograph was converted to H_2 before entry into the IRMS.

The enrichment of the tracer was measured by monitoring the Ion masses 2 and 3 to determine the $^2H/^1H$ ratios in the samples and referenced to the calibration curve. The calibration curve consisted of a series of known concentrations of d4-alanine and was applied to assess both the linearity of the mass spectrometer and to control for the loss of tracer. The isotopic abundances were expressed as the delta notation, δ^2H per mil (‰) deviation from VSMOW standard. Values of delta per mil given by the IRMS were transformed into MPE.

2.8.3 Muscle RNA extraction

In **Chapters 3, 4, 5** and **6**, skeletal muscle total RNA was extracted from ~20 mg of wet muscle using TRI Reagent® (Thermo Fisher Scientific, Waltham, Massachusetts, USA), a mono-phasic solution of phenol and guanidine isothiocyanate, according to the manufacturer's protocol. The sample was homogenised in 800 µL of TRIzol reagent with a power homogeniser (PowerGen 700, Fisherbrand, Fisher Scientific Ltd, Leicestershire, UK) for 30 seconds to disrupt cellular membrane and dissolve cellular components. The homogenisation pestle was sequentially washed in 0.3 M NaOH, DDH_2O and TRI reagent® between samples, and frequently replaced to maintain homogenising efficiency. Samples were incubated at room temperature for 5 mins before 160 µL of chloroform:iso-amyl alcohol (49:1) was added, and samples were shaken vigorously for 30 seconds followed by a brief vortex and left to incubate at room temperature for 15 mins. Samples were then centrifuged (12,000 *xg* at 4°C for 15 min) to separate the solution into an aqueous phase (exclusively RNA) and interphase (genomic DNA) and an organic phase (other contaminants). 400 µL of the aqueous phase was removed with care taken not to disturb the inter or

organic phase and aliquoted into a new microcentrifuge tube to which an equal volume of ice-cold iso-propanol was added before being left overnight at -20ºC to precipitate RNA. The following morning, samples were centrifuged (12000 *xg* at 4ºC for 15 min) and the pellets were washed twice in 75% ethanol diluted with RNase free water with centrifugation (10,000 *xg* at 4ºC for 10 min) in between washes. Samples were then redissolved in 30 µL of RNase free water for subsequent quantification. The concentration of the total RNA was determined spectrophotometrically at a wavelength of 260 nm and RNA purity was determined as the ratio of readings at 260/280 nm which was required to be between 1.8 and 2.1 (NanoDrop ND-2000 Spectrophotometer; Thermo Fisher Scientific).

2.8.4 Reverse transcription-polymerase chain reaction

The skeletal muscle mRNA expression of 224 genes was determined in the muscle RNA extracts via reverse transcription-polymerase chain reaction (RT-PCR). RT-PCR couples the conversion of mRNA into first strand complementary DNA (cDNA) which is then amplified and quantified to provide an expression value for the gene(s) of interest. Reverse transcription of skeletal muscle was performed using a commercially available kit (SuperScript™ VILO™ cDNA Synthesis Kit, Thermo Fischer Scientific, Waltham, Massachusetts, USA). Firstly, extracted mRNA was diluted with RNase free water to achieve a final concentration of 142.9 ng/µL. Four µL of a reaction mix including random primers and deoxyribonucleotide triphosphate nucleotides (dNPTs), and 2 µL of an enzyme mix containing a thermally stable reverse transcriptase engineered from Moloney Murine Leukaemia Virus (M–MLV) was added to individual wells of a 96 well plate. Fourteen µL of diluted sample was then added, and the plate was sealed, vortexed and centrifuged (700 rpm for 10 seconds) to consolidate the well contents. The 96 well plate was then run on a thermal cycler for 10 min at 25ºC, 60 min at 42ºC and the reaction was finally terminated at 85ºC for 5 min. cDNA was then stored at -20ºC for subsequent PCR.

PCR was performed using custom-designed 224 gene nanofluidic OpenArray™ plates (Thermo Fisher Scientific). Each OpenArray™ plate contains 3,072 through-holes divided into 48 subarrays of 64 through-holes. Thus, in the 224 gene format, 12 samples can be run on each OpenArray™ plate as eight through-

holes per subarray are not loaded. OpenArray™ plates are supplied with the individual assays pre-loaded to the hydrophilic surface of each through-hole. In preparation for loading the cards, 7.75 µL of OpenArray™ Master Mix (ThermoFisher Scientific) and 3.88 µL of ddH_2O was added to each well of a 384 well plate. The cDNA samples were first diluted 1:1 in ddH_2O, and then 3.1 µL of diluted cDNA was added to each well of the 384 well plate. The plate was sealed, vortexed and centrifuged (2000 rpm for 1 minute) to consolidate the well contents. The plate was positioned into an OpenArray™ AccuFill™ System (ThermoFisher Scientific) which robotically loads 33 nL of the sample from the 384 well plate into each through-hole of the OpenArray™ plate. The plate is then sealed and placed into a QuantStudio 12K Flex Real-Time PCR system (Thermo Fischer Scientific, Waltham, Massachusetts, USA) for subsequent PCR thermal cycling. Fluorescent emission data were captured at a critical threshold (C_t) which was used to quantify mRNA concentrations relative to a control leg (**Chapters 3, 4** and **5**) or a nominal control participant who represented the median cohort age (**Chapter 6**). In **Chapters 3, 4** and **6**, a heterogenous cDNA mix from all samples was prepared and loaded onto each OpenArray™ plate to correct for reaction efficiency between plates. This was not possible in the study reported in **Chapter 5** as all the wells on the OpenArray™ plates were required for samples. To compensate for input variations in mRNA quantity and reaction efficiency, the skeletal muscle mRNA expression of the housekeeping genes *ACTA1*, *ACTB* and *B2M* were determined. In studies presented in **Chapters 3** and **4**, the expression of alpha actin (*ACTA1*), beta actin (*ACTB*) and beta-2 microglobulin (*B2M*) remained stable throughout the intervention. In **Chapters 5** and **6**, the skeletal muscle mRNA expression *ACTA1* (**Chapter 5** and **6**) *ACTB* (**Chapter 5**) and *B2M* (**Chapter 5**) was not stable, so Acetyl-CoA carboxylase 2 (*ACACB*) and Solute Carrier Family 38 Member 4 (*SLC38A4*) were used in **Chapter 5** and *ACTB* and *B2M* were used in **Chapter 6**. Housekeeping gene expression is presented in the **Appendix**. The relative fold change in mRNA expression of the genes of interest was calculated using the $2^{-\Delta\Delta CT}$ method (**calculation 2**).

2.8.5 Gene ontology analysis

Gene ontology analysis was performed in **Chapters 3, 4, 5** and **6**. The relative expression of each gene obtained from the OpenArray™ plates was analysed

individually, and the significant gene list was adjusted with a Benjamini-Hochberg false discovery rate (FDR) correction (*FDR*<0.05) to control for type I errors (201). The resulting significant gene list was compared against Gene Ontology biological processes and Reactome pathways using the PANTHER overrepresentation test (binomial test with no correction; released 20190711) (PANTHER™ version 15.0) accessed via Gene Ontology (http://geneontology.org/). The original gene list, as opposed to the whole human genome, was used as a background for the enrichment analysis to eliminate any bias from the selection of genes.

2.9 Magnetic resonance imaging

Skeletal muscle volume was measured in **Chapters 5** and **6**. A 1.5 tesla (T) magnetic resonance imaging (MRI) scanner (Intera, Phillips, The Netherlands) was used to obtain images of both thighs in the axial plane over the full length of the femur. A T1-weighted 3D turbo spin echo sequence was used (field of view 500 x 500 mm, reconstructed matrix 512 x 512 mm, echo time 15 ms, repetition time 645 ms, slice thickness 5 mm, slice gap 5 mm) with the subject lying still in the supine position. A 4-element sense body radiofrequency (RF) coil was wrapped around both thighs. During the first scan, a specified distance from a bony landmark (femoral condyle) in the frontal plane was used to centre the axial plane images (202). This same distance was used on all subsequent MRI scans to ensure the axial images were in the same location along the length of the thigh on all scans.

Slicer software (version 4.10.0; https://www.slicer.org/) (203) was used to analyse the images obtained in the axial plane. The top 25% (from the greater trochanter working distally) and bottom 25% (from the distal end of the femur working proximally) were excluded so that the middle 50% region of the femur (approximately 20 cm depending on the height of the participant) was used for automated thresholding of skeletal muscle tissue with the volume of the thresholded area representing thigh volume. Knee extensor volume was determined by manually erasing the thresholding of non-knee extensor muscles. The volume of the non-knee extensor region was obtained by manually removing the knee extensor thresholding from the whole thigh thresholding.

2.10 Calculations

2.10.1 Myofibrillar protein fractional synthetic rate

The fractional synthetic rate of myofibrillar proteins was calculated based on the incorporation of [^{2}H]alanine into myofibrillar protein and mean body water deuterium enrichment between muscle biopsy time points using the standard precursor-product equation (204) (calculation 1).

$$Msyfobrillar\ FSR\ (\% \cdot h^{-1}\ or\ \% \cdot d^{-1}) = \left[\frac{[\Delta E_{product}]}{[E_{precursor}] \times [T]}\right] \times 100 \quad [1]$$

Where:

[$\Delta E_{product}$] = the increment in [^{2}H]alanine enrichment of myofibrillar protein between two biopsies

[$E_{precursor}$] = the average area under the body water deuterium enrichment curve between two biopsies corrected by a factor of 3.7 based upon the deuterium labelling of alanine during de novo synthesis (196)

[T] = indicates the tracer incorporation time between two muscle biopsies in hours or days.

2.10.2 Relative mRNA expression

The relive fold change in mRNA expression of the genes of interest was calculated using the $2^{-\Delta\Delta CT}$ method (calculation 2).

$$mRNA_{GI} = 2^{-([GI]-([\bar{x}\ HKs]))-([ConGI]-([\bar{x}ConHKs]))} \quad [2]$$

Where:

$mRNA_{GI}$ = the fold change in mRNA expression of the gene of interest

[GI] = the C_t value of gene of interest

[$\bar{x}$ HKs] = the geometric mean C_t value of housekeeping genes

[ConGI] = the C_t value of GI in the control leg (**Chapters 3, 4** and **5**) or control participant (**Chapter 6**)

[$\bar{x}$ ConHKs] = the geometric mean C_t value of the HK genes in the control leg (**Chapters 3, 4** and **5**) or control participant (**Chapter 6**)

2.11 Statistical procedures

All studies presented within this thesis (**Chapters 3-7**) employed a parallel groups design. A repeated-measures two-factor (condition *x* time) analysis of variance (ANOVA) was used to identify the main effect of time and condition, or the interaction between conditions over time. A mixed-effects model was applied when analysing data sets with missing values. A repeated-measures three-factor (treatment *x* time x leg) ANOVA was used to identify the main effect of, and interactions between, time, condition and leg when legs received separate interventions. Single-factor comparisons between treatments were performed with a t test or a one-factor ANOVA. Gene expression was log-transformed before being analysed using a mixed-effects model. All data were tested for sphericity, and where violations occurred, the Greenhouse-Geisser correction was applied. When a significant main or interaction effect was identified, a Sidak post hoc test was performed to locate individual differences. Statistical significance was set at $P<0.05$. Calculations were performed using GraphPad Prism 8.3.0. All data presented in text, tables and figures within this thesis are expressed as means ± standard error of the mean (SEM).

Chapter 3

Nutritionally attenuating the decline of skeletal muscle function after injury occurs independently of an increase in daily myofibrillar protein synthesis rates.

3.1 Introduction

Eccentric muscle contractions induce substantial and immediate ultrastructural injury to myofibrillar structures which is directly related to the impairment in muscle contractile function (47) and typically requires up to 7 days to be restored (41). Microarray approaches coupled with gene ontology analysis have identified a widespread and coordinated upregulation in skeletal muscle mRNA expression after eccentric contractions primarily associated with inflammatory NF-kB transcription factor signalling and protein synthesis and breakdown pathways (86, 171, 205, 206). Collectively, these studies have illustrated the likely importance of these processes in regulating the remodelling of myofibrillar injury. However, there are currently no intervention studies in humans that have directly and quantitatively investigated the temporal association between inflammation and protein turnover. Thus, our understanding of the interaction between these processes during the remodelling of myofibrillar injury and the recovery of muscle function is incomplete.

Consistent with the transcriptional response in skeletal muscle (205), eccentric contractions increase post-exercise myofibrillar protein synthesis rates for at least 8.5 h and by 30% more compared to non-injurious concentric contractions (6). This “snapshot” approach to measuring myofibrillar protein synthesis rates between serial muscle biopsies 8.5 h apart only captures a small proportion (~5%) of the total remodelling process (which presumably occurs for 24 h per day). Consequently, the effects of all lifestyle factors that may contribute to altered myofibrillar remodelling (e.g., repeated postabsorptive, and postprandial and overnight sleep periods, habitual physical activity, hormonal and diurnal metabolic fluctuations, etc.) are not incorporated. Indeed, muscle protein synthesis rates are elevated for at least 120 h after an *in vivo* eccentric contraction protocol in mice (45) and blocking mTOR and p70s6k phosphorylation three days after eccentric contractions using rapamycin attenuates the recovery of muscle function by ~20% on day seven (46). This illustrates that the capacity to upregulate myofibrillar protein synthesis rates for up to 7 days after eccentric contractions might be fundamental to the effective remodelling of injured skeletal muscle (46). Recently, deuterated water has been applied to measure integrative daily myofibrillar protein synthesis rates for up to

7 days after traditional resistance-type exercise (49, 207). However, there are currently no data concerning the time course of myofibrillar protein synthesis rates after eccentric contractions.

Eccentric contractions initiate a biphasic time course of inflammatory events in skeletal muscle. This is characterised by an early (0-72 h) pro-inflammatory phase, which transitions to a late (72-168 h) anti-inflammatory and pro-resolution phase (172). The NF-kB transcription factor is central to this process and undergoes rapid (3 h) and sustained (8 d) phosphorylation and DNA binding after eccentric contractions (86, 164, 165). NF-kB regulates the up- and downstream expression of an abundance of pro-inflammatory effectors after eccentric contractions, including membrane receptors, transcription factors and cytokines such as IL-6 (86, 206). There is evidence to suggest that an increase in systemic inflammation achieved by the intravenous infusion of IL-6 and endotoxin, which are associated with the activation of NF-kB signalling, decreases hourly resting mixed muscle protein synthesis rates in healthy humans (4, 124, 208). In light of these studies, it is conceivable that the induction of NF-kB signalling after eccentric contractions could impair the capacity to upregulate myofibrillar protein synthesis rates and as a consequence, delay the recovery of muscle function. In support of this, previous studies have shown that daily ingestion of supplemental protein accelerates the recovery of muscle function concomitant with reduced NF-kB phosphorylation 48 h after eccentric contractions (164). Furthermore, polyphenols, including those derived from cherry or pomegranate, accelerate the recovery of muscle function after eccentric contractions and have been shown to reduce the systemic plasma concentration of a panel of cytokines associated with NF-kB signalling (181–185, 209). However, it is yet to be determined if protein and polyphenol ingestion expedite the recovery of muscle function by attenuating NF-kB signalling and increasing myofibrillar protein synthesis rates as a consequence.

The current study, therefore, took a deuterated water approach to measure daily myofibrillar protein synthesis rates during both the early (24-72 h) and late (72-168 h) remodelling periods after unilateral eccentric contractions with the contralateral leg acting as a non-injured control. This was combined with a quantitative RT-PCR gene array with gene ontology analysis and a targeted

investigation of NF-kB-mediated transcription. A combined protein-polyphenol nutrition approach was then used to accelerate the recovery of muscle function and determine accompanying changes in myofibrillar protein synthesis rates and gene expression. It was hypothesised that protein-polyphenol ingestion would accelerate the recovery of muscle function which would be associated with a reduction in NF-kB transcriptional signalling and an increase in myofibrillar protein syntheses rates 24-72 h after eccentric contractions.

3.2 Methods

Participants

Eighteen young, healthy males (*n*=11) and females (*n*=7) (age: 23±1 y, BMI: 23.9±1 kg·m^{-2}) volunteered to participate in the present study. Participant characteristics are displayed in **Table 3.1**. The study was approved by the Sport and Health Sciences ethics committee of the University of Exeter (REF NO. 161026/B/06). The study is registered at ClinicalTrials.Gov (ID: NCT02980900).

Table 3.1. Participant characteristics.

	PLA (*n*=9)	**PP** (*n*=9)
Sex	M = 6, F = 3	M = 5, F = 4
Age **(y)**	22±1	23±2
Body mass **(kg)**	78±4	68±4
Height **(cm)**	176±1	174±3
BMI **(kg·m^{-2})**	25±1	23±1
Total eccentric work **(J·kg^{-1})**	642±43	579±44
Habitual protein **(g·kgBM^{-1}·day^{-1})**	1.1±0.1	1.4±0.2
Controlled protein (excluding condition) **(g·kgBM^{-1}·day^{-1})**	1.2±0.1	1.2±0.1
Controlled protein (including condition) **(g·kgBM^{-1}·day^{-1})**	1.2±0.1	1.8±0.1*

Values represent mean±SEM. PLA, Placebo; PP, Protein-polyphenol; BMI, body mass index; M=male; F=Female. Total eccentric work represents the total volume of eccentric work performed normalised for body mass. Habitual protein represents participants average habitual protein intake obtained from a 3-day food diary. Controlled protein represents the 14-day average protein intake provided by the dietary control. Comparisons between PLA and PP were performed with separate unpaired t-tests. * difference in controlled protein intake (including condition) between PLA and PP.

Study protocol

Details of all experimental and analytical procedures are presented in **Chapter 2**. An overview of the experimental protocol is shown in **Figure 3.1**. Participants

were randomly assigned to one of two parallel, fully controlled nutritional intervention groups consisting of either daily post-exercise and pre-bed protein-polyphenol ('PP') or placebo ('PLA') drinks (see **Experimental drinks**) and completed a 14-day study period under fully dietary controlled conditions. At 1900 h on day 1 (baseline) and days 8-14 of the study period, muscle soreness (visual analogue scale (VAS) and pressure pain threshold (PPT)) and knee extensor isometric torque and isokinetic work (collectively referred to as 'functional outcomes') were measured in both legs. At 1900 h on day 7, participants performed a bout of eccentric contractions in one leg ('injured') randomly counterbalanced for leg dominance, with the contralateral leg functioning as a control ('control'). On days 8 – 13, an additional four sets of 30 isokinetic knee extensor contractions were performed by both legs to maximise the post-exercise myofibrillar protein synthetic response. To determine daily myofibrillar protein synthesis rates of injured and control legs under free-living conditions, participants underwent a deuterated water dosing protocol which began with a loading day on day seven and continued with daily maintenance doses on days 8-14 in combination with bilateral vastus lateralis muscle biopsies performed 24, 72 and 168 h after eccentric contractions. Muscle soreness testing was performed before muscle biopsy sampling and exercise was conducted after muscle biopsy sampling.

Experimental drinks

Drinks were provided by the manufacturer (Beachbody LLC, Santa Monica, California, USA) in sachets containing a single serving and sachets were labelled using a coding system to ensure double-blinding. PP and PLA drinks were independently analysed for macronutrient composition (Premier Analytical Services, High Wycombe, Buckinghamshire, UK) and polyphenol content (PP drinks only) (Eurofins Food Testing, Wolverhampton, UK). The detailed nutritional content of a representative drink sample is displayed in **Table 3.2**. The post-exercise PP beverage contained a blend of whey protein isolate, pea protein isolate and micellar casein protein sources and 650 mg of pomegranate extract. The pre-bed PP beverage contained micellar casein protein and 480 mg of tart cherry extract. The post-exercise and pre-bed PLA drinks were taste, texture and energy matched to their respective PP drinks by substituting protein with carbohydrate (maltodextrin). All drinks were prepared by mixing 1 serving (post-

exercise=35.5 g, pre-bed=28.5 g) with 225 mL of water, followed by an additional 50 mL of water to 'wash' the bottle and ensure that all contents were consumed. Post-exercise drinks were consumed in the laboratory under supervision at ~2000 h on day one and days 8 – 14. On days 2 – 6 when participants were not required to attend the laboratory, participants were instructed to consume the drinks at home at 2000 h. Pre-bed drinks were consumed by the participant at home within 30 minutes of going to bed. All drinks were well tolerated, consumed within the allotted time (i.e., 5 min) and resulted in no reported adverse effects during or after the experimental period.

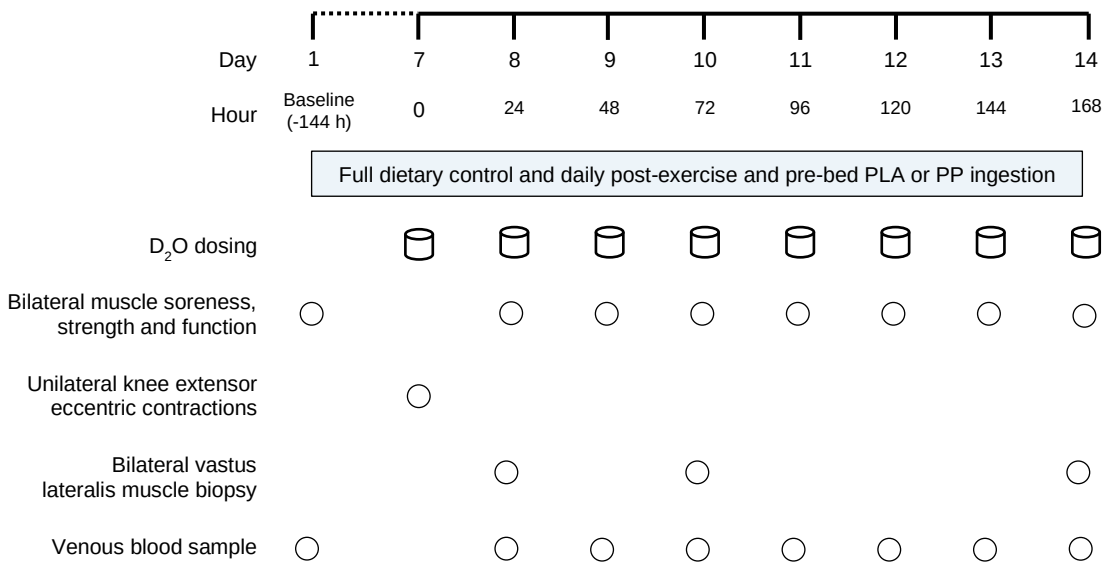

Figure 3.1. Schematic representation of the experimental protocol. Participants were provided with full dietary control (1.2 g·kgBM^{-1}·d^{-1} dietary protein) and post-exercise and pre-bed protein-polyphenol (PP; *n*=9) or isocaloric maltodextrin placebo (PL; *n*=9) drinks were consumed daily. Loading of orally consumed 70% D_2O began at ~0800 h on day 7 with 8 x 6 mL·kg^{-1} doses consumed every 1.5 h and body water enrichment was maintained thereafter with daily doses of 0.54 mL·kg^{-1}. Unilateral eccentric knee extensor contractions were performed at ~1900 h on day 7 (t=0 h) and venous blood samples and muscle soreness, isometric torque and isokinetic work were measured at baseline and at 24 h intervals on days 8 – 14. Bilateral vastus lateralis muscle biopsies were obtained 24, 72 and 168 h following eccentric contractions.

Sample collection and analysis

Ten mL of venous blood from the antecubital vein was collected on day one and days 8 – 14. These samples were analysed at a later date for body water ^2H

enrichment, plasma creatine kinase activity, and plasma cytokine concentrations. Bilateral muscle biopsies of the vastus lateralis were taken 24, 72 and 168 h after eccentric contractions and categorised into “early” (24–72 h) and “late” (72–168 h) remodelling periods. These samples were analysed at a later date for myofibrillar protein-bound [^{2}H]alanine and skeletal muscle mRNA expression.

Table 3.2. The nutritional content of post-exercise and pre-bed placebo and protein-polyphenol drinks.

	PLA		PP	
	Post-exercise	**Pre-bed**	**Post-exercise**	**Pre-bed**
Protein (g/serving)	0.9	0.3	21.1	17.0
Fat (g/serving)	0.3	0.2	0.6	0.3
Carbohydrate (g/serving)	29.2	23.7	8.9	6.1
Fibre (g/serving)	2.3	2.6	1.2	2.4
Energy (kcal/serving)	127.8	103.2	127.8	99.5
Energy (kJ/serving)	541.7	437.2	542.1	421.5
Polyphenol content (GAE/serving)	n/a	n/a	211.2	78.4

PLA, Placebo; PP, Protein-polyphenol; GAE, Gallic acid equivalent.

Statistical analysis

Full statistical procedures and calculations are presented in **Chapter 2**. Differences in subject characteristics and dietary intake between conditions were analysed using unpaired t-tests. PPT, isometric torque and isokinetic work were calculated as a percentage of the control leg (%con) at each time point value before analysis. A repeated-measure two-factor analysis of variance (ANOVA) was used to analyse (condition x time) differences in plasma creatine kinase activity, C-reactive protein concentration, body water ^{2}H enrichment, functional outcomes and skeletal muscle mRNA expression, and (condition x leg) differences in myofibrillar protein synthesis rates. Repeated-measure three-factor AVOVAs were used to analyse (time x condition x leg) differences in myofibrillar protein-bound [^{2}H]alanine mole per cent excess (MPE). A repeated-measure mixed-effects model was used to analyse (condition x time) differences in plasma cytokine concentrations. A false discovery rate (*FDR*<0.05) was applied to adjust

for familywise error before gene ontology analysis (201). Ingenuity pathway analysis software (Quigen) was used to visualise gene expression associated with the NF-kB pathway. Violations of sphericity were corrected with the Greenhouse-Geisser correction. When a significant main and interaction effect was observed, a Sidak post hoc test was performed to locate individual differences. Statistical significance was set at $P<0.05$. Calculations were performed using GraphPad Prism 8.3.0. All data are expressed as means±SEM.

3.3 Results

Participant characteristics and diet

No differences in age, weight, height, BMI, or habitual protein intake were detected between conditions (*P*>0.05; **Table 3.1**). Average daily energy and macronutrient ingestion during the 14-day controlled diet after accounting for adherence (not including condition) was 10775±279 KJ with 52±1% energy as carbohydrate, 33±1% energy as fat and 13±1% energy (1.2±0.1 g·kgBM^{-1}·d^{-1}) as protein, and was not different between conditions (*P*>0.05). PP ingestion increased protein intake compared to PLA (1.8±0.1 *vs* 1.2±0.1 g·kgBM^{-1}·d^{-1}, respectively; *P*<0.001). Total eccentric work performed did not differ between conditions (*P*>0.05). Body water deuterium enrichment and skeletal muscle analysis were performed in *n*=17 unless otherwise stated as one participant (female, PLA) completed the study without muscle biopsies.

Functional outcomes

VAS muscle soreness increased from baseline (*P*<0.001; **Figure 3.2A**), similarly between conditions (*P*=0.898, interaction; *P*=0.316). VAS muscle soreness increased at 24 h (baseline = 1.6±0.7, 24 h = 19.1±2.2 mm; post hoc; *P*<0.001) and peaked at 48 h (36.41±3.5 mm; post hoc; *P*<0.001), before returning to baseline after 120 h (post hoc; *P*<0.0056). Eccentric contractions reduced vastus medialis (*P*<0.001), rectus femoris (*P*<0.001) and vastus lateralis (*P*=0.002) PPT from baseline (**Figure 3.2B-D**). Compared to PLA, vastus medialis PPT was greater (*P*=0.005) with PP ingestion after 72 (PLA = 88±3, PP = 102±3%con, post hoc; *P*=0.045), 120 (PLA = 92±2, PP = 105±3%con; post hoc; *P*=0.014) and 168 (PLA = 96±2, PP = 106±2%con; post hoc; *P*=0.016) h.

Isometric torque decreased relative to baseline (*P*<0.001; **Figure 3.3A**) and to a greater extent with PLA than PP ingestion (*P*=0.022) such that isometric torque was greater at 24 h in PP (103±7%con) compared to PLA (69±7%con) (post hoc; *P*=0.038). Isokinetic work deceased relative to baseline (*P*<0.001; **Figure 3.3B**) and to a greater extent with PLA than PP ingestion (*P*=0.028, interaction; *P*=0.002). With PLA ingestion, isokinetic work decreased from baseline by 36±7%con after 24 h (post hoc; *P*=0.035) and did not return to baseline until 120 h (post hoc; *P*=0.102) following eccentric contractions. With PP ingestion,

isokinetic work did not change from baseline at any point during the time course (post hoc; *P*>0.05). PP ingestion tended to attenuate the impairment in isokinetic work after 24 h relative to PLA (post hoc; *P*=0.076).

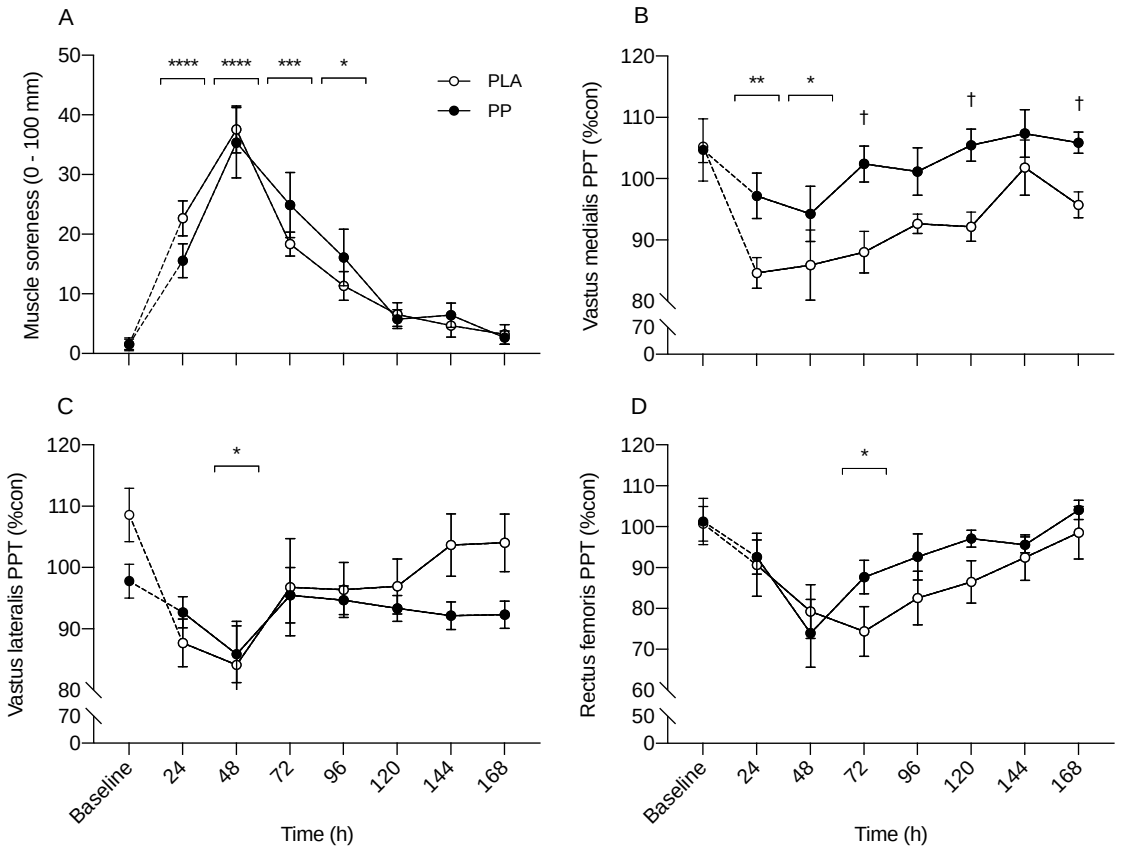

Figure 3.2. Visual analogue scale muscle soreness (**A**) recorded on a 100 mm scale anchored by "No pain" at 0 mm and "Worst possible pain" at 100 mm, and pressure pain threshold (relative to the control leg; PPT; **B-D**) measured using algometry. 300 unilateral eccentric knee extensor contractions were performed at time=0 h. Post-exercise and pre-bed protein-polyphenol (PP; *n*=9; black circles) or isocaloric maltodextrin placebo (PLA; *n*=9; white circles) drinks were ingested for 7 days prior to, and 7 days after eccentric contractions. Data are presented as means with error bars representing standard error. Statistical analysis was performed with separate two-factor ANOVAs. * time points significantly different to baseline (main effect), † PP different to PLA. One symbol *P*<0.05, two symbols *P*<0.01, three symbols *P*<0.001, four symbols *P*<0.0001.

Plasma creatine kinase activity and C-reactive protein concentration

Compared to baseline, plasma creatine kinase activity increased at 96 h (post hoc; *P*=0.011) and 120 h (post hoc; *P*=0.019) and was no longer different to baseline at 144 h (post hoc; *P*=0.0236; **Figure 3.4A**). Creatine kinase activity was not different between conditions (*P*=0.517, interaction; *P*=0.961). C-reactive protein concentration tended to increase over time (*P*=0.098) and was not different between conditions (*P*=0.466, interaction; *P*=0.611; **Figure 3.4B**). C-reactive protein values from 1 participant in the PP condition were below the limits of detection.

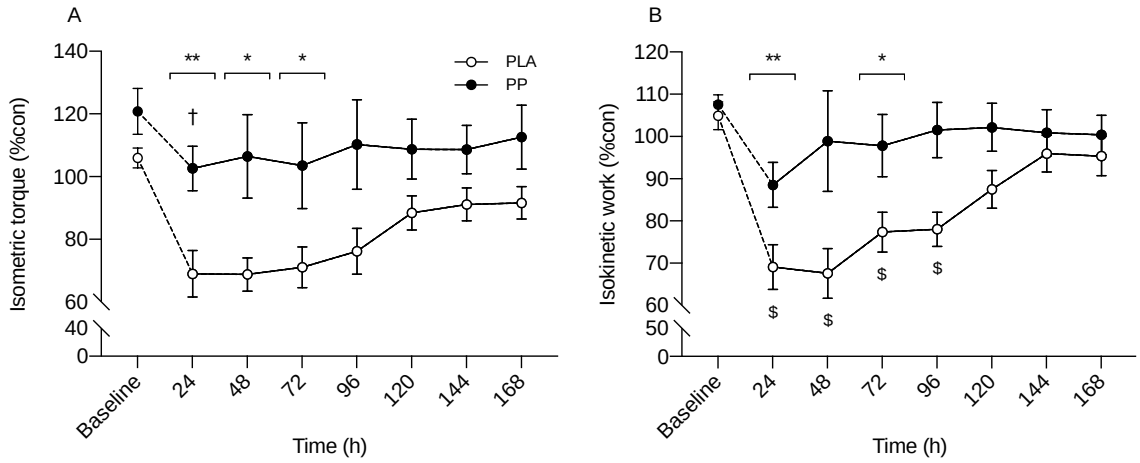

Figure 3.3. Knee extensor isometric torque (**A**) and isokinetic work (**B**) normalised to the control leg. 300 unilateral eccentric knee extensor contractions were performed at time=0 h. Post-exercise and pre-bed protein-polyphenol (PP; *n*=9; black circles) or isocaloric maltodextrin placebo (PLA; *n*=9; white circles) drinks were ingested for 7 days prior to, and 7 days following eccentric contractions. Data are presented as means with error bars representing standard error. Statistical analysis was performed with separate two-factor ANOVAs. * time points significantly different to baseline (main effect), † PP different to PLA, $ time point different to baseline in PLA only. One symbol *P*<0.05, two symbols *P*<0.01.

Plasma cytokine concentrations

Compared to baseline, IL-1RA, IL-1β, and IL-6 concentrations decreased at 24 h (*P*<0.05; **Figure 3.5**). During the early period (24 – 72 h after eccentric contractions), IL-6 concentrations increased, whereas IL-8, CCL2 and TNF-α concentrations decreased (*P*<0.05). TNF-α concentrations then increased during

the late period (72 – 168 h after eccentric contractions) (P<0.001). There was no effect of condition on plasma cytokine concentrations (P>0.05), nor did cytokine concentrations interact between conditions (P>0.05). IL-1β (PLA n=8, PP n=5), IL-6 (PLA n=7, PP n=3) and IL-33 (PLA n=4, PP n=2) did not contain full data sets due to several samples which were below the limits of detection.

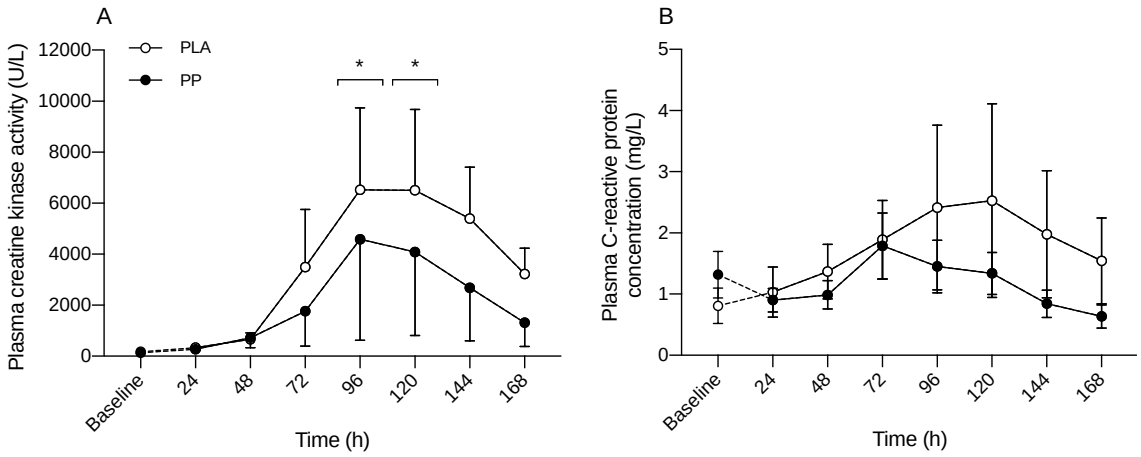

Figure 3.4. Plasma creatine kinase activity (**A**) and C-reactive protein concentration (CRP; **B**). 300 unilateral eccentric knee extensor contractions were performed at time=0 h. Post-exercise and pre-bed protein-polyphenol (PP; n=9 (n=8 for CRP as values from 1 participant were below the limits of detection); black circles) or isocaloric maltodextrin placebo (PLA; n=9; white circles) drinks were ingested for 7 days prior to, and 7 days after eccentric contractions. Data are presented as means with error bars representing standard error. Statistical analysis was performed with separate two-factor ANOVAs. * time points significantly different to baseline (main effect). One symbol P<0.05.

Body water deuterium enrichment

Baseline body water deuterium enrichment did not differ between PLA (0.03±0.01%) and PP (0.02±0.01%) (P>0.05). Deuterated water loading increased body water deuterium enrichment (P<0.001) to a similar extent in PLA (0.63±0.04%) and PP (0.58±0.03%) (interaction; P=0.363; **Figure 3.6A**) conditions. Body water deuterium enrichment did not change during the seven days of maintenance dosing (P=0.718), averaging 0.63±0.02% in PLA and 0.52±0.03% in PP, with no difference between conditions (P=0.120).

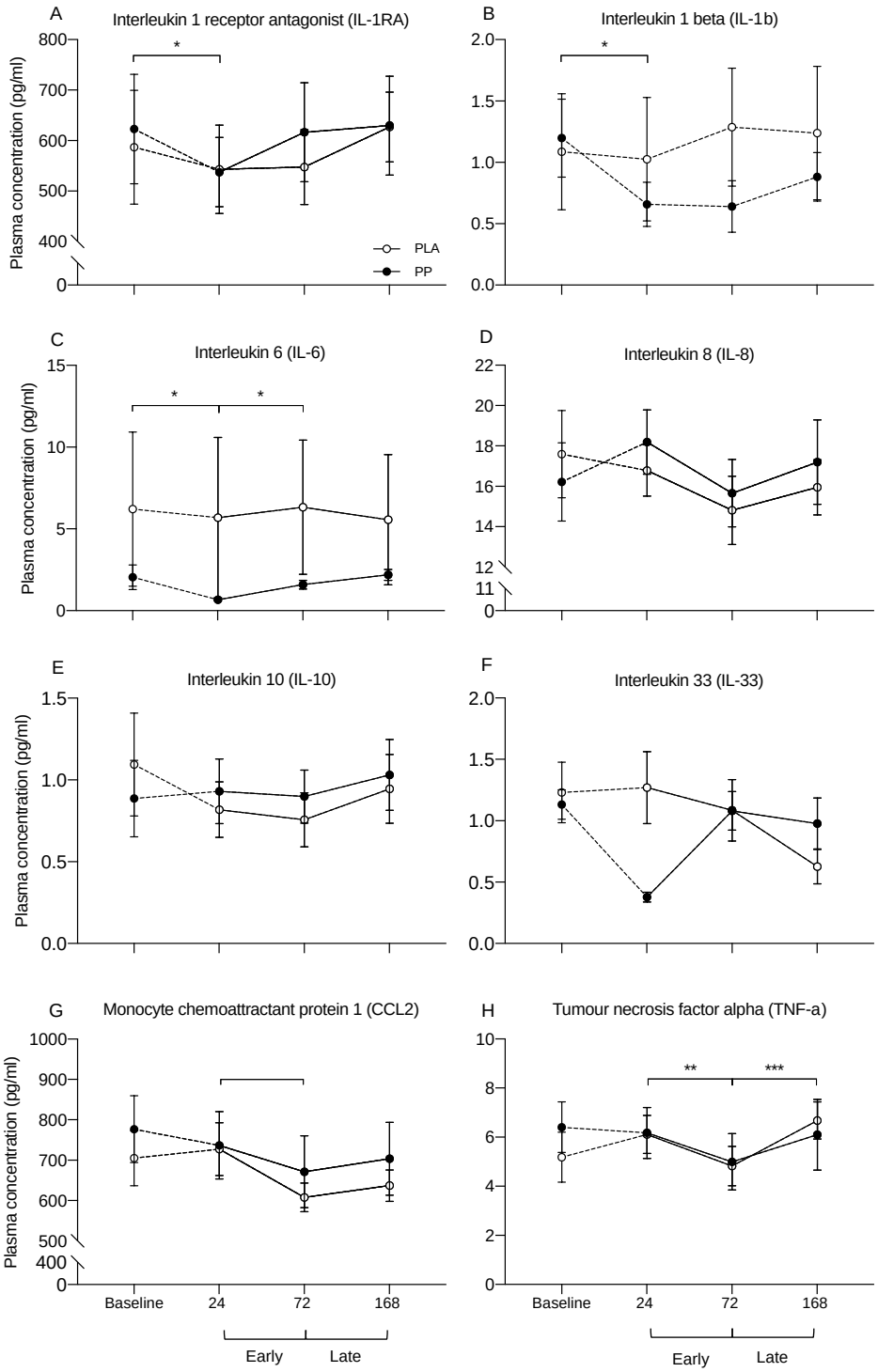

Figure 3.5. Plasma cytokine concentration. 300 unilateral eccentric knee extensor contractions were performed at time=0 h. Post-exercise and pre-bed protein-polyphenol (PP; *n*=9; black circles) or isocaloric maltodextrin placebo (PLA; *n*=9; white circles) drinks were ingested for 7 days prior to, and 7 days following eccentric contractions. IL-1β (PLA *n*=8, PP *n*=5), IL-6 (PLA *n*=7, PP *n*=3) and IL-33 (PLA *n*=4, PP *n*=2) did not contain full data sets due to a number of samples which were below the limits of detection. Data are presented as means with error bars representing standard error. Statistical analysis was performed with separate mixed effect models. * difference between time points (main effect). One symbol *P*<0.05, two symbols *P*<0.01, three symbols *P*<0.001.

Myofibrillar protein-bound [^{2}H]alanine enrichment and synthesis rates

Myofibrillar protein-bound [^{2}H]alanine enrichments increased during both early and late incorporation periods (P<0.001). The increase in early myofibrillar [^{2}H]alanine enrichment was greater in the injured (0.103±0.001 MPE) compared to the control (0.092±0.004 MPE) leg (interaction; P=0.047). The increase in late myofibrillar [^{2}H]alanine enrichment was not different between control (0.132±0.010 MPE) or injured (0.127±0.011) legs (interaction; P=0.746). There were no condition effects on the increase in MPE during early (interaction; P=0.533) and late (interaction; P=0.793) incorporation periods.

Early myofibrillar protein synthesis rates were higher in the injured (2.308±0.081%·d^{-1}) than in the control (2.023±0.079%·d^{-1}) leg (P=0.006; **Figure 3.6C**). Early myofibrillar protein synthesis rates were not affected by condition (P=0.998, interaction; P=0.618). Myofibrillar protein synthesis rates decreased (P<0.001) from the early to the late period similarly between control (from 2.023±0.079 to 1.520±0.096%·d^{-1}, respectively) and injured (from 2.308±0.081 to 1.575±0.147%·d^{-1}, respectively) legs (interaction; P=0.102). Late myofibrillar protein synthesis rates were not different between control (1.520±0.096%·d^{-1}) and injured legs (1.575±0.147%·d^{-1}) legs (P=0.737) or conditions (P=0.150, interaction; P=0.855; **Figure 3.6D**).

Correlations

There was no relationship between the difference in early myofibrillar FSR and the mean change of isometric torque from baseline during the early period in PLA or PP (**Figure 3.7A**). There tended to be a negative association between the difference in early myofibrillar FSR and the mean change of isokinetic work from baseline during the early period in PLA (R^2=0.468; P=0.062) but not in PP (R^2=0.004; P=0.868) (**Figure 3.7B**).

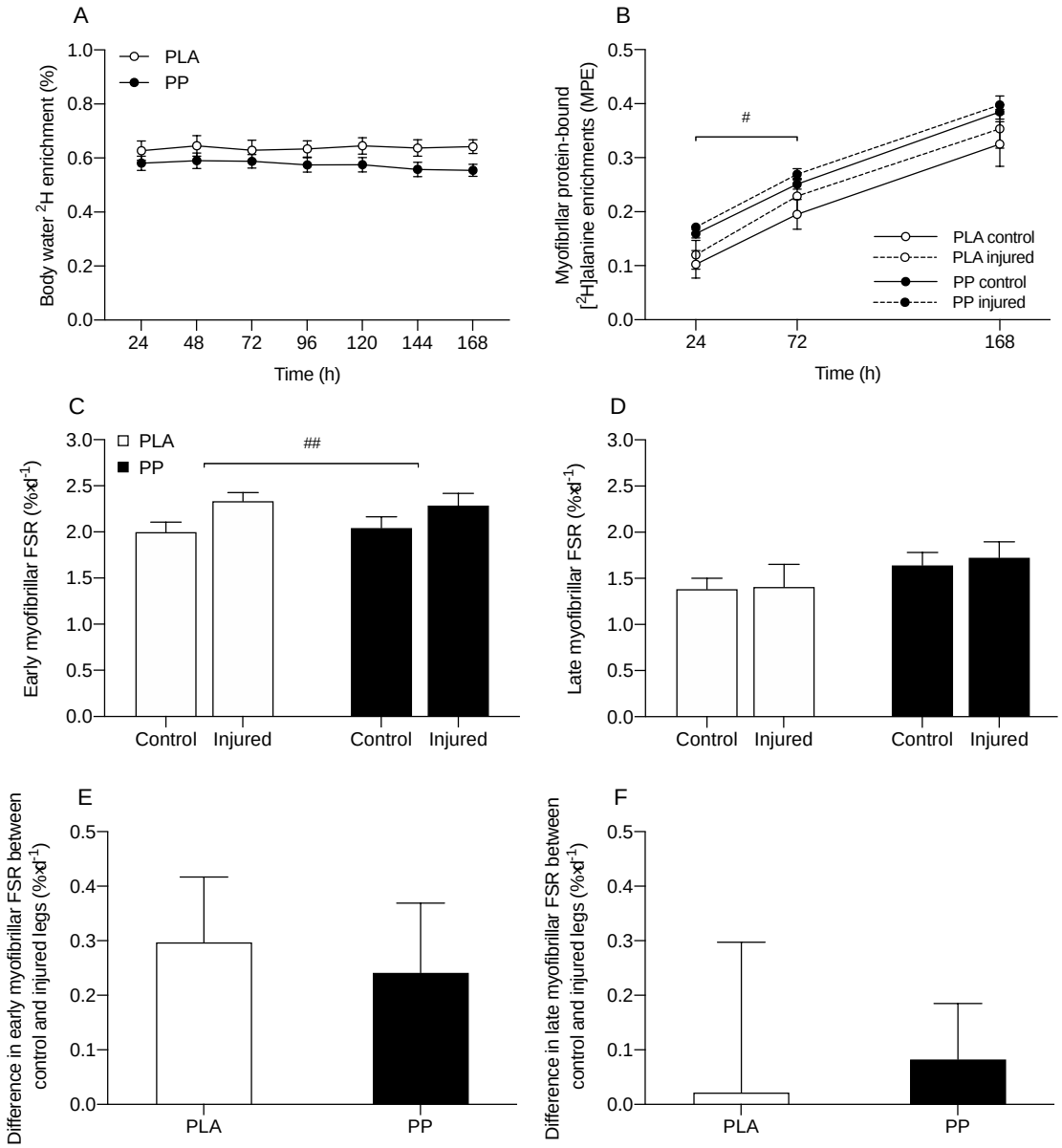

Figure 3.6. Body water ^{2}H enrichment (%) measured in blood plasma (**A**), myofibrillar bound [^{2}H]alanine enrichment (**B**), and early (24 – 72 h) (**C**) and late (72 – 168 h) (**D**) myofibrillar fractional synthetic rates (FSR), and the difference in FSR between control and injured legs during early (**E**) and late (**F**) periods. 300 unilateral knee extensor contractions were performed at time=0. Protein-polyphenol (PP; *n*=9; black bars/circles) or isocaloric maltodextrin placebo (PLA; *n*=9; white bars/circles) drinks were ingested for 7 days prior to, and 7 days after eccentric contractions. Data are presented as means with error bars representing standard error. Data were analysed with separate two-factor (**A**, **C** & **D**) and three-factor (**B**) ANOVAs, and unpaired t-tests (**E** & **F**). $^{\#}$difference between control and injured legs. One symbol $P<0.05$, two symbols $P<0.01$.

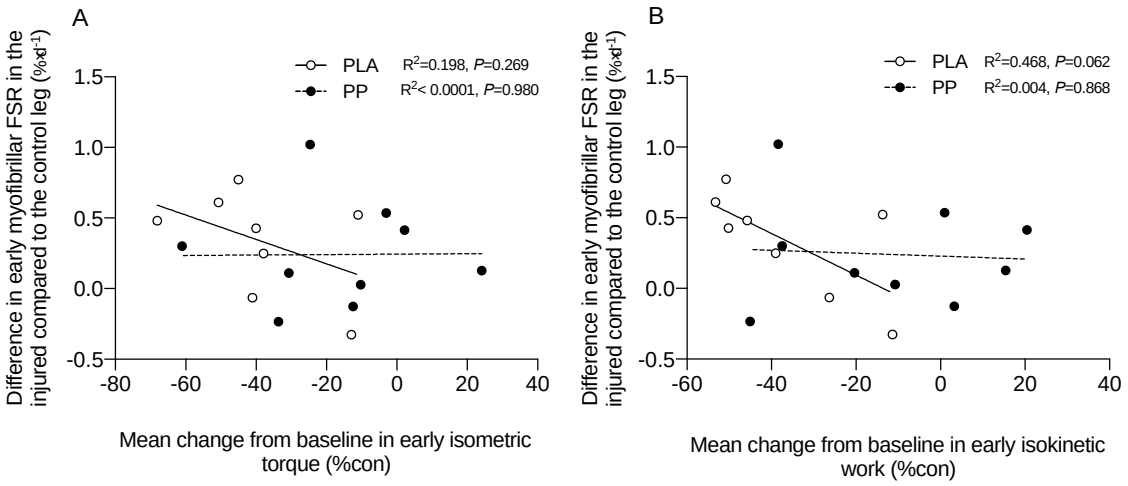

Figure 3.7. The relationship between the difference in myofibrillar fractional synthetic rate (FSR) between the injured compared to the control leg during the early (24-72 h) period and the mean loss of isometric torque (**A**) and isokinetic work (**B**) from baseline during the early (24-72 h post-exercise) period. Correlations were performed using a Pearson's correlation.

Skeletal muscle mRNA expression associated with NF-κB signalling

Gene expression was calculated for *n*=15 (PLA *n*=7, PP *n*=8) due to limited remaining tissue.

The skeletal muscle mRNA expression of all nine upstream (*IL18, IL1R1, IL1RL1, NFKB1, RELT, TGFB2, TNFRSF10B, TNFRSF12A* and *TRAFD1*; *P*<0.05; **Figure 3.8**) and five downstream (*ATF3, CCL2, CEBPD, CXCL1* and *SOCS3*; *P*<0.05; **Figure 3.9**) genes associated with NF-κB were differentially expressed during the early period. *CXCL2* tended to be downregulated during the early period (downstream; *P*=0.055). The early mRNA expression of *IL1R1* was downregulated in PP (0.80±0.36 log_2 fold change) compared to PLA (2.11±0.44 log_2 fold change) (*P*=0.020; **Figure 3.8B**), and *IL18* (*P*=0.069), *TGFB2* (*P*=0.058) and *TNFRSF12A* (*P*=0.091) mRNA expression tended to be downregulated in PP compared to PLA. *TNFRSF10B* and *TNFRSF12A* interacted between conditions during the early period such that mRNA expression of *TNFRSF10B* and *TNFRSF12A* did not change in PLA but decreased in PP (*P*<0.05; **Figure 3.8G-H**).

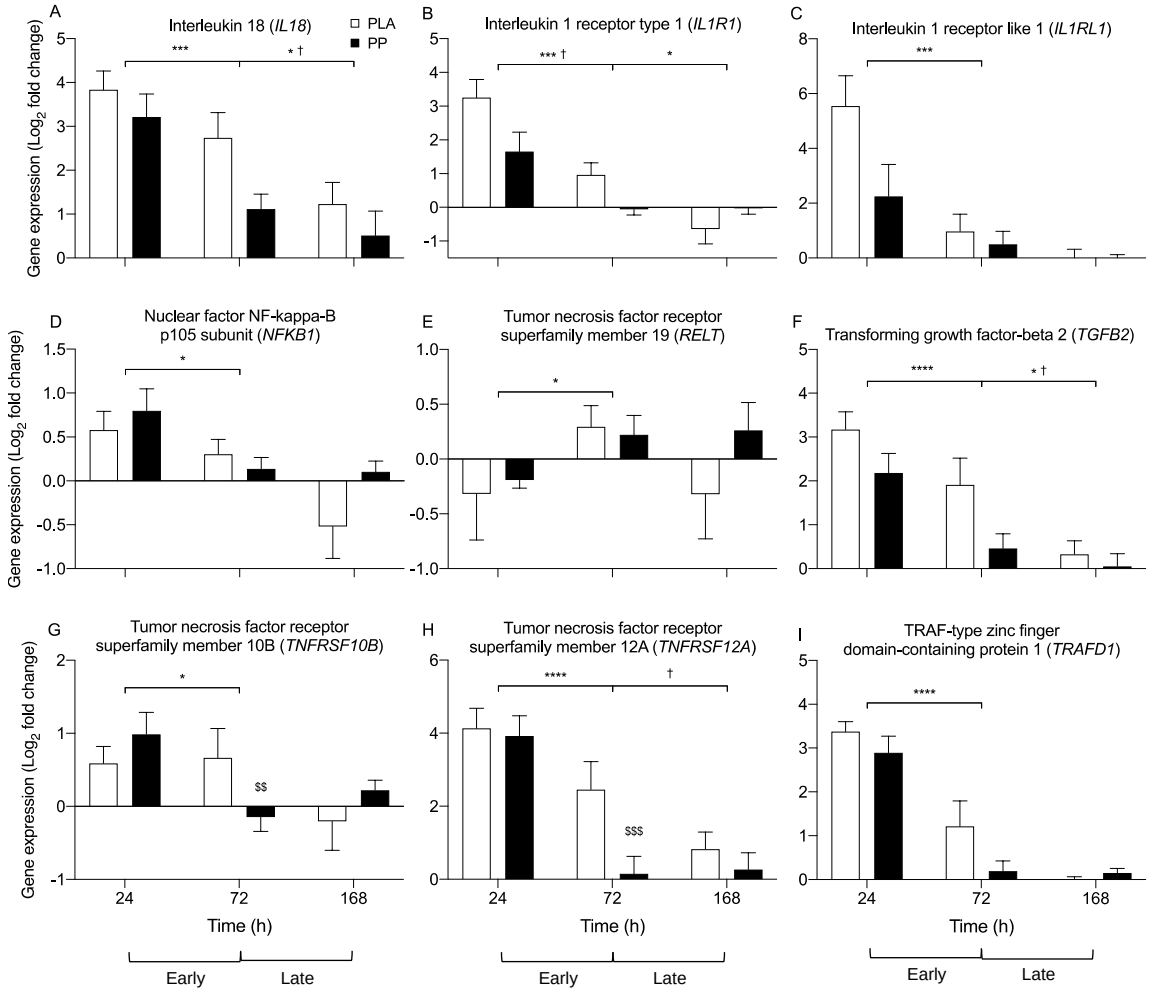

Figure 3.8. Skeletal muscle mRNA expression of 9 genes (**A**-**I**) upstream of NF-κB signalling during early (24 – 72 h) and late (72 – 168 h) periods after 300 unilateral eccentric knee extensor contractions (time=0 h). Post-exercise and pre-bed protein-polyphenol (PP; *n*=9; black bars) or isocaloric maltodextrin placebo (PLA; *n*=9; white bars) drinks were ingested for 7 days prior to, and 7 days following eccentric contractions. All individual expression values are expressed as a fold change from the contralateral control leg at the same timepoint, with log_2 transformation applied. Data are presented as means with error bars representing standard error. Statistical analysis was performed with separate two-factor ANOVAs. * significant difference between time points (main effect), † PP different to PLA, $ change from previous time point in PP only. One symbol $P<0.05$, two symbols $P<0.01$, three symbols $P<0.001$, four symbols $P<0.0001$.

The skeletal muscle mRNA expression of 3 upstream (*IL18, IL1R1* and *TGFB2*; *P*<0.05; **Figure 3.9)** genes associated with NF-κB were differentially expressed during the late period. *NFKB1* (*P*=0.075)*, TRAFD1* (*P*=0.077) and *VEGFA* (*P*=0.082) tended to be differentially expressed. Relative to PLA, PP downregulated the late mRNA expression of *IL18* (PLA = 1.98.42, PP = 0.81±0.33 log_2 fold change; *P*=0.047; **Figure 3.8A**)*, TGFB2* (PLA = 1.12±0.39, PP = 0.26±0.22 log_2 fold change; *P*=0.041; **Figure 3.8F**)*, TNFRSF12A* (PLA = 1.64±0.49, PP = 0.21±0.32 log_2 fold change; *P*=0.023; **Figure 3.8H)** and *ATF3* (PLA = 1.10±0.29, PP = 0.09±0.26 log_2 fold change; *P*=0.011; **Figure 3.8A**)*. IL1R1* mRNA expression decreased in PLA but did not change in PP (*P*=0.026; **Figure 3.8B**), which also tended to occur with the mRNA expression of *TRAFD1* (*P*=0.093).

Gene ontology analysis

Only genes which showed a significant change in expression over time passed the FDR cut-off of 5% and were included in the gene ontology analysis. During the early period there were 5 genes which showed main effect of condition and 37 genes which interacted between conditions (*P*<0.05). During the late period there were 6 genes which showed a main effect of condition and 18 genes which interacted between conditions (*P*<0.05). However, these genes did not pass the FDR cut off of 5% so were not included in gene ontology analysis. The mRNA expression of all 213 genes and their respective *P* values is provided in **Appendices 6.1** for reference.

The skeletal muscle mRNA expression of 49 genes was downregulated during the early period, and an additional 19 genes were upregulated during the early period after applying an *FDR* cut-off of 5%. The result of the gene ontology analysis is presented in **Table 3.3,** and hierarchal clustering is presented in **Figure 3.10A.** Five genes were differentially expressed between conditions (*CAPN3, CCL8, HSF4, IL1R1* and *PENK*; *P*<0.05) and 37 genes interacted between conditions (*AKT2*, *C5, CAPN2, CASP8, CEBPB, COL1A1, CREB1, CXCL3, DNAJB6, EIF2B2, GSR, GYS1, HRAS, HSF2, IKBKB, IL10RB, ITGA7, JAK1, MEF2C, MTOR, PAX7, PENK, PTGS1, RHOB, RHOQ, SGCA, SLC7A8, SMAD2, TFEB, TNFRSF10B, TNFRSF12A, TRAF6, TRIM32, TSC2, USP1,*

USP2 and *WDR24*; *P*<0.05). However, these genes did not meet the FDR cut off of 5% but are shown in **Appendices 6.1** for reference.

The skeletal muscle mRNA expression of seven genes was downregulated during the late period, and no genes were upregulated using an *FDR* cut-off of 5%. The result of the gene ontology analysis is presented in **Table 3.4,** and hierarchal clustering is presented in **Figure 3.10B.** Six genes were differentially expressed between conditions (*ATF3, ANKRD1, DNAJA1, IL18, TGFB2* and *TNFRSF12A*; *P*<0.05) and 18 genes interacted between conditions (*ABL1, C5, CCL8, CSF1, GSR, HIF1A, ICAM1, IFRD1, IL1R1, IL4R, JAK1, MAP2K1, PENK, PTGS1, STAT6, TNFRSF10B, TNFRSF1A* and *TRAFD1*; *P*<0.05). However, these genes did not meet the FDR cut off of 5% but are shown in **Appendices 6.1** for reference.

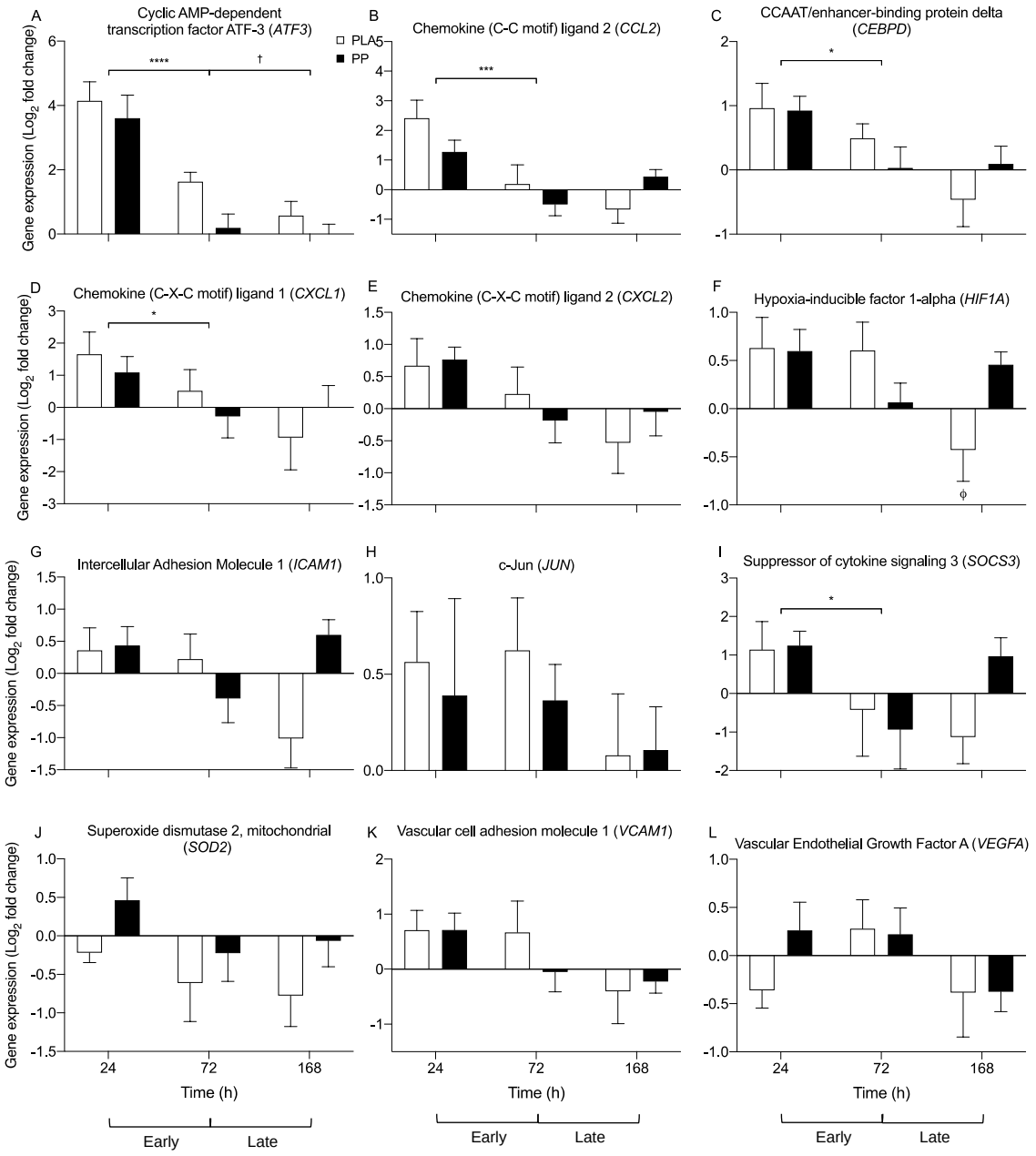

Figure 3.9. Skeletal muscle mRNA expression of 12 genes (**A**-**L**) downstream of NF-κB signalling during early (24 – 72 h) and late (72 – 168 h) periods after 300 unilateral eccentric knee extensor contractions (time=0 h). Post-exercise and pre-bed protein-polyphenol (PP; *n*=9; black bars) or isocaloric maltodextrin placebo (PLA; *n*=9; white bars) drinks were ingested for 7 days prior to, and 7 days following eccentric contractions. All individual expression values are expressed as a fold change from the contralateral control leg at the same timepoint, with log_2 transformation applied. Data are presented as means with error bars representing standard error. Statistical analysis was performed with separate two-factor ANOVAs. * significant difference between time points, † PP different to PLA. Φ change from previous time point in PLA only. One symbol $P<0.05$, two symbols $P<0.01$, three symbols $P<0.001$, four symbols $P<0.0001$.

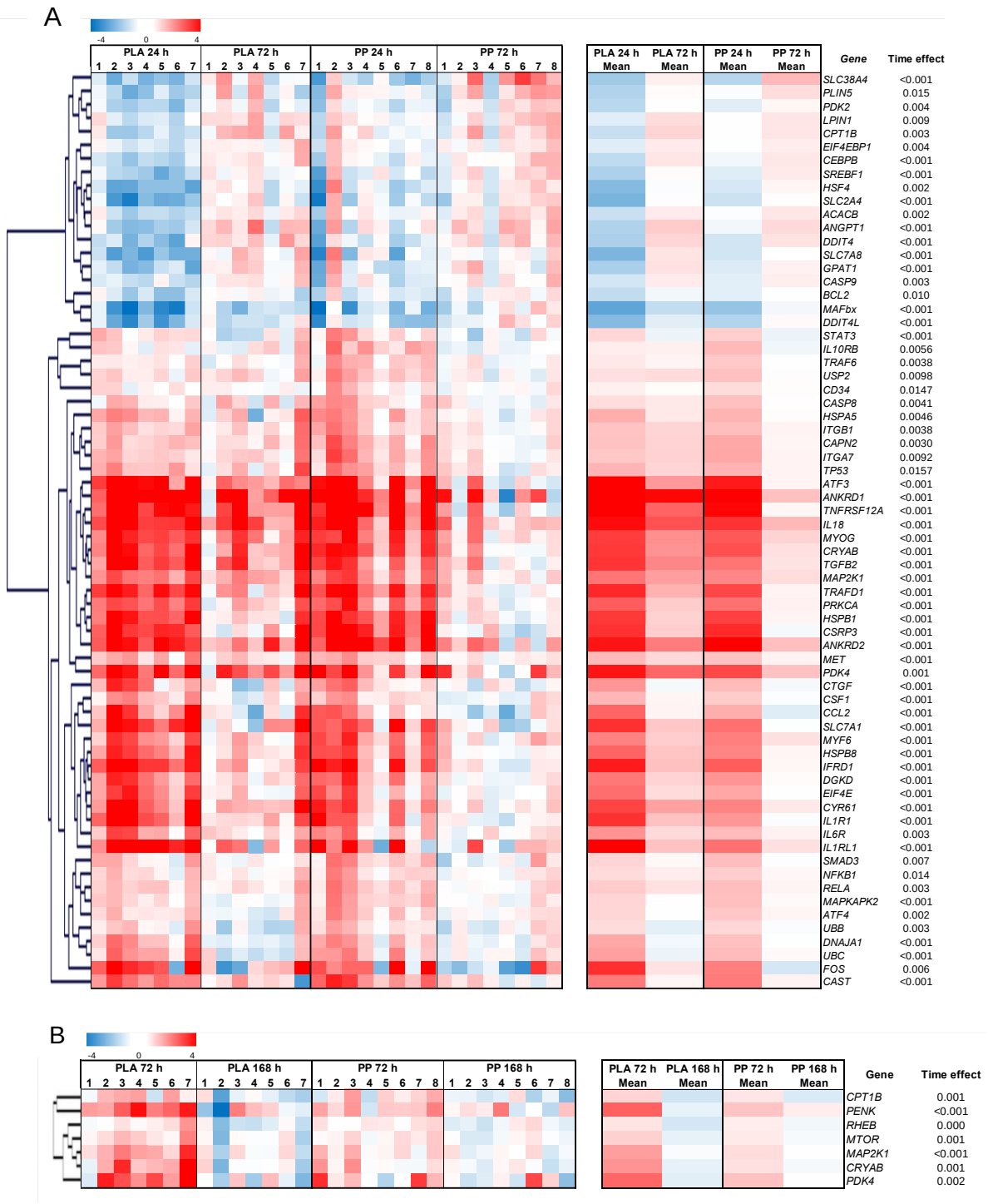

Figure 3.10. Heat maps of skeletal muscle mRNA expression of genes which were differentially expressed during the early (24 – 72 h; **A**) or late (72 – 168 h; **B**) period (time effect; FDR <5%) after 300 unilateral eccentric knee extensor contractions (time=0 h). Post-exercise and pre-bed protein-polyphenol (PP; *n*=9) or isocaloric maltodextrin placebo (PLA; *n*=9) drinks were ingested for 7 days prior to, and 7 days following eccentric contractions. All individual expression values are expressed as a fold change from the contralateral control leg at the same timepoint, with log_2 transformation and hierarchal clustering applied. Statistical analysis was performed with separate two-factor mixed effect models and corrected for familywise errors using a Benjamini-Hochberg correction.

Table 3.3. Gene ontology analysis of genes which were upregulated and downregulated during the early period. The three most enriched processes/pathways are shown.

		List total	List hits	P value	Genes
Up-regulated	**GO biological processes**				
	Transmembrane transport	22	6	0.008	*ACACB*, *BCL2*, *CPT1B*, *SLC2A4*, *SLC38A4, SLC7A8*
	Cellular response to dexamethasone stimulus	10	4	0.009	*CASP9*, *DDIT4*, *EIF4EBP1, MAFbx*
	Carboxylic acid transmembrane transport	10	4	0.009	*ACACB*, *CPT1B*, *SLC38A4, SLC7A8*
	Reactome pathways				
	SLC-mediated transmembrane transport	6	3	0.014	*SLC7A8, SLC2A4*, *SLC38A4*
	Carnitine metabolism	3	2	0.027	*ACACB, CPT1B*
	Triglyceride biosynthesis	3	2	0.027	*GPAT1*, *LPIN1*
Down-regulated	**GO biological processes**				
	Regulation of gene expression	127	39	0.002	*ANKRD1*, *ANKRD2*, *ATF3*, *ATF4*, *CAPN2*, *CASP8*, *CAST*, *CAST*, *CD34*, *CRYAB*, *CSF1*, *CSRP3*, *CTGF*, *CYR61*, *EIF4E*, *FOS*, *HSPA5*, *HSPB1*, *IFRD1*, *IL18*, *IL1R1*, *IL1RL1*, *IL6R*, *MAP2K1*, *MAPKAPK2*, *MET*, *MYF6*, *MYOG*, *NFKB1*, *PRKCA*, *RELA*, *SMAD3*, *STAT3*, *TGFB2*, *TP53*, *TRAF6*, *UBB*, *UBC*, *USP2*
	Regulation of response to stress	77	27	0.004	*ANKRD1*, *ATF3*, *ATF4*, *CD34*, *CRYAB*, *CTGF*, *DNAJA1*, *HSPA5*, *HSPB1*, *HSPB8*, *IL18*, *IL1R1*, *IL1RL1*, *ITGB1*, *MAP2K1*, *MAPKAPK2*, *MET*, NFKB1, *PRKCA*, *RELA*, *SMAD3*, *STAT3*, *TGFB2*, *TNFRSF12A*, *TP53*, TRAF6, *TRAFD1*
	Positive regulation of cell differentiation	63	23	0.006	*ANKRD1*, *CAPN2*, *CASP8*, *CD34*, *CSF1*, *CSRP3*, *CTGF*, *CYR61*, *FOS*, *HSPA5*, *IL18*, *IL6R*, *MAP2K1*, *MYF6*, *MYOG*, *NFKB1*, *PRKCA*, *RELA*, *SMAD3*, *STAT3*, *TGFB2*, *TNFRSF12A*, *TRAF6*
	Reactome pathways				
	Interleukin-1 family signalling	18	11	0.002	*IL1R1, ILR1L1, IL18, MAP2K1, NFKB1, SMAD3, STAT3, TRAF6, UBB, UBC*
	Cellular senescence	14	8	0.013	*FOS, MAPKAPK2, NFKB1, RELA, STAT3, TP53, UBB, UBC*
	NF-κB is activated and signals survival	7	5	0.021	*NFKB1, RELA, TRAF6, UBB, UBC*

List total = number of genes contained on the gene card categorised into that process/pathway. List hits = number of significant genes from the list total.

Table 3.4. Gene ontology analysis of genes which were downregulated during the late period. The three most enriched processes/pathways are shown.

		List total	List hits	P value	Genes
Downregulated	**GO biological processes**				
	Positive regulation of oligodendrocyte differentiation	2	2	0.002	*MTOR*, *RHEB*
	Response to morphine	3	2	0.004	*MTOR*, *PENK*
	Positive regulation of behaviour	3	2	0.004	*MTOR*, *PENK*
	Reactome pathways				
	Signalling by retinoic acid	3	2	0.004	*CPT1B*, *PDK4*
	HSF-1 dependent transactivation	7	2	0.019	*CRYAB*, *MTOR*
	Regulation of PTEN gene transcription	8	2	0.024	*MTOR*, *RHEB*

List total = number of genes contained on the gene card categorised into that process/pathway. List hits = number of significant genes from the list total.

3.4 Discussion

The mechanisms that regulate the recovery of human skeletal muscle function after injury are poorly understood. In the current study, we report several novel findings. Firstly, the individual decline in isokinetic work 24-72 h after eccentric contractions demonstrates a strong tendency to positively correlate with the individual increase in daily myofibrillar protein synthesis rates during the same period (placebo condition; **Figure 3.7B**). This occurred despite a clear increase in inflammatory NF-kB-mediated transcription in skeletal muscle (**Figures 3.8** and **3.9**). Secondly, using a protein-polyphenol nutrition intervention to attenuate the decline in muscle function did not further increase myofibrillar protein synthesis rates, but instead downregulated the skeletal muscle mRNA expression of the membrane receptors TNFRSF10B, TNFRSF12A, and IL1R1 (**Figures 3.8B**, **G** & **H**), which are known to activate NF-kB and regulate proteolytic pathways in skeletal muscle. Consequently, these data suggest the recovery of muscle function after injurious eccentric contractions is dependent on downregulating NF-kB signalling, possibly to prevent aberrant muscle protein breakdown.

Eccentric muscle contractions cause ultrastructural injury to the contractile myofibrillar fraction of skeletal muscle, and this has been directly related to changes in functional outcomes such as isometric torque (6, 42, 47). Consistent with this and with other studies that report functional outcomes after a similar eccentric contraction protocol (41, 159, 160, 164, 165), skeletal muscle isometric torque and isokinetic work were impaired by a respective average of 36% and 34% between 24-72 h after eccentric contractions (placebo condition; **Figure 3.3A** & **B**). This occurred concomitantly with an increase in muscle soreness, a decrease in PPT (placebo condition, 24-96 h; **Figure 3.2**) and preceded the increase in plasma creatine kinase activity (placebo condition, 96-120 h; **Figure 3.4A**). Daily post-exercise and pre-bed protein-polyphenol ingestion attenuated the decline in isometric torque after 24 h, and entirely prevented the decline in isokinetic work from baseline. This is consistent with similar intervention studies in humans whereby protein (164, 173) or polyphenol (182–185) ingestion accelerates the recovery of isometric torque or isokinetic work relative to a placebo 6-72 h after eccentric contractions. Muscle soreness, PPT and creatine kinase activity

was elevated comparably between protein-polyphenol and placebo conditions (although vastus medialis PPT was greater after 72, 120 and 168 h with protein-polyphenol ingestion). This would suggest that the initial myofibrillar injury was consistent between conditions and that protein-ingestion predominantly accelerates the remodelling of contractile components in skeletal muscle.

The recovery of skeletal muscle force production is typically complete within 2-7 days of eccentric contractions (**Figure 3.3**) (41). However, the significance of myofibrillar protein synthesis rates in this process is undetermined. This is primarily because existing studies have used labelled amino acid infusion protocols to provide a 'snapshot' of myofibrillar or mixed muscle protein synthesis rates over a period of hours during bed rest conditions (6, 7, 19, 43). Furthermore, muscle protein synthesis rates have not been measured beyond 48 h post-exercise or in combination with the measurement of any functional outcome. To address this, the current study applied deuterated water to measure average daily myofibrillar protein synthesis rates under free-living conditions in combination with a full dietary control and standardised protein intake. Using a unilateral design, the current study demonstrates for the first time that eccentric contractions increase average daily myofibrillar protein synthesis rates by 17% relative to the contralateral control leg during the early (24-72 h) post-exercise period (placebo condition; **Figure 3.6C**). Underlining the significance of this increase, the individual increase in myofibrillar protein synthesis shows a strong tendency to correlate positively with the average decrease in isokinetic work during the early period (placebo condition; **Figure 3.7B**). This is likely to be a reparative response to provide new myofibrillar protein to restore myofibrillar structure. However, this is in direct contrast to studies that have concluded no relationship exists between free-living myofibrillar protein synthesis rates measured using deuterated water and functional outcomes after traditional resistance-type exercise (51, 210). However, the validity of these conclusions is open to interpretation as Waskiw-Ford and colleagues report that their resistance exercise model failed to induce myofibrillar injury (z-band steaming) in the first instance (210). Consequently, it seems that resistance-type exercise is not a suitable model to investigate the remodelling of myofibrillar injury as substantially less (or no) myofibrillar injury is caused compared to the single joint, maximal eccentric contraction model as used in the current study (41).

It was hypothesised that an increase in myofibrillar protein synthesis rates by protein-polyphenol ingestion would explain the attenuated decline in functional outcomes after eccentric contractions. Contrary to this, daily myofibrillar protein synthesis rates 24-72 h after eccentric contractions increased by 12% in the protein-polyphenol condition, a similar increase to that observed in the placebo condition (17%) (**Figure 3.6C**). Furthermore, the increase in myofibrillar protein synthesis rates did not correlate with the decline in isometric torque or isokinetic work in the protein-polyphenol condition (**Figure 3.7**). Myofibrillar protein synthesis rates were assessed 24-72 h after eccentric contractions. Given isometric torque and isokinetic work had mostly returned to baseline by 24 h with protein-polyphenol ingestion, it is possible that protein-polyphenol ingestion increased myofibrillar protein synthesis rates to the greatest extent before our measurement period (this is discussed further in **Chapter 8**). As such, the 12% increase in myofibrillar protein synthesis rates in the damaged leg is likely to be comprised of both the residual and diminishing stimulation by myofibrillar injury in addition to stimulation by daily resistance exercise combined with protein ingestion. Consequently, the combination of these processes likely resulted in a comparable increase in myofibrillar protein synthesis rates to that seen in the placebo condition, where stimulation by myofibrillar remodelling is ongoing.

It could be argued that daily post-exercise and pre-bed protein polyphenol ingestion was not an effective intervention to increase myofibrillar protein synthesis rates. However, protein-polyphenol ingestion provided an additional ~40 g of daily protein, an increase associated with a greater anabolic response to repeated sessions of resistance exercise (98). Furthermore, protein-polyphenol ingestion provided 20 g of post-exercise protein which maximally potentiates post-exercise muscle protein synthesis rates (95, 96). Indeed, during the late remodelling period (72-168 h after eccentric contractions) once muscle function was largely restored, daily myofibrillar protein synthesis rates in the protein-polyphenol condition were (non-significantly) 20% greater than in the placebo condition (P=0.150). However, a targeted investigation of the acute post-exercise and overnight protein synthetic response to post-exercise and pre-bed protein-polyphenol drinks, respectively, would provide more resolution to delineate the acute increase in myofibrillar protein synthesis.

It has been demonstrated in some (4, 124, 208), but not all (125), studies that an increase in systemic inflammation in healthy individuals achieved by the intravenous infusion of cytokines (e.g. IL-6) and endotoxin, which are associated with the activation of NF-kB signalling, decreases resting mixed muscle protein synthesis rates. Currently, myofibrillar protein synthesis rates were elevated in the placebo condition despite a clear and concomitant upregulation of skeletal muscle mRNA expression associated with NF-kB signalling (**Figures 3.8** and **3.9**). Plasma IL-6 concentrations of ~150 pg/mL following systemic IL-6 overexpression in mice (211) or IL-6 infusion in humans (124) suppress muscle protein synthesis rates. In contrast, plasma TNF-α and IL-6 concentrations of ~17 and ~7 pg/mL, respectively, which are more comparable to concentrations observed presently (~6 and ~2 pg/mL, respectively; **Figure 3.5**), do not. However, cytokine concentrations represent a systemic response, whereas the increase in skeletal muscle mRNA expression associated with NF-kB activation was more robust. Furthermore, daily concentric resistance exercise was performed during the recovery period which stimulates myofibrillar protein synthesis rates and can have anti-inflammatory effects (154). It would therefore be interesting to determine the effect of NF-kB activation on myofibrillar protein synthesis and even muscle mass with the absence of contractions such as occurs during limb immobilisation.

It is interesting to consider what mechanism is driving the protracted increase in myofibrillar protein synthesis rates in the injured leg. Based on studies in burns patients, skeletal muscle assumes the role of an amino acid reservoir and is broken down to meet the increased amino acid demand of wounded tissue and to support remodelling (145). As a consequence, the increase in muscle protein breakdown leads to impaired functional outcomes in these patients (143). Interestingly, 'SLC-mediated transmembrane transport' was enriched during the early period (due to a decrease in expression of genes at 24 h and subsequent return to baseline expression at 72 h; **Table 3.3**). This could represent a feedback response to elevated intracellular amino acid concentrations derived from an increase in muscle protein breakdown. In mice, muscle protein breakdown is elevated for at least 120 h after in vivo eccentric contractions (45). In humans, skeletal muscle proteolytic gene expression, frequently used as a surrogate for a direct measurement of muscle protein breakdown, increases after eccentric contractions (26, 205, 212). It is therefore interesting to speculate if

protein-polyphenol ingestion attenuated an aberrant protein breakdown response to subsequently attenuate the decline in functional outcomes. Indeed, it is well established that protein ingestion attenuates the increase in muscle protein breakdown after resistance exercise (17). Accordingly, the protracted increase in myofibrillar protein synthesis rates with placebo ingestion may be driven by increased muscle protein breakdown, which in turn leads to prolonged functional impairments. An investigation of proteolytic gene expression in response to protein-polyphenol ingestion would therefore be an interesting area for further investigation.

Skeletal muscle mRNA expression associated with NF-kB signalling was first reported to be upregulated three h after eccentric contractions by Hyldahl and colleagues (86). More recently, an increase in NF-kB phosphorylation and DNA binding activity up to eight days after eccentric contractions has been reported (86, 164, 165). In the current study, our unbiased gene ontology approach identified numerous enriched annotations associated with NF-kB activation (**Table 3.3**). Furthermore, we identified a widespread and coordinated upregulation of up and downstream genes associated with NF-kB-mediated transcription (**Figure 3.8** and **3.9**) and based on this gene expression response, the NF-kB pathway was predicted to be activated using Ingenuity Pathway Analysis software (Qiagen; **Figure 3.11**). This response was greatest during the early period, concomitant with the largest impairment in functional outcomes. Accordingly, a major novel finding of the current study is that protein-polyphenol ingestion downregulated the early mRNA expression of the membrane receptors IL1R1, TNFRSF10B and TNFRSF12A (**Figure 3.8 B**, **G** & **H**). The function of these genes has not been characterised in humans in response to myofibrillar injury. However, signalling by the protein product of TNFRSF12A, fibroblast growth factor-inducible 14 (Fn14) and its cognate ligand, tumor necrosis factor (TNF)-like weak inducer of apoptosis (TWEAK) impairs skeletal muscle regeneration and activates NF-kB signalling after cardiotoxin injury in mice (213, 214). Furthermore, the activation of NF-kB by TWEAK/Fn14 signalling in mice causes skeletal muscle atrophy by upregulating the ubiquitin-proteasome system (215). In the current study, TNFRSF10B, TNFRSF12A and IL1R1 mRNA expression was downregulated by protein-polyphenol ingestion predominantly after baseline muscle function was restored. This might suggest that signalling through these receptors may not directly regulate the recovery of muscle function, and rather their expression is a reflection of

the remodelled state of the muscle. Nonetheless, given the reported association between these signalling pathways and muscle protein breakdown, this would support the hypothesis that protein-polyphenol ingestion may attenuate the decline in muscle function by attenuating aberrant muscle protein breakdown.

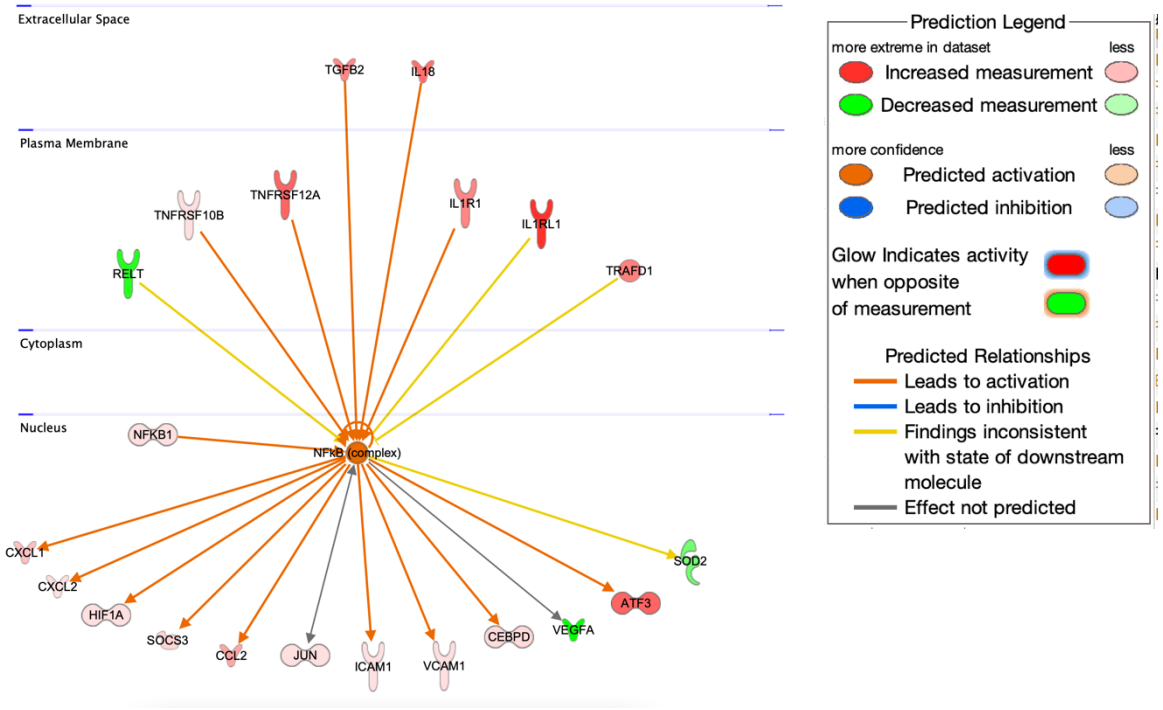

Figure 3.11. Ingenuity Pathway Analysis constructed to demonstrate the predicated activation of the NF-kB complex and its relationship to up- and down-stream muscle gene expression relative to the contralateral control leg 24 h after eccentric contractions in the placebo condition only to demonstrate the effect of eccentric contractions *per se.*

There is a rapidly accumulating evidence base from cell and animal models demonstrating that polyphenols resolve inflammation and accelerate the regeneration of skeletal muscle injury. This has been associated with a reduction in NF-κB activation, cytokine production, ubiquitin-proteasome activation and leukocyte infiltration (216–219). Furthermore, polyphenols promote a phenotypic switch from an inflammatory M1 to a pro-resolution M2 macrophage population in mouse models of obesity and atherosclerosis (220, 221). In humans, M2 macrophages have been shown to resolve inflammation and localise with regenerating skeletal muscle following myofibrillar injury (87). Furthermore, M2 macrophages attenuate the mRNA expression of the E3 ubiquitin ligase MuRF1 and attenuate skeletal muscle protein

breakdown in mice (222). Recently, the phenolic curcumin has been shown to attenuate the mRNA expression of the E3 ubiquitin ligase MAFbx after eccentric treadmill running in humans. Taken together, these data highlight a role for protein-polyphenol ingestion in attenuating the early inflammatory response to myofibrillar injury and reducing aberrant muscle protein breakdown as a consequence. This would explain the subsequent reduction in mRNA expression associated with upstream NF-κB activation 24-72 h after eccentric contractions. Conversely, the protracted increase in inflammation-mediated muscle protein breakdown in the placebo condition would maintain elevated intracellular amino acid availability to support greater myofibrillar protein synthesis rates, and this requires further investigation.

In conclusion, this is the first study to demonstrate that daily myofibrillar protein synthesis rates between 24-72 h after eccentric contractions are likely to be positively associated with the decline in muscle function. However, nutritionally attenuating the decline in muscle function and inflammatory mRNA expression did not increase myofibrillar protein synthesis rates. Instead, reducing inflammatory mRNA expression associated with NF-kB signalling may be important to attenuate the decline in muscle function after eccentric contractions, possibly by attenuating muscle protein breakdown.

Chapter 4

A combined protein-polyphenol nutrition intervention decreases skeletal muscle mRNA expression associated with NF-κB signalling and leukocyte activation after eccentric contractions.

The data presented in this chapter have been published.

Jameson, T. S.*, Pavis, G. F*., Dirks, M. L., Lee, B. P., Abdelrahman, D. R., Murton, A. J., Porter, C., Alamdari, N., Mikus, C. R., Wall, B. T., & Stephens, F. B. (2021). Reducing NF-κB signalling nutritionally is associated with expedited recovery of skeletal muscle function after damage. *The Journal of Clinical Eccrinology and Metabolism*. Doi: 10.1210/clinem/dgab106.

4.1 Introduction

It was demonstrated in **Chapter 3** that eccentric contractions caused a substantial decrease in muscle function between 24-48 h, which was attenuated by post-exercise and pre-bed protein-polyphenol ingestion. This occurred concomitantly with a downregulation in NF-κB associated transcription, which is known to regulate the expression of proteolytic genes in skeletal muscle. It was hypothesised, therefore, that the recovery of muscle function after eccentric contractions may be dependent on a reduction in post-exercise inflammatory and proteolytic events.

The increase in cellular protein degradation after eccentric contractions appears to be regulated by the ubiquitin-proteasome system (UPS) (26) and calcium-dependent calpains (47, 223). UPS activation under pro-inflammatory conditions in skeletal muscle is mediated by cytokines (i.e., TNF-α and IL-6) and NF-κB signalling (111, 224). Ubiquitin mRNA and protein expression and ubiquitin conjugated proteins increase 6-48 h after eccentric contractions (225, 226). The mRNA expression of the E3 ubiquitin ligases MuRF1 and MAFbx increases (MuRF1) (212) decreases (MAFbx) (205) or doesn't change (MAFbx) (227). Recently, ingestion of the phenolic curcumin, which inhibits NF-κB activation (228), has been shown to reduce the protein expression of ubiquitin and MAFbx after an eccentric treadmill running protocol in human volunteers (227). However, no measure of muscle function or protein synthesis was performed, and treadmill running *per se* didn't increase ubiquitin or MAFbx expression, likely because treadmill running induces comparatively less muscle injury than single joint eccentric contractions (41). Therefore, the significance of these results is unclear, and further widespread transcriptional investigation of the effect of nutrition on proteolytic pathways after high-force eccentric contractions is required.

In **Chapter 3**, the RT-PCR gene array and gene ontology approach identified 'SLC-mediated transmembrane transport' as enriched, due to a downregulation in amino acid transporters at 24h. This could represent a feedback response to elevated intracellular amino acid availability from an increase in muscle protein breakdown (229). The mammalian target of rapamycin (mTOR) is an integral protein in the phosphorylation cascade that initiates protein translation in skeletal muscle (31). The availability of intracellular amino acids, which is regulated either by uptake from the

extracellular pool (i.e., plasma) or from the breakdown of muscle protein, is a primary regulatory factor of mTOR signalling (230). Previous work has shown the phosphorylation of proteins in the mTOR1 phosphorylation cascade is increased 3-24 h after eccentric contractions (43, 53), and protein ingestion potentiates the phosphorylation of these proteins after traditional resistance-type exercise (231). However, it is yet to be determined if an intervention which is reasoned to attenuate muscle protein breakdown and intracellular amino acid availability affects mTOR signalling after eccentric contractions.

The current study investigated the post-exercise (24-27 h) and overnight (27-36 h) remodelling periods after a work-matched bout of concentric resistance exercise the day after eccentric contractions. We chose to investigate between 24-36 h after eccentric contractions as this was when the largest loss of muscle function and increase in inflammatory gene expression was observed (**Chapter 3**). The same post-exercise and pre-bed protein-polyphenol nutrition approach used in **Chapter 3** was employed to determine the acute regulation of myofibrillar protein synthesis rates using deuterated water. Based on the conclusions of **Chapter 3,** mTOR phosphorylation and mRNA expression associated with key proteolytic pathways in skeletal muscle were assessed. It was hypothesised that relative to placebo, protein-polyphenol ingestion would downregulate resting mTOR phosphorylation and skeletal muscle mRNA expression associated with muscle protein breakdown.

4.2 Methods

Participants

Seventeen young, healthy males (*n*=11) and females (*n*=6) (age: 23±1 y, BMI: 23.9±0.8 $kg \cdot m^{-2}$) volunteered to participate in the present study. Participants' characteristics are displayed in **Table 4.1**. Female participants not using an oral contraceptive were at day 6-12 of a regular menstrual cycle (i.e. mid-follicular phase) during days 7-9 of the study protocol. The study was approved by the Sport and Health Sciences ethics committee of the University of Exeter (REF NO. 161026/B/06). The study is registered at ClinicalTrials.Gov (ID: NCT02980900).

Table 4.1. Participant characteristics.

	PLA (*n*=8)	**PP** (*n*=9)
Sex	M=6, F=2	M=5, F=4
Age (y)	22±1	23±2
Body mass (kg)	79±4	68±4
Height (cm)	176±1	174±3
BMI ($kg \cdot m^{-2}$)	25±2	23±1
Total eccentric work ($J \cdot kg^{-1}$)	614±36	579±44
Habitual protein ($g \cdot kgBM^{-1} \cdot day^{-1}$)	1.2±0.2	1.4±0.2
Controlled protein (excluding condition) ($g \cdot kgBM^{-1} \cdot day^{-1}$)	1.2±0.1	1.2±0.1
Controlled protein (including condition) ($g \cdot kgBM^{-1} \cdot day^{-1}$)	1.2±0.1	1.8±0.1*

Values represent mean±SEM. PLA, Placebo; PP, Protein-polyphenol; BMI, body mass index; M = male; F = Female; NS, nonsignificant (P>0.05). Total eccentric work represents the total volume of eccentric work performed during the unilateral eccentric exercise protocol on day 7 of the experimental period normalised for body mass. Habitual protein represents participants 3-day average habitual protein intake obtained from a food diary. Controlled protein represents the 14-day average protein intake provided by the dietary control. Comparisons between PLA and PP were performed with separate unpaired t-tests. *difference in controlled protein intake (including condition) between PLA and PP.

Study protocol

Details of all experimental and analytical procedures are presented in **Chapter 2**. An overview of the experimental protocol is shown in **Figure 4.1**. Participants were randomly assigned to one of two parallel, fully controlled nutritional intervention groups consisting of either daily post-exercise and pre-bed protein-polyphenol ('PP') or placebo ('PLA') drinks (see **Chapter 3.2 – Experimental drinks**) and completed a nine-day study period under fully dietary controlled conditions. At 1900 h on day seven, participants performed a bout of eccentric contractions in one leg ('injured') randomly counterbalanced for leg dominance, with the contralateral leg functioned as a control ('control'). To determine free-living myofibrillar protein synthesis rates of injured and control legs, participants underwent a deuterated water dosing protocol which began with a loading day on day seven and continued with daily maintenance doses on days eight and nine.

On day eight, participants arrived at the laboratory at 1730 h, 1.5 h following ingestion of the controlled evening meal whereupon rested bilateral muscle biopsies were sampled (i.e., 24 h following eccentric contractions). Participants then performed five sets of 30 maximal knee extensor concentric contractions in the injured leg, followed by as many maximal repetitions as necessary (in sets of 30) in the control leg to match this volume of maximal work. Participants then ingested a post-exercise drink according to their condition randomisation and rested semi-supine in the laboratory for a 3-hour post-exercise period when the second set of bilateral muscle biopsies were sampled (i.e., 27 h following eccentric contractions). Participants then ingested a pre-bed drink according to their condition randomisation and returned home to sleep, returning to the laboratory the following morning when the third set of bilateral muscle biopsies were sampled (i.e., 36 h following eccentric contractions) exactly nine h after ingestion of the pre-bed beverage. No additional nutrition or physical activity was permitted during these periods.

Sample collection and analysis

Ten mL of venous blood from the antecubital vein was collected on days 8 and 9. These samples were analysed at a later date for body water ^{2}H enrichment. Bilateral muscle biopsies of the vastus lateralis were taken 24, 27 and 36 h following eccentric contractions and categorised into "post-exercise" (24 – 27 h) and "overnight" (27 – 36

h) recovery periods. These samples were analysed at a later date for myofibrillar protein-bound [^{2}H]alanine, skeletal muscle mRNA expression and total and phosphorylated mTOR protein expression.

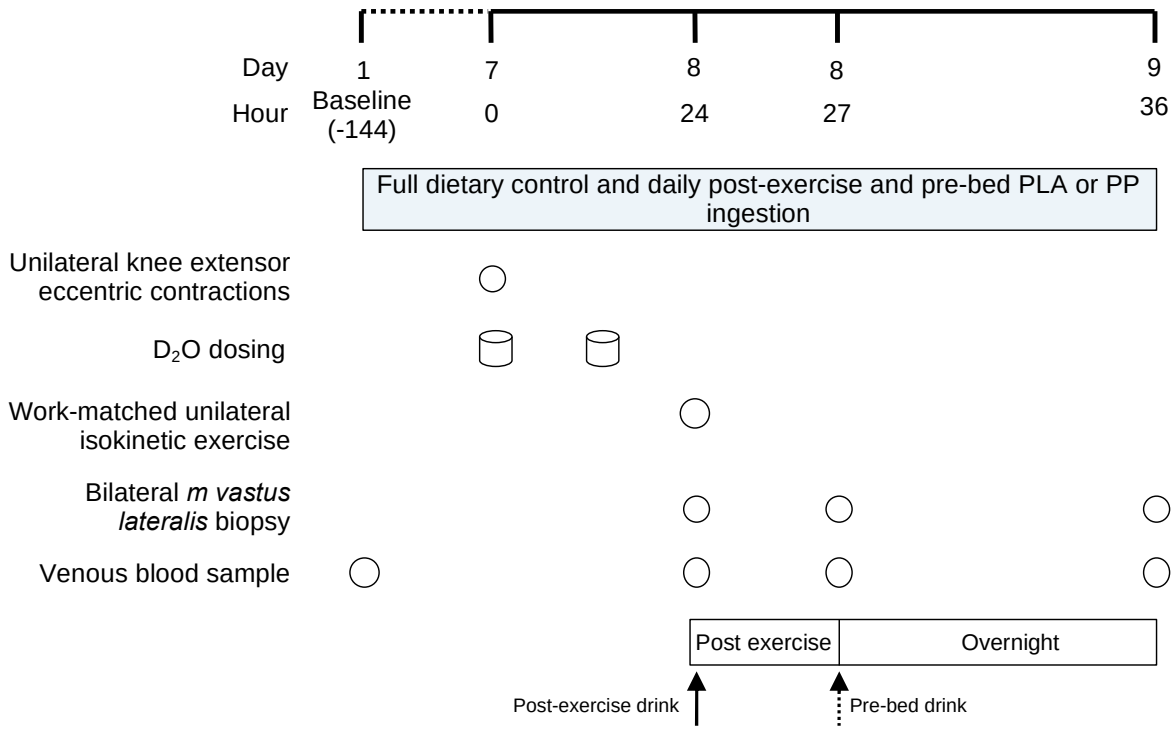

Figure 4.1. Schematic representation of the experimental protocol. Participants were provided with full dietary control (1.2 g·kgBM^{-1}·d^{-1} dietary protein) and post-exercise and pre-bed protein-polyphenol (PP; *n*=9) or isocaloric maltodextrin placebo (PLA; *n*=8) drinks were consumed daily. Loading of orally consumed 70% D_2O began at ~0800 h on day 7 with 8 x 6 mL·kg^{-1} doses consumed every 1.5 h and body water enrichment was maintained thereafter with daily doses of 0.54 mL·kg^{-1}. Unilateral eccentric knee extensor contractions were performed at ~1900 h on day 7 (t=0 h) and a work matched bout of concentric isokinetic contractions were performed unilaterally 24 h later. Bilateral muscle biopsies and a venous blood samples were obtained immediately before the work-matched exercise (24 h), 3 hours after ingestion of the post-exercise drink (27 h) and 9 hours after ingestion of the pre-bed drink (36 h). An additional venous blood sample was obtained at baseline.

Statistical analysis

Full statistical procedures and calculations are presented in **Chapter 2.** A repeated-measure two-factor analysis of variance (ANOVA) was used to analyse (condition *x* time) differences in body water ^{2}H enrichment and skeletal muscle mRNA expression associated with NF-κB and proteolytic pathways (condition *x* leg) differences in myofibrillar protein synthesis rates. Repeated-measure three-factor AVOVAs were used to analyse (time x condition x leg) differences in myofibrillar mole per cent excess (MPE) and post-exercise and overnight total mTOR, $mTOR^{Ser2448}$ and mTOR phosphorylation status. A repeated-measure mixed-effects model was used to analyse (condition x time) differences in post-exercise and overnight gene expression. A false discovery rate (*FDR*<0.05) was applied to adjust for familywise error before functional gene enrichment analysis (201). Violations of sphericity were corrected with the Greenhouse-Geisser correction. When a significant main and interaction effect was observed, a Sidak post hoc test was performed to locate individual differences. Statistical significance was set at *P*<0.05. Calculations were performed using GraphPad Prism 8.3.0. All data are expressed as means±SEM.

4.3 Results

Participant characteristics

No differences in age, weight, height, BMI, or habitual protein intake were detected between conditions (P>0.05; **Table 4.1**). Average daily energy and macronutrient ingestion during the 9-day controlled diet after accounting for adherence (not including condition) not different between conditions (P>0.05). PP ingestion increased protein intake compared to PLA (1.8±0.1 *vs* 1.2±0.1 g·kgBM^{-1}·d^{-1}, respectively; P<0.001). Total eccentric work performed did not differ between conditions (P>0.05).

Plasma creatine kinase activity and C-reactive protein concentration

Compared to baseline, plasma creatine kinase activity was increased after 24, 27 and 36 h (post hoc; P<0.05) (**Figure 4.2A**). Creatine kinase activity was not different between conditions (P=0.823, interaction; P=0.711). C-reactive protein concentration did not change over time (P=0.463) and was not different between conditions (P=0.757, interaction; P=0.142) (**Figure 4.2B**). C-reactive protein values from one participant in the PP condition were below the limits of detection.

Body water deuterium enrichment

Baseline body water deuterium enrichment did not differ between PLA (0.03±0.01%) and PP (0.02±0.01%) (P>0.05). Deuterated water loading increased body water deuterium enrichment (P<0.001) to a similar extent in PLA and PP (to 0.63±0.04% and to 0.58±0.03%, respectively; interaction; P=0.363) (**Figure 4.3A**). Body water deuterium enrichment showed a slight increase during the two days of maintenance dosing (P<0.001), averaging 0.64±0.02% in PLA and 0.59±0.02% in PP, with no difference between conditions (P=0.276).

Myofibrillar protein-bound [^{2}H]alanine enrichment and myofibrillar protein synthesis rates

Myofibrillar protein-bound [^{2}H]alanine enrichments increased during the post-exercise and overnight incorporation periods (P<0.001) (**Figure 4.3C-D**). The increase in post-exercise myofibrillar [^{2}H]alanine enrichment tended to be higher in the injured (∆0.012±0.001 MPE) compared to the control (∆0.010±0.001 MPE) leg (interaction;

P=0.07) (**Figure 4.3C**), and post-exercise MPE tended to be higher in the protein-polyphenol compared to placebo condition (*P*=0.058). The increase in overnight myofibrillar [^{2}H]alanine enrichment was higher in the injured (Δ0.020±0.002) compared to the control (Δ0.016±0.002 MPE) leg (interaction; *P*=0.042) (**Figure 4.3D**) and overnight MPE tended to be higher in the protein-polyphenol compared to placebo condition (*P*=0.051).

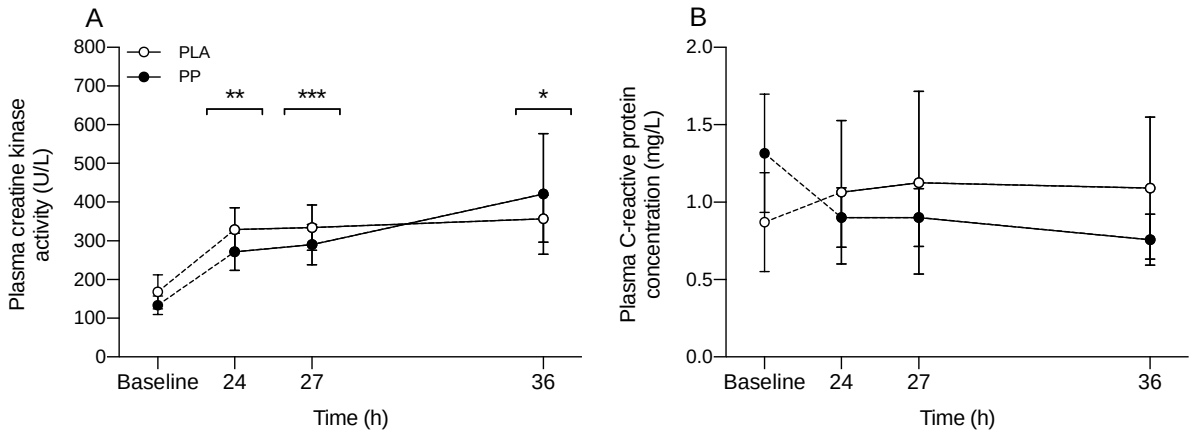

Figure 4.2. Plasma creatine kinase activity (**A**) and C-reactive protein content (**B**) measured at baseline and 24, 27 and 36 h following a bout of 300 unilateral eccentric knee extensor contractions (time=0 h). Post-exercise and pre-bed protein-polyphenol (PP; *n*=9 (*n*=8 for CRP as values from 1 participant were below the limits of detection); black circles) or isocaloric maltodextrin placebo (PLA; *n*=9; open circles) drinks were ingested for 7 days prior to, and 1 day following eccentric contractions. Data are presented as means with error bars representing standard error. Statistical analysis was performed with separate two-factor ANOVAs. * time point different to baseline (main effect of time). One symbol *P*<0.05, two symbols *P*<0.01, three symbols *P*<0.001.

Post-exercise myofibrillar protein synthesis rates following a work-matched bout of concentric exercise tended to be higher in the injured (0.139±0.013%·h^{-1}) than in the control (0.112±0.012%·h^{-1}) leg (*P*=0.075) (**Figure 4.4A**). Post-exercise myofibrillar protein synthesis rates were not affected by condition (*P*=0.412, interaction; *P*=0.313). Myofibrillar protein synthesis rates decreased (*P*=0.027) from the post-exercise to the overnight period similarly between control (from 0.112±0.012 to 0.075±0.008%·h^{-1}, respectively) and injured (from 0.139±0.013 to 0.096±0.007%·h^{-1}, respectively) legs (interaction; P=0.820). Overnight myofibrillar protein synthesis rates were higher in the

injured (0.096±0.007%·h^{-1}) compared to the control (0.075±0.008%·h^{-1}) leg (*P*=0.020) but were not affected by condition (*P*=0.908, interaction; *P*=0.385) (**Figure 4.4B**).

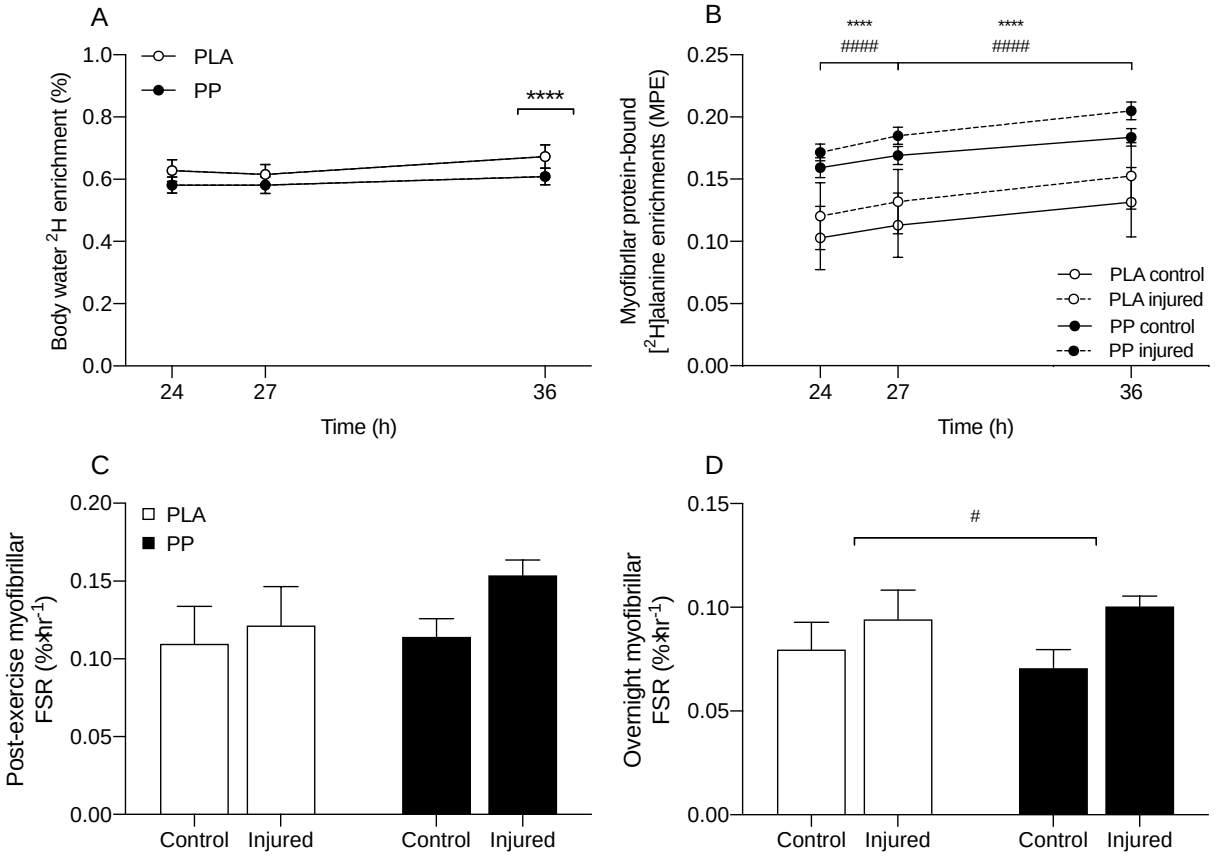

Figure 4.3. Body water ^{2}H enrichment (%) measured in blood plasma (**A**), myofibrillar bound [^{2}H]alanine enrichment a control (solid line) and eccentrically contracted (dashed line) leg (**B**), and post-exercise (24 – 27 h) (**C**) and overnight (27 – 36 h) (**D**) myofibrillar fractional synthetic rates (FSR). 300 unilateral knee extensor contractions were performed at time=0. Protein-polyphenol (PP; *n*=9; black bars/circles) or isocaloric maltodextrin placebo (PLA; *n*=9; white bars/circles) drinks were ingested for 7 days prior to, and 1 day after eccentric contractions. Data are presented as means with error bars representing standard error. Body water ^{2}H enrichment and early and ate FSRs were analysed with two-factor ANOVAs. * difference between time points (difference to 24 h in **A**), # difference between control and injured legs. One symbol *P*<0.05, two symbols *P*<0.01, three symbols *P*<0.001, four symbols

mTOR protein content and phosphorylation status

Western blotting was performed in *n*=16 (PLA *n*=8, PP *n*=8) due to limited remaining tissue.

Skeletal muscle total mTOR protein content did not change during the post-exercise period (*P*=0.448) (**Figure 4.4A**) and was not different between control and injured legs (*P*=0.119). Post-exercise total mTOR protein content was higher in PLA than PP (*P*=0.038). Skeletal muscle mTOR^{Ser2448} protein content did not change during the post-exercise period (*P*=0.127) (**Figure 4.4C**) but tended to be higher in the injured compared to the control leg (*P*=0.053), tended to be higher in PLA compared to PP (*P*=0.070) and tended to interact between leg and condition (interaction; *P*=0.068). Skeletal muscle mTOR^{Ser2448} phosphorylation status decreased by 7±5% during the post-exercise period (*P*=0.035) (**Figure 4.4E**) and was 19±8% higher in the injured compared to the control leg (*P*=0.002). Post-exercise mTOR^{Ser2448} phosphorylation status was not different between conditions (*P*=0.230, interaction effect; *P*=0.496).

Skeletal muscle total mTOR protein content did not change during the overnight period (*P*=0.133) (**Figure 4.4B**) and tended to be different between legs (*P*=0.059) and conditions (*P*=0.057). Overnight skeletal muscle mTOR^{Ser2448} protein content decreased during the overnight period (*P*=0.006) (**Figure 4.4D**) and was higher in the injured than control leg (*P*=0.009). Overnight mTOR^{Ser2448} protein content was not different between conditions (*P*=0.104, interaction; *P*=0.390). Skeletal muscle mTOR^{Ser2448} phosphorylation status decreased by 15±4% during the overnight period (*P*=0.001) (**Figure 4.4F**) and was 25±7% higher in the injured compared to the control leg (*P*<0.001). Overnight mTOR^{Ser2448} phosphorylation status was not different between conditions (*P*=0.222, interaction; *P*=0.775).

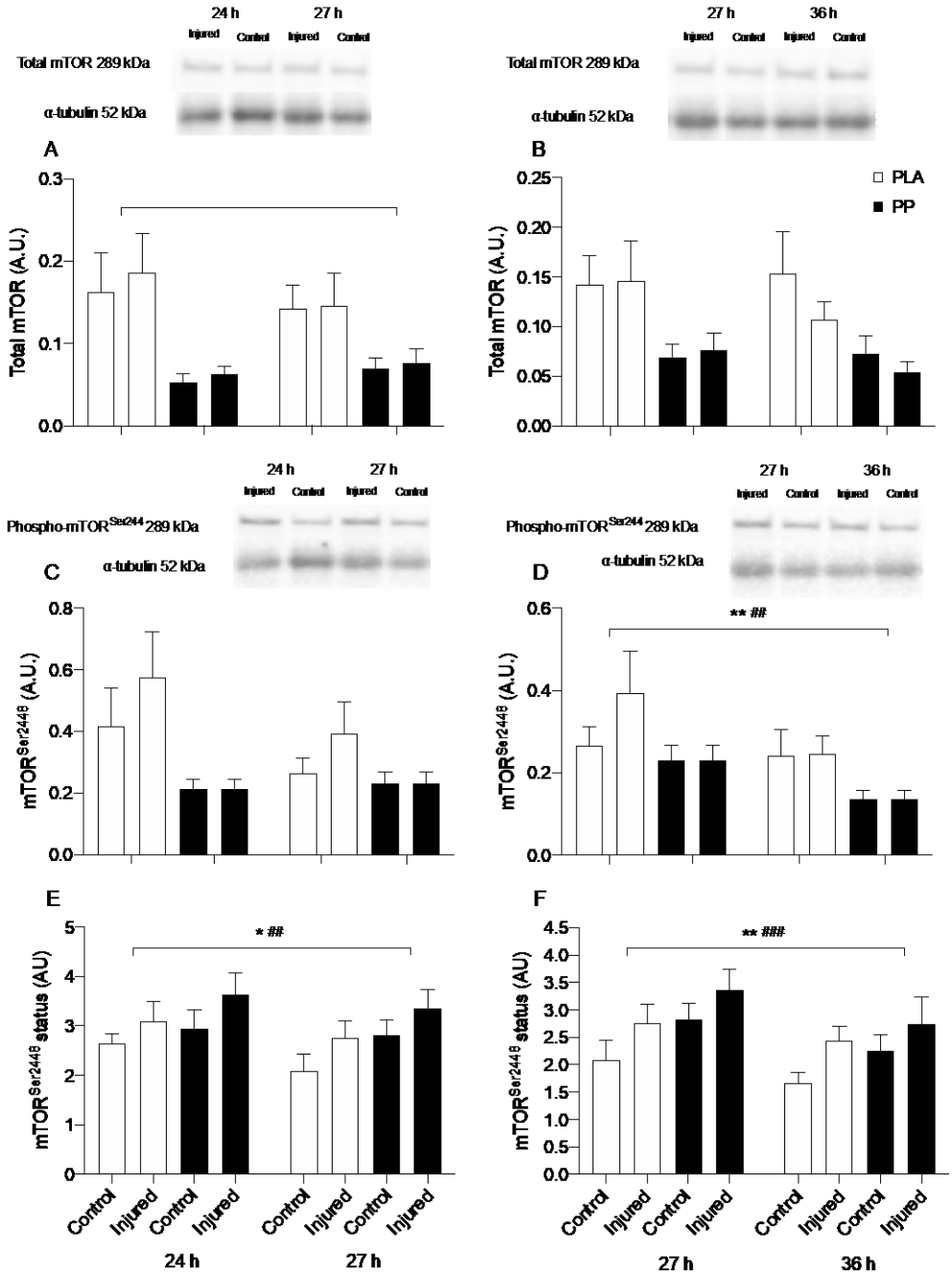

Figure 4.4. Skeletal muscle (mTOR) phosphorylation status, presented as total mTOR protein during post-exercise (**A**) and overnight (**B**) periods; mTOR phosphorylated at Ser2448 during post-exercise (**C**) and overnight (**D**) periods; and the ratio of phosphorylated to total protein during post-exercise (**E**) and overnight (**F**) periods following 300 unilateral eccentric knee extensor contractions (time=0 h). The post-exercise period incorporated a work-matched bout of maximal concentric knee extensor contractions and ingestion of either a post-exercise protein-polyphenol (PP; *n*=8; black bars) or isocaloric maltodextrin placebo (PLA; *n*=8; white bars) drink. The subsequent 9 h overnight period began with ingestion of either a pre-bed protein-polyphenol (PP; *n*=9) or isocaloric maltodextrin placebo (PLA; *n*=8) drink. α-tubulin was used as a loading control. Images obtained from a single representative participant in PLA. Data are presented as means with error bars representing standard error. Statistical analysis was performed with separate two-factor ANOVAs. * difference between time points, # difference between control and injured legs, † difference between conditions. One symbol $P<0.05$, two symbols $P<0.01$, three symbols $P<0.001$.

Skeletal muscle mRNA expression associated with NF-κB signalling

mRNA expression was calculated for *n*=15 (PLA *n*=7, PP *n*=8) due to limited remaining tissue.

The mRNA expression of seven upstream (*IL18*, *IL1R1*, *IL1RL1*, *RELT*, *TGFB2*, *TNFRSF12A* and *TRAFD1*; *P*<0.05) (**Figure 4.5**) and seven downstream (*ATF3*, *CCL2*, *CEBPD*, *CXCL2*, *SOCS3*, SOD2 and *VEGFA*; *P*<0.05) (**Figure 4.6**) genes associated with NF-κB activation were differentially expressed during the post-exercise period. *NFKB1* (upstream, *P*=0.065) and *HIF1A* (downstream, *P*=0.079) tended to be downregulated during the post-exercise period. Relative to PLA, PP ingestion downregulated the post-exercise expression of *IL1R1* (upstream, mean post-exercise $\log_2$ fold change; PLA=2.81±0.37, PP= 1.12±0.36; *P*=0.024, **Figure 4.5B**) and *IL1RL1* (upstream, mean post-exercise $\log_2$ fold change; PLA=4.92±0.74, PP=1.61±0.77; *P*=0.044, **Figure 4.5C**). PP ingestion also tended to downregulate *TGFB2* expression (*P*=0.072) and attenuate the downregulation of *VEGFA* (*P*=0.064). No NF-κB associated genes interacted between conditions over the course of the post-exercise period, but *TNFRSF10B* showed a tendency to (*P*=0.078).

The mRNA expression of four upstream (*IL18, TRAFD1, NFKB1, TNFRSF10B* and *TGFB2; P*<0.05) (**Figure 4.5**) and one downstream (*VCAM1; P*<0.05) (**Figure 4.6**) gene associated with NF-κB activation was differentially expressed during the overnight period. Relative to PLA, PP ingestion further downregulated the overnight expression of *IL1R1* (upstream, mean overnight $\log_2$ fold change; PLA=1.87±0.37, PP=0.31±0.36; *P*=0.018) (**Figure 4.5B**) and *IL1RL1* (upstream, mean overnight $\log_2$ fold change; PLA=3.71±0.61, PP=0.67±0.72; *P*=0.025) (**Figure 4.5C**). PP ingestion also downregulated the overnight expression of *CXCL1* (downstream, mean overnight $\log_2$ fold change; PLA=1.34±0.65, PP=-0.56±0.42; *P*=0.028) (**Figure 4.6D**), *CEBPD* (downstream, mean overnight $\log_2$ fold change; PLA=0.09±0.36, PP= -0.96±0.32; *P*=0.010) (**Figure 4.6C**) and *ICAM1* (downstream, mean overnight $\log_2$ fold change; PLA=-0.15±0.37, PP=-1.11±0.36; *P* = 0.044) (**Figure 4.6G**) relative to PLA. Overnight *VCAM1* mRNA expression (downstream) was downregulated with PLA ingestion (from 1.14±0.62 at 27 h to -0.99±0.60 at 36 h) but did not change with PP ingestion (from 0.17±0.20 at 27 h to -0.33±0.55 at 36 h) (interaction; *P*=0.041) (**Figure 4.6K**).

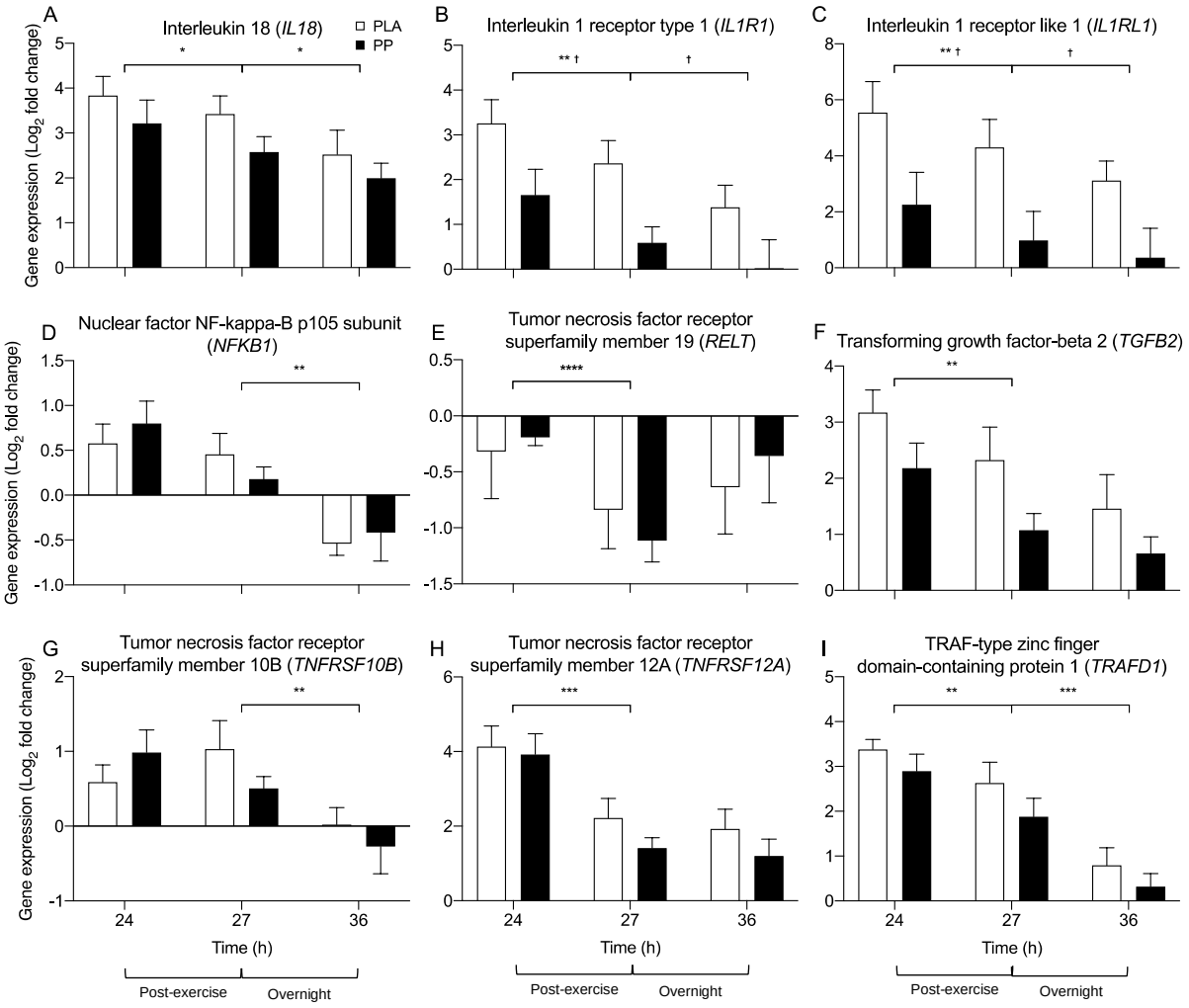

Figure 4.5. Skeletal muscle mRNA expression of 9 genes (**A**-**I**) upstream of NF-κB signalling during post-exercise (24 – 27 h) and overnight (27 – 36 h) periods after 300 unilateral eccentric knee extensor contractions (time=0 h). Post-exercise and pre-bed protein-polyphenol (PP; *n*=8; black bars) or isocaloric maltodextrin placebo (PLA; *n*=7; white bars) drinks were ingested for 7 days prior to, and 1 day following eccentric contractions. All individual expression values are expressed as a fold change from the contralateral control leg at the same timepoint, with log2 transformation applied. Data are presented as means with error bars representing standard error. Statistical analysis was performed with separate two-factor ANOVAs. * difference between time points, † difference between conditions. One symbol $P<0.05$, two symbols $P<0.01$, three symbols $P<0.001$, four symbols $P<0.0001$.

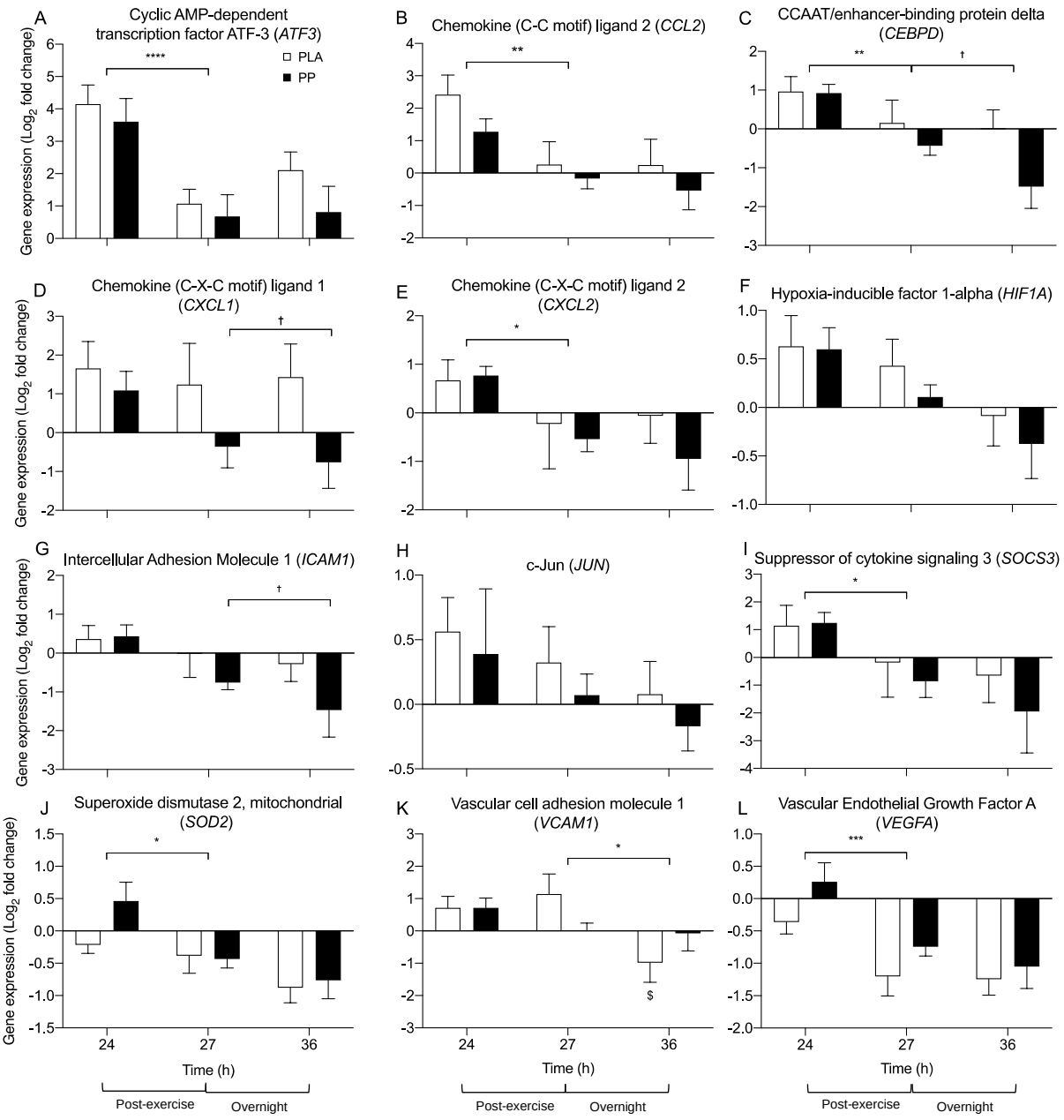

Figure 4.6. Skeletal muscle mRNA expression of 12 genes (**A**-**L**) downstream of NF-κB signalling during post-exercise (24 – 27 h) and overnight (27 – 36 h) periods after 300 unilateral eccentric knee extensor contractions (time=0 h). Post-exercise and pre-bed protein-polyphenol (PP; *n*=8; black bars) or isocaloric maltodextrin placebo (PLA; *n*=7; white bars) drinks were ingested for 7 days prior to, and 1 day following eccentric contractions. All individual expression values are expressed as a fold change from the contralateral control leg at the same timepoint, with log2 transformation applied. Data are presented as means with error bars representing standard error. Statistical analysis was performed with separate two-factor ANOVAs. * difference between time points, † difference between conditions, $ change in placebo condition only. One symbol $P<0.05$, two symbols $P<0.01$, three symbols $P<0.001$, four symbols $P<0.0001$.

Skeletal muscle mRNA expression associated with proteolysis

Post-exercise *FOXO1* and *MuRF1* mRNA expression was downregulated (P<0.05) to a similar extent between conditions (*P*>0.05; **Figure 4.7A** & **C**). Post-exercise *CAPN3* mRNA expression was higher in the protein-polyphenol condition (mean log_2 fold change -0.01±0.25) compared to the placebo condition (mean log_2 fold change -0.59±0.17; *P*=0.048; **Figure 4.7H**). Overnight *BECN1* mRNA expression was downregulated (P<0.05) to a similar extent between conditions (*P*<0.05, interaction; *P*<0.05; **Figure 4.7J**).

Gene ontology analysis

Only genes which showed a significant change in expression over time passed the FDR cut-off of 5% and were included in the gene ontology analysis. During the post-exercise period there were 14 genes which showed main effect of condition and 7 genes which interacted between conditions (*P*<0.05). During the overnight period there were 9 genes which showed a main effect of condition and 3 genes which interacted between conditions (*P*<0.05). However, these genes did not pass the FDR cut off of 5% so were not included in gene ontology analysis. The mRNA expression of all 213 genes and their respective *P* values are provided in **Appendix 7.1** for reference.

The skeletal muscle mRNA expression of 42 genes was downregulated during the post-exercise period, and no genes were upregulated after applying an *FDR* cut-off of 5%. The result of the gene ontology analysis is presented in **Table 4.2,** and hierarchal clustering is presented in **Figure 4.7A.** Fourteen genes were differentially expressed between conditions (*ACACB*, *AKT2*, *C5*, *CAPN3*, *CEBPB*, CASP8, *CFLAR*, *EIF4EBP1*, *HK2*, *HSF4*, *IL10RB*, *IL1R1*, *IL1RL1*, *PAX7* and *PPARGC1A*; *P*<0.05) and 7 genes were differentially expressed between conditions over the post-exercise period (*RHOB*, *SLC38A2*, *TFAM*, *TRAF6*, *TRIM32*, *TYK2* and *UBB*; interaction; *P*<0.05). However, these genes did not meet the FDR cut off of 5% but are shown in **Appendix 7.1** for reference.

Overnight

The expression of 11 genes was downregulated, and the expression of two genes was upregulated during the overnight period after applying an *FDR* cut-off of 5%. The result

of the gene ontology analysis of downregulated genes is presented in **Table 4.3,** and hierarchal clustering is presented in **Figure 4.8B.** The 2 upregulated genes *SLC38A4* (amino acid transport, upregulated overnight exclusively) and *NR4A1* (transcription factor, downregulated post-exercise and upregulated overnight). Nine genes were differentially expressed between conditions (*CAST*, *CEBPD*, *CXCL1*, *FOS*, *HK2*, *HSPA5*, *IL1R1*, *IL1RL1*, *PPARGC1A*; $P<0.05$) and three genes interacted between conditions throughout the overnight period (*CASP9*, *MSTN*, *VCAM1*; interaction; $P<0.05$). However, these genes did not meet the FDR cut off of 5% but are shown in **Appendix 7.1** for reference.

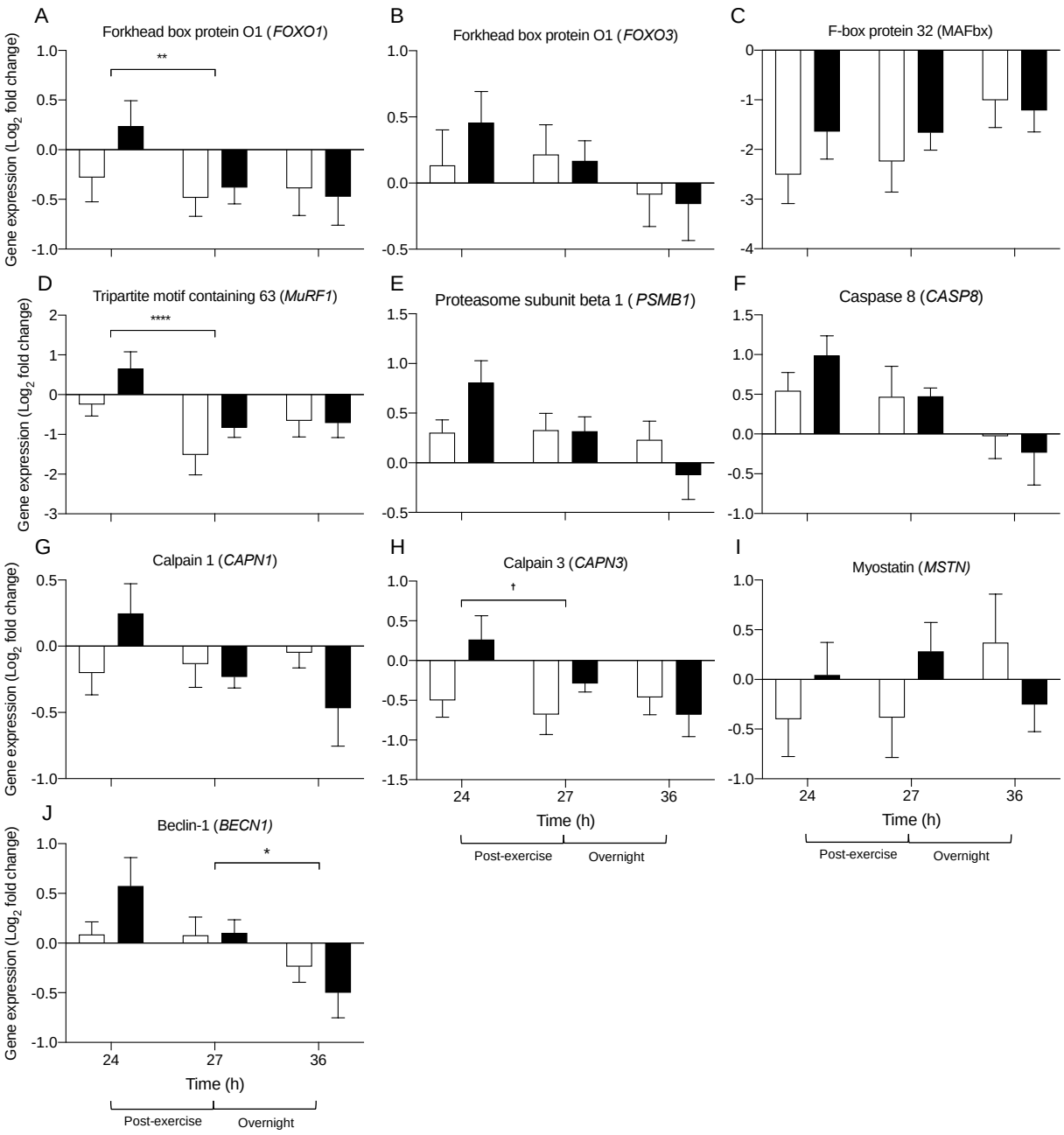

Figure 4.7. Skeletal muscle mRNA expression of genes belonging to pathways associated with skeletal muscle protein breakdown during post-exercise (24 – 27 h) and overnight (27 – 36 h) periods after 300 unilateral eccentric knee extensor contractions (time=0 h). Post-exercise and pre-bed protein-polyphenol (PP; *n*=8; black bars) or isocaloric maltodextrin placebo (PLA; *n*=7; white bars) drinks were ingested for 7 days prior to, and 1 day following eccentric contractions. All individual expression values are expressed as a fold change from the contralateral control leg at the same timepoint, with log2 transformation applied. Data are presented as means with error bars representing standard error. Statistical analysis was performed with separate two-factor ANOVAs. * difference between time points. One symbol *P*<0.05, two symbols *P*<0.01, three symbols *P*<0.001, four symbols *P*<0.0001.

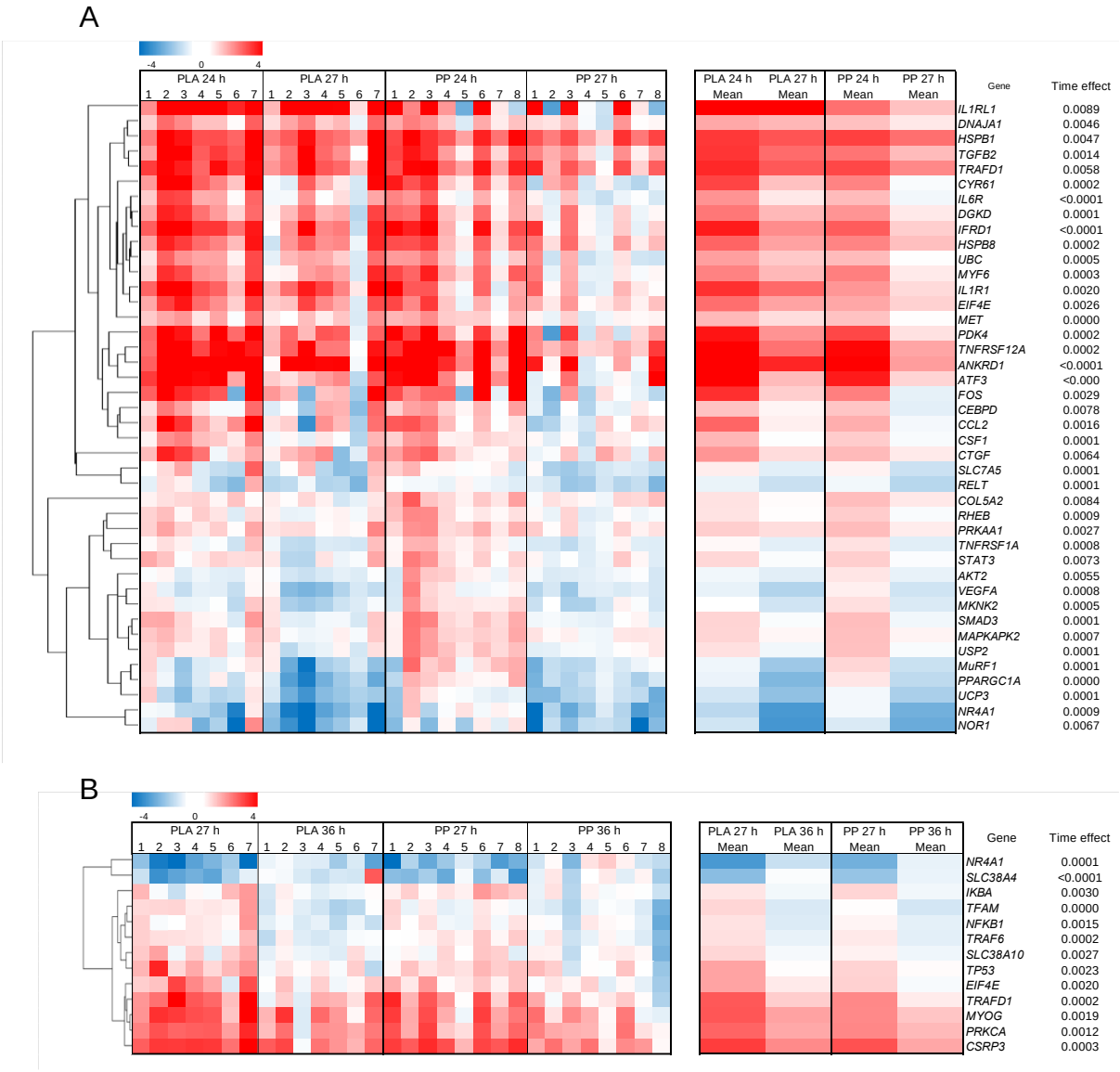

Figure 4.7. Skeletal muscle mRNA expression of genes which were differentially expressed during the post-exercise (24 – 27 h; **A**) or overnight (27 – 36 h; **B**) period (time effect; FDR <5%) following 300 unilateral eccentric knee extensor contractions (time=0 h). Post-exercise and pre-bed protein-polyphenol (PP; *n*=8) or isocaloric maltodextrin placebo (PLA; *n*=7) drinks were ingested for 7 days prior to, and 1 day following eccentric contractions. All individual expression values are expressed as a fold change from the contralateral control leg at the same timepoint, with $\log_2$ transformation and hierarchal clustering applied. Statistical analysis was performed with separate two-factor mixed effect models and corrected for familywise errors using a Benjamini-Hochberg correction.

Table 4.2. Gene ontology analysis of genes which were downregulated during the post-exercise period and genes which were downregulated exclusively during the post-exercise period and not overnight. No genes were upregulated. The three most enriched processes/pathways are shown.

Post-exercise		List total	List hits	P value	Genes
Downregulated	**GO biological processes**				
	Positive regulation of endothelial cell chemotaxis	3	3	0.019	*HSPB1*, *MET*, *VEGFA*
	Transcription by RNA polymerase II	12	6	0.025	*CEBPD*, *CTGF*, *FOS*, *NR4A1*, *PPARGC1A*, *STAT3*
	Regulation of membrane lipid metabolic processes	4	3	0.040	*CTGF*, *NR4A1*, *PPARGC1A*
	Reactome pathways				
Downregulated exclusively post-exercise	**GO biological processes**				
	Xenobiotic transport	2	2	0.019	*NOR1*, *SLC7A5*
	Response to caffeine	2	2	0.025	*PPARGC1A*, *PRKAA1*
	Adaptive thermogenesis	2	2	0.040	*PPARGC1A*, *UCP3*
	Reactome pathways				
	FOXO-mediated transcription	14	3	0.045	*AKT2*, *MURF1*, *PPARGC1A*

List total = number of genes contained on the gene card categorised into that process/pathway. List hits = number of significant genes from the list total.

Table 4.3. Gene ontology analysis of genes which were downregulated during the overnight period and genes which were downregulated exclusively during the overnight period and not post-exercise. The three most enriched processes/pathways are shown.

Overnight		List total	List hits	P value	Genes
Downregulated	**GO biological processes**				
	Positive regulation of lipopolysaccharide-mediated signalling pathway	2	2	0.005	*PRKCA*, *TRAF6*
	Response to dsRNA	3	2	0.011	*IKBA*, *NFKB1*
	Positive regulation of cell cycle arrest	3	2	0.011	*MYOG*, *TP53*
	Reactome pathways				
	TRAF6 mediated NF-κB activation	6	3	0.004	*IKBA*, *NFKB1*, *TRAF6*
	NF-κB is activated and signals survival	7	3	0.006	*IKBA*, *NFKB1*, *TRAF6*
	TAK1 activates NF-κB by phosphorylation and activation of IKKs complex	9	3	0.011	*IKBA*, *NFKB1*, *TRAF6*
Downregulated exclusively overnight	**GO biological processes**				
	Positive regulation of lipopolysaccharide-mediated signalling pathway	2	2	0.004	*PRKCA*, *TRAF6*
	Cellular response to tumor necrosis factor	28	5	0.005	*IKBA*, *MYOG*, *NFKB1*, *TP53*, *TRAF6*
	Response to muscle stretch	9	3	0.007	*CSRP3*, *IKBA*, *NFKB1*
	Reactome pathways				
	TRAF6 mediated NF-κB activation	6	3	0.002	*IKBA*, *NFKB1*, *TRAF6*
	NF-κB is activated and signals survival	7	3	0.003	*IKBA*, *NFKB1*, *TRAF6*
	TAK1 activates NF-κB by phosphorylation and activation of IKKs complex	9	3	0.007	*IKBA*, *NFKB1*, *TRAF6*

List total = number of genes contained on the gene card categorised into that process/pathway. List hits = number of significant genes from the list total.

4.4 Discussion

It was concluded in **Chapter 3** that the recovery from eccentric contraction-induced injury could be dependent on the ability to downregulate inflammatory NF-κB and muscle protein breakdown pathways in skeletal muscle. The principal finding of the current study is that the day after eccentric contractions and after additional concentric exercise, post-exercise and pre-bed protein-polyphenol ingestion downregulated skeletal muscle mRNA expression (post-exercise and overnight) and gene ontology annotations (overnight) associated with NF-κB signalling (**Figures 4.5** & **4.6**; **Table 4.3**). The expression of genes associated with muscle protein breakdown, however, was not downregulated by protein-polyphenol ingestion. This extends upon data presented in **Chapter 3**, suggesting that downregulating NF-κB signalling is associated with an attenuated loss of muscle function after eccentric contractions (**Figure 3.3**), but it is unlikely that this is achieved by downregulating transcriptional pathways associated with muscle protein breakdown.

In **Chapter 3** it was identified that nutritionally attenuating the loss of muscle function after eccentric contractions was associated with downregulated IL1R1, TNFRSF10B and TNFRSF12A mRNA expression 24-72 h post-exercise. These genes are implicated in the activation of muscle protein breakdown pathways leading to the hypothesis that aberrant protein breakdown may delay the recovery from injury. Indeed, skeletal muscle protein breakdown is increased for at least 120 h after eccentric contractions in mice (45). The current study demonstrates that both post-exercise and pre-bed protein-polyphenol ingestion acutely downregulates IL1R1 in addition to IL1RL1 (which has been shown to antagonise IL1R1 signalling (232)) mRNA expression (**Figure 4.5B** and **C**). TNFRSF10B and TNFRSF12A were not acutely regulated by protein-polyphenol ingestion (**Figure 4.5G** and **H)**. Binding of IL-1β and TWEAK to IL1R1 and TNFRSF12A, receptively, activates NF-κB signalling in mouse skeletal muscle atrophy models and upregulates the UPS as a consequence (112, 129). TRAF6 is an E3-ligase involved in IL1R1 and TNFRSF12A signal transduction leading to the activation of NF-κB (233). This was identified by our enriched overnight gene ontology annotation ‘TRAF6 mediated NF-κB activation’ (**Table 4.3)**. Furthermore, the

‘cellular response to tumor necrosis factor’ gene ontology annotation was also enriched overnight (**Table 4.3)**, and TNF-α activates NF-κB leading to downstream UPS activation (234). Whilst we present robust transcriptional evidence of NF-κB activation, the downstream expression of the UPS and other proteolytic pathways is less clear. The mRNA expression of the E3 ubiquitin ligase MuRF1 and its transcription factor FOXO1 deceased during the post-exercise period (placebo; **Figure 4.7A** & **D**) corroborated by the downregulated gene ontology annotation ‘FOXO mediated transcription’ (**Table 4.2**). This is likely to be an acute response to nutrition given insulin signalling phosphorylates (and inactivates) FOXO1 (235). Consistent with a previous investigation (205), MAFbx expression was downregulated 4-fold compared to the control leg 24 h after eccentric contractions but didn’t change either post-exercise or overnight (placebo; **Figure 4.7C**; this is discussed further in **Chapter 8**). Post-exercise or pre-bed protein-polyphenol ingestion did not acutely regulate post-exercise or overnight RNA expression of genes associated with the UPS. This is in contrast to a recent report showing the polyphenol curcumin reduces MAFbx mRNA expression after an eccentric treadmill protocol (227). This discrepancy may be because of a different mechanism of action between curcumin, and the pomegranate and cherry derived polyphenol used presently, or a higher phenolic dose provided by Cardaci and colleagues. Nonetheless, the current data suggest that the contribution of the UPS to the remodelling of injured skeletal muscle and recovery of muscle function is likely to be small. However, changes in the mRNA expression of the representative genes selected may not directly reflect dynamic measures of muscle protein breakdown (17, 236), nor can the possibility of changes in UPS expression 0-24 h after eccentric contractions be excluded.

Skeletal muscle mRNA expression of calpain-3 (CAPN3) has been reported to decrease 24 h after eccentric treadmill running (237). This is consistent with the numerical downregulation of CAPN3 mRNA expression 24, 27 and 36 h after eccentric contractions (placebo condition; **Figure 4.7H**). However, substantial increases in the autolysis (activation) of calpain-3 (after 24 h) and calpain activity (from 0.5 h up to 168 h) have been reported in human skeletal muscle after a similar 300 eccentric contraction protocol as used currently (47, 223). Calpain-3 autolysis was predominantly localised to myofibrillar protein, where it has been suggested to play a role in the proteolysis of myofibrillar protein (223, 238).

However, knock-in of inactive calpain-3 in mouse models exacerbates myofiber degeneration after an eccentric treadmill protocol (239). Furthermore, calpain-3 has been shown to be required for proper Ca^{2+} efflux from the sarcoplasmic reticulum to the cytosol during muscle contraction (240), and calpain-3 deficiency leads to an impairment in muscle contractile capacity. In humans, patients with limb-girdle muscular dystrophy which is characterised by a defect in CAPN3 gene and thereby the production of non-functional calpain-3 present with a greater level of myofiber necrosis and defective sarcomere remodelling (241). Therefore, our observation of increased CAPN3 mRNA expression in the protein-polyphenol condition 24-27 h after eccentric contractions could represent a non-proteolytic function for CAPN3 in maintaining sarcomere integrity and calcium homeostasis after eccentric contractions. This could in part explain the concomitant attenuated loss of muscle function with protein-polyphenol ingestion.

After traditional resistance-type exercise, post-exercise whey protein and pre-bed casein protein ingestion increase myofibrillar protein synthesis rates during the respective post-exercise and overnight sleep periods (96, 101). However, the post-exercise and overnight regulation of myofibrillar protein synthesis rates after prior eccentric contractions in combination with protein ingestion is unknown. During the 3 h incorporation period after a (work-matched) bout of maximal concentric contractions, myofibrillar protein synthesis rates in the injured leg were 10% higher than the contralateral control leg (placebo; **Figure 4.3C**). Absolute myofibrillar protein synthesis rates of $0.139\%\cdot h^{-1}$ measured using deuterated water during the post-exercise period are quantitatively aligned with rates of $0.132\text{-}0.140\%\cdot h^{-1}$ measured elsewhere using isotopically labelled amino acid tracers 24 h after eccentric contractions (7, 43). Ingesting 20 g of post-exercise protein has been shown to maximally potentiate myofibrillar protein synthesis rates after traditional resistance-type exercise (95, 96). However, relative to the placebo condition, ingesting 20 g of protein did not measurably increase post-concentric exercise myofibrillar protein synthesis rates when eccentric contractions were performed 24 h prior. Furthermore, whilst free-living overnight myofibrillar protein synthesis rates 27 – 36 h after eccentric contractions were 18% higher than the contralateral control leg (placebo; **Figure 4.3D**), they were also not further increased by 20 g of pre-sleep casein protein (protein-polyphenol; **Figure 4.3D**). In **Chapter 3** it was shown that the decline in muscle function

strongly tended to correlate positively with the increase in myofibrillar protein synthesis rates after eccentric contractions (**Figure 3.7B**). Muscle function was most impaired after 24 h in the placebo condition (**Figure 3.3B**) which is therefore likely to be driving increased myofibrillar protein synthesis rates during the post-exercise and overnight period. In the protein-polyphenol condition, there was no loss of function after 24 h, but we provided an intervention which is known to increase myofibrillar protein synthesis rates. Accordingly, it seems reasonable to suggest that the stimulation of myofibrillar protein synthesis rates by injury and by nutrition are comparable during 24-37 h after eccentric contractions. Based on the transcriptional response it is unclear if the increase in myofibrillar protein synthesis rates in the placebo condition is driven by an increase in muscle protein breakdown as discussed in **Chapter 3**, or by an alternative mechanism such as injury per se.

mTOR is an integral intracellular amino acid sensitive protein in the phosphorylation cascade that initiates protein translation in skeletal muscle (31). Consistent with previous work (43) and with measured myofibrillar protein synthesis rates (**Figure 4.3B**), post-exercise and overnight mTOR phosphorylation status was 23 and 24% higher, respectively in the injured compared to the control leg (placebo; **Figure 4.4E** & **F**). Despite performing a maximal bout of concentric exercise, mTOR phosphorylation status decreased by 15% during the 3 h post-exercise period and by a further 15% during the subsequent 9 h overnight sleep period. This could suggest that mTOR is refractory to further phosphorylation by contraction for at least 24 h after eccentric contractions. mTOR phosphorylation status also did not increase in the control leg, implicating a systemic response to eccentric contractions. Post-exercise and overnight mTOR phosphorylation status also decreased to a similar extent in the protein-polyphenol condition (**Figure 4.4E** & **F**), suggesting mTOR is also refractory to further stimulation by an increase in extracellular amino acid availability, or the chosen time points (i.e., 3 h post-exercise and 9 h overnight) may have missed the increase in mTOR phosphorylation which has been reported after 2 but not 4 h after resistance exercise with whey protein ingestion (231). Notably, however, and in line with our hypothesis, total mTOR protein content between 24-27 h was higher in the placebo relative to the protein-polyphenol condition. There also strongly tended to be more phosphorylated

mTOR in the placebo condition. This could illustrate the absolute level of mTOR signalling is higher in the placebo compared to the protein-polyphenol condition, which could be attributed to elevated muscle protein breakdown, although changes in gene expression did not support this. While it presents a methodological challenge, it is intriguing for future work to endeavour to make accurate and direct measures of muscle protein breakdown in humans after eccentric contractions to establish a causative role in driving muscle protein synthesis and the decline in muscle function.

As discussed above, gene ontology annotations associated with upstream NF-κB activation by IL1R1 were enriched during the overnight period (**Table 4.3**), and protein-polyphenol ingestion downregulated upstream IL1R1 mRNA expression both post-exercise and overnight (**Figure 4.5B**). In the current study, we extend this by demonstrating that pre-bed protein-polyphenol ingestion downregulated the overnight mRNA expression of CXCL1, ICAM1 and CEBPD, which are sensitive to IL-1β/IL1R1 dependent NF-κB activation (**Figures 4.6C**, **D** & **G**) (242–244). CXCL1, ICAM1 and CEPBD share a common role as mediators of leukocyte (i.e., neutrophil and monocyte) recruitment and extravasation. Indeed, the post-exercise expression of CEPBD clustered with additional mediators of leukocyte activity, CCL2 and CSF1 (**Figure 4.7A**). Collectively, this increase in mRNA expression associated with leukocyte activation is consistent with reports elsewhere of a similar pattern of expression of these genes (86, 171, 206, 245), and direct evidence of a rapid and sustained accumulation of neutrophils and macrophages in skeletal muscle after eccentric contractions (41, 159, 168). However, the current study provides further novel data demonstrating that protein-polyphenol ingestion may downregulate the chemotactic signals to leukocytes which in turn would abate the degenerative inflammatory phase after eccentric contractions. In addition to being major sources of pro-inflammatory cytokine production, leukocytes and in particular neutrophils are considered to cause aberrant damage to skeletal muscle through oxidising reactions associated with the respiratory burst (246–248). However, the significance of neutrophil accumulation after eccentric contractions in humans has been contested (249), whereas the infiltration of macrophages is a more consistent observation (41, 159, 249). Indeed, leukocyte accumulation (predominantly macrophages) in human skeletal muscle tissue is significantly associated with a greater loss of

muscle function after eccentric contractions (159). In support of this notion, CXCL1, ICAM1 and CEPBD mRNA expression was downregulated by protein-polyphenol ingestion (27 – 36 h) concomitantly with the reported peak accumulation of macrophages that express an inflammatory M1 phenotype in injured skeletal muscle (i.e. 24 – 48 h) (172). In contrast, muscle function in the placebo condition plateaued during the same period (24 – 48 h; **Chapter 3, Figure 3.3B**), which may be an indication of protracted M1 macrophage activity and inflammatory events. Polyphenols are purported to scavenge free radicals and suppress reactive oxygen species production (250), which highlights a mechanism by which protein-polyphenol ingestion might attenuate aberrant breakdown of muscle protein caused by leukocytes. In turn, this would interrupt the positive feedback of NF-κB activation as NF-κB is redox sensitive and would explain the observation of downregulated NF-κB associated mRNA expression.

A limitation of skeletal muscle gene expression data presented in the current chapter and in **Chapter 3**, is the absence of baseline gene expression values. We believe that the current approach to correct gene expression values in the injured leg to the contralateral control leg is a more robust approach to reduce inter-individual variation than correcting to a baseline value obtained in the same leg. However, due to the inherent limitations and participant burden of repeated muscle biopsy sampling in the same individual, we chose to maximise the number of post-exercise muscle biopsy time points and therefore chose not to perform an additional muscle biopsy at baseline. Consequently, although we attempted to mitigate for differences in baseline gene expression between legs by counterbalancing for leg dominance, the effect of possible differences in baseline gene expression between legs on post-eccentric contraction gene expression is not clear.

In conclusion, the current study demonstrates that post-exercise and pre-bed protein-polyphenol ingestion does not downregulate mRNA expression associated with muscle protein breakdown after eccentric contractions. Indeed, calpain-3 may play an important non-proteolytic role possibly to maintain sarcomere integrity after eccentric contractions. Protein-polyphenol ingestion may instead interrupt the positive feedback loop between NF-κB signalling and leukocyte recruitment and attenuate aberrant damage to skeletal muscle as a

consequence. A time-course investigation of leukocyte accumulation in combination with protein-polyphenol ingestion is required to investigate this, and to elucidate if leukocytes cause a loss of muscle function after eccentric contractions.

Chapter 5

Short-term immobilisation of injured skeletal muscle causes a greater increase in proteolytic gene expression than in non-injured but immobilised skeletal muscle, with comparable declines in daily myofibrillar protein synthesis rates and muscle volume.

5.1 Introduction

In **Chapters 3** and **4** it was demonstrated that eccentric knee extensor contractions which induce myofibrillar injury (47) cause a peak decline in isometric strength and isokinetic work within 48 h. This was accompanied by an increase in daily myofibrillar protein synthesis rates and changes in skeletal muscle mRNA expression associated with NF-kB signalling and the ubiquitin-proteasome, calpain and autophagy protein degradation pathways. Between 48-168 h, isometric strength and isokinetic work recovered to baseline and myofibrillar protein synthesis rates and the change in mRNA expression of these pathways subsequently also returned to similar levels as measured in the contralateral non-injured control leg. However, in **Chapters 3** and **4** participants continued to perform daily maximal isokinetic exercise for seven days after eccentric contractions. Conversely, the recovery from a musculoskeletal injury, which is frequently sustained during athletic training or competition, often requires otherwise healthy individuals to undergo a period of short term (≤ one week) limb immobilisation thereby removing muscle contraction entirely.

One week of limb immobilisation also leads to a decline in quadriceps muscle function (132). This is likely due to muscle atrophy which occurs rapidly with immobilisation and positively correlates with the decline in quadriceps strength after seven days of immobilisation (132). Our group have recently applied deuterated water to measure a decline in free-living myofibrillar protein synthesis rates after 3 and 7 days of unilateral leg immobilisation (63, 64). However, this decline was calculated to only account for 25 and 47% of the decline in quadriceps volume after 2 and 7 days of immobilisation, respectively (63). This illustrates that an increase in muscle protein breakdown is likely to play an equal or greater role than the decline in myofibrillar protein synthesis in regulating the decline in quadriceps volume and subsequently muscle strength during disuse. However, this has not been measured directly or addressed in detail. It was hypothesised in **Chapters 3** and **4** that the loss of isometric strength and isokinetic work after eccentric contractions is attributable to an increase in muscle protein breakdown which may be driven by an increase in NF-kB signalling. It was also hypothesised that an increase in muscle protein breakdown might drive the increase in myofibrillar protein synthesis rates after eccentric contraction-induced injury.

Consequently, an upregulated inflammatory and muscle protein breakdown response associated with musculoskeletal injury may exacerbate skeletal muscle atrophy and the loss of muscle strength during immobilisation. However, this has not been investigated in humans.

The aim of the current study, therefore, was to investigate further the role of myofibrillar protein synthesis rates and inflammatory and proteolytic gene expression in governing the early (0-48 h) and late (48-168 h) remodelling of injury by using a limb immobilisation model which rapidly induces muscle atrophy. This was achieved using a unilateral approach whereby both legs performed eccentric contractions, immediately after which one leg was immobilised for seven days with the contralateral leg remaining ambulant. This unilateral immobilisation model allows us to identify the interaction between injury and immobilisation and reduce interindividual variation by comparing the difference between the immobilised and contralateral non-immobilised (but injured) leg. The response of participants in this condition was then compared to a control condition undergoing unilateral leg immobilisation without injury. It was hypothesised that compared to the control condition, injury would accelerate the rate of decline in quadriceps volume with immobilisation. It was further hypothesised that this would be paralleled by a greater increase in gene expression associated with NF-kB and proteolytic pathways and a greater reduction in myofibrillar protein synthesis rates.

5.2 Methods

Participants

Twenty-two young, healthy males (age: 20±1 y, BMI: 23.5±1.0 $kg \cdot m^{-2}$) volunteered to take part in the present study. Only young males were included in the present study as both age (251) and sex (252) can influence the rate of muscle disuse atrophy. Participants' characteristics are displayed in **Table 5.1**. The study was approved by the Sport and Health Sciences ethics committee of the University of Exeter (REF NOs. 151021/B/02 and 171206/B/08). The study was registered at ClinicalTrials.Gov (IDs: NCT02984332 and NCT03559452).

Table 5.1. Participant characteristics.

	Control (*n*=11)	**Injured** (*n*=11)
Age (y)	21±1	20±1
Body mass (kg)	73±3	74±5
Height (cm)	178±2	176±2
BMI ($kg \cdot m^{-2}$)	23±1	24±2

Values represent mean ± SEM. Control participants performed no eccentric contractions whereas injured participants performed 300 maximal eccentric knee extensor contractions in both legs immediately before the start of a seven day unilateral leg immobilisation period. BMI, body mass index.

Experimental protocol

Details of all experimental and analytical procedures are presented in **Chapter 2.** An overview of the experimental protocol is shown in **Figure 5.1**. All participants performed a detailed familiarisation of the exercise protocols at least 48 hrs before beginning the study period. The study involved five laboratory visits during an 11-day study period which included a seven day period of unilateral leg immobilisation using a leg brace with ambulation using crutches. At 0800 h on day five of the experimental protocol, bilateral vastus lateralis muscle biopsies were collected, and an MRI scan of both thigh muscles was performed (0830 h; participants were transported to and from the MRI scanner via a wheelchair to ensure no contraction or weight bearing on the

immobilised leg). Then, participants were assigned to either perform no exercise (control; *n*=11), or perform 300 maximal, unilateral knee extensor contractions in both legs (injured; *n*=11). Eccentric contractions (matched for volume and intensity) were performed in both the immobilised and non-immobilised leg, and muscle volume and gene expression in the immobilised leg was expressed relative to the non-immobilised leg to delineate the effect of immobilisation *per se* on these outcomes. Immediately afterwards, a seven day immobilisation period was started at ~0900-1000 hrs. After two and seven days of immobilisation, subjects returned to the laboratory at 0800 h for additional bilateral vastus lateralis muscle biopsies and MRI scans. To measure daily free-living myofibrillar protein synthesis rates, participants underwent a deuterated water dosing protocol which began with a loading day on day one and continued with daily maintenance doses thereafter on days 2-11. Saliva samples were collected daily either in the laboratory (days 5, 7 and 12) or by the participant at home (days 6, 8-11) and frozen at -80^{o} until subsequent body water ^{2}H enrichment analysis. Knee extensor 1 repetition maximum (1-RM) testing of both legs was performed on day one and immediately following the end of the immobilisation period on day 11. Participants were only allowed to bear weight on the immobilised leg following completion of 1-RM testing on day 11. Habitual dietary intake was recorded using a 3-day diet diary before immobilisation, and by two further 2-day diet diaries in the first and last two days of immobilisation which were averaged to create an 'immobilisation diet'.

Immobilisation protocol

Leg immobilisation was administered using a unilateral leg brace (X-ACT Donjoy brace, DJO global, Vista, CA, USA) with the participant ambulating on crutches (after receiving instructions) during the immobilisation period. The immobilised leg was counterbalanced for leg dominance, and the non-immobilised leg acted as a within-participant control. The knee was fixed at an angle of 40° flexion (i.e., full knee extension being considered as 0°) by the locking hinge of the brace to ensure no weight-bearing occurred. Participants were instructed that all ground contact and muscle contraction (except for ankle rotation exercises twice per day to activate the venous muscle pump) in the immobilised leg were forbidden. Adhesive tape with the experimenters signature inscribed was placed around the straps of the brace and breaking of the tape would indicate tampering and result in exclusion from the study

(251), however this did not occur. Participants were provided with a plastic cover to wear over the brace when showering. Daily contact was maintained with the participants throughout the entire study to ensure compliance, and any adjustments to the fitting of the brace were only made by an experimenter.

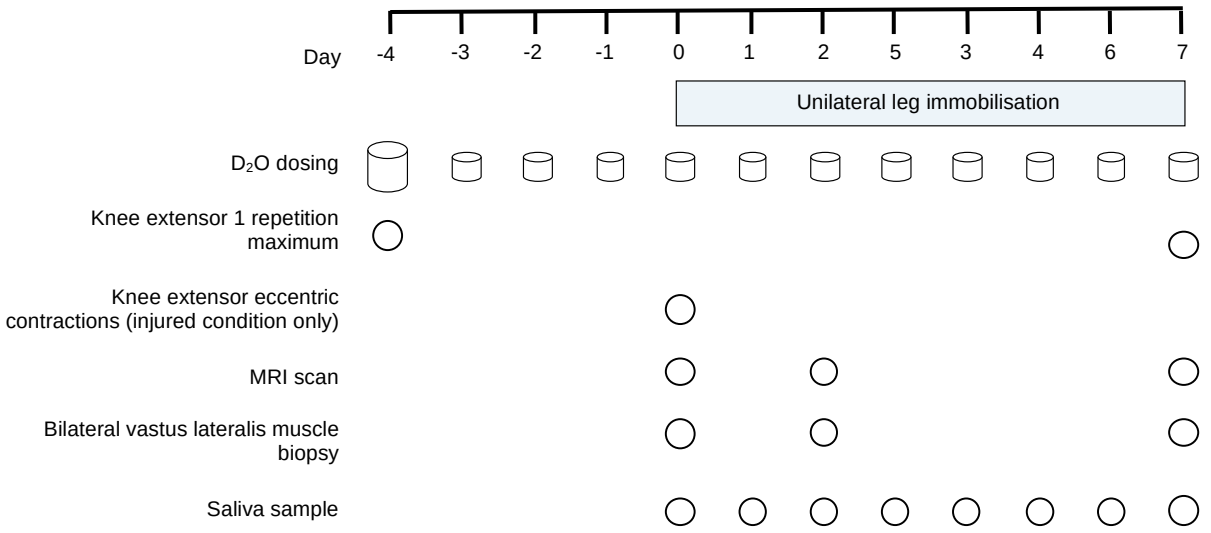

Figure 5.1. Schematic representation of the experimental protocol. Participants underwent a seven day unilateral leg immobilisation protocol which was immediately preceded by no exercise (control) or 300 maximal knee extensor eccentric contractions performed in both legs (injured). Loading of orally consumed 70% D_2O began at ~0800 h on day one with eight x 50 mL doses consumed every 1.5 h and body water enrichment was maintained thereafter with daily doses of 50 mL. Vastus lateralis muscle biopsies and MRI scans were performed before, and after two and seven days of immobilisation, and knee extensor 1-RM was measured before and after immobilisation. Saliva samples were collected daily to measure body water ^2H enrichment.

Statistics

Full statistical procedures and calculations are presented in **Chapter 2**. Differences in subject characteristics and dietary intake between conditions were analysed using unpaired t-tests. Absolute muscle volumes were analysed using a repeated-measure three-factor (condition x time x immobilisation) analysis of variance (ANOVA). The change in muscle volume and 1-RM strength in the immobilised leg was first expressed as a change from the non-immobilised leg (%N-IMM) before statistical analysis. A repeated-measure two-factor ANOVA was used to analyse (condition x

time) differences in body water ^{2}H enrichment, myofibrillar protein synthesis rates and leg extension 1-RM. A repeated-measure three-factor ANOVA was used to analyse (condition x time x immobilisation) differences in myofibrillar-protein bound [^{2}H]alanine. Skeletal muscle mRNA expression was calculated as a fold change from the non-immobilised leg and analysed using a mixed-effects model. A false discovery rate (*FDR*<0.05) was applied to adjust for familywise error before gene ontology analysis (201). Correlations between 1-RM and myofibrillar protein synthesis rates and quadriceps volume were analysed with a Pearson's correlation coefficient. Violations of sphericity were corrected with the Greenhouse-Geisser correction. When a significant main and interaction effect was observed, a Sidak post hoc test was performed to locate individual differences. Statistical significance was set at *P*<0.05. Calculations were performed using GraphPad Prism 8.3.0. All data are expressed as means±SEM.

5.3 Results

One participant in the injured condition withdrew from the study after 2 days of immobilisation due to the inconvenience of immobilisation. Therefore, comparisons between the control and injured conditions are for *n*=11 and *n*=10, respectively, unless otherwise stated.

Participants' characteristics and diet

No differences in age, weight, height or BMI were detected between conditions (*P*>0.05) (**Table 5.1),** and average habitual and immobilised energy and macronutrient intake were not different between conditions (*P*>0.05) (**Table 5.2**).

Table 5.2. Average dietary intake during a habitual period and during one week of unilateral leg immobilisation.

	Control		**Injured**	
	Pre	**During**	**Pre**	**During**
Energy (KJ)	10954±641	11251±715	11973±993	9551±1053
Protein (g·day^{-1})	120±6	111±7	119±13	98±14
Protein (g·kgBM·day^{-1})	1.7±0.1	1.5±0.1	1.7±0.2	1.3±0.1
Protein (%En)	19±1	17±1	17±1	18±1
Carbohydrate (g·day^{-1})	293±23	311±24	288±29	254±26
Carbohydrate (%En)	45±2	46±2	41±3	45±3
Fat (g·day^{-1})	103±8	108±8	123±12	97±11
Fat (%En)	35±2	36±2	38±2	36±2

Values represent mean ± SEM. Control participants performed no eccentric contractions (*n*=11) whereas injured (*n*=10) participants performed 300 maximal eccentric knee extensor contractions in both legs immediately prior to the start of a seve day unilateral leg immobilisation period. 'Pre' represents average three day dietary intake before immobilisation; 'During' represents average dietary intake during the first and last two days of immobilisation.

Skeletal muscle volume

Absolute muscle volumes are presented in **Figure 5.2A**, **C** & **E**. Interactions were observed for muscle volumes over each time period (i.e., early [day 0-2], late [day 2-7] and full [day 0-7] immobilisation periods) whereby muscle volumes decreased in the immobilised leg but remained unchanged in the non-immobilised leg (P<0.05). This was not observed in the non-quadriceps muscle region between days 0-7 (P>0.05; **Figure 5.2E**). A significant 3-way interaction was observed in early (day 0-2) quadriceps muscle volume whereby muscle volume decreased between day 0-2 in the immobilised leg of control condition, but not in the immobilised leg of the injured condition or the non-immobilised leg of either condition (P=0.020; **Figure 5.2C**).

Muscle volumes of the immobilised leg presented relative to the non-immobilised leg are presented in **Figure 5.2B**, **D** & **F** in order to correct for oedematous swelling induced by eccentric contraction. Thigh muscle volume decreased between day 0 (100.8±0.9%N-IMM) and day two (99.3±0.8%N-IMM) (P=0.001) and decreased further between days two and seven (97.2±1.1%N-IMM) (P=0.015; **Figure 5.2B**). The decrease in thigh muscle volume between day 0 and day two (P=0.471) and day two and day seven (P=0.802) was similar between control and injured conditions.

Quadriceps muscle volume did not change between day 0 (100.8±1.3%N-IMM) and day two (100.4±1.2%N-IMM) (P=0.427) but interacted between conditions such that it tended to decrease in the control (day 0 = 100.8±1.6, day two = 99.4±1.1%N-IMM; P=0.061) but not the injured condition (day 0 = 100.8±1.8, day 2 = 101.4±1.8%N-IMM; P=0.490; **Figure 5.2D**). Quadriceps muscle volume decreased between days two and seven (96.2±1.7%N-IMM) (P=0.001) similarly between conditions (P=0.660).

Non-quadriceps muscle volume decreased between day 0 (100.9±0.7%N-IMM) and day two (98.2±1.1%N-IMM) (P=0.002) but did not change further between days two and seven (98.3±1.0%N-IMM) (P=0.951; **Figure 5.2F**). The decrease in non-quadriceps muscle volume between day 0 and day two (P=0.736) and day two and day seven (P=0.882) was similar between control and injured conditions.

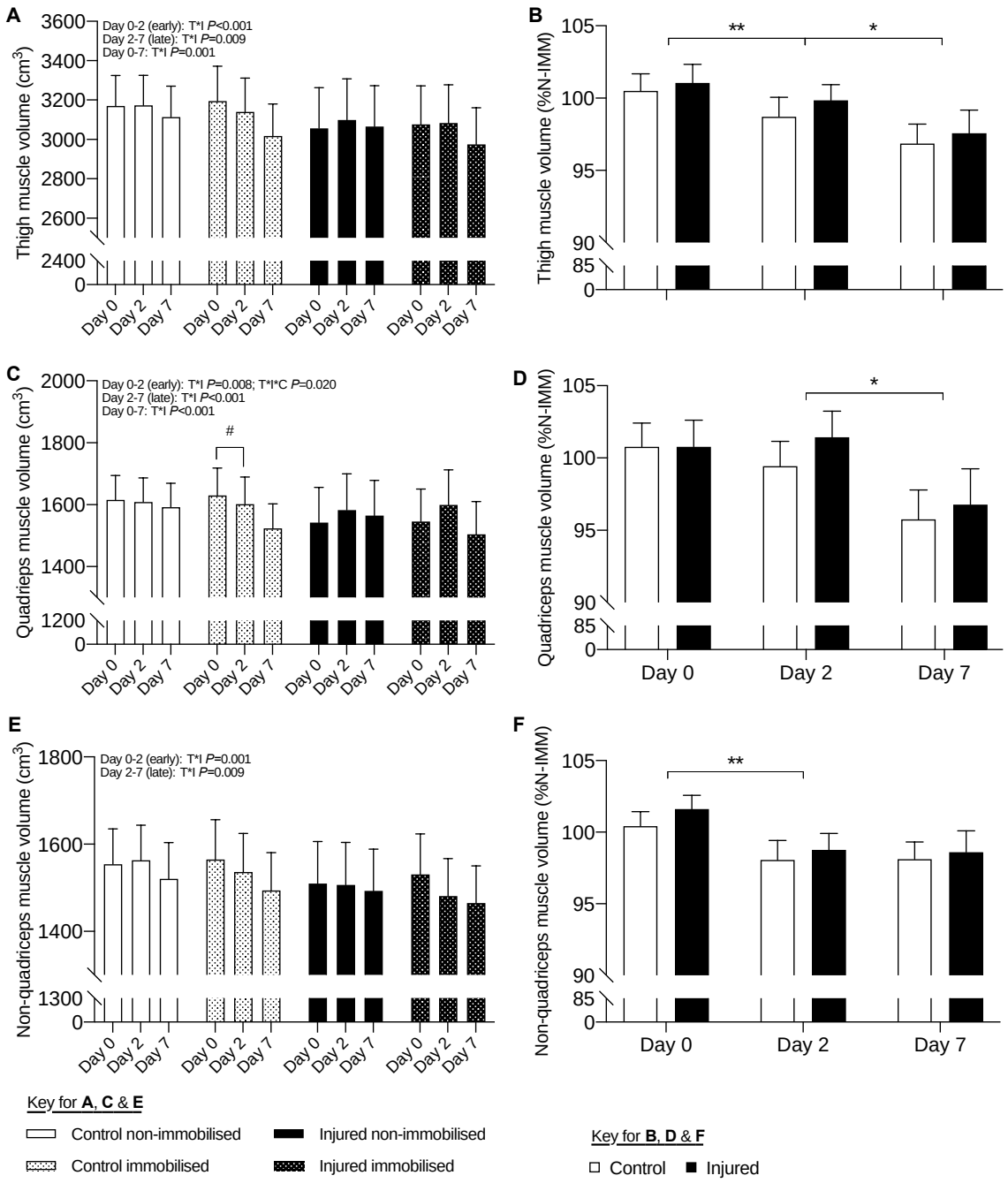

Figure 5.2. Thigh (**A** & **B**), quadriceps (**C** & **D**) and non-quadriceps (**E** & **F**) muscle volume determined using MRI at day 0 and after two and seven days of unilateral leg immobilisation following either no exercise (control; *n*=11), or 300 eccentric knee extensor contractions performed in both legs immediately before immobilisation (injured; *n*=10). Raw data is displayed in **A**, **C** and **E** and data are presented normalised to the non-immobilised leg (N-IMM) in **B**, **D** and **F**. All data are presented as means with error bars representing the SEM. Statistical analysis was performed with separate three-factor (**A**, **C**, **E**) and two-factor (**B**, **D**, **F**) ANOVAs. * difference between time points. T = time, I = immobilisation status, C = condition. Day 0-2 is classified as the early immobilisation period and days 2-7 is classified as the late immobilisation period. One symbol $P<0.05$, two symbols $P<0.01$.

Body water deuterium enrichment

A participant in the injured condition was not deuterium naïve. Therefore, myofibrillar protein synthesis data are presented as control *n*=11 and injured *n*=9 due to the potential confounding factor of deuterium recycling. Body water deuterium enrichment (**Figure 5.3A)** averaged 0.78±0.01%, 0.85±0.01% and 0.88±0.01% between days 0 and two, days 0 and seven and days two and seven, respectively. Body water deuterium enrichment showed a modest increase over the immobilisation period (*P*<0.001).

Myofibrillar protein-bound [^{2}H]alanine enrichment and myofibrillar protein synthesis rates

After two days of immobilisation, myofibrillar protein-bound [^{2}H] enrichments were not different between non-immobilised and immobilised legs (*P*=0.508) (**Figure 5.3B**). After seven days of immobilisation, myofibrillar protein-bound [^{2}H] enrichments increased more in the non-immobilised (Δ0.3953±0.0268 MPE) compared to the immobilised (Δ0.2819±0.0252 MPE) leg (*P*<0.001). Between 2 and 7 days of immobilisation, myofibrillar protein-bound [^{2}H] enrichments increased more in the non-immobilised (Δ0.2746±0.0219 MPE) compared to the immobilised (Δ0.1817±0.0217 MPE) leg (*P*<0.001). The change in myofibrillar protein-bound [^{2}H]alanine enrichments in immobilised and non-immobilised legs between days 0 and two, 0 and seven and two and seven was not different between control and injured conditions (*P*>0.05).

Average daily myofibrillar protein synthesis rates during the full seven day immobilisation period (**Figure 5.3C**) were 34% lower in the immobilised (1.37±0.12%·d^{-1}) compared to the non-immobilised (1.94±0.13%·d^{-1}) leg (*P*<0.001), and the difference between the immobilised and non-immobilised leg was similar between the injured (Δ0.34±0.14%·d^{-1}) and control (Δ0.72±0.13%·d^{-1}) conditions (*P*=0.127; **Figure 5.3E**). Average daily myofibrillar protein synthesis rates between days 0 and two (**Figure 5.3D**) were not different between the immobilised (1.83±0.22%·d^{-1}) and non-immobilised (2.19±0.16%·d^{-1}) legs (*P*=0.109), and the difference between the immobilised and non-immobilised legs was similar between the injured (Δ0.25±0.36%·d^{-1}) and control (Δ0.47±0.25%·d^{-1}) conditions (*P*=0.614; **Figure 5.3E**). Average daily myofibrillar protein synthesis

rates between days two and seven (**Figure 5.3D**) were 41% lower in the immobilised (1.24±0.15%·d^{-1}) compared to the non-immobilised (1.88±0.14%·d^{-1}) leg (*P*<0.001) and the difference between the immobilised and non-immobilised leg was similar between the injured (Δ0.37±0.26%·d^{-1}) and control (Δ0.82±0.14%·d^{-1}) conditions (*P*=0.339; **Figure 5.3E**).

The difference in myofibrillar protein synthesis rates between the immobilised and non-immobilised leg correlated positively with the change in quadriceps volume between day 0 and seven (R^2=0.403, *P*=0.036; **Figure 5.9A**) and days two and seven (R^2=0.437, *P*=0.027; **Figure 5.9C**) in the control condition. Conversely, the difference in myofibrillar protein synthesis rates between the immobilised and non-immobilised leg correlated negatively with the change in quadriceps volume between day 0 and seven in the injured condition (R^2=0.483, *P*=0.037; **Figure 5.9A**).

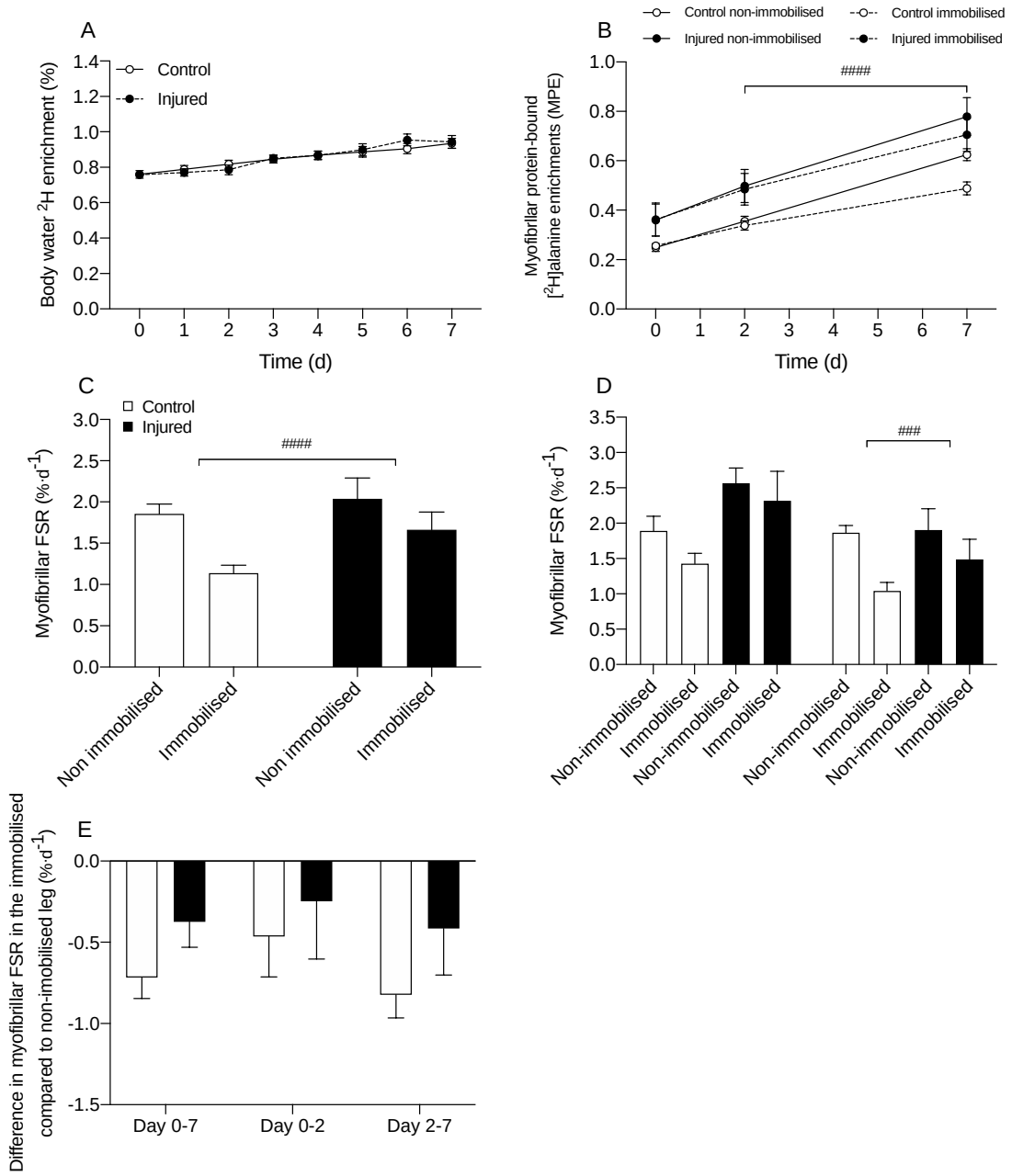

Figure 5.3. Body water ^{2}H enrichment measured in saliva is shown in **A**, and myofibrillar protein-bound [^{2}H]alanine enrichments are shown in **B**. Myofibrillar FSRs were calculated using body water ^{2}H enrichment as the precursor and daily myofibrillar FSRs during seven days of unilateral leg immobilisation in the non-immobilised and immobilised leg are shown in **C**, whereas daily FSRs during the first two days and subsequent five days are displayed in **D**. The difference in myofibrillar protein synthesis rates between the non-immobilised and immobilised legs are displayed in **E**. Participants either performed no eccentric contractions prior to immobilisation (*n*=11; control; white bars/circles) or performed 300 unilateral eccentric knee extensor contractions in both legs immediately before the 7 day immobilisation period (*n*=9; injured; black bars/circles). Data are presented as means with error bars representing the SEM. Statistical analysis was performed with separate two-factor (**A**, **C** & **D**), three-factor ANOVAS (**B**) and unpaired t-tests (**E**). # difference between immobilised and non-immobilised legs. One symbol *P*<0.05, two symbols *P*<0.01, three symbols *P*<0.001, four symbols *P*<0.0001.

Targeted skeletal muscle mRNA expression associated with NF-κB signalling

Skeletal muscle mRNA expression associated with NF-κB signalling did not change between days 0 and two of immobilisation (**Figure 5.4** and **5.5**). *CXCL2* (*P*=0.052; **Figure 5.5E**), *VCAM1* (*P*=0.080; **Figure 5.5K**) and *VEGFA* (*P*=0.059; **Figure 5.5L**) mRNA expression tended to be higher in the injured compared to the control condition between days 0 and two of immobilisation. *VEGFA* mRNA expression tended to decrease in the control condition (post hoc; *P*=0.081) and not change in the injured condition (post hoc; *P*=0.139; **Figure 5.5L**) between days 0 and two of immobilisation.

The skeletal muscle mRNA expression of four upstream (*IL18*, *NFKB1*, *TGFB2*, *TNFRSF12A;* **Figure 5.4**) and one downstream (*SOD2*; **Figure 5.5**) genes associated with NF-κB signalling increased between days two and seven of immobilisation (*P*<0.05). *HIF1A* mRNA expression tended to be higher in the injured condition (*P*=0.068; **Figure 5.5F**) between days two and seven of immobilisation. The mRNA expression of *VEGFA* decreased in the injured condition (post hoc; *P*=0.013) but did not change in the control condition (post hoc; *P*=0.816; **Figure 5.5L**) between days two and seven of immobilisation.

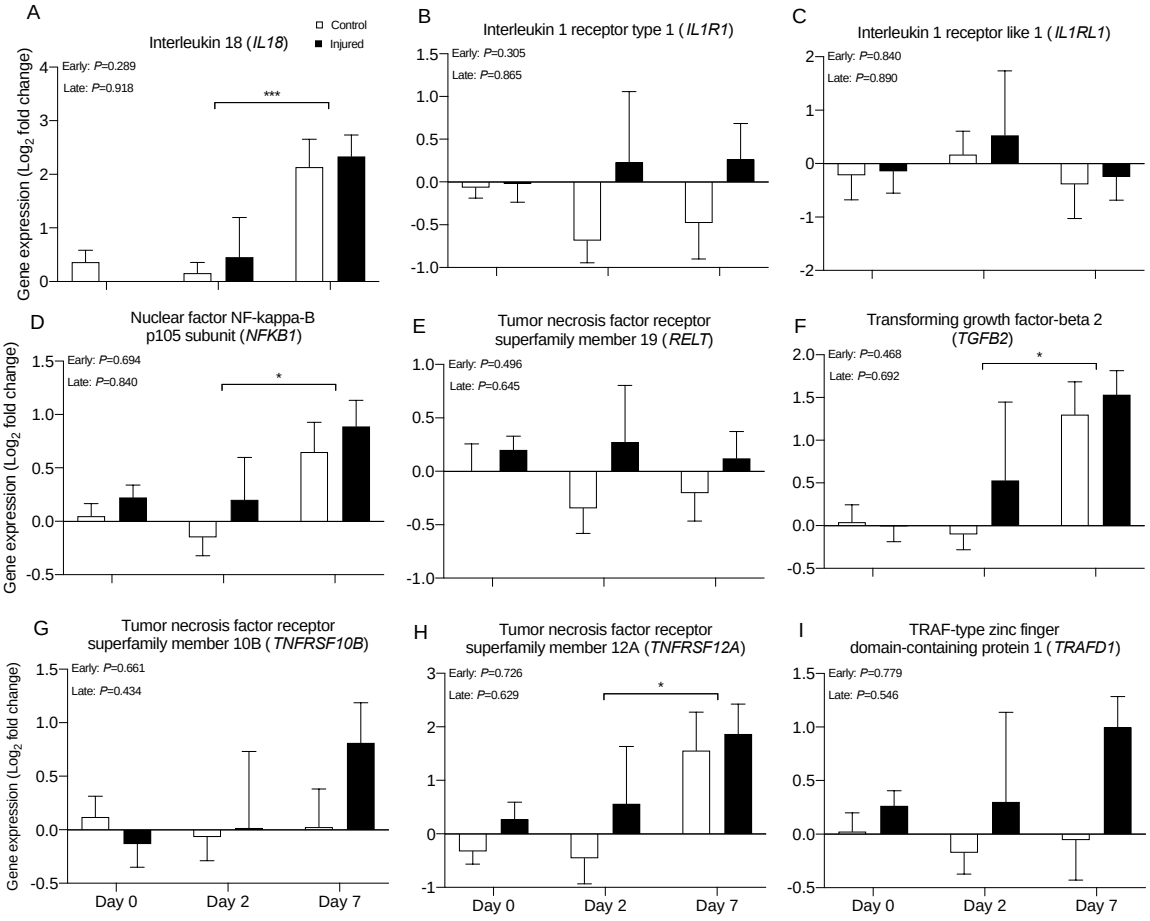

Figure 5.4. Skeletal muscle mRNA expression of nine genes (**A**-**I**) associated with upstream activation of NF-κB signalling. mRNA expression was compared between days 0 and two and two and seven of unilateral leg immobilisation. Participants either performed no eccentric contractions prior to immobilisation (control; *n*=11; white bars) or performed 300 unilateral eccentric knee extensor contractions in both legs immediately before the seven day immobilisation period (injured; *n*=10; black bars). All individual expression values are expressed as a fold change from the contralateral non-immobilised leg at the same timepoint, with $\log_2$ transformation applied. Data are presented as means with error bars representing the SEM. Interaction *P* values are displayed on each graph. Statistical analysis was performed with separate two-factor ANOVAs. * Difference between time points. One symbol *P*<0.05, two symbols *P*<0.01, three symbols *P*<0.001, four symbols *P*<0.0001.

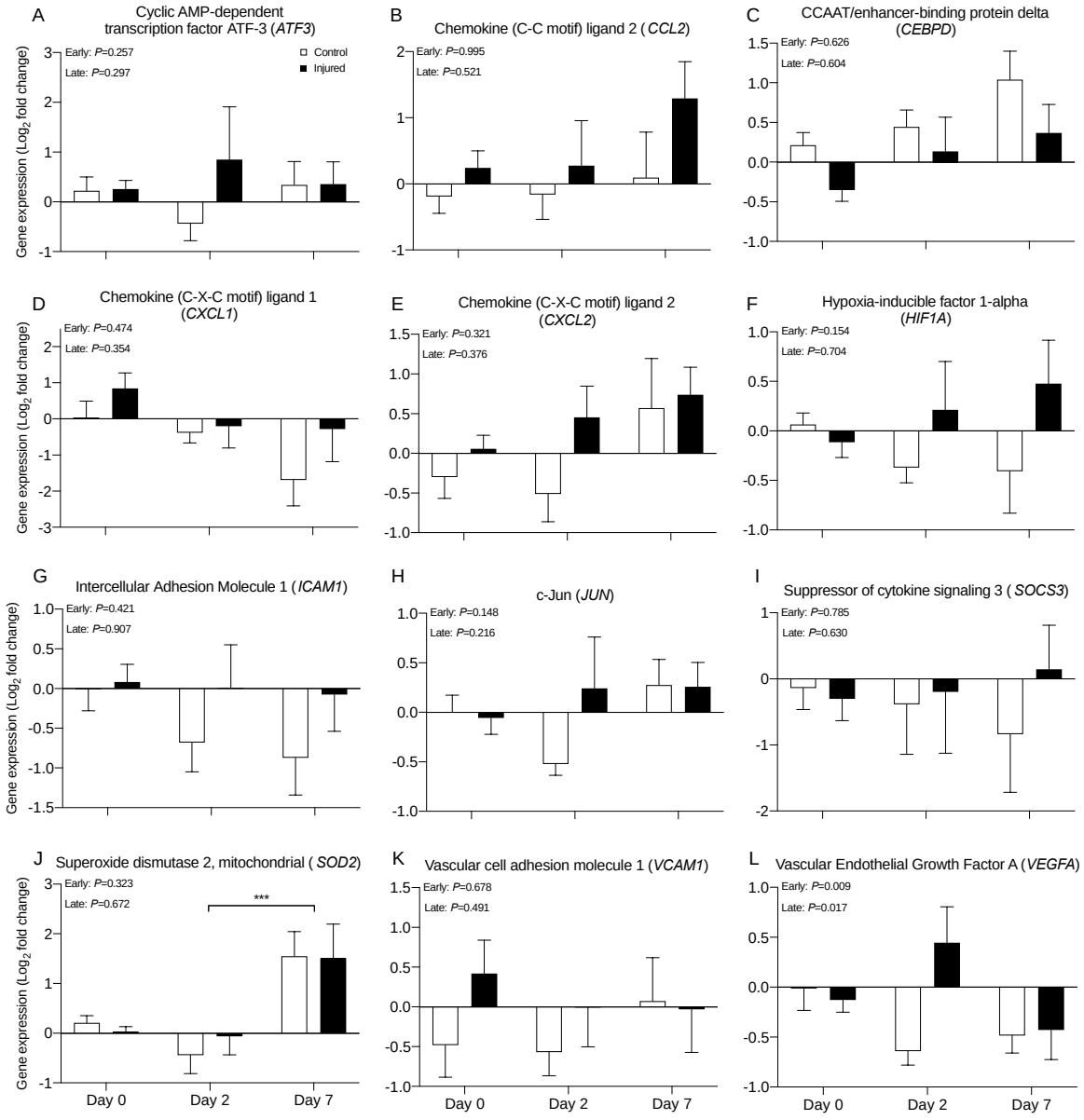

Figure 5.5. Skeletal muscle mRNA expression of 12 genes (**A**-**L**) associated with downstream activation of NF-κB signalling. mRNA expression was compared between days 0 and two and two and seven of unilateral leg immobilisation. Participants either performed no eccentric contractions prior to immobilisation (control; *n*=11; white bars) or performed 300 unilateral eccentric knee extensor contractions in both legs immediately before the 7-day immobilisation period (injured; *n*=10; black bars). All individual expression values are expressed as a fold change from the contralateral non-immobilised leg at the same timepoint, with log_2 transformation applied. Data are presented as means with error bars representing the SEM. Interaction *P* vales are displayed on each graph. Statistical analysis was performed with separate two-factor ANOVAs. * difference between time points, † change from previous time point in the injured condition only. One symbol *P*<0.05, two symbols *P*<0.01, three symbols *P*<0.001, four symbols *P*<0.0001.

Targeted skeletal muscle mRNA expression associated with muscle proteolysis

Skeletal muscle mRNA expression associated with muscle protein breakdown pathways did not change over the first two days of immobilisation (**Figure 5.6**). Between days two and seven of immobilisation, *FOXO1*, *MAFbx*, *MuRF1*, *PSMB1,* and *BECN1* mRNA expression increased (P<0.05), whereas *CAPN3* mRNA expression decreased (P=0.035). Between days two and seven of immobilisation the mRNA expression of *FOXO1* (control = -0.11±0.14, injured = 0.55±0.22; P=0.008; **Figure 5.6A**) and *CASP8* (control = -0.39±0.23, injured = 0.34±0.32; P=0.030; **Figure 5.6F**) was higher in the injured condition compared to the control condition, whereas the mRNA expression of *MSTN* (control = 0.24±0.12, injured = -0.13±0.17; P=0.049; **Figure 5.6I**) was lower in the injured compared to the control condition.

Gene ontology analysis

Between days 0 and two of immobilisation, the skeletal muscle mRNA expression of 6 genes changed with time (*ACAT1*, *CTGF*, *GYS1*, *HSF4*, *IGF1* and *NR4A1*), 24 genes were differentially expressed between conditions (*ACTN3*, *ANGPT1*, *CD34*, *COL1A1*, *COL3A1*, *COL5A2*, *COL6A1*, *CTGF*, *CXCL3*, *GAPDH*, *GYS1*, *IGF1*, *MMP2*, *MYOD1*, *NOR1*, *NR4A1*, *PENK*, *PPARGC1A*, *RHOQ*, *SCD1*, *SDHA*, *SLC7A8*, *SREBF1* and *TGFB1*) and 24 genes interacted between conditions between days 0 and two of immobilisation (*ACAT1*, *ANKRD2*, *CPT1B*, *DNM1L*, *GYS1*, *HADHB*, *MCAD*, *MKNK2*, *MYOD1*, *NOR1*, *NR4A1*, *PAX7*, *PLIN2*, *PLIN5*, *PNPLA2*, *PPARGC1A*, *PRKCQ*, *PTGS1*, *PYGM*, *SDHA*, *SLC2A4*, *SLC7A8*, *SMAD3* and *VEGFA*) (P<0.05). However, these genes did not meet the FDR cut off of 5% but are shown in **Appendices 8.1** for reference.

Between days two and seven of immobilisation, the skeletal muscle mRNA expression of six genes increased, and six genes decreased after applying an *FDR* cut-off of 5%. The result of the gene ontology analysis is presented in **Table 5.3,** and hierarchal clustering is presented in **Figure 5.8.** The mRNA expression of 22 genes was differentially expressed between conditions (*ANKRD2*, *CTSL1*, *DDIT3*, *DDIT4L*, *DNM1L*, *EIF4EBP1*, *FOXO1*, *GYS1*, *IGF1*, *MYD88*, *NOR1*, *NR4A1*, *PAX7*, *PLIN2*, *PNPLA2*, *PPARGC1A*, *PTGS1*, *RHOQ*, *SLC7A8*, *SMAD3*, *TFAM* and *TRAF6*) and five genes interacted between conditions during

the late period (*ACTN3*, *ANGPT1*, *SDHA*, *SOD1* and *VEGFA*) (P<0.05). However, these genes did not meet the FDR cut off of 5% but are shown in **Appendices 8.1** for reference.

1 repetition maximum strength

Knee extensor 1-RM strength decreased by 22% between day 0 (100±3%N-IMM) and day 7 (79±3%N-IMM) (P<0.001). 1-RM after seven days of immobilisation was greater in the control (85±3%N-IMM) compared to in the injured condition (71±5%N-IMM) (P=0.014, post hoc; P=0.013; **Figure 5.7A**). The decrease in 1-RM positively correlated with the decrease in quadriceps volume after seven days of immobilisation in the control (R^2=0.0405, P=0.035) but not the injured (R^2=0.051, P=0.560; **Figure 5.7B**) condition.

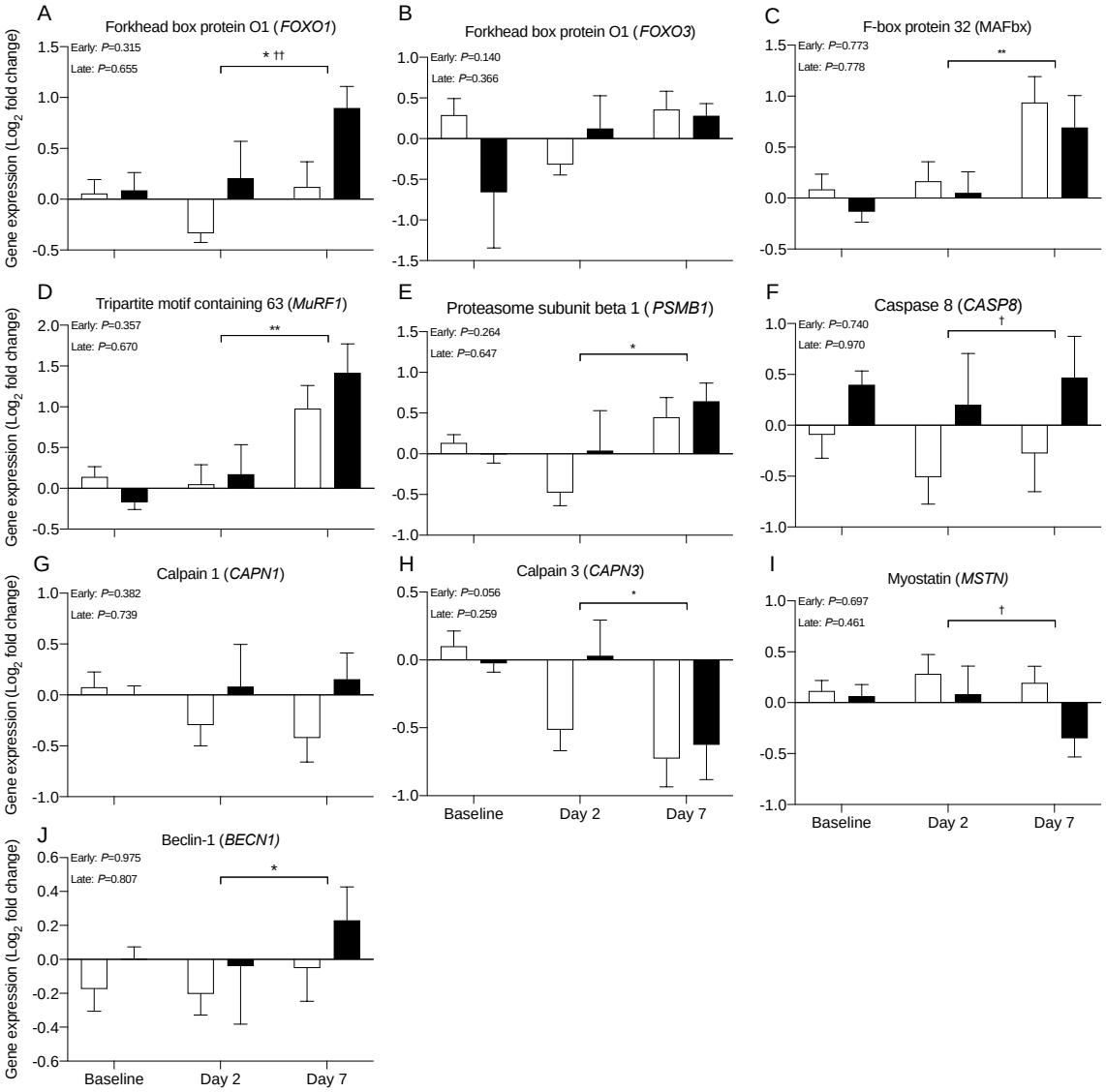

Figure 5.6. Skeletal muscle mRNA expression of genes belonging to pathways associated with skeletal muscle protein breakdown. mRNA expression was compared between days 0 and two and two and seven of unilateral leg immobilisation. Participants either performed no eccentric contractions prior to immobilisation (control; *n*=11; white bars) or performed 300 unilateral eccentric knee extensor contractions in both legs immediately before the seven day immobilisation period (injured; *n*=10; black bars). All individual expression values are expressed as a fold change from the contralateral non-immobilised leg at the same timepoint, with $\log_2$ transformation applied. Data are presented as means with error bars representing the SEM. Interaction *P* values are displayed on each graph. Statistical analysis was performed with separate two-factor ANOVAs. * difference between time points, † difference between injured and control conditions. One symbol *P*<0.05, two symbols *P*<0.01, three symbols *P*<0.001, four svmbols *P*<0.0001.

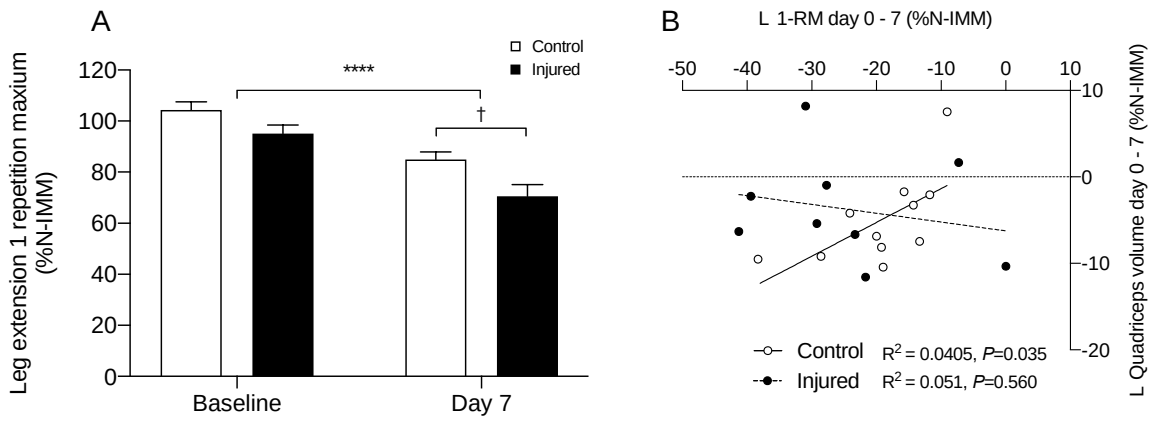

Figure 5.7. Leg extension 1 repetition maximum (**A**) and the corelation between the individual change in leg extension 1-RM and the individual change in quadriceps volume (**B**) after seven days of unilateral leg immobilisation. Participants either performed no prior eccentric contractions (control; *n*=11; white bars and circles) or performed 300 unilateral eccentric knee extensor contractions in both legs immediately before the seven day immobilisation period (injured; *n*=9; black bars and circles). Data are normalised to the non-immobilised leg (N-IMM) and in **A** data are presented as means with error bars representing standard error. Statistical analysis was performed with a two-factor ANOVA (**A**) and Pearson's correlation (**B**). * difference between time points, † difference between conditions. One symbol *P*<0.05, two symbols *P*<0.01, three symbols *P*<0.001, four symbols *P*<0.0001.

Table 5.3. Gene ontology analysis of genes which were upregulated and downregulated between days two and seven of immobilisation. The three most enriched processes/pathways are shown.

		List total	List hits	P value	Genes
Up-regulated	**GO biological processes**				
	Skeletal muscle atrophy	6	2	<0.001	*CFLAR*, *MuRF1*
	Positive regulation of NF-kappaB transcription factor activity	20	3	0.011	*CFLAR*, *IL18*, *NFKB2*
	Lipid homeostasis	7	2	0.013	*IL18*, *PNPLA2*
	Reactome pathways				
	Interleukin 1 processing	5	2	0.028	*IL18*, *NFKB2*
	FOXO-mediated transcription of oxidative stress, metabolic and neuronal genes	9	3	0.011	*MuRF1*, *PPARGC1A*, *SOD2*
Down-regulated	**GO biological processes**				
	Regulation of glucose metabolic processes	16	4	<0.001	*ACTN3*, *IRS1*, *PDK2*, *PPARGC1A*
	Regulation of cellular carbohydrate metabolic process	19	4	<0.001	*ACTN3*, *IRS1*, *PDK2*, *PPARGC1A*
	Positive regulation of glucose metabolic process	8	3	<0.001	*ACTN3*, *IRS1*, *PPARGC1A*
	Reactome pathways				
	IRS activation	1	1	0.026	*IRS1*
	RAF/MAP kinase cascade	12	2	0.037	*ANGPT1*, *IRS1*

List total = number of genes contained on the gene card categorised into that process/pathway. List hits = number of significant genes from the list total.

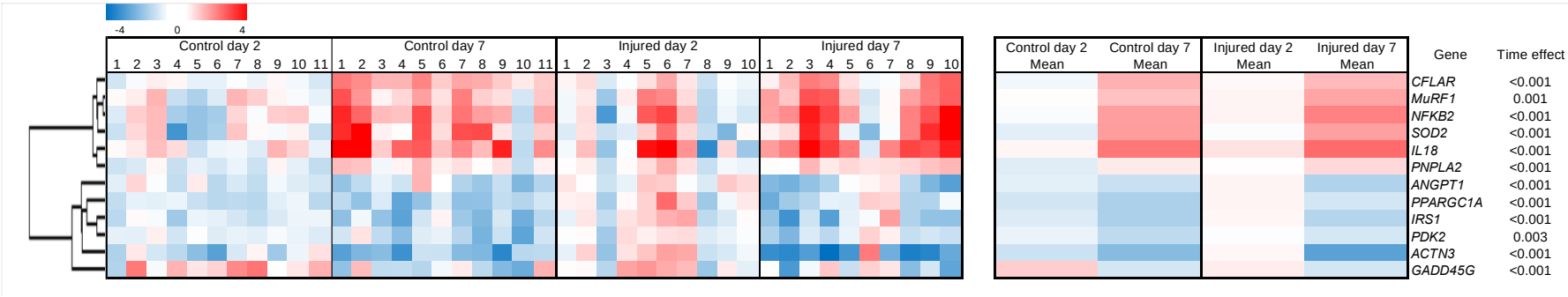

Figure 5.8. Skeletal muscle mRNA expression of genes which were differentially expressed between days two and seven of immobilisation (time effect; FDR <5%). Participants either performed no eccentric contractions prior to immobilisation (control; *n*=11) or performed 300 unilateral eccentric knee extensor contractions in both legs immediately before the seven day immobilisation period (injured; *n*=10). All individual expression values are expressed as a fold change from the contralateral non-immobilised leg at the same timepoint, with $\log_2$ transformation and hierarchal clustering applied. Statistical analysis was performed with separate two-factor mixed effect models and corrected for familywise errors using a Benjamini-Hochberg correction.

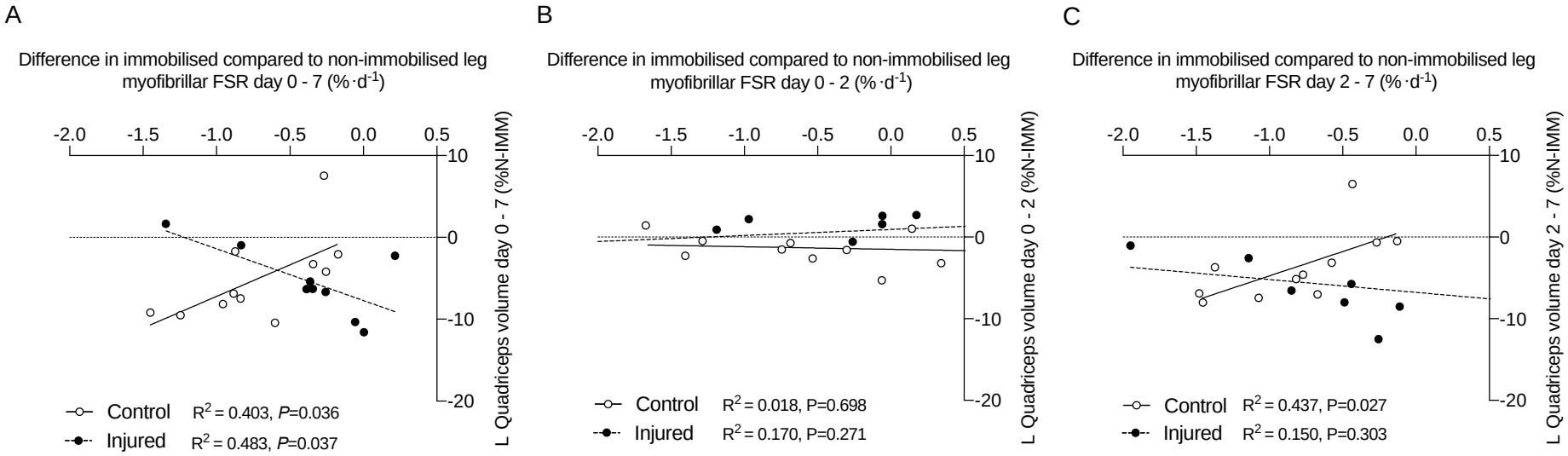

Figure 5.9. Correlations between the change in quadriceps volume in the immobilised leg corrected for the non-immobilised leg, and the difference in myofibrillar FSR between the non-immobilised and immobilised leg across the following timepoints; **A**: days 0 to seven, **B**: days 0 to two, **C**: days two to seven in either a control condition where no prior eccentric contractions were performed (control; *n*=11; white circles), or injured condition where 300 unilateral eccentric knee extensor contractions were performed in both legs immediately before the seven day immobilisation period (injured; *n*=9; black circles). Statistical analysis was performed with a Pearson's correlation and R^2 and *P* values are displayed on each graph.

5.4 Discussion

Musculoskeletal injuries frequently occur during athletic training or competition, mandating a short-term period of limb immobilisation. To elucidate how injury affects skeletal muscle strength and volume during a period of short-term limb immobilisation, the current study used eccentric contractions to induce myofibrillar injury bilaterally immediately before seven days of unilateral leg immobilisation. The primary novel finding of this investigation is that loss of muscle strength after 7 days of immobilisation was greater in the injured compared to the control condition (**Figure 5.7A**). This was associated with an increase in FOXO1 and CASP8 mRNA expression between days 2 and 7 of immobilisation (**Figure 5.6A** & **F**), which are known to be involved in muscle protein breakdown. Contrary to the hypothesis, injury did not affect myofibrillar protein synthesis rates (**Figure 5.3**) or the decline in muscle volume (**Figure 5.2**), suggesting the decline in myofibrillar protein synthesis rates likely does not determine the loss of muscle strength after injury.

Uncomplicated short-term (< 7 days) skeletal muscle disuse in healthy volunteers leads to a decline in skeletal muscle volume (3, 63, 68, 132, 253, 254) and impairs muscle strength. Consistent with these observations, thigh muscle volume declined by 1.8 and 3.7% after 2 and 7 days of unilateral leg immobilisation, respectively, representing ~184 g of total muscle tissue lost from the immobilised leg (control; **Figure 5.2B**). This was mainly attributable to a decline in quadriceps muscle volume of 1.3 and 5% after 2 and 7 days of immobilisation, respectively, which comprised 60% (~111 g) of the total loss of muscle tissue from the immobilised leg (control; **Figure 5.2D**). Moreover, the functional consequences of the loss of quadriceps muscle volume manifested as a 19% reduction in quadriceps 1-RM, evidenced by a strong positive correlation between the individual decrease in quadriceps 1-RM and the individual decline in quadriceps volume after 7 days of immobilisation (control; **Figure 5.7**).

Our group and others have recently applied deuterated water to demonstrate a chronic (presumably for 24 h per day) decline in integrative free-living muscle protein synthesis rates during short-term immobilisation (63, 64, 67). Currently, free-living daily

myofibrillar protein synthesis rates declined by 37% (or 5% per day) over one week of immobilisation (control; **Figure 5.3C**). This decline manifested similarly between days 0 to 2 (6% per day, or 11% in total) and days 2 to 7 (9% per day, or 43% in total) of immobilisation (control; **Figure 5.3D**). Underlining the role the decline in myofibrillar protein synthesis rates are believed to play in driving uncomplicated disuse atrophy in humans, the individual decline in myofibrillar protein synthesis rates was strongly positively correlated with the decrease in quadriceps muscle volume between days 0 to 7 and 2 to 7 (control; **Figure 5.8A** & **C**). This relationship wasn't evident between days 0 to 2, possibly due to a greater contribution of muscle protein breakdown to the loss of muscle volume during the first few days of immobilisation.

Eccentric contractions cause myofibrillar injury (47). In **Chapters 3** and **4** we demonstrated that eccentric contractions impair isometric torque and isokinetic work, increase daily myofibrillar protein synthesis rates and modulate gene expression associated with inflammation and muscle protein breakdown predominantly 24-72 h post eccentric contractions. However, participants in these chapters continued to perform a daily bout of maximal exercise, so it is unknown how these factors affect the remodelling of skeletal muscle in the absence of contraction. Currently, the decline in myofibrillar protein synthesis relative to the contralateral leg after seven days of immobilisation in the injured condition (18%) was not different to the decline in the control condition (37%). Furthermore, the decline was not statistically different between days 0 to 2 (12 vs 11%) and days 2 to 7 (19 vs 43%) in injured and control conditions, respectively (**Figure 5.3E**). It has been demonstrated that intravenous infusions of IL-6 or endotoxin, which promote an inflammatory response centred around NF-kB activation suppress hourly resting mixed muscle protein synthesis rates in healthy humans (4, 124, 208). In the current study under disuse conditions which promote a rapid and chronic decline in myofibrillar protein synthesis rates, NF-kB activation at the onset of immobilisation did not decrease myofibrillar protein synthesis rates further during the subsequent immobilisation period. Consequently, NF-kB signalling does not appear to regulate the decline in myofibrillar protein synthesis rates during disuse.

The decline in whole thigh, quadriceps and non-quadriceps volume were similar between injured and control conditions (**Figure 5.2**). We did detect a trend for

eccentric contractions to attenuate the decline in quadriceps volume (+0.6%) after two days of immobilisation compared to the control condition (-1.4%). However, given the similar decline in myofibrillar protein synthesis rates between conditions over the first two days, this is likely to reflect the additive effect of the removal of contraction on the accumulation of intramuscular fluid induced by injury (52). We have attempted to correct for this against the contralateral ambulant and injured leg. However, our data may underrepresent the magnitude of disuse atrophy attributable to protein loss with injury. With this in mind, we did not observe a correlation between the decline in 1-RM and quadriceps volume in the injured condition, however quadriceps 1-RM in the injured condition was impaired by 18% more than in the control condition after 7 days of immobilisation (**Figure 5.7A**). Given myofibrillar protein synthesis rates declined similarly between conditions during the 7-day immobilisation period, this supports the conclusion made in **Chapters 3** and **4** that myofibrillar protein synthesis rates do not determine the loss (or recovery) of muscle function after injury. Consequently, muscle protein breakdown might be important.

Given the challenges associated with measuring in vivo muscle protein breakdown rates within the current study design, skeletal muscle mRNA expression of genes implicated in the breakdown of muscle protein was determined. Using an unbiased gene ontology approach, the decline in muscle volume measured directly using MRI was paralleled by the upregulated transcriptional 'skeletal muscle atrophy' ontological annotation in immobilised skeletal muscle between two and seven days of immobilisation (**Table 5.3**)**.** The ubiquitin-proteasome system is regarded as a principal regulator of skeletal muscle atrophy under immobilisation conditions (23, 26), and the ubiquitin ligases MAFbx and MuRF1 are considered central to this process (23, 24). In the current study, the mRNA expression of MuRF1 (control; **Figure 5.6D**) and MAFbx (control; **Figure 5.6C**) and their key transcription factor FOXO1 (control; **Figure 5.6A**) increased between 2 and 7, but not 0 and 2 days of immobilisation. This was substantiated at the pathway level as the 'FOXO-mediated transcription of oxidative stress, metabolic and neuronal genes' annotation was upregulated concomitantly. These data are in line with previous findings of elevated mRNA expression associated with the UPS after 5, 7 and 14 days of leg immobilisation, demonstrating genes specifically involved in the ubiquitination and degradation of myofibrillar protein are upregulated rapidly, but not immediately with disuse (63, 65,

66, 254). Calpain-3 (CAPN3) expression decreased between 2 and 7 days of immobilisation, and this decrease tended to occur earlier (days 0-2) in the control condition (**Figure 5.6H**). Given that calcium accumulates intracellularly after eccentric contractions, this could explain the attenuated downregulation in the injured condition (255). However, a downregulation in calpain-3 after 7 days of immobilisation would support the non-proteolytic role of calpain-3 discussed in **Chapter 4.**

Immobilisation induces rapid insulin resistance after 48 h in skeletal muscle (256). Consistent with this, IRS1 and PDK2 skeletal muscle mRNA expression was downregulated in the immobilised leg by 1.7 and 1.6-fold, respectively, between 2 and 7 of immobilisation (control; **Figure 5.8)**. Furthermore, 'IRS activation' and processes associated with glucose utilisation were among the most downregulated gene ontology annotations during the same period (**Table 5.3**). FOXO1 is phosphorylated (inactivated) by insulin-sensitive AKT signalling (257) and is considered to be the integration point of insulin resistance with muscle protein breakdown (258). A primary novel finding of the current study is that FOXO1 mRNA expression was higher in the injured condition between 2 and 7 days of immobilisation. Eccentric contractions also cause insulin resistance after 48 h (259, 260). Consequently, our observation of elevated FOXO1 expression could be illustrative of compounded insulin resistance induced by immobilisation and injury which could result in elevated muscle protein breakdown by the UPS, although direct evidence that FOXO1 and subsequent ubiquitin-mediated muscle protein breakdown is regulated by insulin during disuse is lacking (258). Downstream of FOXO1, MAFbx or MuRF1 expression was not different between conditions, but changes in the expression and genes being used as a proxy for changes in muscle protein breakdown do not necessarily represent actual changes in muscle protein breakdown or translate to changes in muscle mass (103, 142). Conversely, caspase-8 (CASP8) expression was increased concomitant with FOXO1 in the injured condition. CASP8 is involved in cell apoptosis, and the FOXO transcription factors have been identified as promoters of apoptotic signalling, although the contribution of apoptosis to skeletal muscle disuse atrophy is not clear (261). The increase in FOXO1 is likely to occur via a myostatin independent mechanism given myostatin (MSTN) was downregulated in the injured condition at the same time. Indeed, downregulated MSTN expression in the injured condition may reflect an attempt to restrain muscle loss from excessive muscle protein breakdown

and may be an anabolic response to elevated amino acid availability derived from muscle protein breakdown.

Aside from insulin, TNF-α or IL1 signalling, which converges on NF-kB can lead to FOXO1 activation and upregulate the UPS (258). Consistent with this, the 'positive regulation of NF-kappaB transcription factor activity' and 'interleukin 1 processing' ontology annotations were enriched between days 2 and 7 of immobilisation (**Table 5.3**). At the individual gene level, four upstream (of nine; **Figure 5.4**) and one downstream (of 12**, Figure 5.5**) genes associated with NF-kB activation were increased after seven, but not two days of immobilisation. Nonetheless, injury did not upregulate the expression of any of these genes. Consequently, whilst NF-kB may be an important signalling cascade leading to FOXO1 and subsequent UPS activation under disuse conditions, the greater increase in FOXO1 mRNA expression or indeed the greater loss of muscle strength in the injured condition does not appear to be dependent on NF-kB signalling.

In view of the increase in FOXO1 expression with injury, the observation that injury inverted the relationship between the decline in quadriceps volume and the decline in myofibrillar protein synthesis rates (day 0 to 7; **Figure 5.8A**) could represent a manifestation of elevated muscle protein breakdown. As discussed in **Chapters 3** and **4**, eccentric contractions may increase muscle protein breakdown such that it starts driving an increase in myofibrillar protein synthesis in a similar way as is observed in other models of tissue injury (i.e., burns) (145). Given that the decline in muscle function was greater with injury, this would further corroborate the hypothesis that the loss (and subsequent recovery) of muscle function after injury is regulated by changes in muscle protein breakdown and not myofibrillar protein synthesis rates. Given the limitations of measuring indirect markers of muscle protein breakdown (i.e., gene expression), it is necessary for future work to endeavour to make direct measurements of muscle protein breakdown to establish any causative role in muscle function.

In conclusion, myofibrillar injury induced by eccentric contractions exacerbates the loss of muscle strength after seven days of unilateral leg immobilisation. Injury did not affect the decline in myofibrillar protein synthesis rates or the increase in NF-kB signalling during immobilisation but did increase FOXO1 and CASP8 mRNA

expression between 2 and 7 days of immobilisation. Consequently, this provides further evidence that muscle protein breakdown as opposed to myofibrillar protein synthesis rates may be important in determining changes in muscle function of injured skeletal muscle.

Chapter 6

Functional electrical stimulation-assisted cycle ergometry administered to critically ill patients during intensive care unit residency increases gene expression associated with fibrosis and impairs functional outcomes associated with intensive care unit acquired weakness in survivors.

6.1 Introduction

Survivors of critical illness frequently experience significant fatiguability and decreased aerobic performance, termed intensive care unit acquired weakness (ICUAW). This can persist for at least five years after ICU admission and is the primary determinant of failed functional outcomes in survivors (136, 137). Transcriptomic approaches have identified perturbations in skeletal muscle mRNA expression in critically ill patients during ICU residency associated with muscle protein remodelling, including the ubiquitin-proteasome, calpain, autophagy and translation initiation factor pathways (262). Furthermore, protracted perturbations in skeletal muscle transcription associated with skeletal muscle remodelling and inflammation are evident six months after ICU admission in patients that present with ICUAW (263). Indeed, previous work has demonstrated that differences in gene expression at ICU admission are predictive of subsequent survival outcomes in critically ill patients (264).

Skeletal muscle atrophy during ICU residency is a primary risk factor for the subsequent development of ICUAW (265). Skeletal muscle atrophy is primarily driven by an increase in muscle protein breakdown (5, 140). This is believed to occur as a consequence of elevated skeletal muscle mRNA expression of components of the ubiquitin-proteasome system, including MuRF1, MAFbx and their transcription factor FOXO1 (131, 140, 142, 262). Microarray investigations of whole blood or peripheral blood mononuclear cells have identified enriched pathways associated with the activation of the inflammatory NF-κB transcription factor during critical illness (266). In rodent skeletal muscle, inhibiting NF-κB activity reduces denervation and tumor-induced muscle loss and decreases muscle protein breakdown, concomitantly with a decrease (267) or no change (152) in MuRF1 mRNA expression. As a consequence, inflammation has been identified as one of the main risk factors that contribute to the development of ICUAW (139). However, there is a paucity of prospective time course data quantifying mRNA expression associated with skeletal muscle remodelling and NF-κB signalling in skeletal muscle of critically ill patients during ICU residency or after six months of convalescence.

Strategies to reduce ICUAW target the preservation of muscle mass during ICU residency by restoring muscle contraction through exercise (268), passive loading (269) or neuromuscular electrical stimulation (NMES) (142, 270). Indeed, muscle contraction is associated with a reduction in inflammation and all-cause mortality in healthy individuals and patients with chronic disease (151, 153, 154). Exercise, (i.e., cycling ergometry), and NMES can be combined to produce a coordinated pattern of movement independent of the patient's level of consciousness; a method termed functional electrical stimulation-assisted cycle ergometry (FESCE). FESCE performed during ICU residency has been shown to improve functional outcomes and decrease the length of ICU and hospital stay in sedated and mechanically ventilated critically ill patients (271). However, it is unknown how FESCE performed during critical illness affects skeletal muscle transcription associated with remodelling and NF-κB pathways, both during ICU residency and six months after ICU admission in survivors.

The aim of the current study was to apply a quantitative RT-PCR gene array and gene ontology approach to quantify the relative change in expression of skeletal muscle genes associated with inflammation and skeletal muscle remodelling in critically ill patients after seven days of either standard care (SC) or FESCE administered during ICU residency. This was prospectively repeated six months after ICU admission in survivors. It was hypothesised that seven days of a FESCE intervention would downregulate skeletal muscle gene expression associated with NF-κB signalling and skeletal muscle protein breakdown relative to patients that received seven days of SC, and this would be associated with improved quality of life outcomes six months after ICU admission.

6.2 Methods

Patients

Thirty patients (males *n*=21, females *n*=9) meeting the eligibility criteria were recruited from four ICUs at FNKV University Hospital, Prague, Czech Republic (**Table 6.1**). All patients admitted to participating ICUs were screened daily by research nurses and all eligible patients or their representatives are approached by investigators within 72 hours of ICU admission. Eligibility criteria included participants requiring mechanical ventilation, or imminent need of mechanical ventilation at presentation and a predicted ICU length of stay of more than seven days. Exclusion criteria included a known primary systemic neuromuscular disease or spinal cord lesion at admission; severe lower limb injury or amputation; bedridden premorbid state (Charleston Comorbidity Score > 4); approaching imminent death or withdrawal of medical treatment within 24 h; pregnancy; the presence of external fixator or superficial metallic implants in lower limbs; open wounds or skin abrasions at electrode application points; the presence of a pacemaker, implanted defibrillator, or other implanted electronic medical device; predicted as unable to receive first rehabilitation session within 72 h of admission or transferred from another ICU after more than 24 h of mechanical ventilation; the presence of another condition preventing the use of FESCE or considered unsuitable for the study by a responsible medical team and prior participation in another functional outcome-based intervention research study. An additional 17 age, sex (males *n*=9, females *n*=8) and BMI-matched metabolically healthy control participants undergoing elective hip surgery at the Department of Orthopaedic Surgery were also recruited. The study was approved by the Královské Vinohrady University Hospital Research Ethics Board in accordance with standards for human research as outlined in the declaration of Helsinki. The study is part of a larger investigation registered at ClinicalTrials.Gov (ID: NCT02864745). The full protocol has been published (272).

Experimental protocol

Prior to randomisation, a muscle biopsy of the vastus lateralis was obtained within 72 hours after ICU admission (day 1). Participants were randomised to receive either standard care (*n*=15; males *n*=10) or a FESCE intervention (*n*=15; males *n*=11). Randomization was stratified according to the presence of sepsis (standard care *n*=5;

FESCE *n*=5). On day seven of ICU residency after at least five days of intervention, a second muscle biopsy of the vastus lateralis was obtained. Standard care or FESCE interventions were administered for a total of 28 days or until ICU admission, whichever came sooner. Six months following ICU admission, an additional muscle biopsy of the vastus lateralis was obtained from survivors.

Standard care

The standard care condition included daily sedation holds when applicable and delirium 12-hourly monitoring (273) with management as usual in routine practice. Respiratory physiotherapy was also delivered without alterations. Patients in the standard care condition underwent mobilisation and rehabilitation treatment in the usual routine way, which was recorded but not protocoled.

FESCE

In addition to standard care as detailed above, participants randomised into the FESCE condition received early goal-directed rehabilitation protocolled according to patients' condition and safety criteria (274). This was initiated within 72 h of ICU admission and after muscle biopsy sampling on day 1. The FESCE intervention aimed to deliver a total of 90 min of active exercise daily until ICU admission or day 28, whichever occurred earlier. The FESCE intervention protocol has been published elsewhere (271). Briefly, FESCE involved a supine motorized cycle ergometer attached to a current-controlled stimulator (RT-300 supine model and SAGE stimulator; Restorative Therapies, Ltd, Baltimore, MD). Disposable adhesive gel electrodes were placed over the quadriceps, hamstrings, gluteals, and calf muscles. Muscles were stimulated at specific stages throughout the cycling phase based on normal muscular activation patterns regulated by the bicycle software. The intensity of muscle stimulation was delivered at a level able to cause visible contractions (confirmed by palpation if uncertain) in all muscle groups without causing undue pain or discomfort to the patient. Once the patient was more alert and able to participate, they were encouraged to engage in therapy. Resistance and cycling cadence was increased incrementally to increase the intervention workload.

Quality of life

The Short Form health survey (SF-36) (275) was administered as part of the wider investigation of which the current study is a part of. The SF-36 was completed by all surviving patients 6 months after hospital admission (SC *n*=40, FESCE *n*=36). The SF-36 is recommended for use in critical care situations (276). The SF-36 has 36 questions and generates a health profile of eight subscales: physical functioning, role limitations due to physical health, role limitations due to emotional problems, energy/fatigue, emotional well-being, social functioning, pain, general health. The scores on all the subscales are transformed to a scale from 0 = unfavourable health state to 100 = favourable health state (277).

Calculations and statistics

Full statistical procedures and calculations are presented in **Chapter 2**. Gene expression between elective hip surgery patients (control) and critically ill patients upon ICU admission was determined using a Welch's unpaired T-test, and only genes which were significantly different were included in subsequent analysis, with the exception of a comparison between critically ill patients at six months after ICU admission and control patients when all genes were included and analysed using a T-test. Changes in gene expression between day one and day seven and day one and month 6 in SC and FESCE conditions were determined using a mixed-effects model. Gene expression between control patients and SC and FESCE treated critically ill patients at six months was analysed using a one-way ANOVA. Gene expression at day 1 in critically ill patients was collapsed across conditions and compared between patients who survived six months and those who did not survive using a Welch's unpaired T-test. SF-36 scores were compared between conditions with a Wilcoxon rank sum test. Violations of sphericity were corrected with the Greenhouse-Geisser correction. When a significant main and interaction effect was observed, a Sidak post hoc test was performed to locate individual differences. Statistical significance was set at $P<0.05$. Calculations were performed using GraphPad Prism 8.3.0. All data are expressed as means±SEM.

6.3 Results

Patient characteristics

Patients in the FESCE condition presented with a higher Acute Physiology and Chronic Health Evaluation (APACHE) II score on ICU admission (*P*=0.022; **Table 6.1**). There was no difference between conditions in Rapid Assessment of Physical Activity (RAPA) 1 scores (*P*=0.765) and Charlson comorbidity (*P*=0.306) scores.

SF-36

Scores from the eight subscales of the SF-36 are presented in **Table 6.2** with the corresponding FESCE vs SC *P*-value presented.

Table 6.2. RAND 36-Item Short Form Survey response six months after ICU admission in survivors. N.B. A higher score defines a more favourable health state.

	SC	FESCE	P-value
	***n*=40**	***n*=36**	
Physical functioning	53±5	50±6	0.449
Role limitations due to physical health	51±6	28±6	0.011
Role limitations due to emotional problems	78±6	57±7	0.018
Energy/fatigue	53±8	46±8	0.072
Emotional well-being	68±3	57±3	0.002
Social functioning	72±5	49±5	0.001
Pain	74±4	67±4	0.204
General health	51±4	41±4	0.083

Values represent mean±SEM. SC standard care; FESCE, functional electrically stimulated cycling ergometry.

Table 6.1. Patient characteristics.

Condition	Age	Sex	Diagnosis	Sepsis	APACHE	CCS	Length of ICU stay	Survived	Average daily SC or FESCE (min)	Included in analysis		
										Day 1	Day 7	Mon 6
SC	70	M	Medical	No	26	3	25	Yes	67		Y	Y
SC	53	F	Medical	No	23	3	7	Yes	52	Y	Y	
SC	71	F	Medical	Yes	20	7	18	Yes	48			Y
SC	78	M	Medical	Yes	37	5	22	Yes	46	Y		Y
SC	43	F	Trauma	No	27	0	17	No	56	Y	Y	
SC	69	M	Trauma	No	16	2	22	Yes	53	Y	Y	Y
SC	51	M	Medical	No	17	1	12	Yes	19	Y	Y	
SC	79	M	Surgery non trauma	No	23	11	2	No	0	Y		
SC	55	M	Trauma	No	26	2	14	Yes	23	Y	Y	
SC	73	F	Medical	Yes	31	4	14	Yes	45	Y	Y	Y
SC	68	M	Medical	No	34	3	25	No	44	Y	Y	
SC	66	M	Medical	No	31	3	10	Yes	46	Y	Y	
SC	66	M	Medical	Yes	40	7	18	Yes	18	Y	Y	Y
SC	53	F	Trauma	No	29	2	14	Yes	43	Y	Y	Y
SC	71	M	Medical	Yes	20	3	4	No	15	Y		
FESCE	55	M	Trauma	No	23	5	3	Yes	21	Y		Y
FESCE	72	F	Medical	No	16	5	13	No	17	Y	Y	
FESCE	24	M	Trauma	Yes	19	0	27	Yes	27	Y	Y	
FESCE	71	F	Surgery non trauma	Yes	33	4	1	No	0	Y		
FESCE	74	M	Trauma	No	28	4	1	No	0	Y		
FESCE	71	F	Medical	No	16	5	18	No	28	Y	Y	
FESCE	34	M	Trauma	No	20	0	11	Yes	13	Y	Y	Y
FESCE	74	M	Surgery non trauma	Yes	19	6	6	Yes	13	Y		
FESCE	65	M	Trauma	No	16	3	16	No	40	Y	Y	
FESCE	21	M	Trauma	No	23	0	15	Yes	27	Y	Y	Y
FESCE	63	M	Medical	Yes	25	3	28	No	28	Y	Y	
FESCE	69	M	Trauma	No	27	3	9	No	42	Y	Y	
FESCE	62	M	Surgery non trauma	Yes	21	2	17	No	8	Y	Y	
FESCE	51	F	Medical	Yes	25	1	20	Yes	36	Y	Y	Y
FESCE	54	M	Trauma	No	19	1	7	No	6	Y	Y	

Values represent mean±SEM. SC standard care; FESCE, functional electrically stimulated cycling ergometry; APACHE II, Acute Physiology and Chronic Health Evaluation; M=male, F=female; CCS, Charlson comorbidity score Y = yes.

Skeletal muscle mRNA expression

Protein synthesis

AKT2, *DDIT4*, *EIF4EBP1*, *MTOR*, *RPS6KB1* and *SLC7A5* mRNA expression was upregulated (*P*<0.05), whereas *GDF11* mRNA tended to be upregulated (*P*=0.063) and *IRS1* mRNA expression was downregulated (*P*=0.015) at ICU admission compared to control patients (**Figure 6.1**). After one week in the ICU, the mRNA expression of *AKT2*, *GDF11*, *IRS1*, *MTOR*, *RPS6KB1*, *SLC7A5* did not change (*P*>0.05) whereas the mRNA expression of *DDIT4*, *EIF4EBP1* and *PIK3R1* decreased to a similar extent in SC and FESCE conditions (*P*<0.05, interaction; *P*>0.05). At six months following ICU admission, the mRNA expression of IRS1 increased to a similar extent in SC and FESCE conditions (*P*=0.047). The mRNA expression of *DDIT4*, *EIF4EBP1*, *MTOR* and *SLC7A5* decreased to a similar extent in SC and FESCE conditions (*P*<0.05, interaction; *P*>0.05), apart from MTOR which decreased in FESCE (from 0.72±0.32 to -0.56±0.75 log_2 fold change; *P*=0.018) but not SC (from 0.42±0.43 to -0.99±0.44 log_2 fold change; *P*=0.668) (interaction; *P*=0.049). At six months following ICU admission, *IRS1* mRNA expression was higher in the FESCE condition (1.76±0.27 log_2 fold change) than in control patients (0.52±0.18 log_2 fold change) (*P*=0.006).

Protein breakdown

BECN1, *CAPN1*, *FOXO1*, *MAFbx*, *MSTN*, *MuRF1* and *PSMB1* mRNA expression was upregulated (*P*<0.05) and *CAPN3* and *FOXO3* mRNA expression was not different (*P*>0.05) at ICU admission compared to control patients (**Figure 6.2**). After one week in the ICU, the mRNA expression of *BECN1, CAPN1*, *CAPN3* and *PSMB1* did not change (*P*>0.05) whereas the mRNA expression of *FOXO1*, *FOXO3*, *MAFbx*, *MSTN* and *MuRF1* decreased to a similar extent in SC and FESCE conditions (*P*<0.05, interaction; *P*>0.05). At six months following ICU admission, the mRNA expression of *BECN1*, *CAPN1*, *CAPN3*, *FOXO1*, *MAFbx*, *MuRF1* and *PSMB1* decreased with respect to ICU admission (*P*<0.05) and *FOXO3* and *MSTN* mRNA expression was unchanged (*P*>0.05). At 6 months following ICU admission, the mRNA expression of *CAPN1* (SC=-0.60±0.06, control=-0.18±0.06 log_2 fold change; *P*<0.001), *MAFbx* (SC=-1.43±0.31, control=-0.09±0.17 log_2 fold change; *P*=0.003) and *MuRF1* (SC=-1.71±0.38, control=-0.65±0.19 log_2 fold change; *P*=0.028), was lower in the

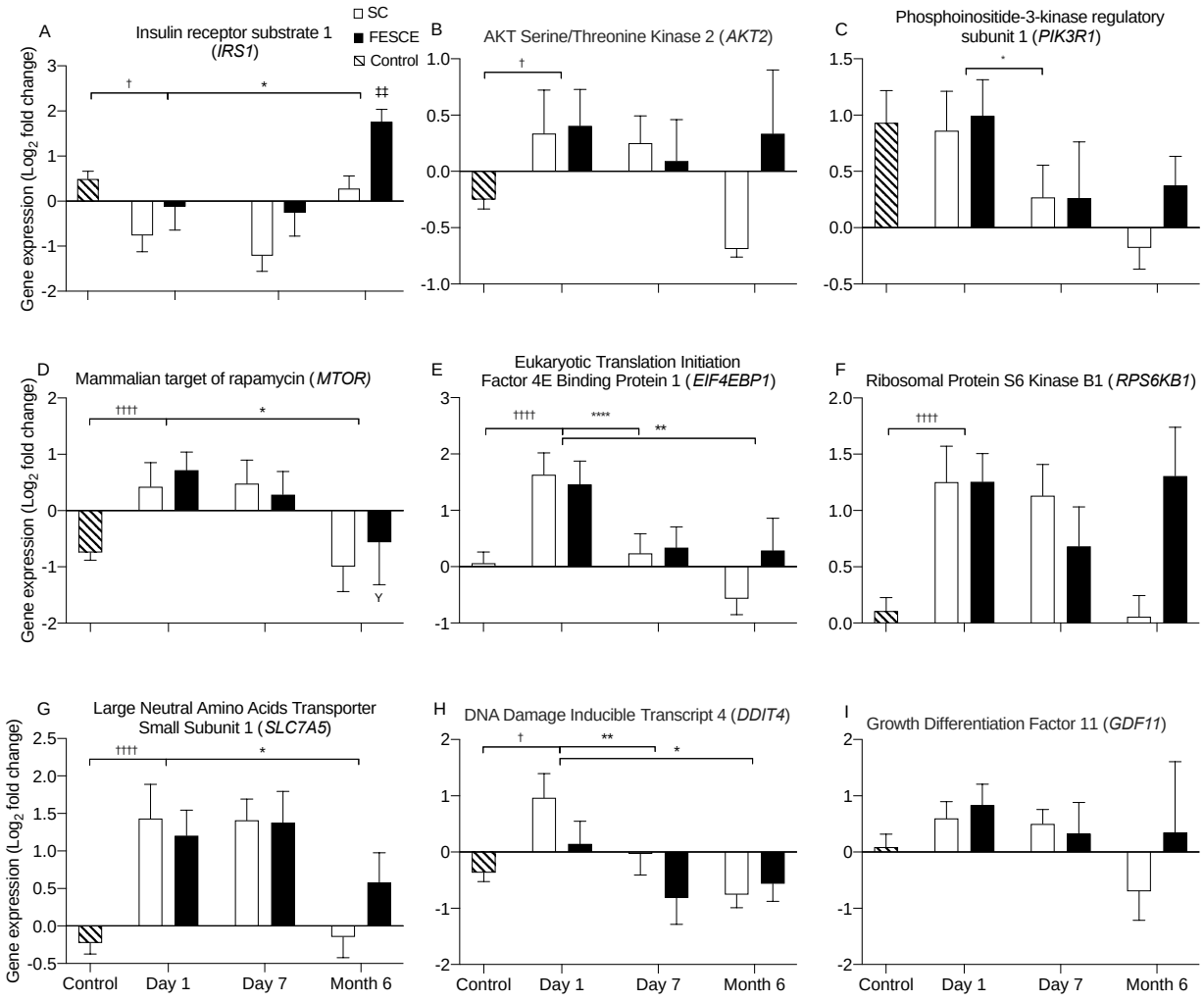

Figure 6.1. Skeletal muscle mRNA expression associated with skeletal muscle protein synthesis in elective hip surgery control patients (control n=17) and critically ill patients resident in an ICU who received either standard care (SC; white bars) or a functional electronically stimulated cycle ergometry intervention (FESCE; black bars). Muscle biopsies were taken at ICU admission (day one; SC *n*=13, FESCE *n*=14), seven days after ICU admission (day seven; SC *n*=11, FESCE *n*=11) and six months after ICU admission (six months; SC *n*=7, FESCE *n*=4). mRNA expression is calculated as a fold change from a nominal control patient and log_2 transformed. mRNA expression in control vs critically ill patients at day one was analysed using a t-test, the change in mRNA expression in critically ill patients between day one and seven and day one and month six was analysed using a mixed effects model and mRNA expression in control vs critically ill patients at six months was analysed using a one-way ANOVA. † = difference between control and critically ill patients at day one, * difference between time points in critically ill patients, ‡ difference between SC or FESCE at six months and controls, Ψ change in mRNA expression between day seven and month six in FESCE only. One symbol *P*<0.05, two symbols *P*<0.01, three symbols *P*<0.001, four symbols *P*<0.0001.

SC condition than in control patients. *CAPN3* (FESCE=0.90±0.39, control=-0.04±0.10 log_2 fold change; *P*=0.002) and *MSTN* (FESCE=2.11±0.68, control=0.55±0.18 log_2 fold change; *P*=0.013) mRNA expression was higher in the FESCE conditions than in control patients.

NF-κB signalling

IL18, *IL1R1*, *NFKB1*, *TNFRSF10B*, *TNFRSF12A* and *TRAFD1* mRNA expression upstream of NF-κB (**Figure 6.3**) and *ATF3*, *CCL2*, *CEBPD*, *CXCL1*, *CXCL2*, *HIF1A*, *SOCS3*, *SOD2* and *VEGFA* mRNA expression downstream of NF-κB (**Figure 6.4**) was upregulated at ICU admission compared to control patients (*P*<0.05). After one week in the ICU, the expression of *CEBPD, ICAM1, IL1R1, IL1RL1, RELT*, *SOD2* and *VEGFA* was downregulated whereas the expression of *IL18*, *TGFB2* and *TNFRSF12A* was upregulated to a similar extent in SC and FESCE conditions (*P*<0.05, interaction; *P*>0.05). The expression of *NFKB1* between day one and seven in the ICU tended to be downregulated in the FESCE condition but not the SC condition (interaction; *P*=0.044). At six months following ICU admission, the mRNA expression of *ATF3* and *TGFB2* was further upregulated (*P*<0.05). In contrast, the mRNA expression of *CEBPD*, *CXCL2*, *IL1R1*, *SOCS3*, *SOD2*, and *TRAFD1* was downregulated (*P*<0.05) relative to ICU admission, and to a similar extent in SC and FESCE conditions (*P*<0.05, interaction; *P*<0.05). The expression of *RELT* six months following ICU admission was not different to ICU admission in the FESCE condition (-1.31±0.58 vs -0.77±0.28 log_2 fold change, respectively; *P*=0.256), but remained downregulated in the SC condition (-2.13±0.36 vs 0.47±0.42 log_2 fold change, respectively; *P*=0.010). At six months following ICU admission, the mRNA expression of *CXCL1* (FESCE=-1.53±0.30, control=0.25±0.15 log_2 fold change; *P*=0.004), *HIF1A* (FESCE=1.03±0.68, control=0.09±0.06 log_2 fold change; *P*=0.012), *TGFB2* (FESCE=2.25±0.71, control=-0.52±0.14 log_2 fold change; *P*<0.001), *TNFRSF10B* (FESCE=1.00±0.50, control=-0.11±0.18 log_2 fold change; *P*=0.006) and *VEGFA* (FESCE=0.38±0.42, control=-0.71±0.11 log_2 fold change; *P*=0.002) was higher in the FESCE condition only than in control patients, whereas *ATF3* (FESCE=2.64±1.12; *P*<0.001, SC=1.62±0.34; *P*<0.001, control=-0.45±0.18 log_2 fold change) and *TNFRSF12A* (FESCE=2.47±1.05; *P*<0.001, SC=1.65±0.20; *P*<0.001, control=-0.27±0.21 log_2 fold change; *P*=0.028) mRNA expression was higher in both FESCE and SC conditions compared to control patients. *CEBPD* (FESCE=-2.42±0.71, control=-0.65±0.19 log_2 fold change; *P*=0.004)

mRNA expression was lower in the FESCE condition only and RELT (SC=-2.13±0.36, control=-0.99±0.16 log_2 fold change; *P*=0.009) mRNA expression was lower in the SC condition only compared to control patients.

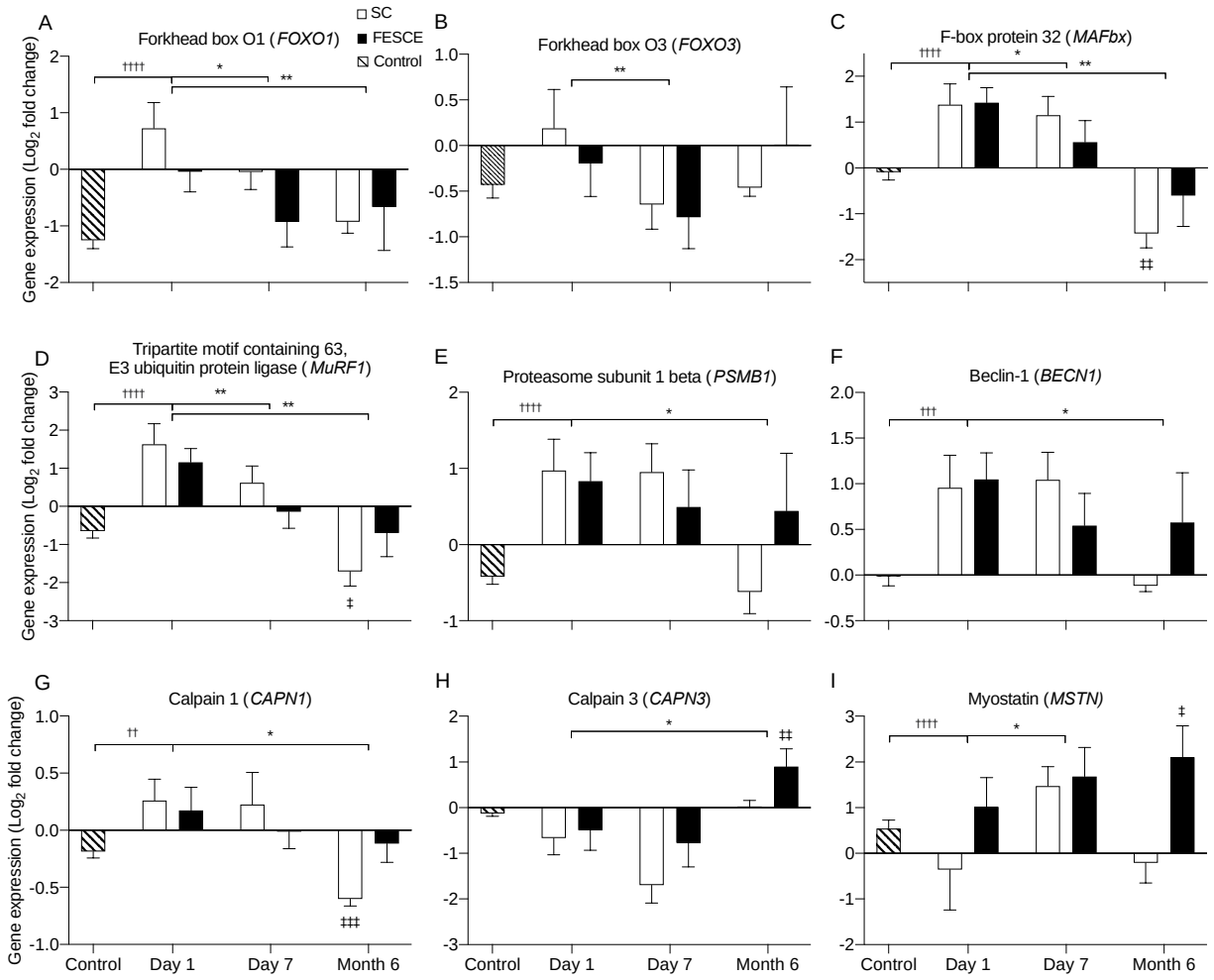

Figure 6.2. Skeletal muscle mRNA expression associated with skeletal muscle protein breakdown in elective hip surgery control patients (control *n*=17) and critically ill patients resident in an ICU who received either standard care (SC; white bars) or a functional electronically stimulated cycle ergometry intervention (FESCE; black bars). Muscle biopsies were taken at ICU admission (day one; SC *n*=13, FESCE *n*=14), seven days after ICU admission (day seven; SC *n*=11, FESCE *n*=11) and six months after ICU admission (six months; SC *n*=7, FESCE *n*=4). mRNA expression is calculated as a fold change from a nominal control patient and log_2 transformed. mRNA expression in control vs critically ill patients at day one was analysed using a t-test, the change in mRNA expression in critically ill patients between day one and seven and day one and month six was analysed using a mixed effects model and mRNA expression in control vs critically ill patients at six months was analysed using a one-way ANOVA. † = difference between control and critically ill patients at day one, * difference between time points in critically ill patients, ‡ difference between SC or FESCE at six months and controls. One symbol *P*<0.05, two symbols *P*<0.01, three symbols *P*<0.001, four symbols *P*<0.0001.

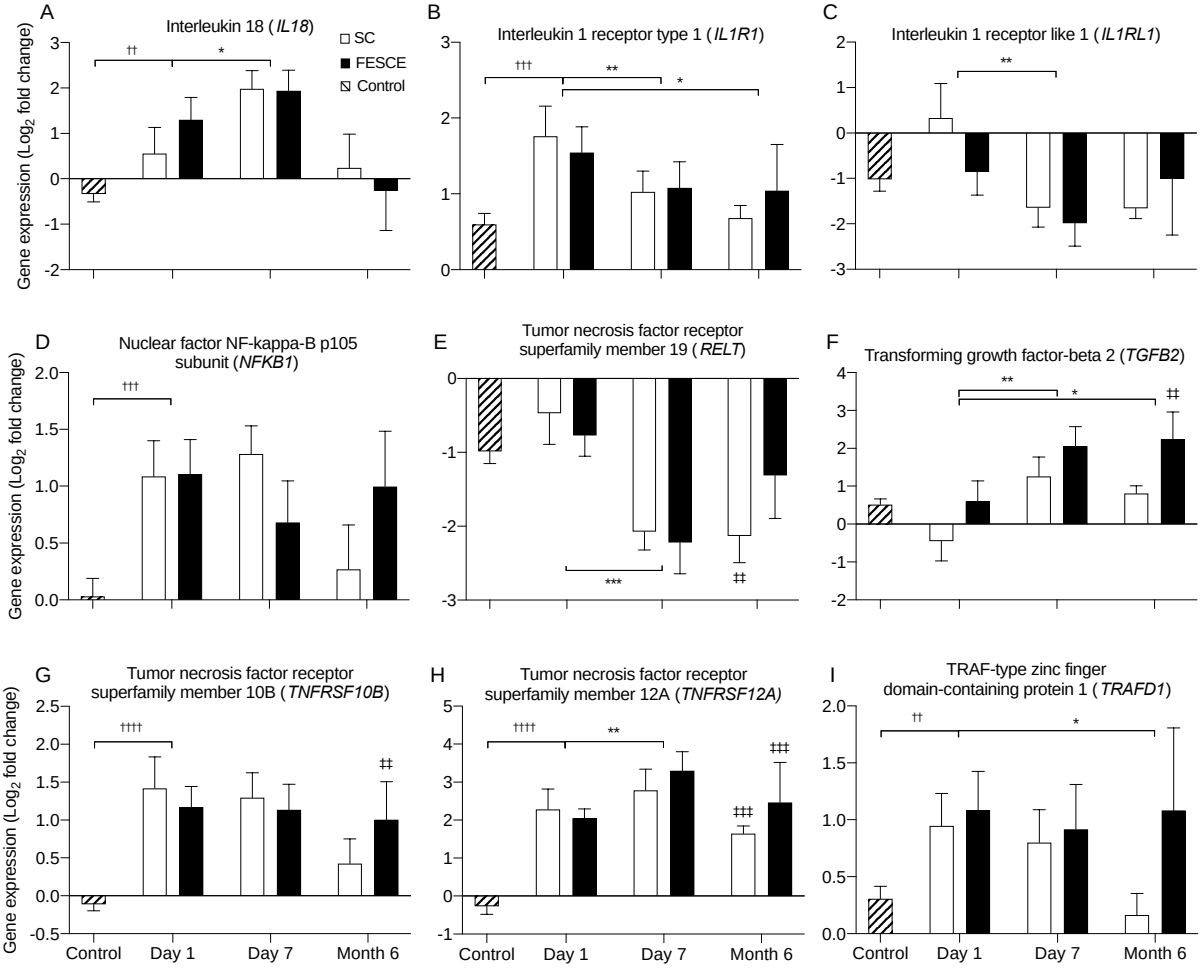

Figure 6.3. Skeletal muscle mRNA expression associated with upstream activation of NF-κB in elective hip surgery control patients (control n=17) and critically ill patients resident in an ICU who received either standard care (SC; white bars) or a functional electronically stimulated cycle ergometry intervention (FESCE; black bars). Muscle biopsies were taken at ICU admission (day one; SC *n*=13, FESCE *n*=14), seven days after ICU admission (day seve; SC *n*=11, FESCE *n*=11) and six months after ICU admission (six months; SC *n*=7, FESCE *n*=4). mRNA expression is calculated as a fold change from a nominal control patient and log_2 transformed. mRNA expression in control vs critically ill patients at day one was analysed using a t-test, the change in mRNA expression in critically ill patients between day one and seven and day one and month six was analysed using a mixed effects model and mRNA expression in control vs critically ill patients at six months was analysed using a one-way ANOVA. † = difference between control and critically ill patients at day one, * difference between time points in critically ill patients, ‡ difference between SC or FESCE at six months and controls. One symbol *P*<0.05, two symbols *P*<0.01, three symbols *P*<0.001, four symbols *P*<0.0001.

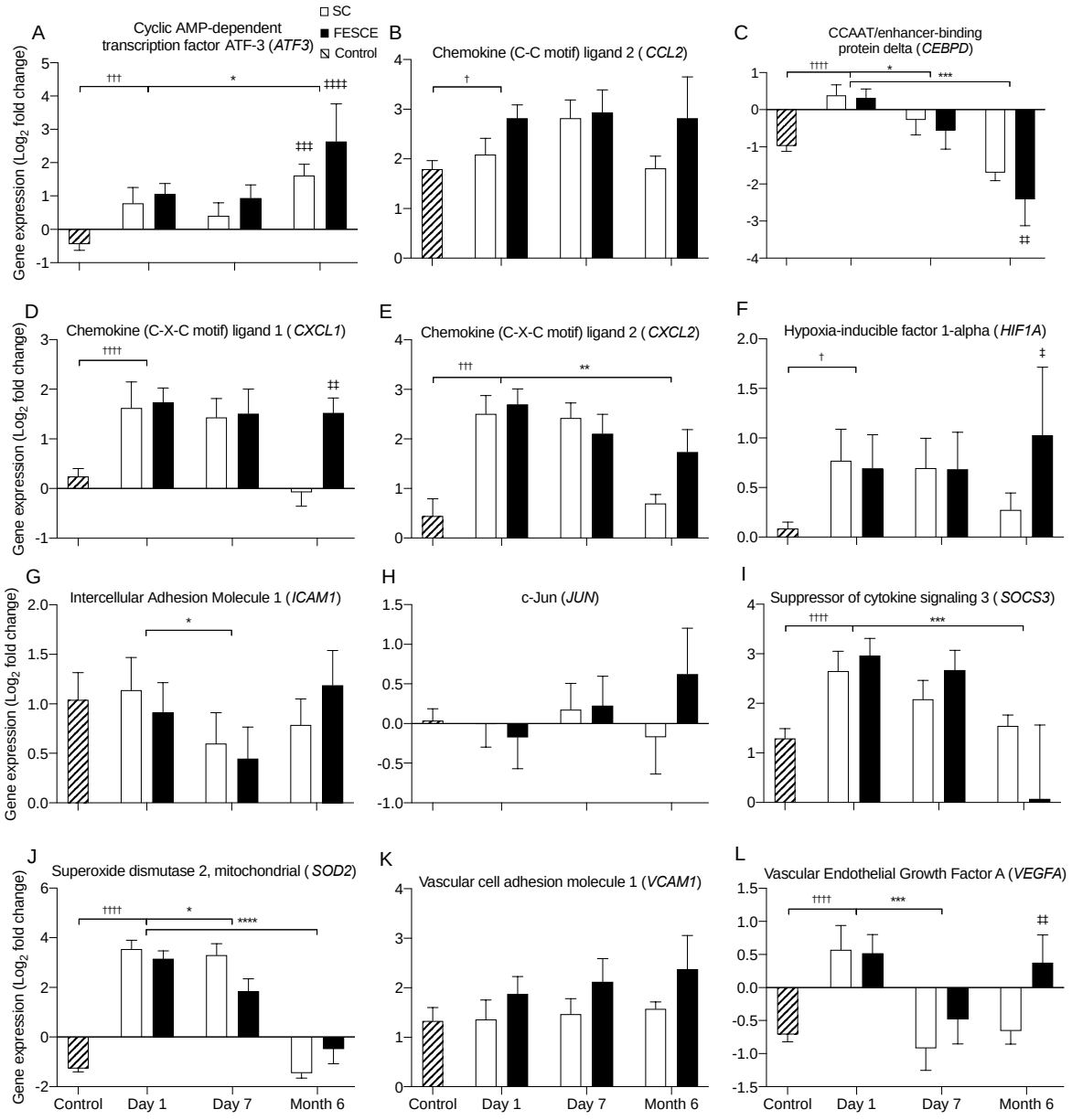

Figure 6.4. Skeletal muscle mRNA expression associated with downstream activation of NF-κB in elective hip surgery control patients (control *n*=17) and critically ill patients resident in an ICU who received either standard care (SC; white bars) or a functional electronically stimulated cycle ergometry intervention (FESCE; black bars). Muscle biopsies were taken at ICU admission (day one; SC *n*=13, FESCE *n*=14), 7 days after ICU admission (day seven; SC *n*=11, FESCE *n*=11) and six months after ICU admission (six months; SC *n*=7, FESCE *n*=4). mRNA expression is calculated as a fold change from a nominal control patient and $\log_2$ transformed. mRNA expression in control vs critically ill patients at day one was analysed using a t-test, the change in mRNA expression in critically ill patients between day one and seven and day one and month six was analysed using a mixed effects model and mRNA expression in control vs critically ill patients at six months was analysed using a one-way ANOVA. † = difference between control and critically ill patients at day one, * difference between time points in critically ill patients, ‡ difference between SC or FESCE at six months and controls. One symbol $P<0.05$, two symbols $P<0.01$, three symbols $P<0.001$, four symbols $P<0.0001$.

Survivors vs non-survivors

The expression of all genes associated with NF-κB signalling and muscle protein turnover at ICU admission was not different between eventual survivors and non-survivors at six months after ICU admission (*P*>0.05). However, MTOR (survivors=0.14±0.39, non-survivors 1.07±0.31 log_2 fold change; *P*=0.075) and PIK3R1 (survivors=0.49±0.34, non-survivors 1.37±0.35 log_2 fold change; *P*=0.085) tended to be downregulated more in survivors compared to non-survivors.

Gene ontology

Only genes which showed a significant change in expression over time passed the FDR cut-off of 5% and were included in the gene ontology analysis. The mRNA expression of all genes and their respective *P*-values are provided in **Appendices 9.1** for reference.

The skeletal muscle mRNA expression of 124 genes was upregulated, and 13 genes were downregulated at ICU admission relative to controls and after applying an *FDR* cut-off of 5% (**Table 6.3**, **Figure 6.5**). The mRNA expression of six genes were further upregulated between day one and day 7 of ICU residency and 30 were downregulated (**Table 6.4**, **Figure 6.6**). Six months after ICU admission, the mRNA expression of 27 genes were downregulated, and five were upregulated relative to ICU admission (**Table 6.5**, **Figure 6.7**).

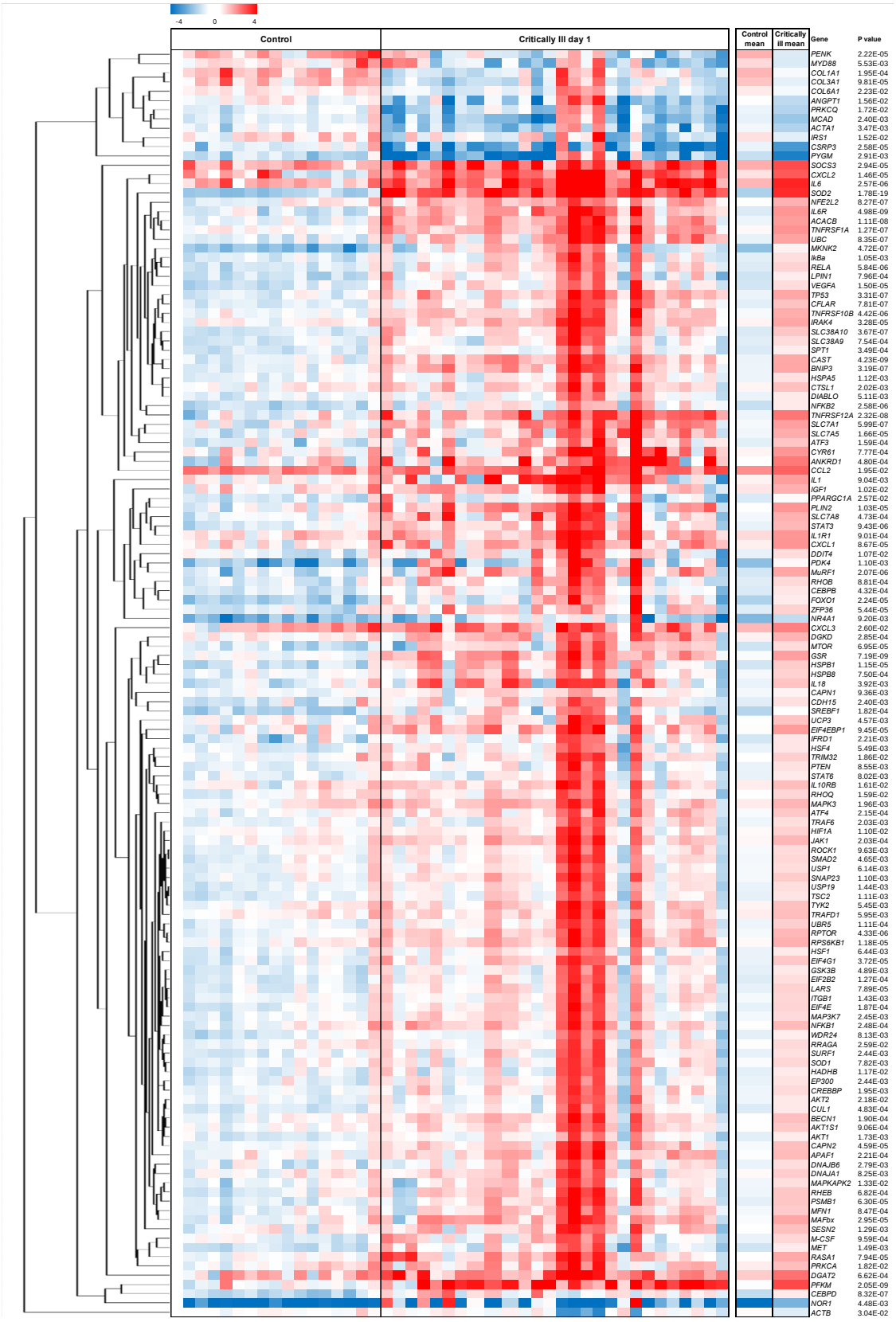

Figure 6.5. Heat map of skeletal muscle mRNA expression which was differentially expressed between control patients (*n*=17) and critically ill patients on ICU admission (day one; *n*=27). mRNA expression is calculated as a fold change from a nominal control patient and $\log_2$ transformed with hierarchal clustering applied. mRNA expression in control vs critically ill patients at day one was analysed using a t-test and corrected for familywise errors using a Benjamini-Hochberg correction (false discovery rate <5%).

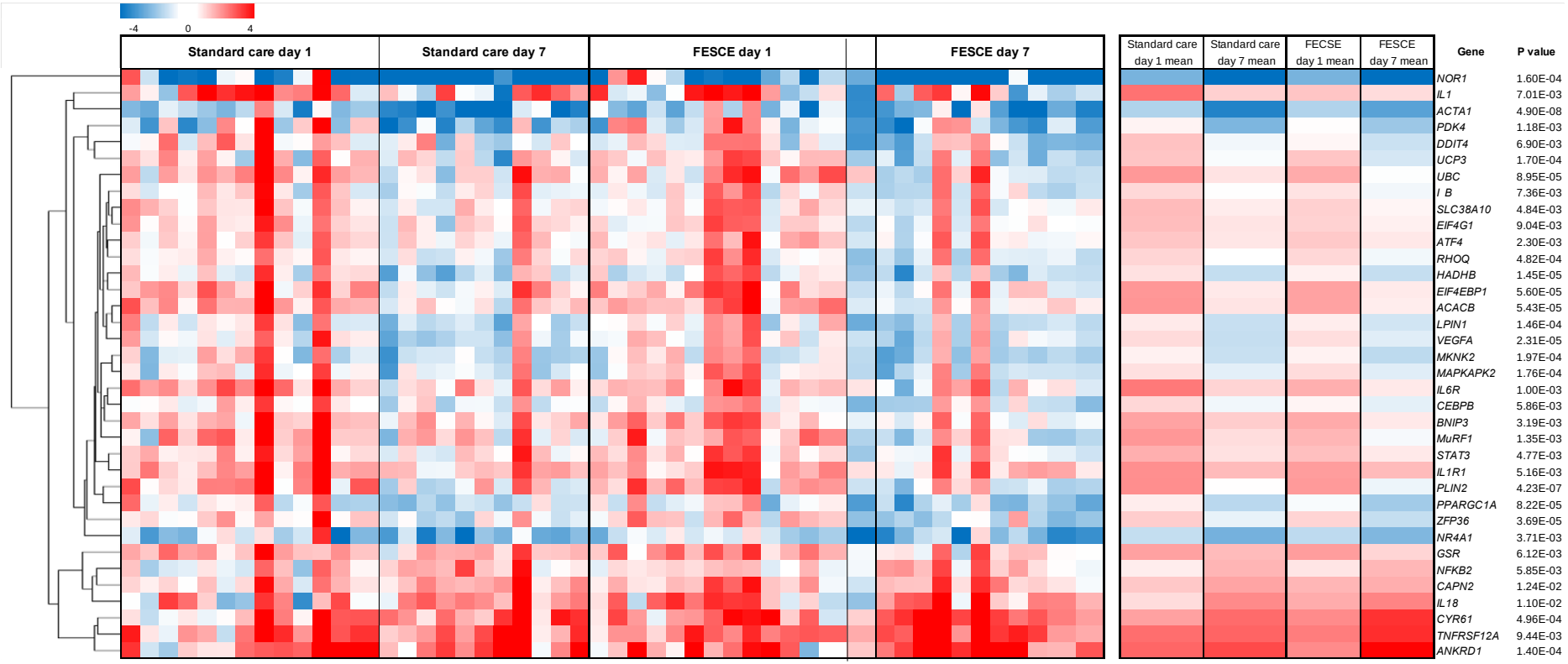

Figure 6.6. Heat map of skeletal muscle mRNA expression in critically ill patients which was differentially expressed between day one (*n*=27) and day seven (*n*=22) collapsed for condition (SC or FESCE). mRNA expression is calculated as a fold change from a nominal control patient and $\log_2$ transformed with hierarchal clustering applied. mRNA expression was analysed using a mixed-effects model and corrected for familywise errors using a Benjamini-Hochberg correction (false discovery rate <5%).

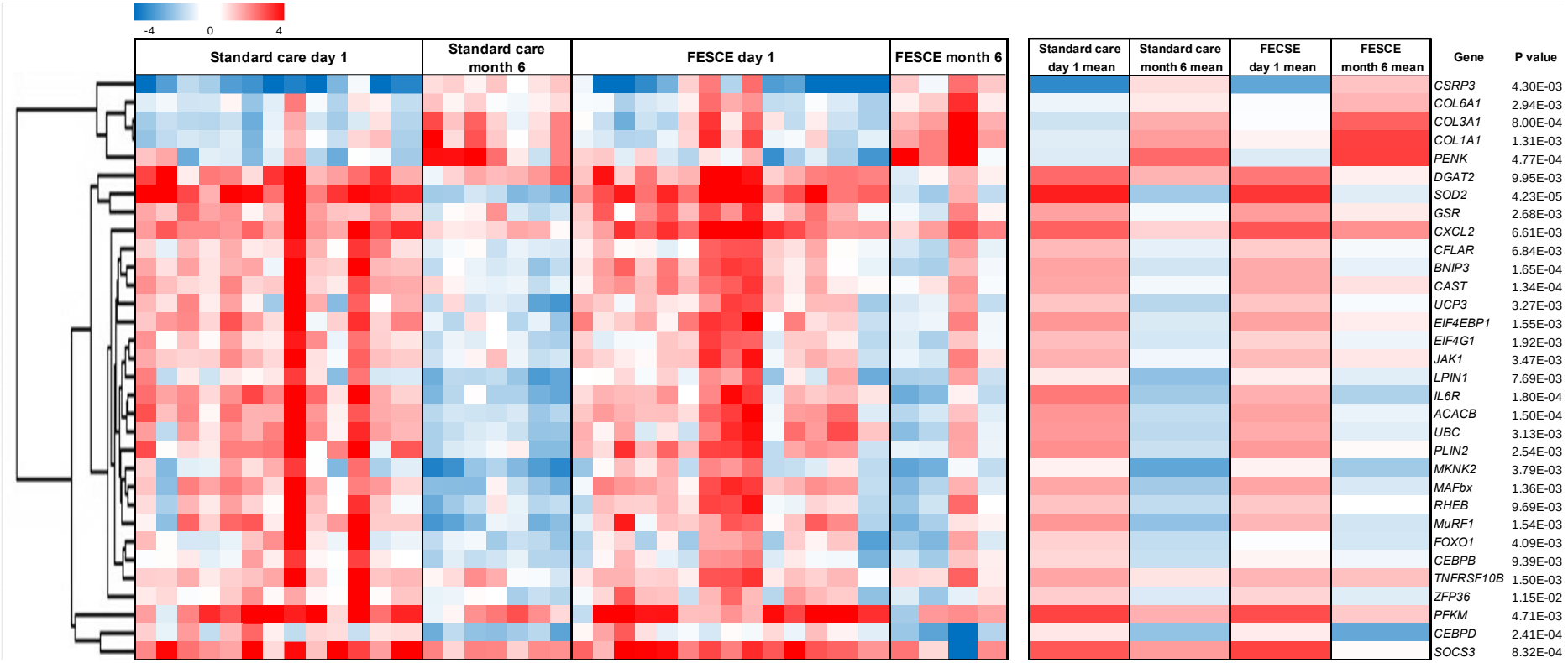

Figure 6.7. Heat map of skeletal muscle mRNA expression in critically ill patients which was differentially expressed between day 1 (*n*=27) and month 6 (*n*=11) collapsed for condition (SC or FESCE). mRNA expression is calculated as a fold change from a nominal control patient and $\log_2$ transformed with hierarchal clustering applied. mRNA was analysed using a mixed-effects model and corrected for familywise errors using a Benjamini-Hochberg correction (false discovery rate <5%).

Table 6.3. Gene ontology analysis of genes which were upregulated and downregulated between critically ill patients at ICU admission and control patients. The three most enriched processes/pathways are shown.

		List total	List hits	P-value	Genes
Up-regulated	**GO biological processes**				
	Regulation of cellular amide metabolic processes	34	27	0.034	*AKT1*, *AKT2*, *ATF3*, *ATF4*, *CEBPB*, *CYR6*, *EIF2B2*, *EIF4E*, *EIF4EBP1*, *EIF4G1*, *HSPB1*, *IGF1*, IL6, *MAPK3*, *MKNK2*, *MTOR*, *NFE2L2*, *PDK4*, *RELA*, *ROCK1*, *RPS6KB1*, *SESN2*, *STAT3*, *TNFRSF1A*, *TP53*, *ZFP36*
	Interspecies interactions between organisms	87	59	0.034	*AKT1*, *ANKRD1*, *BECN1*, *BNIP3*, *CAPN2*, *CCL2*, *CEBPB*, *CEBPD*, *CFLAR*, *CREBBP*, *CTSL1*, *CUL1*, *CXCL1*, *CXCL2*, *CXCL3*, *DDIT4*, *EIF4E*, *EIF4G1*, *EP300*, *GSK3B*, *HIF1A*, *HSF1*, *HSPB1*, *IkBa*, *IL10RB*, *IL18*, *IL1b*, *IL6*, *IL6R*, *IRAK4*, *ITGB1*, *JAK1*, *M-CSF*, *MAP3K7*, *MAPK3*, *MAPK3*, *MAPKAPK2*, *MET*, *NFE2L2*, *NFKB1*, *NFKB2*, *NR4A1*, *PPARGC1A*, *PSMB1*, *RELA*, *RPTOR*, *RRAGA*, *SOD2*, *STAT3*, *TNFRSF1A*, *TP53*, *TRAF6*, *TRIM32*, *TSC2*, *TYK2*, *UBC*, *UBR5*, *ZFP36*
	Reactome pathways				
	Cellular response to stress	41	33	0.016	*AKT1S1*, *ATF3*, *ATF4*, *CEBPB*, *CREBBP*, *DNAJA1*, *DNAJB6*, *EP300*, *GSK3B*, *HIF1A*, *HSF1*, *HSPA5*, *HSPB8*, *IL6*, *MAPK3*, *MAPK3*, *MAPKAPK2*, *MTOR*, *NFKB1*, *PSMB1*, *RELA*, *RHEB*, *RPTOR*, *RRAGA*, *SESN2*, *SLC38A9*, *SOD1*, *SOD2*, *STAT3*, *TP53*, *UBC*, *VEGFA*, *WDR24*
Down-regulated	**GO biological processes**				
	Platelet activation	12	4	0.004	*ACTB*, *COL1A1*, *COL3A1*, *PRKCQ*
	Cellular response to vitamin	2	2	0.006	*COL1A1*, *PENK*
	Osteoblast differentiation	7	3	0.007	*COL1A1*, *COL6A1*, *PENK*
	Reactome pathways				
	Collagen chain trimerization	4	3	0.001	*COL1A1, COL3A1, COL6A1*
	Collagen formation	5	3	0.003	*COL1A1, COL3A1, COL6A1*
	Collagen degradation	6	3	0.004	*COL1A1, COL3A1, COL6A1*

List total = number of genes contained on the gene card categorised into that process/pathway. List hits = number of significant genes from the list total.

Table 6.4. Gene ontology analysis of genes which were upregulated and downregulated in critically ill patients between day one and day seven of ICU residency. The three most enriched processes/pathways are shown.

		List total	List hits	P-value	Genes
Up-regulated	**GO biological processes**				
	Anatomical structure formation involved in morphogenesis	51	6	<0.001	*ANKRD1*, *CAPN2*, *CYR61*, *IL18*, *NFKB2*, *TNFRSF12A*
	Response to molecule of bacterial origin	41	4	0.012	*ANKRD1*, *CAPN2*, *IL18*, *NFKB2*
	Extracellular structure organisation	21	3	0.013	*CAPN2*, *CYR61*, *NFKB2*
	Reactome pathways				
	Interleukin 1 processing	5	2	0.007	*IL18*, *NFKB2*
	TNFR2 non-canonical NF-κB pathway	8	2	0.017	*NFKB2, TNFRSF12A*
	Interleukin 18 signalling	1	1	0.026	*IL18*
Down-regulated	**GO biological processes**				
	mRNA metabolic process	9	5	0.001	*ATF4*, *EIF4G1*, *PPARGC1A*, *STAT3*, *ZFP36*
	Acute phase response	5	4	0.005	*CEBPB, IL1B*, *IL6R*, *STAT3*
	Positive regulation of lipid localisation	6	4	0.009	*ACACB*, *IL1B*, *IKBA*, *PLIN2*
	Reactome pathways				
	Transcriptional regulation of granulopoiesis	5	3	0.030	*CEBPB*, *IL6R*, *STAT3*
	Tristetraprolin (TTP, ZFP36) binds and destabilizes mRNA	2	2	0.030	*MAPKAPK2, ZFP36*

List total = number of genes contained on the gene card categorised into that process/pathway. List hits = number of significant genes from the list total.

Table 6.5. Gene ontology analysis of genes which were upregulated and downregulated in critically ill patients between ICU admission and six months after ICU admission. The three most enriched processes/pathways are shown.

		List total	List hits	P-value	Genes
Up-regulated	**GO biological processes**				
	Osteoblast differentiation	7	3	<0.001	*COL1A1*, *COL6A1*, *PENK*
	Cellular response to vitamin	2	2	<0.001	*COL1A1*, *PENK*
	Cellular response to organonitrogen compound	60	5	0.001	*COL1A1*, *COL3A1*, *COL6A1*, *CSRP3*, *PENK*
	Reactome pathways				
	Collagen chain trimerization	4	3	<0.001	*COL1A1, COL3A1, COL6A1*
	Assembly of collagen fibrils and other multimeric structures	5	3	<0.001	*COL1A1, COL3A1, COL6A1*
	Collagen degradation	6	3	<0.001	*COL1A1, COL3A1, COL6A1*
Down-regulated	**GO biological processes**				
	mRNA catabolic process	3	2	0.006	*EIF4G1*, *ZFP36*
	Response to hyperoxia	4	3	0.014	*BNIP3, FOXO1*, *SOD2*
	Response to glucocorticoid	21	7	0.014	*CFLAR*, *EIF4EBP1*, *FOXO1*, *MAFbx*, *MuRF1*, *UCP3*, *ZFP36*
	Reactome pathways				
	Antigen processing: Ubiquitination & Proteasome degradation	8	4	0.018	*MAFbx*, *MuRF1*, *SOCS3*, *UBC*
	FOXO-mediated transcription of oxidative stress, metabolic and neuronal genes	9	4	0.026	*FOXO1*, *MAFbx*, *MuRF1*, *SOD2*
	Regulation of IFGN signalling	6	3	0.027	*JAK1*, *SOCS3*

List total = number of genes contained on the gene card categorised into that process/pathway. List hits = number of significant genes from the list total.

6.4 Discussion

Critical illness induces widespread alterations to the skeletal muscle transcriptome associated with muscle protein turnover and inflammation with perturbations evident for six months after ICU admission in patients presenting with ICUAW (262, 263). A pilot study has demonstrated FESCE administered during ICU residency improves functional outcomes and decreases the length of ICU and hospital stay in crucially ill patients (271). The current study demonstrates for the first time that FESCE exercise performed during ICU residency does not affect the expression of genes associated with muscle protein turnover and inflammation (**Figure 6.1**-**6.4**). Furthermore, six months after ICU admission FESCE performed during ICU residency led to upregulated mRNA expression of genes associated with skeletal muscle fibrosis which was not evident in patients that received standard care. This was associated with a consistent pattern of more unfavourable quality of life outcomes in patients that received FESCE compared to SC (**Table 6.2**). These data suggest exercise performed during critical illness may be detrimental to long term skeletal muscle morphology, which could lead to exacerbated ICUAW in these patients.

Consistent with the metabolic stress response and catabolic state associated with critical illness (5, 131, 140), skeletal muscle mRNA expression of MuRF1, MAFbx, PSMB1 and FOXO1 was upregulated at ICU admission (**Figure 6.2**) (140, 142, 262). This occurred concomitantly with the enriched 'cellular response to stress' annotation (**Table 6.3**) illustrating a rapid transcriptional response characteristic of increased muscle protein breakdown at ICU admission. The expression of FOXO3 did not change (**Figure 6.2B**), consistent with a previous report in critically ill patients (141). Skeletal muscle protein breakdown is known to be inhibited by physiological insulin action (102), and the sensitivity of skeletal muscle to insulin is profoundly impaired in critically ill patients (278). Indeed, IRS-1 mRNA expression was downregulated at the onset of critical illness relative to control patients (**Figure 6.1A**). However, in spite of this, expression of genes belonging to the anabolic PI3K/AKT/mTOR insulin sensitive pathway, which is downstream of IRS-1 signalling, was upregulated at ICU admission (**Figure 6.1**). As discussed previously (**Chapters 3-5**), in skeletal muscle of burns patients the pathophysiological increase in muscle protein breakdown leads to an increase in

intracellular amino acid availability. The increase in protein synthesis rates observed in burns patients has been attributed to this increased intracellular amino acid availability (279), and intracellular amino acids can activate mTOR independently of the IRS/PI3K/AKT pathway (280, 281). In light of studies that have directly measured an increase in muscle protein breakdown in critically ill patients (131, 140), the resulting increase in intracellular amino acid availability would explain the observed (insulin independent) increase in mTOR/EIF4EBP1/RSP6KB1 mRNA expression at ICU admission in spite of concomitant IRS-1 downregulation, and presumably, increased insulin resistance. Moreover, the observation of sustained mRNA expression of mTOR and RPS6KB1, and downregulated expression of EIF4EBP1 (a repressor of translation initiation) after seven days of ICU residency (**Figure 6.1**), agrees with studies that have directly measured increased muscle protein breakdown rates for at least seven days in critically ill patients (131, 140). However, these proteins are also regulated post-translationally in critically ill patients (282).

Microarray analysis has consistently identified enriched pathways in whole blood and peripheral blood mononuclear cells associated with the activation of the NF-κB transcription factor during critical illness (266). Furthermore, inflammatory pathways in skeletal muscle are enriched seven days and six months after ICU admission in patients presenting with ICUAW (263). Given NF-κB signalling in skeletal muscle is a primary driver of muscle loss in rodents (129, 283) and NF-κB mediated transcription is upregulated concomitantly with muscle atrophy during immobilisation in humans (**Chapter 5**) the current study targeted mRNA expression associated with NF-κB signalling to address the paucity of data related to NF-κB signalling in skeletal muscle during critical illness. Our observation that 15 of 21 (6 upstream (**Figure 6.4**) and 9 downstream (**Figure 6.5**)) genes associated with NF-κB signalling were upregulated at ICU admission compared to control patients demonstrates a robust and coordinated activation of NF-κB signalling by critical illness. After seven days of ICU residency, NF-κB associated mRNA expression either remained upregulated (9 genes), was downregulated (4 genes) or was upregulated further (2 genes). IL18 and TNFRSF12A, which were further upregulated, clustered with CYR61, CAPN2, ANKRD1 and NFKB2 (**Figure 6.7**) which were contained within the enriched ‘Extracellular structure organisation’ and ‘Anatomical structure formation involved

in morphogenesis' process (**Table 6.4**). TNFRSF12A encodes fibroblast growth factor-inducible 14 (Fn14) which is the receptor of tumor necrosis factor-like weak inducer of apoptosis (TWEAK). TWEAK/Fn14 is engaged in response to tissue injury and disease, and is considered to impair skeletal muscle regeneration, promote muscle atrophy and promote muscle fibrosis (214). Consistent with this, Fn14 expression can be induced by transforming growth factor beta (TGF-β) (214), and TGF-β2 was transcriptionally upregulated between ICU admission and day 7 (**Figure 6.3F**). Although less is known about the direct effects of the TGF-β2 isoform, TGF-β1, which binds to the same SMAD 2/3 receptors as TGF-β2, is a potent pro-fibrotic cytokine and has been cited as a potential candidate for causing impaired functional outcomes in patients suffering from myopathies (284). This would agree with the high incidence rate of myofiber necrosis in critically ill patients after seven days of ICU residency (131), and a preliminary suggestion of increased fibrosis inferred from ultrasonography in ICU patients (285). Indeed, we identified perturbations in ontological annotations associated with collagen turnover (**Table 6.3**). Given fibrosis is associated with impaired skeletal muscle regeneration, NF-κB/TGF-β/Fn14 is a candidate signalling pathway which may contribute to the development of ICUAW.

We identified no effect of 7 days of FESCE on the expression of genes involved in skeletal muscle protein turnover and inflammation relative to patients that received routine standard care. This is consistent with recently published RNA sequencing data that demonstrated ten hours of daily passive mechanical loading for an average of 9 days, reversed the expression of only 46 of 6257 genes identified to be differentially expressed in critically ill patients compared to controls (262). Furthermore, seven days of NMES (without cycle ergometry) has been reported to not affect the mRNA expression of MAFbx, MuRF1 or RPS6K1B in critically ill patients, despite preventing muscle atrophy (142). In contrast, NMES in healthy volunteers has been shown to downregulate the mRNA expression of FOXO1 and MuRF1 in older individuals (286) and MAFbx expression in immobilised muscle of young individuals (68). Taken together, these data suggest that the transcriptional response to the critically ill condition could be overwhelming that which might be affected by 7 days of FESCE. If this were true, the effect of continued FESCE on gene expression is more likely to be

seen at the point at which the critically ill condition subsides and the patient is discharged from ICU, and this warrants investigation.

Six months after ICU admission, pathways associated with FOXO transcription and the ubiquitin-proteasome system were downregulated (**Table 6.5**), likely as a consequence of improved insulin sensitivity (257). Interestingly, relative to control patients, the FOXO regulated genes MuRF1 and MAFbx were downregulated at six months after ICU admission in patients that received SC but not FESCE. Whether this represents an adaptive response to maintain muscle mass or a maladaptive response that may impair the normal turnover and remodelling of skeletal muscle in patients that received SC is not clear, as no direct measure of muscle protein turnover has been made in patients after ICU admission. Furthermore, six months after ICU admission TGF-β2 (**Figure 6.3F**) and myostatin (**Figure 6.2I**) mRNA expression remained upregulated but only in patients that received FESCE. It is possible that this represents a protracted fibrotic response and maladaptation to exercise performed during critical illness given myostatin and TGF-β2 are both associated with skeletal muscle fibrosis (284, 287). This would align with HIF1A (**Figure 6.4F**) and VEGFA (**Figure 6.4L**) mRNA expression, which was also increased only in FESCE patients six months after ICU admission. HIF1A and VEGA are elevated in fibrotic tissue (288, 289) and regulated by myostatin (287). Aberrant collagen deposition has been reported in patients presenting with ICUAW 6 months after ICU admission and gene clusters associated with extracellular matrix remodelling identified using a microarray approach inversely correlate with muscle strength in these patients (263). Our gene ontology approach identified upregulated annotations associated with collagen deposition after six months (**Table 6.5**)**,** which interestingly was markedly driven by an increase in expression in the FESCE condition only (data not shown). These quantitative gene expression data are inconsistent with pilot data demonstrating a numerical improvement in some functional outcomes (i.e., earlier return to functional independence) in patients that received FESCE (*n*=8) compared to SC (*n*=8) (271). However, these patients were only monitored for up to 30 days after ICU admission, so the longer-term effects on functional outcomes are unclear. Indeed, our well powered (SC *n*=40, FESCE *n*=36) SF-36 response data performed six months after hospital admission demonstrates a consistent pattern of less favourable outcomes in patients that received FESCE compared

with patients that received SC. Collectively, these data illustrate the need for further direct investigations of the effect of FESCE on muscle remodelling and fibrosis after ICU admission.

Plasma myostatin concentrations at ICU admission have also been positively correlated with survival in ICU patients, although it was undetermined if skeletal muscle was the source of plasma myostatin (290). Consistent with this, whilst we saw no association between myostatin mRNA expression at ICU admission and subsequent survival, PIK3R1 mRNA, which inhibits myostatin, tended to be lower in survivors. Furthermore, MTOR mRNA expression, which is inhibited by myostatin, also tended to be lower in survivors. However, the increase in myostatin mRNA expression in patients that received FESCE did not associate with improved survival in this patient group. Indeed, survival was numerically lower in patients that received FESCE (6 out of 15) compared to SC (11 out of 15) (**Table 6.1**), and as discussed SF-36 outcomes were less favourable in this participant cohort. Worthy of consideration, however, is the relatively small number of samples (SC *n*=7, FESCE *n*=4) included in the analysis 6 months after ICU admission, due to patient survival and tissue availability.

In conclusion, the current study provides a quantitative examination of skeletal muscle mRNA expression associated with candidate remodelling and inflammatory pathways in combination with a novel FESCE exercise intervention during ICU residency. This has demonstrated for the first time that FESCE performed during critical illness does not affect skeletal muscle mRNA expression associated with muscle protein turnover and inflammation during intensive care residency. The current study also demonstrates that FESCE performed during ICU residency upregulated mRNA expression of genes associated with skeletal muscle fibrosis six months after ICU admission, which was not evident in patients that received standard care. This is associated with less favourable health outcomes in FESCE patients. Based on these findings, further direct evidence of skeletal muscle extracellular matrix remodelling is required to determine the long-term effect of FESCE on skeletal muscle structure and functional outcomes in survivors of critical illness.

Chapter 7

A combined protein-polyphenol nutrition intervention does not accelerate early gains in muscle function or increase skeletal muscle hypertrophy after 30 sessions of resistance exercise training.

7.1 Introduction

Isokinetic and isotonic resistance exercise training comprised of concentric and eccentric loading phases results in skeletal muscle hypertrophy and an increase in muscle function (55, 106, 291–293). Skeletal muscle hypertrophy is considered to be underpinned by the accumulation of transient increases in the rate of myofibrillar protein synthesis in response to each resistance exercise session. Unaccustomed resistance exercise performed at the onset of training can cause myofibrillar injury (42, 51) and an acute decline in muscle function (51). Accordingly, it has been hypothesised that the increase in post-exercise myofibrillar protein synthesis rates at the onset of training is directed at repairing myofibrillar injury which may delay hypertrophic adaptations and persist for up to the first three weeks of training (51). Whist exercise-induced oedematous swelling may confound measurements of hypertrophy for at least three weeks of resistance exercise training (52), adaptations in training volume and muscle function can be easily measured at a high resolution to provide a time course of how functional adaptations to resistance exercise training occur. Nonetheless, studies typically only report pre- to post-training changes in muscle function, so the temporal increase in muscle function at the onset of training is poorly characterised.

In **Chapter 3** it was demonstrated that a whey protein and polyphenol drink ingested post-exercise and a casein protein and polyphenol drink ingested before bed markedly attenuated the decline in isometric torque and isokinetic work after a single bout of injurious eccentric contractions (**Figure 3.3**). This facilitated a greater daily volume of concentric isokinetic work to be performed during the following 7-day post-eccentric contraction period. Indeed, training volume is one of the primary factors that determine strength and hypertrophic gains after a period of resistance exercise training (106, 294). Accordingly, if myofibrillar injury delays the adaptation to resistance exercise training, it could be reasoned a protein-polyphenol nutrition intervention would accelerate gains in training volume and muscle function during the early (1-3 weeks) resistance training period. Post-training gains in muscle function and skeletal muscle hypertrophy might then be greater as a consequence.

Supplemental whey protein ingested post-exercise and casein protein ingested before bed have been shown to potentiate gains in muscle function and hypertrophy when consumed in combination with resistance exercise training (55, 97, 98, 295). Traditionally this is believed to result from the accumulation of greater increases in protein synthesis rates after each training session. In **Chapters 3** and **4** it was demonstrated that protein-polyphenol ingestion did not increase daily (**Figure 3.6C** & **D**), post-exercise (**Figure 4.3C**) or overnight (**Figure 4.3D**) myofibrillar protein synthesis rates after eccentric contractions. This was likely because a greater magnitude of injury in the placebo condition increased myofibrillar protein synthesis rates to a similar extent as protein-polyphenol ingestion. Once habituated to resistance exercise after ten weeks of resistance exercise training, post-exercise myofibrillar injury is markedly attenuated (51). Therefore, it is of interest to determine if protein-polyphenol ingestion increases post-exercise myofibrillar protein synthesis rates after ten weeks of resistance exercise training, and to what extent this explains skeletal muscle hypertrophy. Indeed, without a dietary protein intervention, the increase in post-exercise myofibrillar protein synthesis rates assessed over a 48 h free-living period after ten weeks of training positively correlates with the post-training increase in quadriceps volume (51). However, it remains to be determined if this is also the case when resistance exercise training is combined with protein ingestion.

The aim of the current study, therefore, was to perform a tightly controlled, 30 session (~10 weeks) unilateral leg extension resistance exercise training study in untrained individuals. Muscle function was measured every three sessions in the trained and contralateral control leg to provide a high-resolution time course of early (sessions 1-10 corresponding to week ~1 to 3) and late (sessions 10-30 corresponding to week ~3 to 10) changes in training volume and muscle function in response to resistance exercise training. A post-exercise and pre-bed protein polyphenol nutrition approach was then used to determine if this accelerated the early increase in training volume and muscle function. After 30 training sessions, muscle volume was measured concomitant with myofibrillar protein synthesis rates assessed using deuterated water during a 48 h post-exercise period. It was hypothesised that protein-polyphenol ingestion would accelerate the increase in training volume and muscle function during the early training period. Furthermore,

it was hypothesised that this would lead to a greater increase in quadriceps muscle volume, and this would be associated with greater post-exercise myofibrillar protein synthesis rates after 30 resistance exercise training sessions.

7.2 Methods

Participants

Twenty-nine young, healthy males (*n*=14) and females (*n*=15) (age: 24±1 y, BMI: 23.1±0.6 $kg \cdot m^{-2}$) volunteered to participate in the present study. Participants' characteristics are displayed in **Table 7.1**. The study was approved by the Sport and Health Sciences ethics committee of the University of Exeter (Ref No: 171206/B/09). The study was registered at ClinicalTrials.Gov (ID: NCT03918395).

Table 7.1. Participant characteristics.

	PLA		PP	
	All participants (*n*=14)	Biopsy subgroup (*n*=9)	All participants (*n*=15)	Biopsy subgroup (*n*=9)
Sex	M=7, F=7	M=5, F=4	M=7, F=8	M=5, F=4
Age (y)	25±2	24±2	24±1	25±2
Height (cm)	168.5±2.9	170.5±3.2	170.3±2.8	169.9±3.9
BMI ($kg \cdot m^{-2}$)	23.9±1.0	23.4±1.0	22.0±0.7	21.8±0.9
Body mass pre training (kg)	67.3±2.5	67.7±3.0	64.1±3.4	63.4±5.0
Body mass post training (kg)	67.9±2.4	68.6±2.8	65.0±3.3	64.1±4.7
Time between training sessions (days)	2.4±0.1	2.4±0.1	2.4±0.1	2.5±0.1
Supplement adherence (%)	98±1	99±1	99±1	99±1

Values represent mean ± SEM. Subgroup represents participant who underwent muscle biopsy sampling. BMI, body mass index.

Study protocol

Details of all experimental and analytical procedures are presented in **Chapter 2**. An overview of the experimental protocol is shown in **Figure 7.1**. Participants were randomly assigned to one of two parallel nutritional intervention groups consisting of either daily post-exercise and pre-bed protein-polyphenol ('PP') or

placebo ('PLA') drinks (post-exercise drinks were consumed at breakfast on non-training days; see **Chapter 3.2** – **Experimental drinks**) and completed a 30-session unilateral resistance exercise training protocol over a ~ ten week period. Experimental drinks were ingested every day, beginning after the first training session and concluding after the post-training measure of myofibrillar protein synthesis. Prior to the commencement of training, a bilateral MRI scan of the thighs was performed, and muscle function was measured under overnight fasted conditions. A minimum of 48 h later, participants began the 30 session resistance exercise training period with the aim of completing all 30 sessions within ten weeks. A second post-training bilateral MRI scan and muscle function measurement was performed 48 h after the 30^{th} training session under overnight fasted conditions. A further 48 h after this, a subgroup of participants underwent a deuterated water dosing protocol with muscle biopsy sampling and performed a final (31^{st}) training session to determine free-living daily myofibrillar protein synthesis rates of the trained and control leg during a 48 h post-exercise period in combination with a full dietary control (1.2g·Kg body mass·day^{-1} protein). To determine the temporal response to resistance exercise training, muscle function was measured in both trained and control legs prior to every third training session. Habitual dietary intake was assessed using three day dietary diaries completed pre- and post-training, in addition to after every six training sessions. Participants completed two separate familiarisation visits to become familiarised with all exercise procedures prior to participation.

Resistance exercise training

Supervised resistance exercise training was performed unilaterally on an isokinetic dynamometer. Each training session consisted of 5 sets of 30 isokinetic knee extensor contractions. Each set alternated between concentric and eccentric contractions, always starting with concentric contractions so 90 concentric and 60 eccentric repetitions were performed in total. Each contraction was performed across an 80^{o} range of motion equidistant from maximal voluntary knee extension and flexion. Concentric contractions were performed at 75^{o}·$sec^{-1,}$ and eccentric contractions were performed at 60^{o}·sec^{-1}. Participants were instructed to perform each repetition maximally and were provided with verbal encouragement throughout. Each set was separated by 2 minutes of rest. Exercise was performed in one leg only ('trained') whilst the other leg acted as

an internal untrained control (‘control’). The trained leg was counterbalanced for leg dominance.

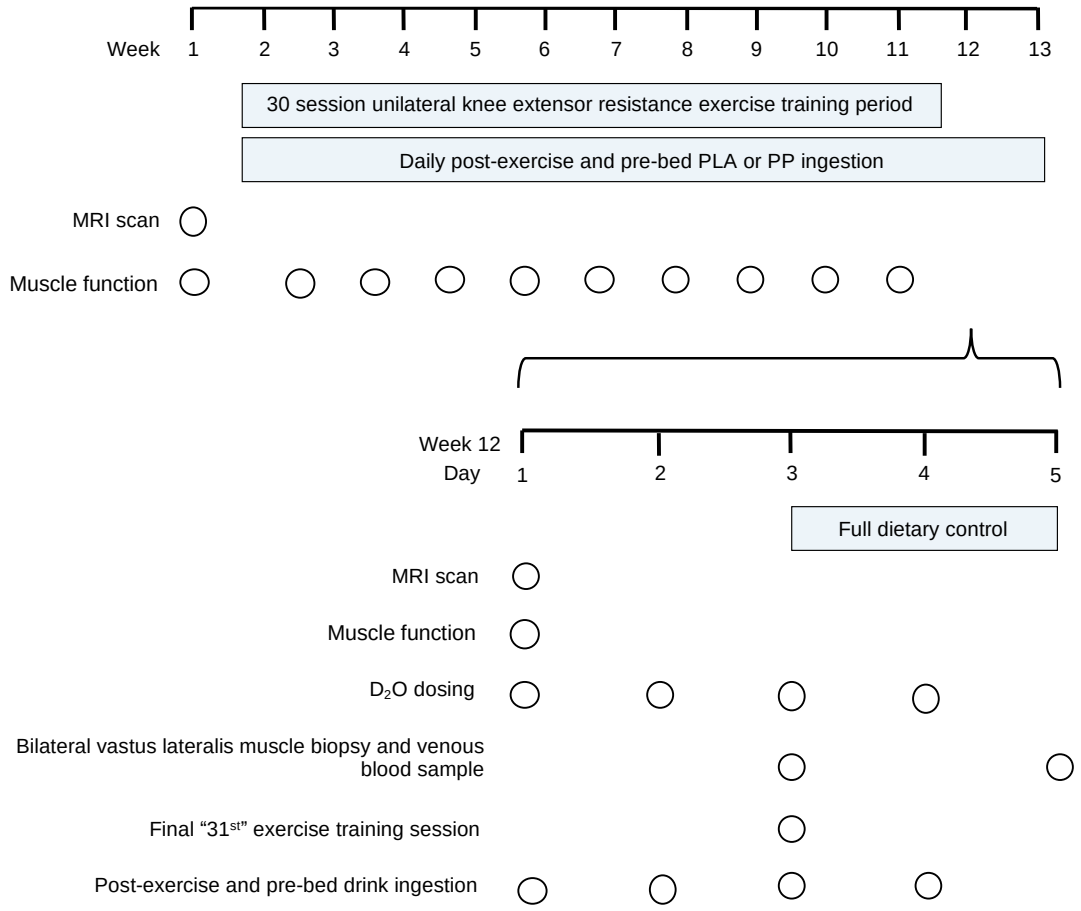

Figure 7.1. Schematic representation of the experimental protocol. Participants completed 30 unliteral knee extensor resistance exercise training sessions in combination with either daily post-exercise and pre-bed protein-polyphenol (PP; *n*=15) or isocaloric maltodextrin placebo (PLA; *n*=14) drinks. MRI scans were performed pre- and post-training. Muscle function was measured in both legs pre- and post-training and every three training sessions during the training period. A subgroup of participants (PLA *n*=9, PP *n*=9) consumed 70% D_2O at the start of week 12 and myofibrillar protein synthesis rates were measured in the trained and control leg during a 48 h post-exercise period after a 31st training session in combination with full dietary control.

Muscle function

Knee extensor isometric torque, isokinetic torque and isokinetic work was measured on an isokinetic dynamometer in both the control and trained leg. Pre- and post-training muscle function was measured after an overnight fast and after

a minimum 48 h abstinence from exercise. Additional muscle function measurements were performed prior to every third training session.

Muscle biopsy sampling

Fourty-eight hours after the post-training MRI scan and strength assessment, a subgroup of participants arrived at the laboratory overnight fasted and a bilateral muscle biopsy of the vastus lateralis and a ten mL venous blood sample from the antecubital vein was taken. Next, participants performed a final (31^{st}) training session in the trained leg whilst the control leg rested followed post-exercise drink ingestion depending on condition randomisation. For the subsequent 48 h period, participants were provided with a full dietary control (1.2g·Kg body mass·day^{-1} protein) and continued to ingest daily post-exercise and pre-bed drinks and refrain from structured exercise. At the end of this period, participants returned to the laboratory overnight fasted for a final bilateral muscle biopsy of the vastus lateralis and ten mL venous blood sample. Blood and muscle samples were frozen at -80^{o}C and analysed at a later date for body water ^{2}H enrichment and myofibrillar protein-bound [^{2}H]alanine enrichment, respectively.

Statistics

Full statistical procedures and calculations are presented in **Chapter 2**. Differences in subject characteristics and dietary intake between conditions were analysed using unpaired t-tests. Muscle function and volume was corrected to the control leg prior to analysis. The average session-by-session increase in training volume and muscle function during early and late periods was compared using a t-test. A repeated-measure two-factor analysis of variance (ANOVA) was used to analyse (condition x time) differences in early and late training volume, early and late muscle function, body water ^{2}H enrichment, and skeletal muscle volumes, and (condition x leg) differences in myofibrillar protein synthesis rates. Repeated-measure three-factor AVOVAs were used to analyse (time x condition x leg) differences in myofibrillar protein-bound [^{2}H]alanine mole per cent excess (MPE). When a significant main and interaction effect was observed, a Sidak post hoc test was performed to locate individual differences. Statistical significance was set at $P<0.05$. Calculations were performed using GraphPad Prism 8.3.0. All data are expressed as means±SEM.

7.3 Results

Participant characteristics and diet

No differences in age, weight, height or BMI were detected between conditions (*P*>0.05; **Table 7.1**). Body mass increased between pre- (66.2±2.1 kg) and post-training (66.9±2.0 kg) (*P*=0.046) and to a similar extent between PLA and PP (*P*=0.901). Inclusive of condition, relative carbohydrate and fat intake during the resistance exercise training period (training diet) were not different between conditions (*P*>0.05) (**Table 7.2**) whereas relative protein intake during the resistance exercise training period was higher in the PP compared to PLA (*P*<0.001) (**Table 7.2**). During the controlled diet period, relative carbohydrate intake was higher in PLA compared to PP (*P*<0.001), whereas relative protein intake was higher in PP compared to PLA (*P*<0.001) (**Table 7.2**).

Training volume

During the early training period, total training volume increased from session 1 (12623±738 J) to session 10 (14865±827 J) (*P*<0.001; **Figure 7.2A**) which was comprised of an increase in concentric work (from 5853±369 to 6360±342 J; *P*=0.005; **Figure 7.2D**) and eccentric work (from 6770±413 to 8504±543 J*;* *P*<0.001; **Figure 7.2G**). During the late training period total training volume increased from session 10 to session 30 (to 15636±827 J; *P*=0.024; **Figure 7.2B**) which was comprised of an increase in concentric work (to 6611±352 J; *P*=0.039; **Figure 7.2E**) whereas eccentric work did not change (*P*=0.119; **Figure 7.2H**). The increase in training volume was similar between PLA and PP (*P*>0.05).

The increase in total work (*P*<0.001; **Figure 7.2B**), concentric work (*P*=0.005; **Figure 7.2D**) and eccentric work (*P*=0.002; **Figure 7.2F**) performed was greater during the early compared to the late training period, and this was similar between PLA and PP (*P*>0.05).

Muscle function

During the early training period, isometric torque increased from pre-training (98.9±2.1%con) to session 10 (106.4±2.9%con) (*P*=0.016; **Figure 7.3A**) and the increase was similar between PLA and PP (*P*=0.176). The early increase in isokinetic work interacted between conditions (*P*=0.045) with post-hoc testing

revealing that relative to pre training, isokinetic work did not increase in PLA, but tended to increase after session 10 in PP (*P*=0.090; **Figure 7.3G**).

Table 7.2. Average dietary intake during training and controlled periods. All values are inclusive of the post-exercise and pre-bed maltodextrin placebo (PLA) or protein-polyphenol (PP) drinks.

	PLA		PP	
	Training diet	**Controlled diet**	**Training diet**	**Controlled diet**
Energy (KJ)	9466±603	11263±350	8678±583	10123±381
Protein ($g \cdot day^{-1}$)	90±9	84±2	111±6	113±4
Protein ($g \cdot kgBM \cdot day^{-1}$)	1.34±0.13 [a]	1.25±0.02 [b]	1.75±0.10 [a]	1.77±0.03 [b]
Protein (%En)	16±1 [a]	13±1 [b]	22±1 [a]	19±1 [b]
Carbohydrate ($g \cdot day^{-1}$)	273±19	352±14	232±16	291±12
Carbohydrate (%En)	49±2	52±1 [a]	45±1	48±1 [a]
Fat ($g \cdot day^{-1}$)	85±6	96±3	76±7	81±4
Fat (%En)	33±1	32±1	32±1	30±1

Training diet represents the mean habitual dietary intake recorded via a three day diet diary completed pre-and post-training and after sessions 7, 13, 19 and 25. Controlled diet represents the mean dietary intake during the 48 h controlled diet (accounting for adherence) period provided to a subgroup of participants that underwent muscle biopsy sampling. En = energy. Comparisons in relative macronutrient intake were made using t-tests. Values presented in the same row with the same superscript letter are different (*P*<0.05). Values represent mean ± SEM.

During the late training period isokinetic work increased from session 10 (105.8±2.0%con) to post-training (112.2±3.2%con) (*P*<0.001; **Figure 7.3H**) and the increase was similar between PLA and PP (*P*=0.634). Isometric torque (*P*=0.547; **Figure 7.3B**) and isokinetic torque (*P*=0.381; **Figure 7.3E**) did not increase during the late period.

The increase in isometric torque (*P*=0.078; **Figure 7.3C**), isokinetic torque (*P*=0.203; **Figure 7.3F**) and isokinetic work (*P*=0.785; **Figure 7.3I**) was not different between the early and late training periods.

Skeletal muscle volume

Thigh muscle volume increased between pre-training (98.9±0.6%con) and post-training (102.6±0.8%con) (*P*<0.001; **Figure 7.4A**). The increase in thigh muscle volume was similar between PLA (3.3±0.8%con) and PP (4.1±1.0%con) (*P*=0.700). Quadriceps muscle volume increased between pre-training (99.1±0.8%con) and post-training (104.3±1.3%con) (*P*=0.001; **Figure 7.4B**). The increase in quadriceps muscle volume was similar between PLA (4.8±1.2%con) and PP (5.6±1.5%con) (*P*=0.781).

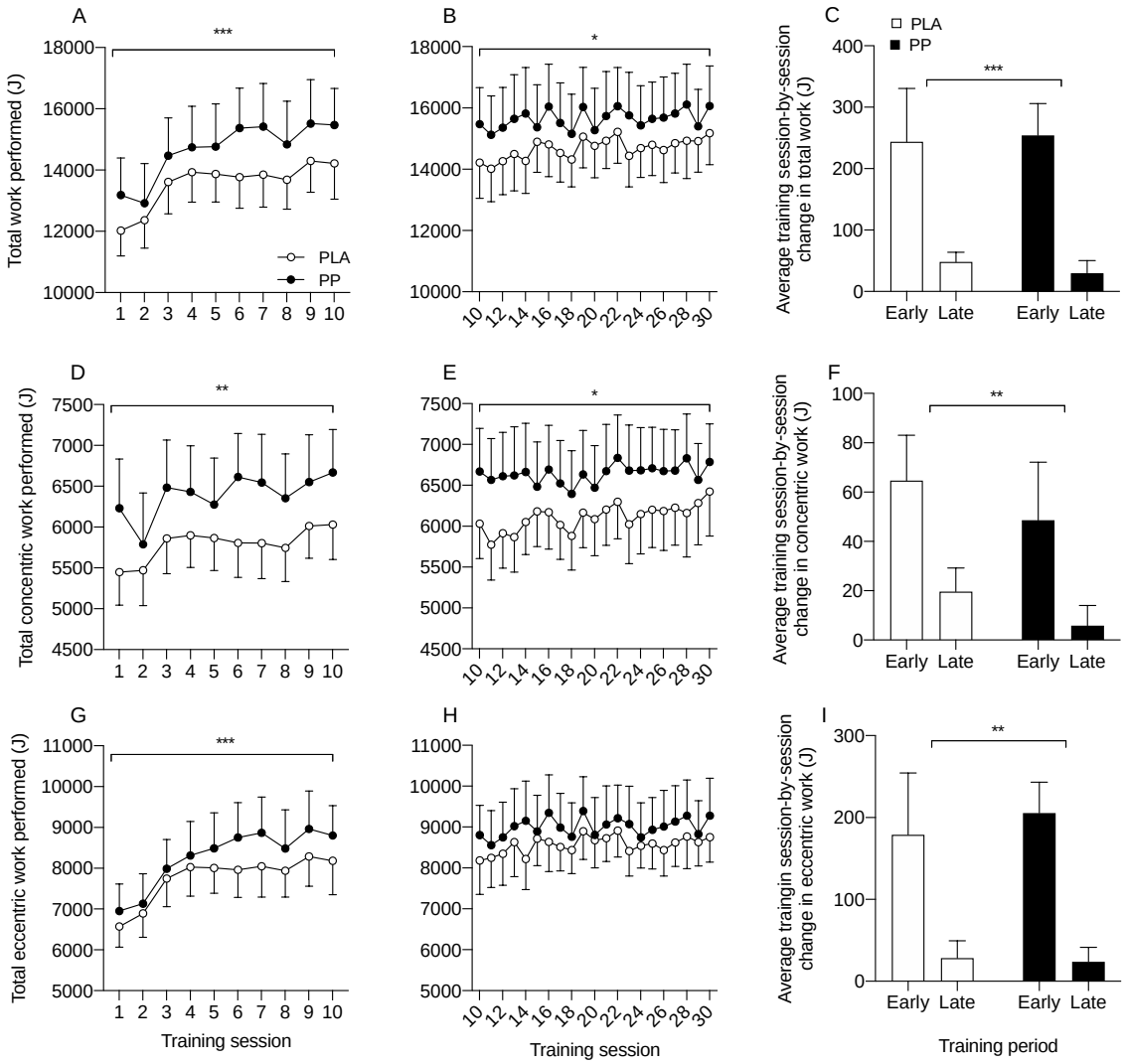

Figure 7.2. Total (**A**-**C**), concentric (**D**-**F**) and eccentric (**G**-**I**) training volume during early (sessions one to ten; left panels) and late (session ten to 30; central panels) training periods. The right panels present a comparison between early and late periods. Post-exercise and pre-bed protein-polyphenol (PP; *n*=15; black circles/bars) or isocaloric maltodextrin placebo (PLA; *n*=14; white circles/bar) drinks were ingested daily throughout the training period. Data are presented as means with error bars representing standard error. Statistical analysis was performed with separate two-factor ANOVAs. * change over time (main effect). One symbol *P*<0.05, two symbols *P*<0.01, three symbols *P*<0.001, four symbols *P*<0.0001.

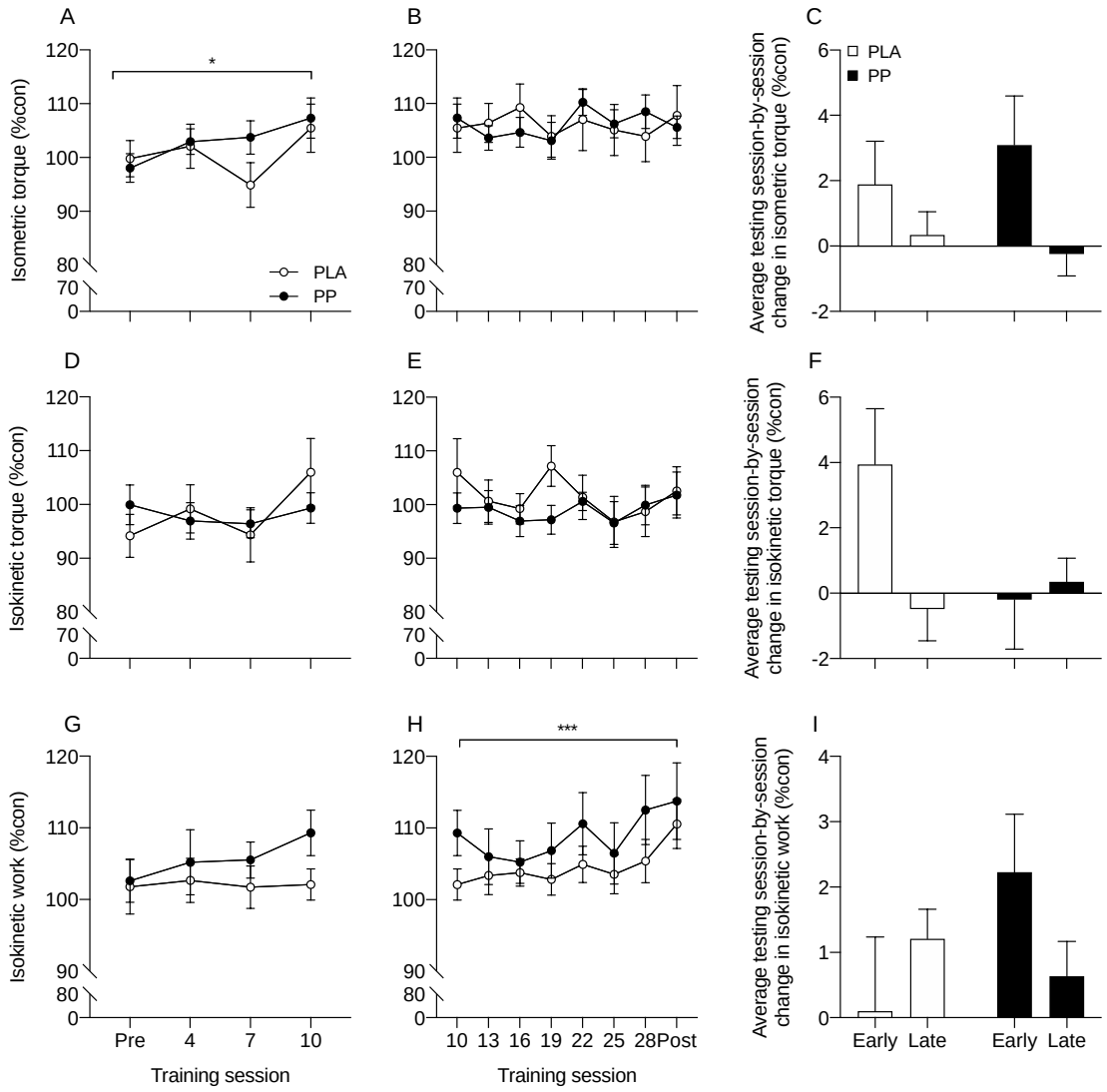

Figure 7.3. Isometric torque (**A**-**C**), isokinetic toque (**D**-**F**) and isokinetic work (**G**-**I**) performed in each testing session during early (pre-training-session ten; left panels) and late (session ten-post-training; central panels) training periods. The right panels present a comparison between early and late periods. Post-exercise and pre-bed protein-polyphenol (PP; *n*=15; black circles/bars) or isocaloric maltodextrin placebo (PLA; *n*=14; white circles/bars) drinks were ingested daily throughout the training period. Data are presented as means with error bars representing standard error. Statistical analysis was performed with separate two-factor ANOVAs. * change over time (main effect). One symbol *P*<0.05, two symbols *P*<0.01.

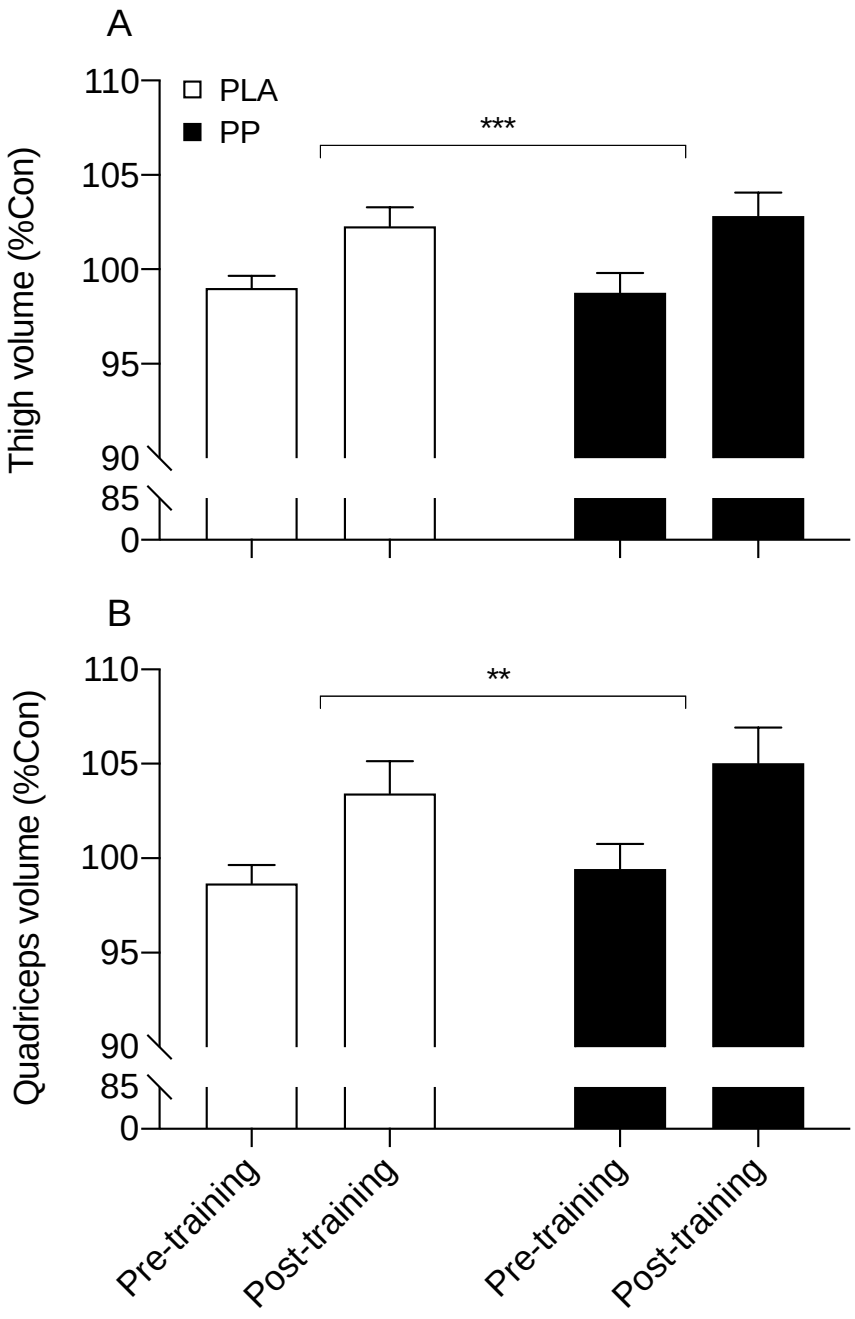

Figure 7.4. Whole thigh (**A**) and quadriceps (**B**) muscle volume measured using MRI pre- and post-training. Post-exercise and pre-bed protein-polyphenol (PP; *n*=15; black bars) or isocaloric maltodextrin placebo (PLA; *n*=14; white bars) drinks were ingested daily throughout the training period. Data are expressed as a % from the contralateral untrained leg and are presented as means with error bars representing standard error. Statistical analysis was performed with separate two-factor ANOVAs. * difference between pre- and post-training (main effect). Two symbols *P*<0.01, three symbols *P*<0.001.

Body water deuterium enrichment

Body water deuterium enrichment averaged 0.68±0.01% between days 3 and 5 in week 12 and did not change over this time (*P*=0.234) and was similar between PLA (0.70±0.02%) and PP (0.66±0.01%) (*P*=0.301; **Figure 7.5A**).

Myofibrillar protein-bound [^{2}H]alanine enrichment and myofibrillar protein synthesis rates

Myofibrillar protein-bound [^{2}H] enrichments tended to increase more in the trained (Δ0.1259±0.0174 MPE) compared to the control (Δ0.0782±0.0162 MPE) leg (*P*=0.023) (**Figure 7.5B**). The increase in MPE was similar between PLA and PP (*P*=0.354).

Average daily post-exercise myofibrillar protein synthesis rates during days three to five in week 12 tended to be higher in the trained (2.53±0.35%·d^{-1}) compared to the control (1.54±0.32%·d^{-1}) leg (*P*=0.075; **Figure 7.5C**). Average daily post-exercise myofibrillar protein synthesis rates were similar between PLA and PP (*P*=0.462, interaction; *P*=0.188; **Figure 7.5C** & **D**).

The difference in myofibrillar FSR between the control and trained leg did not correlate with the difference in quadriceps volume between the control and trained leg in PLA (R^2=0.001, *P*=0.942) or PP (R^2=0.015, *P*=0.750) conditions.

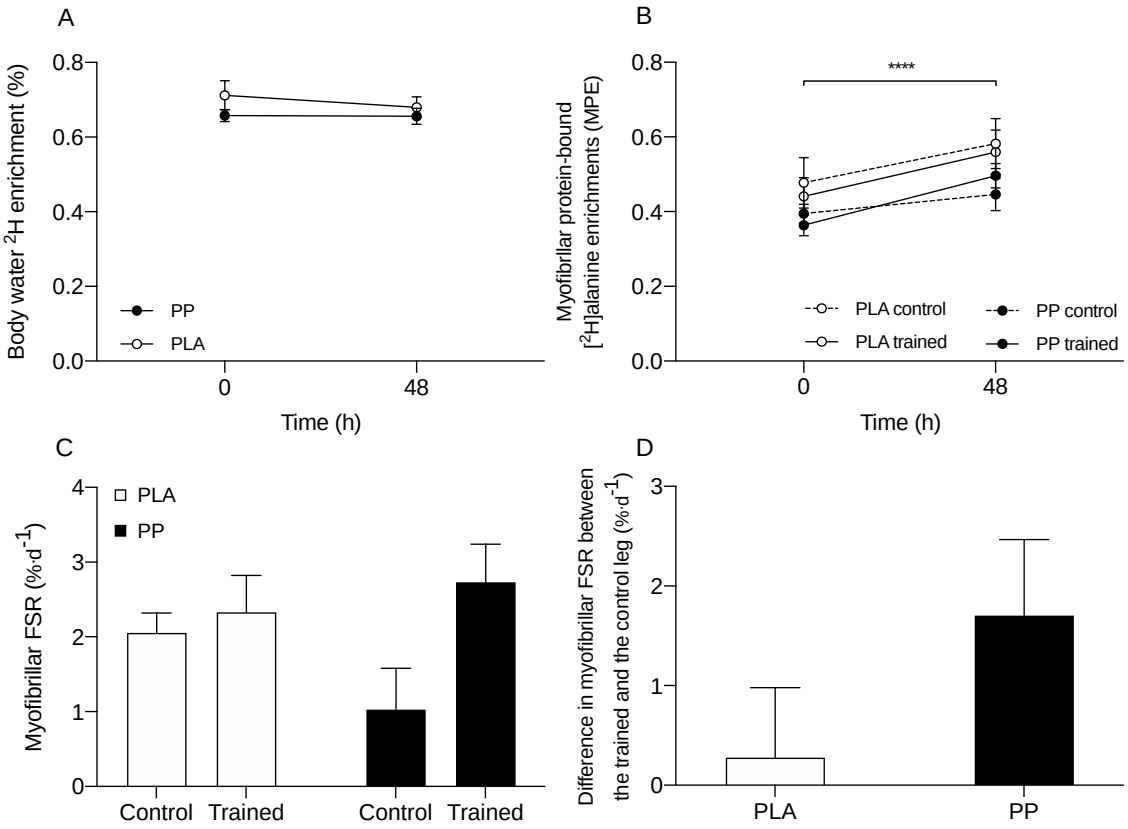

Figure 7.5. Body water ^{2}H enrichment (%) measured in blood plasma (**A**), myofibrillar bound [^{2}H]alanine enrichment (**B**), myofibrillar fractional synthetic rates (FSR) (**C**), and the difference in FSR between control and trained legs (**D**) periods. Post-exercise and pre-bed protein-polyphenol (PP; *n*=9; black bars) or isocaloric maltodextrin placebo (PLA; *n*=9; white bars) drinks were ingested daily throughout the training period. Data are presented as means with error bars representing standard error. Data were analysed with separate two-factor (**A**, **C**) and three-factor (**B**) ANOVAs, and unpaired t-tests (**D**). * Increase over time (main effect). Four symbols *P*<0.0001.

7.4 Discussion

It has been suggested that myofibrillar injury caused by unaccustomed resistance exercise at the onset (1-3 weeks) of a resistance exercise training programme delays hypertrophic adaptations due to the requirement to prioritise the remodelling of myofibrillar injury (51). However, there are currently no intervention studies that have investigated this. The current study demonstrates that a protein-polyphenol nutrition intervention which was shown in **Chapter 3** to attenuate the decline in isokinetic work after injurious eccentric contractions, potentiates the increase in isokinetic work during the first 10 resistance exercise training sessions relative to a carbohydrate placebo. However, this did not translate to greater gains in isokinetic work or skeletal muscle hypertrophy at the end of the 30-session resistance exercise training period.

It has been well established that long-term resistance-type exercise training leads to an increase in muscle function (106). However, typically only pre- to post-training changes in muscle function are reported, so the time course is less well understood. The current study used a unilateral isokinetic training paradigm with a standardised number of maximal concentric and eccentric repetitions in each training session. Consequently, session-by-session changes in work performed represent a functional adaptation. The increase in training volume during the early period (20%) was higher than during the late training period (6%), and the increase in early training volume was comprised of a numerically greater increase in eccentric work (29%) relative to a smaller increase in concentric work (11%) (placebo; **Figure 7.2**). This discrepancy between contraction modalities is likely explained by an early refinement in neuromuscular activity during eccentric contractions (296), which is both larger and more variable than during concentric contractions (297). Indeed, the increase in training volume between early and late training phases in response to isotonic leg resistance training is reported to be more linear. This is likely because less eccentric work is performed during these movements compared to the maximal eccentric sets performed currently (52). After correcting to the untrained contralateral leg, isometric torque increased by 6% during the early period, and this tended to be greater than the increase during the late period (placebo; **Figure 7.3**). This is in line with the 8-11% increase in isometric torque reported after six to ten weeks of isotonic resistance exercise

(52, 60). Isokinetic work increased during the late period only, and isokinetic torque didn't change (placebo; **Figure 7.3**) which is consistent with similar isokinetic resistance training studies which report no increase in isokinetic torque (298, 299). This would suggest that the late increase in isokinetic work occurs primarily by attenuating the decline in peak torque over consecutive repetitions rather than increasing peak torque *per se*.

The current study used a post-exercise and pre-bed protein-polyphenol nutrition approach based on data in **Chapter 3** demonstrating this attenuated the decline in isometric torque and isokinetic work after injurious eccentric contractions. This allowed significantly more isokinetic work to be performed during the subsequent seven days of recovery (**Figure 3.3B**). Furthermore, in **Chapter 3** the greatest decline in isometric torque and isokinetic work occurred 48 h after eccentric contractions, so the current study prescribed (on average) a training session every 48 h (**Table 7.1**). In the current chapter, protein-polyphenol ingestion also accelerated the increase in isokinetic work during the early phase of a resistance exercise training programme. Specifically, isokinetic work after 10 training sessions tended to increase by 7.3% in the protein-polyphenol condition (P=0.090) but was unchanged (-1.9%) in the placebo condition (P=0.998) (**Figure 7.3G**). Unaccustomed resistance exercise, especially when comprised of a substantial eccentric component as is the case in the current chapter, is accompanied by myofibrillar injury and a transient decline in contractile function. Consequently, the current findings extend upon the conclusions of **Chapter 3** demonstrating protein-polyphenol ingestion likely accelerates recovery after each subsequent bout of damaging exercise, thereby allowing a greater volume of work to be performed. This did not manifest as a more rapid increase in training volume, however, possibly as training volume is not corrected to the contralateral control leg, and therefore true increases in contractile function may be confounded by a larger and more variable familiarisation effect during the early training period.

Muscle volume was measured before and after 30 sessions (~10 weeks) of training as an increase in skeletal muscle CSA after ten weeks is considered to reflect true skeletal muscle hypertrophy. Muscle volume at the end of the early training period (i.e., 3 weeks) would likely be confounded by oedematous muscle

swelling (52). Relative to the contralateral control leg, there was an increase in quadriceps (4.7%) and whole thigh (3.3%) muscle volume (placebo; **Figure 7.4**). This is in line with the 3.4 to 6.0% increase in quadriceps volume, and CSA reported after 8-12 weeks of isotonic resistance exercise training (53, 301). Meta-analyses have demonstrated that protein supplementation which is associated with an increase in dietary protein intake from 1.4 to 1.8 g·kgBM·day^{-1} potentiates gains in fat-free mass and muscle CSA after >6 weeks of resistance exercise training in untrained individuals (97, 98). Muscle hypertrophy is potentiated by both post-exercise whey protein (56) and pre-bed casein protein (55) ingestion. In the current study, both post-exercise whey protein and pre-bed casein protein were provided in an attempt to maximally stimulate hypertrophic adaptations. This approach increased daily protein intake from 1.3 g·kgBM·day^{-1} in the placebo condition to 1.8 g·kgBM·day^{-1} in the protein-polyphenol condition (**Table 7.2**). Currently, the increase in quadriceps (5.6%) and whole thigh (4.2%) muscle volume in the protein-polyphenol condition was similar to the placebo condition. Whilst protein supplementation has been shown to increase lean mass after 6 weeks of resistance training (302), it may require 12 or more weeks of training to realise a greater hypertrophic effect of post-exercise whey protein or pre-bed casein protein supplementation at the individual muscle CSA level (53, 55, 303–305). Nevertheless, this is not a consistent observation in untrained individuals (306–308). Using an isokinetic training paradigm allowed us to prescribe a maximal intensity, a high-frequency training programme which matched total training load between conditions (30 sessions of 150 repetitions per session). Furthermore, to reduce the large variability in hypertrophic responses between individuals, a unilateral model was used (59). Despite what should be a potent stimulus to promote training adaptations and a high level of control, 10 weeks of training may be an inadequate duration to realise an additional benefit of protein supplementation at increasing muscle volume.

Protein ingestion results in a greater myofibrillar protein synthetic response after a single bout of resistance exercise which is believed to underpin greater rates of skeletal muscle hypertrophy when resistance exercise training is combined with supplementary protein ingestion (96, 97). Concomitant to assessing post-training muscle volume, the current study assessed integrative myofibrillar protein synthesis rates using deuterated water over a 48 h free-living period after a 31st

training session in a sub-group of participants. Consistent with the increase in quadriceps volume, post-exercise myofibrillar protein synthesis rates were 13% higher in the trained compared to the untrained leg, which tended towards significance (placebo; **Figure 7.5C**). Furthermore, consistent with the similar hypertrophic response between conditions, the increase in post-exercise myofibrillar protein synthesis rates were similar between conditions (**Figure 7.5C**). Worthy of note however is that the increase in myofibrillar protein synthesis rates with respect to the control leg was numerically larger in the protein-polyphenol (1.70%·d^{-1}) compared to the placebo (0.28%·d^{-1}) condition. Whilst this could reflect a larger anabolic response with protein-polyphenol ingestion, the large variability in responses to resistance exercise training makes this hard to identify, especially when habitual protein intake is sufficient (59).

The increase in myofibrillar protein synthesis rates did not correlate with the increase in quadriceps volume. This is in contrast to data by Damas and colleagues who reported a correlation between myofibrillar protein synthesis rates also assessed over a 48 h period using deuterated water and the increase in vastus lateralis CSA after 19 resistance exercise training sessions (10 weeks) (51). This discrepancy could be due to differences in the adaptation to training between studies given we prescribed 30 training sessions in a similar 10 week period. This illustrates the limitation of a ‘snapshot’ approach to measuring training adaptations at a single post-training time point. A time-course approach such as we have provided currently with respect to training volume and muscle function is needed to elucidate the temporal relationship between myofibrillar protein synthesis rates and hypertrophy with resistance exercise training.

In conclusion, daily post-exercise and pre-bed protein-polyphenol ingestion increased the gains in muscle function but not training volume during the early phase of a resistance exercise training period. After 30 sessions of training, the increase in muscle volume and post-exercise myofibrillar protein synthesis rates are not augmented by protein-polyphenol supplementation. These findings suggest that protein-polyphenol ingestion may accelerate recovery of muscle function during the early resistance exercise training period, but this is not an effective strategy to augment functional and hypertrophic adaptations after 30 sessions of resistance exercise training. s

Chapter 8

General discussion

Skeletal muscle mass and function are dynamically regulated in response to injury, illness and an increase (i.e., resistance exercise training) or decrease (i.e., immobilisation) in the demand for contractile work. The principle aim of this thesis was to investigate myofibrillar protein synthesis and inflammatory signalling in regulating muscle mass and function under these impositions. This was achieved using controlled time course experiments whereby changes in skeletal muscle function was determined alongside changes in free-living myofibrillar protein synthesis rates and transcriptional inflammatory signalling which were measured directly by sampling vastus lateralis muscle tissue in healthy and critically ill human volunteers.

8.1 The effects of myofibrillar protein synthesis rates on muscle function

The remodelling of skeletal muscle, particularly in response to injury and disuse is likely to be a continuous process that is ongoing 24 h per day for multiple days. The primary aim of this thesis was to measure myofibrillar protein synthesis rates over multiple days of free-living using deuterated water in order to provide novel insight into the regulation of muscle function by changes in myofibrillar protein synthesis rates during a period of muscle remodelling.

The aim of **Chapter 3** was to test the hypothesis that myofibrillar protein synthesis rates are associated with the recovery of muscle function after eccentric contractions. Consistent with evidence in mice (45), daily myofibrillar protein synthesis rates increased by 17% relative to the contralateral control leg 24-72 h after eccentric contractions (**Figure 3.6C**). This incorporation period began 24 h after eccentric contractions and included two additional bouts of maximal concentric exercise (at 24 and 48 h). Consequently, the fact that myofibrillar protein synthesis rates in the injured leg remained greater for at least three days in parallel with the largest decline in muscle function demonstrates that this increase is likely to be largely driven by injury and not contraction. Indeed, a novel finding from **Chapter 3** was that the individual increase in myofibrillar protein synthesis rates in the injured leg strongly tended (P=0.062) to positively correlate with the individual decline in isokinetic work during the 24-72 h post-eccentric contraction period (placebo; **Figure 3.7B**). This is intuitively satisfying given that a supply of new myofibrillar protein would surely be required to repair injured

myofibers. Nonetheless, whether myofibrillar protein synthesis rates determined the rate of muscle function recovery was unclear.

To answer this, a combined protein-polyphenol nutrition approach was used, which profoundly prevented the decline in isokinetic work 24h after eccentric contractions (**Figure 3.3 B**). This response exceeded the effectiveness of single-ingredient nutritional intervention studies using a similar eccentric contraction model. For example, in a similar fashion to **Chapter 3**, Draginidis and colleagues have shown that isometric torque is impaired for 120 h after 300 eccentric knee extensor contractions with the daily ingestion of a carbohydrate placebo. They then demonstrated that daily ingestion of 20 g of milk protein (80 g on the day of eccentric contractions) also resulted in a decline in isometric torque for 48 h, before recovering to baseline by 96 h (164). In **Chapters 3**, **4** and **7** a combined protein-polyphenol approach was used in an attempt to robustly accelerate the recovery of muscle function so the underpinning regulatorily mechanisms could clearly be delineated. It was not the aim of this thesis to establish the independent contribution of protein and polyphenols to this response. It does none the less generate interesting questions for future research to determine if protein and polyphenol co-ingestion act via an additive or synergistic mechanism to prevent the loss of muscle contractile function after eccentric contractions.

In **Chapters 3** and **4**, the decision to measure myofibrillar protein synthesis rates 24-72 h after eccentric contractions was in part based on the observation that muscle function typically plateaus between 24-72 h after 300 eccentric contractions (41, 47, 159, 164, 165). Indeed, this is in line with data presented in **Chapter 3** (**Figure 3.3A** and **B**), suggesting that the 24-72 h window could be crucial for myofibrillar remodelling and determine the subsequent rate of recovery of muscle function. Nonetheless, isokinetic work was not statistically different from baseline after 24 h in the protein-polyphenol condition, and isometric torque was markedly greater relative to the placebo condition also after 24h. This would suggest that the bulk of remodelling that contributed to the recovery of muscle function had been completed by 24 h. An outstanding question from **Chapters 3** and **4**, therefore, is did protein-polyphenol ingestion increase myofibrillar protein synthesis rates during the first 24 h after eccentric contractions, and to what

extent might this explain the attenuated decline in muscle function that was measured after 24 h?

To address this, myofibrillar protein synthesis rates can speculatively be calculated between -11 h (the beginning of deuterated water ingestion) and 24 h by assuming parity between baseline body water and myofibrillar protein-bound [^{2}H]alanine enrichment as all participants were deuterium naïve. This method has previously been used to publish baseline data sets using deuterated water (107), and is considered a valid method to calculate myofibrillar protein synthesis rates using intravenous stable isotope labelled amino acid infusions (309). This calculation yields a similar increase in myofibrillar protein synthesis rates between the injured and control leg in placebo (16%) and protein-polyphenol (10%) conditions. These calculations are based on the understanding that no participant had received oral or intravenous deuterium previously, and that body water enrichment kinetics were equal between participants as deuterated water was dosed relative to estimations of the size of the body water pool (see **Chapter 2**). Data published elsewhere during the preparation of this thesis also shows that a protein nutrition intervention attenuates the loss of isometric torque after unaccustomed resistance exercise, but does not increase myofibrillar protein synthesis rates when measured using deuterated water during the 0-96 h post-exercise period (210). Collectively, these data corroborate the conclusions made in **Chapters 3** and **4** that myofibrillar protein synthesis rates are unlikely to determine the rate of recovery of muscle function after eccentric contractions.

Myofibrillar injury was not directly measured in **Chapters 3**, **4**, **5** and **7**. **Chapters 3**, **4** and **5** used a 300 eccentric knee extensor contraction protocol which induced a decline in muscle function consistent with other studies using this protocol (41, 47, 159, 164, 165). Furthermore, this protocol has been shown to induce ultrastructural disruptions to sarcomere integrity, which is directly related to changes in muscle function (47). The aim of this thesis was to investigate the regulation of muscle function after eccentric contractions. Nonetheless, given protein-polyphenol ingestion recovers muscle function within the first 24 h, it would suggest that the mechanism of action by which this occurs may be related to the magnitude of the initial injury as opposed to the regulation of post-exercise events that occur in the days following. It has been shown that heat shock

proteins bind to cytoskeletal/myofibrillar proteins immediately after eccentric contractions, possibly to stabilise myofibrillar structures and attenuate the unfolding and subsequent degradation of damaged proteins (310). Recently, a role for both dietary protein and polyphenols in modulating heat shock protein expression predominantly in animal models has been discussed (311). There is currently no direct evidence for this in human skeletal muscle. Still, it is interesting for future research to investigate the regulation of heat shock proteins by nutrition after eccentric contractions.

A notable observation from **Chapter 4** was the under expression of MAFbx mRNA 24-36 h after eccentric contractions (mean log_2 fold change = -1.92±0.35; **Figure 4.7C**). MAFbx is an E3 ubiquitin ligase that is upregulated in skeletal muscle under atrophic conditions (24, 63, 65, 66). Indeed, in **Chapter 5** MAFbx mRNA expression was upregulated between 2-7 days of leg immobilisation (mean log_2 fold change = 0.94±0.25; **Figure 5.6C**) and in **Chapter 6** at ICU admission in critically ill patients (mean log_2 fold change = 1.41±0.27; **Figure 6.2C**). In congruence with the data presented in **Chapter 4,** downregulated MAFbx mRNA expression has been reported 2-24 h after resistance exercise (312–315). In vitro, MAFbx ubiquitinates the myogenic transcription factor MyoD and suppresses MyoD induced myoblast differentiation and inhibits myotube formation (316). In humans, the time course of increased MyoD mRNA expression and $MyoD^+$ cells aligns with downregulated MAFbx mRNA expression after exercise (73, 312, 313, 315). In **Chapters 3** and **4**, MYOD1 mRNA expression did not change (possibly because MYOD1 expression had returned to baseline within 24 h), but its target gene myogenin (MYOG) which is also ubiquitinated by MAFbx (317) was robustly upregulated 24 h after eccentric contractions. Indeed, 'Positive regulation of cell differentiation' was an enriched gene ontology annotation during the early remodelling period in **Chapter 3** (**Table 3.3**). Furthermore, the progressive downregulation in MYOG expression (**Figure 3.10A** and **4.7B**) was temporally coupled with the progressive upregulation of MAFbx (**Figure 4.7C**). The attenuated decline in muscle function with protein-polyphenol ingestion was not paralleled by a change in the magnitude or duration of expression of MAFbx or MYOG. In **Chapter 4**, although not passing the FDR correction, post-exercise PAX7 mRNA expression was higher in the protein-polyphenol (mean log_2 fold change = 0.63±0.17) compared to placebo (mean log_2

fold change = -0.14±0.14) condition. Whey protein has been shown to increase $PAX7^+$ satellite cells 2-fold 24 h following eccentric contractions. However, whey protein did not accelerate the recovery of muscle function (176). Furthermore, protein-polyphenol ingestion did not augment skeletal muscle hypertrophic adaptations to 30 sessions of resistance exercise training (**Chapter 7**; **Figure 7.4**), and it is suggested satellite cells play a key role in skeletal muscle hypertrophy (318). With the caveat in mind that changes in gene expression may not reflect changes in satellite cell status, it seems unlikely that protein-polyphenol ingestion attenuates the decline in muscle function after eccentric contractions by enhancing myogenic processes.

It has been calculated that only 25-50% of the loss of muscle volume after seven days of leg immobilisation can be explained by a decline in myofibrillar protein synthesis rates (63). Consequently, muscle protein breakdown could be an important contributor to muscle atrophy and the resulting decline in muscle function during short term disuse. An interesting observation from **Chapter 5** was the significant correlation showing that higher myofibrillar protein synthesis rates were associated with a greater decline in quadriceps volume when immobilisation is preceded by eccentric contractions (**Figure 5.9A**). This correlation might be explained by a compounding of muscle protein breakdown induced by eccentric contractions and by immobilisation such that the increase in free amino acid availability may start driving the increase in myofibrillar protein synthesis rates. The observation that prior eccentric contractions increased FOXO1 mRNA expression between 2 and 7 days of immobilisation could be used to support this argument, but this is not congruent with the mRNA expression of the E3 ubiquitin ligases MuRF1 and MAFbx downstream of FOXO1 which not increase further with prior eccentric contractions. However, it should be noted that a (absence of) change in the expression of genes does not necessarily represent dynamic changes in muscle protein breakdown or translate to changes in muscle mass (68, 103). Aside from ubiquitin-mediated protein degradation, the observation that calpain-3 mRNA expression showed a strong tendency not to be downregulated with prior eccentric contractions during the first two days of immobilisation could suggest that increased intracellular Ca^{2+} caused by eccentric contractions is activating calpain-mediated protein degradation to increase the early breakdown of muscle protein which did not occur in the control condition (255). This could

increase the availability of disrupted protein to be ubiquitinated and degraded at the 26S proteasome.

FOXO1 activation during immobilisation is likely to be regulated in part by insulin sensitive AKT signalling (258). The primary candidate mechanisms driving insulin resistance induced by immobilisation *per se* are a reduction in insulin stimulated glucose uptake via GLUT4 (319), and intramyocellular lipid (IMCL) accumulation possibly due to impaired insulin signalling and a decline in lipid oxidation (258), respectively. However, IMCL content or muscle oxidative capacity does not change after five days of leg immobilisation or seven days of bed rest despite elevated insulin resistance and skeletal muscle atrophy suggesting IMCL accumulation *per se* doesn't contribute to short-term immobilisation induced insulin resistance, but IMCL metabolites might (30, 31). In light of this, an interesting observation from **Chapter 5** is that the preponderance of genes which decreased in the control condition but didn't change in the injured condition between days 0 and two of immobilisation were associated with lipid metabolism and insulin signalling ($P<0.05$, $FDR>0.05$). Ontological analysis of these interacting genes identifies "metabolism of lipids' (*PLIN2*, *PTGS1*, *MCAD*, *HADHB*, *PNPLA2*, *PPARGC1A*, *ACAT1*, *CPT1B*; $P=0.002$) as the most enriched annotation. Also worthy of note was the downregulation in GLUT4 (*SLC2A4*) in the control but not injured condition. The downregulation in gene expression associated with lipid metabolism is consistent with reports elsewhere in response to short term disuse (63, 320). The maintenance of lipid metabolism in the injured condition could, therefore, reflect increased fatty acid uptake in response to contraction. Given that the leg is then immobilised this would result in the accumulation of lipid which would sustain higher rates of lipid metabolism for the first two days of immobilisation.

The process whereby muscle protein breakdown drives an increase in protein synthesis is described in burns patients where there is a large catabolism of skeletal muscle in order to provide amino acids to stimulate wound healing (145). Indeed, in **Chapter 6** a pattern of gene expression consistent with elevated muscle protein breakdown and elevated translation initiation was demonstrated in critically ill patients. Whilst muscle mass was not measured in this chapter, critical illness is associated with profound loss of muscle mass likely to be induced

by both immobilisation (i.e., bed rest) (321) and an increase in amino acid requirements to support the immune response (322). This catabolism of skeletal muscle contributes to impaired functional outcomes in burns patients, and intensive care unit acquired weakness in critically ill patients (143, 265). The breakdown of muscle protein to stimulate the repair of myofibrillar injury in healthy individuals has not yet been described in non-pathological states, and the scale of protein breakdown is expected to be considerably lower after exercise-induced injury relative to burns injury or critical illness. Nonetheless, it is plausible that the currently held belief that myofibrillar protein synthesis rates are increased after eccentric contractions as a direct response to injury is in fact misguided (51, 210), and rather the increase occurs secondary to an increase in muscle protein breakdown. This would consequently explain the impaired muscle function during the recovery from eccentric contractions, both during ambulation (**Chapter 3**) and immobilisation (**Chapter 5**). Whether muscle protein breakdown is intentionally upregulated to increase amino acid availability for the repair of injured tissue, or if increased amino acid availability is a by-product of the degradation of damaged and unfolded proteins induced by physical strain of eccentric contractions, or perhaps due to inflammatory events, is not clear. It is clear however that developing and applying stable isotope tracer methodologies to directly measure dynamic changes in the breakdown of muscle protein after eccentric contractions both during ambulation and during disuse is a key area for future research.

8.2 The effects of skeletal muscle inflammation on muscle function

A secondary aim of this thesis was to investigate the involvement of the NF-κB signalling pathway in the remodelling of skeletal muscle. NF-κB was first identified as a candidate pathway in the remodelling of skeletal muscle three hours after eccentric contractions by Hyldahl and colleagues in 2011 using a microarray approach (86). This thesis took a semi-targeted RT-PCR gene array approach to quantitatively investigate NF-κB associated genes identified by Hyldahl and colleagues further and in relation to other candidate remodelling pathways. The data presented in this thesis demonstrate that NF-κB-mediated transcription is likely to play a key role in the remodelling of skeletal muscle in response to eccentric contractions (**Chapters 3** and **4**), immobilisation (**Chapter 5**) and critical illness (**Chapter 6**). It should be noted that the protein expression or DNA binding

activity of NF-κB subunits (i.e., p50 or p65) or the expression of proteins associated with NF-κB signalling were not measured. However, given NF-κB is a transcription factor and the regulation and significance of NF-κB signalling in skeletal muscle remodelling remain unclear, a widespread investigation of genes known to regulate and be regulated by NF-κB transcription seemed a more appropriate approach to take, thereby generating candidate proteins for a future targeted investigation.

Attenuating the decline in muscle function using nutrition in **Chapters 3** and **4**, was associated with a more transient increase in mRNA expression linked to up and downstream NF-κB signalling (**Figures 3.8**, **3.9**, **4.5** and **4.6**). Nonetheless as demonstrated during the early remodelling period in **Chapter 3**, the initial increase in mRNA expression of all genes (except IL1R1) was similar between conditions 24 h after eccentric contractions, despite the pronounced difference in muscle function between conditions measured at the same time point. It is therefore hard to argue that the vast majority of the NF-κB targeted genes in **Chapters 3** and **4** are directly responsible for the attenuated recovery of muscle function. Whilst inflammatory processes are undoubtedly indispensable for proper remodelling of injured skeletal muscle, it seems most likely that the decline in muscle function is predominantly determined by the initial injury and immediate post-exercise events (i.e., protein breakdown and loss of sarcomere integrity). The magnitude of the subsequent inflammatory response, therefore, is likely to primarily reflect the magnitude of the initial injury. However, there may still be an important role for inflammatory processes in exacerbating the early degenerative processes and therefore contribute to the loss of muscle function as demonstrated by the plateau in muscle function 24-48 h after eccentric contractions in the placebo condition (**Figure 3.3**).

An exception to this could be IL1R1-mediated signalling which demonstrated a condition effect during the early remodelling period whereby it was less expressed in the protein-polyphenol condition at 24 and 72 h after eccentric contractions (**Figure 3.8B**). This could suggest that the expression of IL1R1 was also downregulated by protein-polyphenol ingestion prior to the recovery of muscle function and thereby could be, to some extent, driving this process. An increase in IL1R1 mRNA expression has been reported before in response to

eccentric contractions (86, 206, 245), but this is the first known report demonstrating a reduction in IL1R1 expression concomitant with an attenuated decline in muscle function. In **Chapter 3** the plasma protein expression of IL-1β which activates NF-κB signalling via IL1R1, and IL1-RA which inhibits IL1R1 signalling was not statistically different between conditions (**Figure 3.5**), however the protein expression of IL-1β in skeletal muscle increases immediately and six hours after eccentric contractions which would be consistent with a role for protein-polyphenol ingestion in attenuating IL1R1 mediated activation of NF-κB signalling within the first 24 h of eccentric contractions. Conversely, in **Chapter 5**, prior eccentric contractions resulted in a greater decline in muscle function after seven days of immobilisation without a greater increase in IL1R1 mRNA expression (**Figure 5.4B**) or indeed the mRNA expression of any of the targeted genes associated with NF-κB signalling (**Figures 5.4** and **5.5**). This demonstrates the strength of the unilateral model whereby the interaction between injury and immobilisation can be identified consequently showing that IL1R1 signalling may not actually be important in regulating changes in muscle function, at least under disuse conditions. Indeed, in **Chapter 6** IL1R1 mRNA expression was not different between SC or FESCE conditions either after seven days of intervention or six months after ICU admission (**Figure 6.3B**) and consequently is not associated with the pattern of less favourable functional outcomes in survivors of critical illness. Nonetheless, it would be interesting for future studies to take a targeted pharmaceutical approach using an IL-1 receptor antagonist, such as is used to safely treat rheumatoid arthritis (323), to establish if a causative role of IL1R1 signalling exists in the recovery of muscle function after injury.

In **Chapter 6**, a transcriptional signature in skeletal muscle characteristic of fibrosis was identified in critically ill patients during ICU residency. Fibrosis is characterised by aberrant extracellular matrix (ECM) deposition, primarily collagen, which can be exacerbated by altered activity of ECM degradation proteins such as matrix metalloproteinases (MMP) (324). There are numerous processes which might lead to increased collagen deposition during ICU residency, including inflammation, oxidative stress and bed rest (284). Indeed, inflammatory signalling pathways (**Figures 6.3** and **6.4**) and the 'response to hyperoxia' annotation (**Table 6.5**) were enriched in critically ill patients in **Chapter 6**. However, an important and unresolved question from **Chapter 6** is why does

FESCE administered during ICU residency appear to induce a protracted fibrotic response six months after ICU admission?

In young healthy individuals, a single bout of resistance exercise increases collagen synthesis (1, 6) and nine weeks of neuromuscular electrical stimulation training increases collagen mRNA expression without evidence of fibrosis in elderly healthy individuals (325). Therefore, it is possible that FESCE increases collagen synthesis, but immobilisation or illness (or both) may attenuate MMP activity leading to collagen accumulation. Indeed, skeletal muscle MMP mRNA expression decreases after two days of leg immobilisation (70) and neuromuscular electrical stimulation in critically ill patients has been shown not to attenuate the numerical decline in plasma MMP concentrations (326). Alternatively, in critically ill patients where the limb is inactive and devoid of contraction for ~22 h per day, the abrupt muscle stretch and contraction induced by electrical stimulation is likely to cause sarcomere injury (such as eccentric contraction-induced injury that was investigated in **Chapters 3**, **4** and **5**) which in the pathophysiological state of critical illness could become fibrotic, especially when exercise is repeated daily (327). Furthermore, the average ~25 minutes per day of FESCE (**Table 6.1**) is unlikely to optimally stimulate myofibrillar protein synthesis which under bed rest conditions may favour a larger turnover of collagen leading to progressive fibrosis and diminishing myofibrillar protein content. It is clear that further and direct investigations of fibrosis in a larger patient cohort is required to elucidate any causative role of FESCE on fibrosis and subsequent ICUAW. It is also clear that a holistic approach should be taken given fibrosis may just be one consequence of FESCE, and the effect of FECSE on direct measurements of insulin sensitivity, mitochondrial function and muscle protein turnover, for example, is still to be elucidated. Nonetheless, the implications of administering an exercise intervention to a critically ill patient have the potential to be great. While strategies to preserve the quality of life and indeed life itself should be at the forefront of research endeavours, these data highlight the potential dangers of a short-sighted focus without an awareness of long-term consequences.

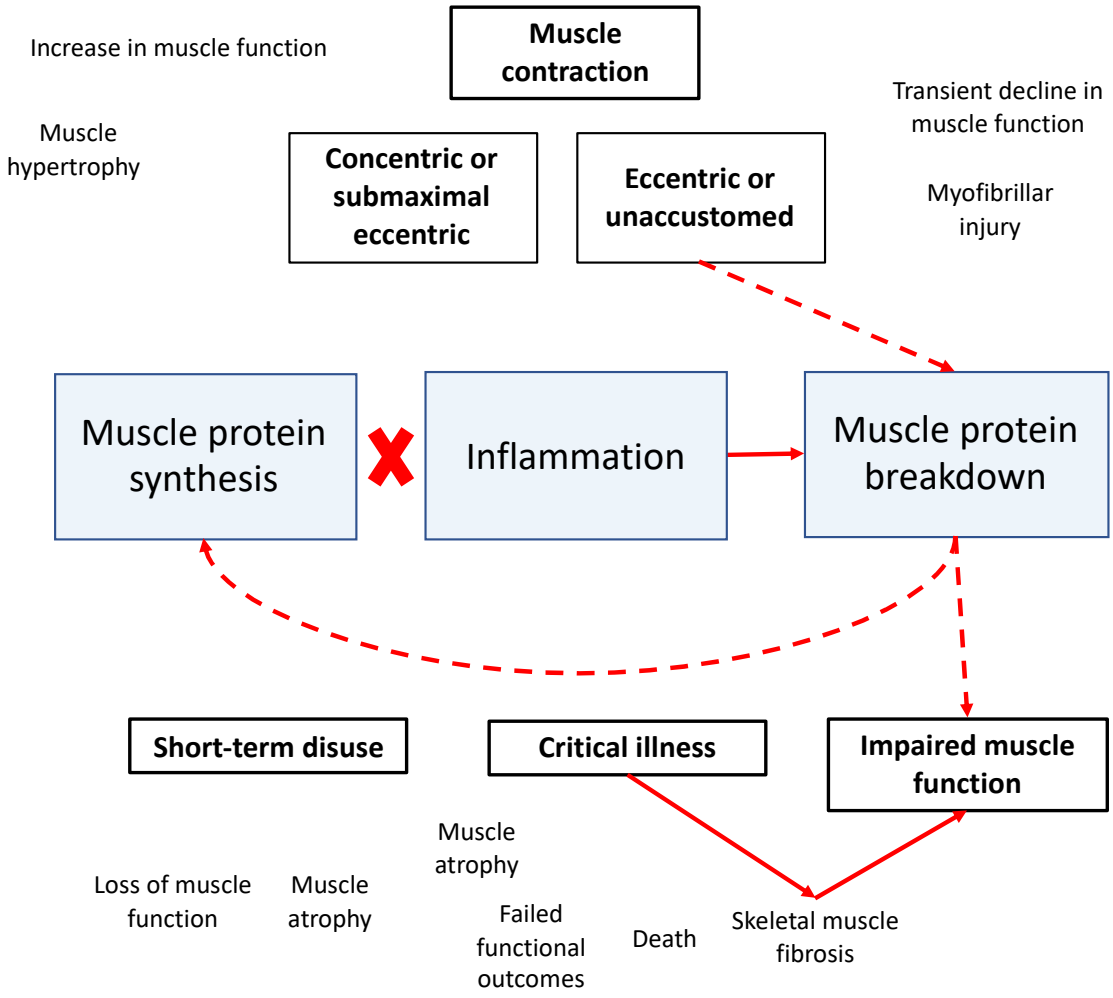

Figure 8.1. An updated schematic to illustrate the new proposed relationship between muscle protein synthesis, muscle protein breakdown and inflammation in skeletal muscle. Grey lines represent associations based on previous literature with arrows ending in a flat line representing a downregulation. Red lines represent new proposed relationships based on the data presented in this thesis. The cross symbolises that this thesis has demonstrated inflammation that occurs consequent with injury does not suppress myofibrillar protein synthesis rates in healthy individuals. Dashed red lines highlight areas for future research that have been identified by the studies presented in this thesis.

8.3 Conclusions

The studies presented in this thesis have investigated for the first time the association between myofibrillar protein synthesis rates and inflammation in the regulation of muscle function in humans. This was achieved by performing controlled time course intervention studies using a robust unilateral design across multiple different instances of skeletal muscle remodelling. Contemporary methodologies were employed to measure myofibrillar protein synthesis rates over multiple days in combination with a semi-targeted quantitative gene array and ontology approach in consideration of the multitude of interacting processes that promote successful remodelling and adaptation of skeletal muscle.

The studies presented in this thesis have established that myofibrillar protein synthesis rates are increased for at least 72 h after eccentric contractions and subsequently demonstrated for the first time that the increase in myofibrillar protein synthesis rates is directly related to the loss of muscle function after eccentric contractions. Using nutrition and immobilisation models to manipulate the change in muscle function after eccentric contractions, we have provided novel data to show that changes in muscle function can occur independently of changes in myofibrillar protein synthesis rates. Based on this we hypothesise that an early increase in muscle protein breakdown may be responsible for the loss of muscle function after eccentric contractions with or without subsequent immobilisation and may, in turn, govern changes in myofibrillar protein synthesis rates by increasing amino acid availability. It is therefore of interest for future intervention studies to target attenuating the breakdown of muscle protein to determine any causative role of muscle protein breakdown in driving the decline in muscle function after injury and with disuse. Such investigations will need to sensitively and directly measure muscle protein breakdown, and the further application and refinement of these methodologies this is likely to offer a stepwise advancement in our understanding of the regulation of muscle function.

This thesis has also demonstrated that the induction of inflammatory signalling is common to multiple instances of skeletal muscle remodelling, even when the objectives of that remodelling process are markedly different (i.e., to remodel injury, to promote skeletal muscle atrophy during disuse, to preserve life during

critical illness). Crucially, using the unilateral approach and within-subject control leg we can state with confidence and for the first time that transcriptional inflammatory signalling induced by eccentric contractions does not downregulate myofibrillar protein synthesis rates during ambulation and does not exacerbate the decline in myofibrillar protein synthesis rates or skeletal muscle atrophy during short-term limb immobilisation. The regulation of muscle protein breakdown by inflammation in this context is an intriguing area for future research. Furthermore, using a time-course approach this thesis has demonstrated that it is unlikely physiological NF-κB signalling regulates the recovery of muscle function after injury, but it is recommended that further work targets the IL1R1 receptor to investigate this further. A final and pertinent conclusion from this thesis is that a pathophysiological increase in NF-κB signalling during critical illness, especially when combined with exercise performed during ICU residency, could promote impairments in functional outcomes in survivors attributable, at least in part, to failed remodelling of skeletal muscle.

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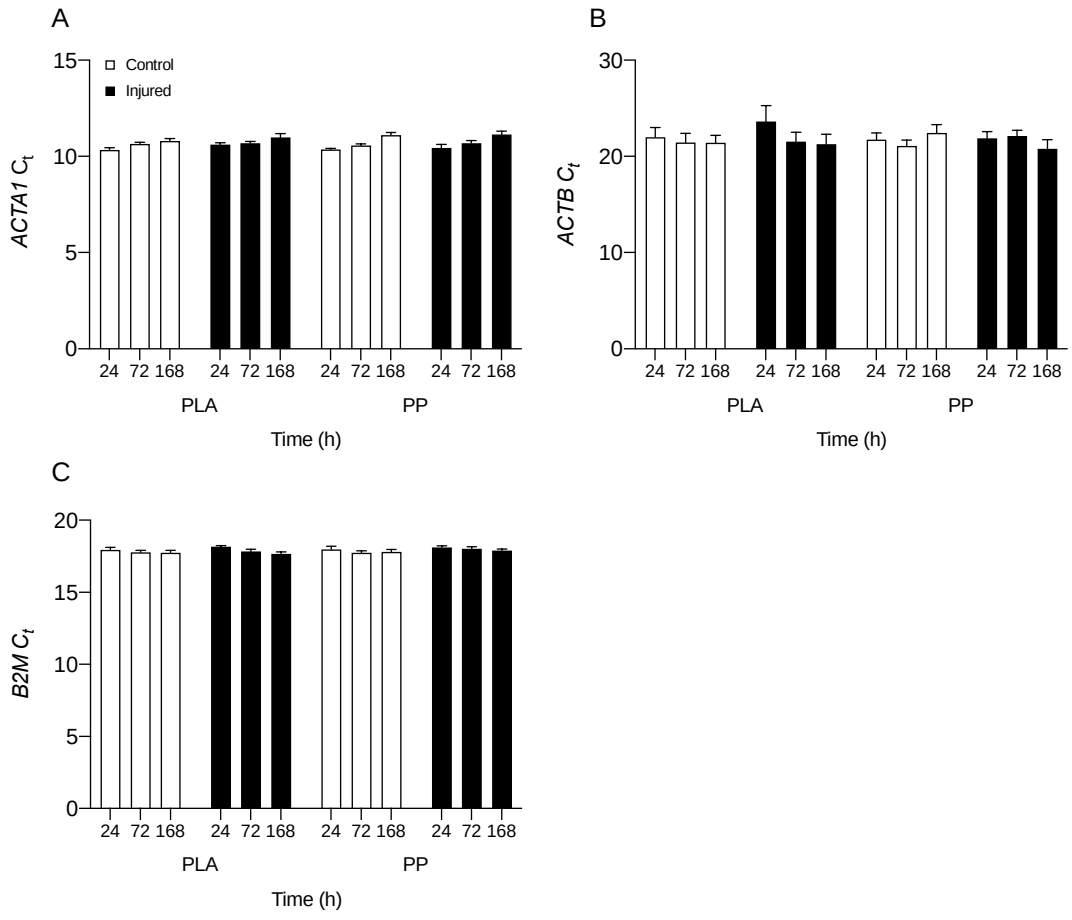
Appendix

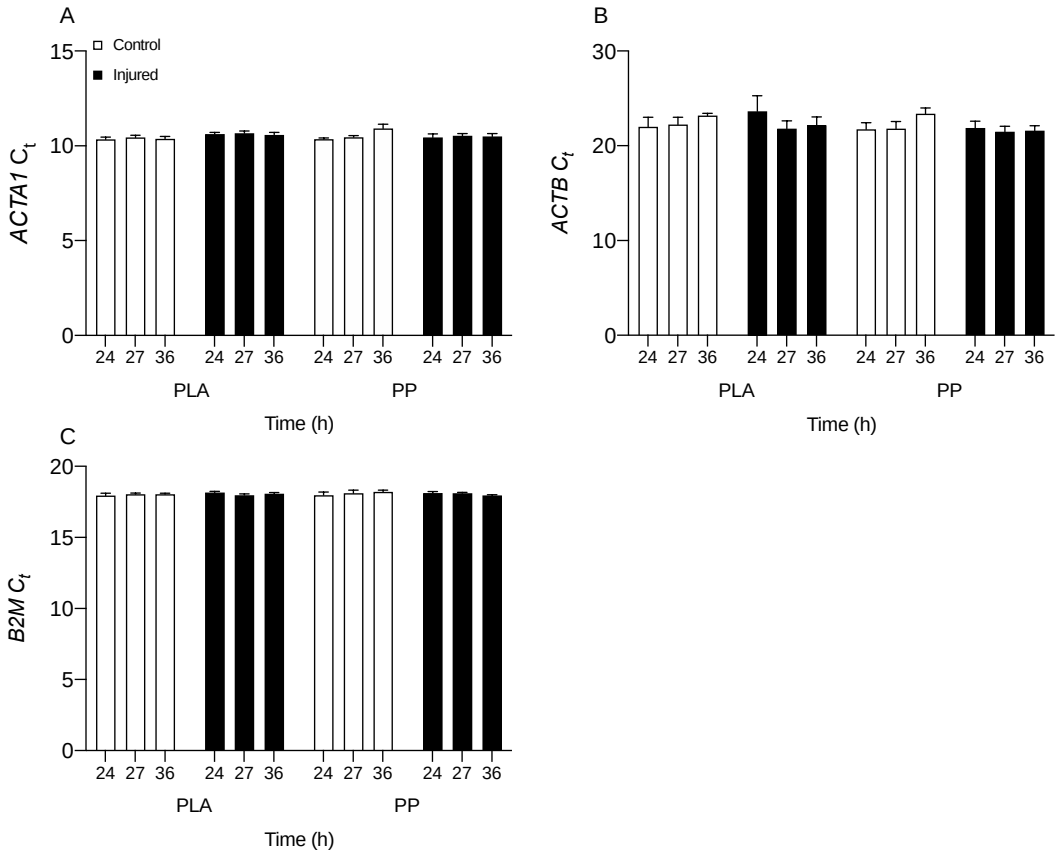
Appendix 1.1

Skeletal muscle mRNA expression of housekeeping genes alpha actin (A), beta actin (B) and beta-2 microglobulin (C) used for normalisation purposes in **Chapter 3**. Genes expression was measured after 300 unilateral eccentric knee extensor contractions (time=0 h). Post-exercise and pre-bed protein-polyphenol (PP; *n*=9; black bars) or isocaloric maltodextrin placebo (PLA; *n*=9; white bars) drinks were ingested for 7 days prior to, and 7 days following eccentric contractions.



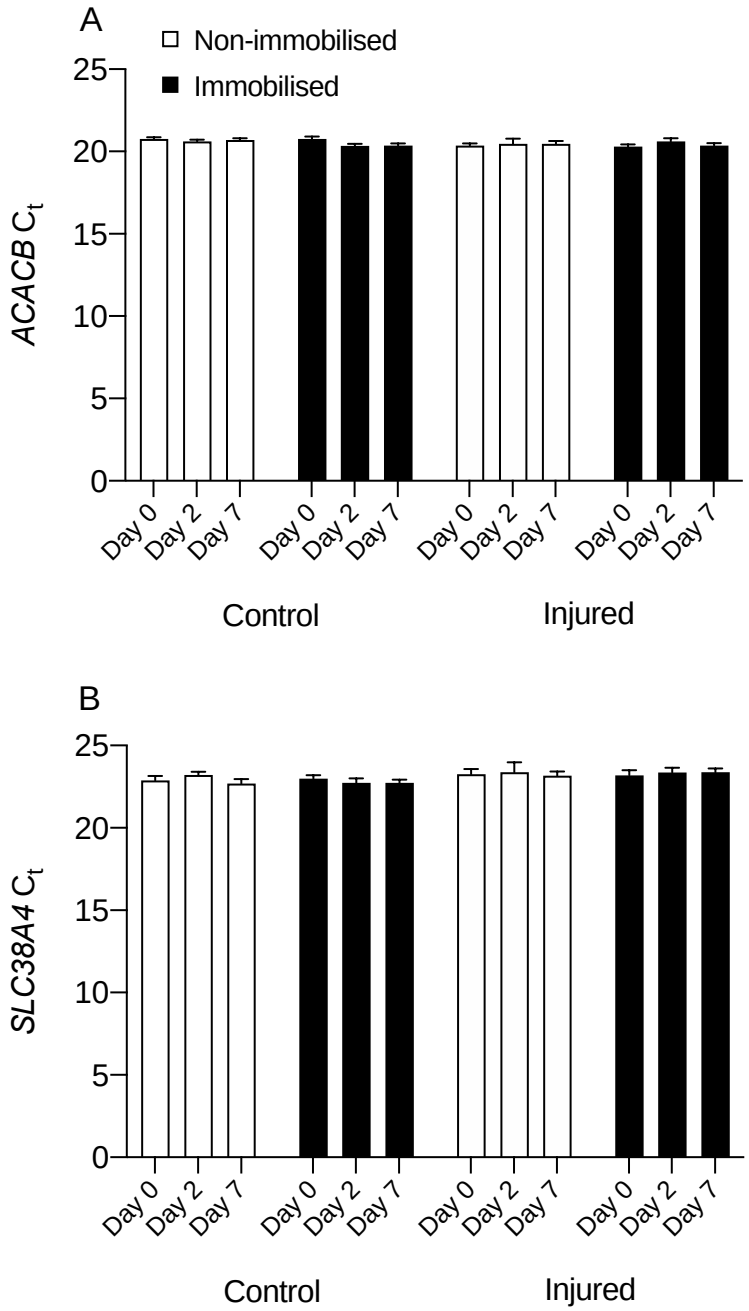
Appendix 2.1

Skeletal muscle mRNA expression of housekeeping genes alpha actin (**A**), beta actin (**B**) and beta-2 microglobulin (**C**) used for normalisation purposes in **Chapter 4**. Genes expression was measured after 300 unilateral eccentric knee extensor contractions (time=0 h). Post-exercise and pre-bed protein-polyphenol (PP; *n*=9) or isocaloric maltodextrin placebo (PLA; *n*=9) drinks were ingested for 7 days prior to, and 7 days following eccentric contractions.



Appendix 3.1

Skeletal muscle mRNA expression of housekeeping genes acetyl-CoA carboxylase 2 (**A**) and solute carrier family 38 member 4 (**B**) used for normalisation purposes in **Chapter 5**. Participants either performed no eccentric contractions (control; *n*=11) or performed 300 unilateral eccentric knee extensor contractions in both legs (injured; *n*=10) immediately before 7-days of unilateral leg immobilisation.



Appendix 4.1

Skeletal muscle mRNA expression of housekeeping genes beta actin (**A**) and beta-2 microglobulin (**B**) used for normalisation purposes in **Chapter 6**. Data was obtained from elective hip surgery control patients (control *n*=17) and critically ill patients resident in an ICU who received either standard care (SC; white bars) or a functional electronically stimulated cycle ergometry intervention (FESCE; black bars). Muscle biopsies were taken at ICU admission (day 1; SC *n*=13, FESCE *n*=14), 7 days after ICU admission (day 7; SC *n*=11, FESCE *n*=11) and 6 months after ICU admission (6 months; SC *n*=7, FESCE *n*=4).