

Investigation of muscle mass and metabolic regulation in human volunteers: the impact of resistance exercise, pharmacological intervention and obesity

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ABSTRACT

Skeletal muscle is the most abundant tissue in the human body, yet is able to adapt to myriad exogenous or endogenous stimuli. Resistance exercise training is known to increase muscle cross-sectional area (CSA), muscle volume and strength. Optimisation of these adaptations is attempted via modalities that include variations in muscle contraction type, and pharmacological intervention. Conversely, in obesity, muscle protein synthetic response to anabolic stimuli is compromised, highlighting the need for interventions to minimise poor health outcomes and disability associated with impairments in muscle mass regulation.

The three experiments described in this thesis aimed to examine the effects of resistance exercise, pharmacological intervention and obesity on muscle mass and metabolic regulation. The first experimental chapter examined the temporal impact of 84 days of maximal combined concentric-eccentric versus maximal concentric training on muscular adaptations and targeted muscle mRNA expression in the young. Despite ~16% greater work done overall during concentric–eccentric training, no differences in muscle CSA, volume or strength were detected between modalities at any time during training. Gene networks linked to inflammation and regulation of myogenesis and muscle metabolism were altered in the initial week of training, but especially with concentric-eccentric training. However, these mRNA responses waned thereafter, regardless of modality. The second experimental chapter examined whether non-steroidal anti-inflammatory drug (NSAID) ingestion over 84 days of resistance training (i) increased training work output above placebo and (ii) impacted upon skeletal muscle adaptation by influencing the early inflammatory response

observed in the young in the previous experimental chapter. Augmentation in muscle CSA and volume in the group consuming NSAIDs was found to be ~120% greater than placebo, occurring mainly from 28 days onward, paralleled by greater alteration in gene networks linked to myogenesis and muscle metabolic regulation. Strength did not increase in the NSAID group above placebo at any time during training, and training work output was similar between groups. The third experimental chapter examined whether forcibly increasing carbohydrate oxidation by oral dichloroacetate (DCA) administration might resolve the anabolic resistance in the elderly obese by increasing the efficiency of mitochondrial ATP production in a condition of lipid-induced muscle metabolic inflexibility. DCA administration increased myofibrillar fractional synthetic rate (FSR) in response to feeding ~40% above placebo. However, it did not increase myofibrillar FSR in response to combined feeding and exercise above placebo.

The resistance training studies in this thesis provided novel insight into the impact of eccentric exercise, and of NSAID administration on skeletal muscle adaptations. In combination with data from targeted gene network bioinformatics analysis, this added valuable knowledge on the impact of resistance exercise and medication on muscle mass regulation. Advances in the understanding of impaired anabolic response in the elderly obese were made by the novel experimental implementation of pharmacological intervention, while simultaneously opening up potential avenues in the management of conditions associated with muscle mass and metabolic dysregulation.

Declaration

All of the experimental data presented in this thesis were generated by me with the assistance of academic and technical staff at the David Greenfield Human Physiology Unit, Queen's Medical Centre, University of Nottingham; the Stable Isotope Mass Spectrometry Facility, Derby Royal Hospital, University of Nottingham; and the Sir Peter Mansfield Imaging Centre, University of Nottingham.

For experiments in Chapters 3 and 4, muscle biopsies were performed mainly by me. Muscle mRNA expression and muscle protein expression analysis were carried out by Dr Joanne Mallinson. MRI scans were performed by Anita French, and MRI data acquisition was done by Dr Martino Franchi. Volunteer resistance exercise training was supervised by Dr Mallinson and me. Bioinformatics analysis was performed by Dr Despina Constantin.

For experiments in Chapter 5, all clinical procedures were performed by me. DEXA scans were performed by technical staff at the David Greenfield Human Physiology Unit. MRI was performed by scanner operators at the Sir Peter Mansfield Imaging Centre. MRI analysis and data acquisition were performed by me, utilising software created by Olivier Mougin. Experimental trial visits were conducted by me with assistance from technical staff at the David Greenfield Human Physiology Unit. Muscle myofibrillar protein FSR and muscle protein expression analysis were performed by colleagues at the Stable Isotope Mass Spectrometry Facility, Derby Royal Hospital, University of Nottingham (Ms Jessica Cegielski, Ms Debbie Rankin, Dr Matthew Brook, Dr Daniel Wilkinson, Professor Kenneth Smith and Professor Phillip Atherton).

Declaration

I hereby declare that the work presented in this thesis, has not been submitted for any degree or diploma, at this, or any other university, and that all of the experiments, unless otherwise stated, were performed by me.

Signed:_____ Tariq Taylor

Date:_____

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****

"Thou reader throbbest life and pride and love the same as I,

Therefore for thee the following chants."

(Walt Whitman, Leaves of Grass)

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List of Abbreviations

3T	3-Tesla
4EBP1, 4E-BPI	Eukaryotic initiation factor 4E-binding protein-1
ABLIM2	Actin binding LIM protein family member 2
ACSA	Anatomical cross-sectional area
ACTC1	Actin, alpha, cardiac muscle 1
ACTN3	Alpha-actinin-3
ACVRC1	Activin A receptor 1
ADORA1	Adenosine A1 receptor
AKT1	Serine-threonine protein kinase 1
ALT	Alanine aminotransferase
AMPK	AMP-activated protein kinase
ANKRD1	Ankyrin repeat domain 1
ANKRD2	Ankyrin repeat domain 2
ASB5	Ankyrin repeat and SOCS box containing 5
ATF3	Activating transcription factor 3
ATGS	Autophagy-related genes
ATP	Adenosine triphosphate
BCAA	Branched-chain amino acid
BNIP3	BCL-2/E1B-19kD-interacting protein-3
BSA	Bovine serum albumin
c-MYC	MYC proto-oncogene
CEBP	CCAAT/enhancer-binding protein
CALCR	Calcitonin receptor
CD34	Hematopoietic progenitor cell antigen CD34
CDH15	Cadherin 15
cDNA	Complementary DNA
CEc r4BPB	CCAAT/enhancer binding protein (C/EBP), beta
CLEC3B	C-type lectin domain family 3, member B
CoASH	Coenzyme A
COX	Cyclooxygenase
CPT1	Carnitine palmitoyl transferase 1
Cr	Creatine
CRP	C-reactive protein
CSA	Cross-sectional area
CT	Computed tomography
CTSL1	Cathepsin L
CYR61	Cysteine-rich angiogenic inducer 61
DCA	Dichloroacetate
DEXA	Dual-energy X-ray absorptiometry
DNAJB4	DnaJ (Hsp40) homolog, subfamily B, member 4
dNTP	Deoxynucleoside triphosphates
DOMS	Delayed-onset muscle soreness
DTT	Dithiothreitol
ECG	Electrocardiogram
ECM	Extracellular matrix
EEF2	Eukaryotic elongation factor 2
EEF2K	Eukaryotic initiation factor 2 kinase

List of Abbreviations

EEG	Electroencephalogram
EFEMP1	EGF containing fibulin-like extracellular matrix protein 1
EIF2B	Eukaryotic translation initiation factor 2B, subunit 2 beta
eIF2B/4A/4B/4E	Eukaryotic initiation factor 2B/4A/4B/4E
EMCL	Extramyocellular lipid
EMG	Electromyography
FABP3	Fatty acid binding protein 3
FAK	Focal adhesion kinase
FBXO32	F-box protein 32
FFA	Free fatty acid
FIGF	Cellular oncogene c-fos
FOXO	Forkhead box-containing protein O
FOXO1	Forkhead box protein O 1
FOXO3	Forkhead box protein O 3
FRET	Fluorescent Resonance Energy Transfer
FSR	Fractional synthetic rate
GADD45a	Growth arrest and DNA damage-inducible protein alpha
GADD45b	Growth arrest and DNA damage-inducible protein
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GC-MS	Gas chromatography-mass spectrometry
GEM	GTP-binding protein
GSK	Glycogen synthase kinase
GSK3b	Glycogen synthase kinase 3 beta
HB	Homogenization buffer
HDAC9	Histone Deacetylase 9
HDL	High density lipoprotein cholesterol
HEMk1	HemK methyltransferase family member 1
HGF	Hepatocyte growth factor
HIF1a	Hypoxia inducible factor 1, alpha subunit
HMBS	Hydroxymethylbilane synthase
HPLC	High performance liquid chromatography
HSPA1a	Heat shock protein (70 kDa) 1A
HSPA1b	Heat shock protein (70 kDa) 1B
iAUC	Incremental total area under the curve
IFRD1	Interferon-related developmental regulator 1
IGF1	Insulin-like growth factor 1
IgG	Immunoglobulin G
IGJ	JCHAIN
IL-1	Interleukin-1
IL-18	Interleukin-18
IL-6	Interleukin-6
IMCL	Intramyocellular lipid
IMTGs	Intramuscular triglycerides
IPA	Ingenuity Pathway Analysis
IRS-1	Insulin receptor substrate 1
ITGA10	Integrin subunit alpha 10
JCHAIN	Joining chain of multimeric IgA And IgM
JKAMP	Mitogen-activated protein kinase 8
Jun	c-Jun
LDL	Low density lipoprotein cholesterol
MAFbx	Muscle atrophy F box

MAFF	MAF bZIP transcription factor F
MAP3K12	Mitogen-activated protein kinase kinase kinase 12
MAPK	Mitogen activated protein kinase
MDF1	MAD (yeast Mitosis arrest deficient) related
MET	MET proto-oncogene
Met1A	DNA (cytosine-5)-methyltransferase
MGB	Minor groove binder
MHC	Myosin heavy chain
MPB	Muscle protein breakdown
MPS	Muscle protein synthesis
MRF	Myogenic regulatory factor
Mrf-4	Myogenin and myogenic factor 6
MRI	Magnetic Resonance Imaging
mRNA	Messenger RNA
MSTN	Myostatin
mTOR	Mammalian target of rapamycin
mTORC1	Mechanistic target of rapamycin complex 1
MU	Motor unit
MuRF1	Muscle ring finger protein 1
MYC	MYC proto-oncogene
MYF5	Myogenic factor 5
MYF6	Myogenic factor 6
MYH1	Myosin heavy chain 1
MYH2	Myosin heavy chain 2
MYH3	Myosin heavy chain 3
MYH4	Myosin heavy chain 4
MYH7	Myosin heavy chain 7
MyoD	Myoblast determination protein 1
MYOG	Myogenin
NEDD4	Neural precursor cell expressed developmentally down-regulated protein 4
NFKb1	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1
NFKb2	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 2
NFQ	Non-fluorescent quencher
NSAID	Non-steroidal anti-inflammatory drugs
NWS	Non-water saturated spectra
PA	Phosphatidic acid
PAX3	Paired box gene 3
PAX7	Paired box gene 7
PCr	Phosphocreatine
PDC	Pyruvate dehydrogenase complex
PDK	Pyruvate dehydrogenase kinase
PDK2	Pyruvate dehydrogenase kinase 2
PDK4	Pyruvate dehydrogenase kinase 4
PDP1	Pyruvate dehyrogenase phosphatase catalytic subunit 1
PFK1	Phosphofructokinase 1
PG	Prostaglandin
PGF2alpha	Prostaglandin F2alpha
PI3K	Phosphoinositide 3-kinase
PIK3R1	Phosphoinositide-3-kinase regulatory subunit 1
PLD	Phospholipase D
PNG	Portable Network Graphics

List of Abbreviations

POL1	RNA polymerase 1
PPARGC1A	Peroxisome proliferator-activated receptor gamma coactivator 1 alpha
PRKAA1	Protein kinase AMP-activated catalytic subunit alpha 1
PSMA1	Proteasome subunit alpha 1
PTGS1	Prostaglandin-endoperoxide synthase 1
PTGS2	Prostaglandin-endoperoxide synthase 2
PTK2	Protein tyrosine kinase 2
qPCR	Real-time PCR
Rabb15	Member RAS oncogene family
rDNA	Ribosomal DNA
REE	Resting energy expenditure
RER	Respiratory exchange ratio
RF	Rectus femoris
RIA	Radioimmunoassay
RM	Repetition-maximum
RPS6	Ribosomal protein S6
RPS6KA	Ribosomal protein S6 kinase
RRAD	Ras related glycolysis inhibitor and calcium channel regulator
rRNA	Ribosomal RNA
RRPTOR	Regulatory associated protein of MTOR, complex 1
RT-PCR	Reverse transcription polymerase chain reaction
S6K1	Ribosomal protein S6 kinase 1
SCD	Stearoyl-Coenzyme A desaturase 1
SEAM	STimulated Echo Acquisition Mode
SIRT1	Sirtuin 1
SL1	Selective factor 1
SNR	Signal-to-noise ratio
SREBF2	Sterol regulatory element binding protein
SRF	Serum response factor
SSC	Stretch shortening cycle
t-BDMS	tert-Butyldimethylsilyl

CHAPTER 1. General Introduction

1.1 Chapter overview

Regular performance of resistance exercise results in well-established muscle mass, functional and metabolic adaptations. This chapter will review these adaptations and their regulation in depth, in order to set the stage for investigations into how they are influenced by specific training modalities and pharmacological interventions. This chapter will also discuss the current evidence on the dysregulation of muscle mass and metabolic adaptations in certain conditions. The suggested mechanisms in the literature that are suspected to drive these impairments will also be examined, so as to contextualise one of the main research questions that this thesis will attempt to answer.

1.2 Resistance exercise training

1.2.1 Background

Mammalian skeletal muscle mass is regulated by a complex process due to the ability of this tissue to respond to unloading by atrophy and overloading by hypertrophy. Atrophy of skeletal muscle can be defined as wasting or decrease in muscle mass due to injury, disuse, or disease. It is characterised by a loss of myofibrils, which in turn results in reduced cross-sectional area of muscle fibres (McKinnell and Rudnicki 2004). Muscle hypertrophy is said to be characterised by “an increase in the size (as opposed to the number) of individual myofibers.” (Glass 2005).

Resistance training is defined as training during which muscle contraction occurs against an external, opposing force (Atha 1981) and when performed frequently results in an increase in strength, mass, maximal power or local endurance of

muscle. Eyster (1927) found that raising tension in a rubber band in canine aorta increased the workload of the heart, consequently causing myocardial hypertrophy; he established thereby that resistance, and not work, induced strength gains and muscle hypertrophy. Later, DeLorme (1945) promoted the use of high load/low repetition training as the ideal means for improving muscle strength and size in the rehabilitation of wounded World War II soldiers, positing that endurance training, or low load/high repetition training, used predominantly at the time for rehabilitation, helped to reduce fatigue, but did not produce gains in muscle strength or size. DeLorme coined the former "progressive resistance exercise".

Thus, the concepts of one-repetition-maximum (1-RM) and ten-repetition-maximum (10-RM) were conceived; 1-RM being the "maximum load that can be lifted one time using proper form" (McArdle WD 2009). This allowed for the establishment of an initial training load, in order to subsequently adjust maximal sets and repetitions as strength increased. Atha (1981) defined muscular strength as 'the ability to develop force against an unyielding resistance in a single contraction of unrestricted duration', and demonstrated that gains in strength occurred when muscle repeatedly generated forces greater than those produced during daily activity (Atha 1981). These increases are proportional to the degree of tension developed in muscle during resistance exercise (Atha 1981; Hortobagyi, Hill, et al. 1996; McArdle WD 2009). Since there is a positive correlation between muscle strength and cross-sectional area (Atha 1981; Jones and Rutherford 1987), force generation over time produces gains in both strength and size of muscle (Atha 1981; Jones and Rutherford 1987).

The last three decades have witnessed an increase in the popularity of resistance exercise (Bird, Tarpenning, and Marino 2005). It is now a proven method to enhance muscle strength (Kraemer and Ratamess 2004), improve athletic performance (Wilson et al. 2012) and is also used towards aesthetic ends (Folland and Williams 2007). Other documented benefits include maintenance of musculoskeletal (Layne and Nelson 1999), mental (Fox 1999) and general (Warburton, Nicol, and Bredin 2006) health and well-being. It is utilised in power sports to increase strength and prevent injury (Roig et al. 2009; Faigenbaum and Myer 2010; Fleck and Falkel 1986) and has clinical applications in conditions like sarcopenia (Burton and Sumukadas 2010) and bone loss (Shackelford et al. 2004).

1.2.2 Adaptations to resistance training

Broadly, adaptations to resistance training can be categorised as (i) morphological, (ii) neuromuscular and (iii) metabolic.

1.2.2.1 Morphological adaptations

The primary morphological adaptations to overloading of muscle are (i) changes in whole muscle size, or muscle volume and (ii) changes in muscle cross-sectional area (CSA). Increase in muscle CSA is the result of an increase in cross-sectional area of muscle fibres (MacDougall et al. 1980). Since hypertrophy of muscle fibres is thus an increase in contractile machinery due to enhanced protein synthesis post-exercise (Glass 2003), the expectation is that this would lead to greater muscle strength. The literature has many examples of reported increases in muscle CSA and muscle volume after resistance exercise

training in both young (Narici et al. 1989) and old adults (Reeves, Narici, and Maganaris 2004; Harridge, Kryger, and Stensgaard 1999; Narici, Maganaris, and Reeves 2005).

Skeletal muscle fibres in humans can be classified based on their contractile properties of "twitch" or "time to peak tension" as (i) slow-twitch, or Type I and (ii) fast twitch or Type IIx (Schiaffino and Reggiani 2011), reflective of their histochemical staining for myofibrillar or myosin ATPase. These isoforms are distinct in terms of their contractile properties, which correspond to their twitch characteristics and ATPase activity. Type is determined genetically, and whether these fibre types might adaptively "transform" from one to the other is controversial (Booth, Laye, and Spangenburg 2010). It is worth noting that there is a differential response in fibre size between fibre types after strength training, in that type II fibres undergo hypertrophy more quickly than type I fibres during training, and atrophy more quickly than type I fibres during detraining (Hather et al. 1991).

1.2.2.2 Neuromuscular adaptations

Voluntary muscle contractions are controlled by the central nervous system, which, via motor unit (MU) recruitment and rate coding, stimulates muscle fibres. Each MU is a group of muscle fibres under the control of a single, common motoneuron. Impulses from the central nervous system course through the axons of these motoneurons to arrive at the fibres. If the impulse exceeds the activation threshold of that specific MU, a muscle twitch is produced.

The magnitude and speed of the twitch depends on the class and number of muscle fibres within a single motor unit. Hence, MUs are differentiated on the basis of both the number of fibres they comprise, as well as their contractile and metabolic properties. Since increase in muscle fibre size is one of the main morphological adaptations to resistance training, increases in strength are influenced both neurologically as well as a result of hypertrophic change.

Thus, during the initial stages of strength training regimens, there is a pattern of temporal divergence between increases in muscle strength, which can be detected within the first few days of training; and increases in muscle mass (Hakkinen et al. 2001; Jones and Rutherford 1987) which are only observable after weeks of training by methods such as dual-energy x-ray absorptiometry (DEXA), computed tomography (CT) or magnetic resonance imaging (MRI). Some studies however, have detected molecular changes in response to muscle loading during resistance training as early as 12 hours (h) after a training bout (Bickel et al. 2005), and architectural signs of hypertrophy within 10 days (Seynnes, de Boer, and Narici 2007).

The primary neural adaptations to resistance training are (i) a neural drive increase, due to greater MU recruitment (Aagaard et al. 2000), and (ii) a firing frequency increase (Sale 1988). A number studies have also shown a reduction in activation of the antagonist muscle following resistance training (Hakkinen et al. 2001), which could potentially be contributory to the strength gains associated with it (Narici et al. 1996).

1.2.2.3 Metabolic and health adaptations

Nearly 40% of total body mass is made up of skeletal muscle in mammals. In adult humans, this accounts for around 30% of resting metabolic rate (Zurlo et al. 1990). A key role is therefore played by skeletal muscle in metabolic homeostasis and glycaemic control, since it is the main site of glucose disposal in insulin stimulated conditions (DeFronzo et al. 1981). Moreover, it serves as the largest organ for glycogen storage, being able to store four times as much glycogen as the liver (Egan and Zierath 2013).

Hence, because aerobic or endurance exercise effects a high repetition, low-power output cost on muscle contraction, while resistance exercise imposes a low repetition, high-power demand, they do not impart identical metabolic and cardiovascular adaptations (Table 1-1). Both modalities of exercise promote significant health benefits, but divergent effects are noted with respect to certain parameters. As an example, while aerobic exercise better benefits the cardiovascular system, resistance training is better at maintaining basal metabolic rate (Table 1-1), by virtue of its ability to promote muscle hypertrophy. Due to its ability to improve insulin sensitivity, improve physical function and maintain lean body mass and increase bone mineral density (Egan and Zierath 2013), it is recommended that resistance exercise be used in combination with aerobic exercise as part of exercise guidelines in the elderly (Davidson et al. 2009), and in conditions that feature metabolic dysregulation (Colberg et al. 2010).

Table 1-1. Metabolic adaptations and health adaptations to aerobic/endurance training versus resistance/strength training. ↑ indicates values increase; ↓ indicated values decrease; ↔ indicates values remain unchanged; ↑ or ↓ indicates a small effect, ↑↑ or ↓↓ indicates a medium effect; ↑↑↑ or ↓↓↓ indicates a large effect, ↔↑ or ↔↓ indicates no change or slight change. Adapted from Egan and Zierath (2013).

	Aerobic (Endurance) Exercise Training	Resistance (Strength) Exercise Training
Bone mineral density	↑↑	↑↑
Body composition		
Percentage body fat	↓↓	↓
Lean body mass	↔	↑↑
Glucose metabolism		
Resting insulin levels	↓	↓
Insulin response to glucose challenge	↓↓	↓↓
Insulin sensitivity	↑↑	↑↑
Inflammatory markers	↓↓	↓
Resting heart rate	↓↓	↔
Stroke volume, resting and maximal	↑↑	↔
Blood pressure at rest		
Systolic	↔↓	↔
Diastolic	↔↓	↔
Cardiovascular risk profile	↓↓↓	↓
Basal metabolic rate	↑	↑↑
Flexibility	↑	↑
Posture	↔	↑
Abilities in activities of daily living	↔↑	↑↑

1.2.3 Modes of muscle contraction

Muscle contraction can be categorised based on tension, length and speed as isotonic, isometric and isokinetic, respectively (Atha 1981; McArdle WD 2009). Isometric (or static) contractions occur when muscle generates force against external resistance that it is unable to overcome, resulting in fibre length remaining unchanged (McArdle WD 2009). Isotonic (or dynamic) contractions occur when the muscle generates force to execute movement of the body part to which the contracting muscle is attached (Atha 1981; McArdle WD 2009). Isotonic loading is the most common method of resistance training, generally employed using standard weight lifting equipment or free weights.

Isokinetic contractions occur when the muscle generates force while the attached body part moves at a constant angular velocity controlled by an external device. The force applied against the device results in an equal and opposite reactive force (Fleck 1997). The device, known as an isokinetic dynamometer, accelerates to a constant, previously set angular velocity when a force is applied against it. Theoretically, this allows for the application of maximal force throughout the range of movement. Hence, maximal intensity contractions occur when the greatest amount of tension is generated and maintained throughout the full range of motion of limb movement (McArdle WD 2009). These can also be performed using conventional weight training (McArdle WD 2009). Sub-maximal contractions occur when tension is generated against a load that is lower than or a specific percentage of 1-RM. These can be performed using conventional weight training or equipment such as bicycle ergometers (Abbott, Bigland, and Ritchie 1952; Bigland-Ritchie and Woods 1976).

Isotonic contractions comprise a concentric (or shortening) phase, and an eccentric (or lengthening) phase. A concentric contraction occurs when the muscle generates tension as it shortens. An eccentric contraction occurs when the muscle generates tension as it lengthens (Figure 1-1).

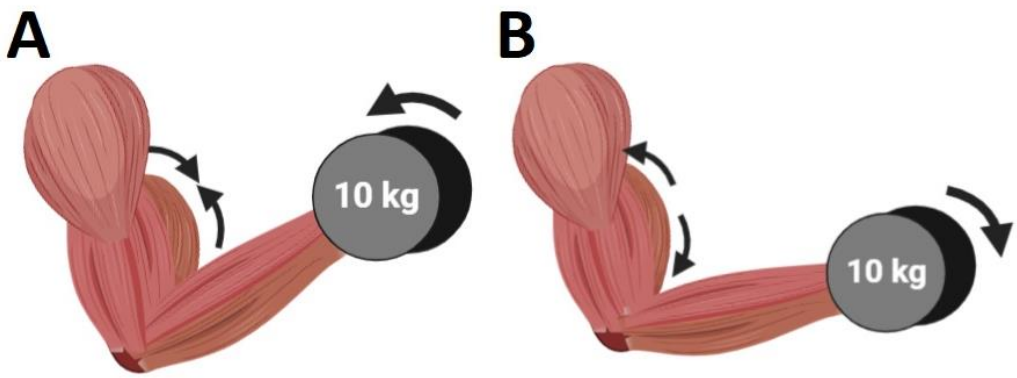

Figure 1-1: Schematic representation of concentric and eccentric contractions of the biceps brachii. Image in the sagittal plane of elbow flexion: A) Concentric contraction of biceps brachii muscle which shortens as weight is lifted upwards under tension. (B) Eccentric contraction of the biceps brachii which lengthens as the weight is brought down under tension.

1.2.3.1 Characteristics of eccentric contractions

Force generation in concentric versus eccentric muscle actions

In accordance with the classical force-velocity relationship of muscle (Hill 1938) (Figure 1-2), force generated during concentric actions of muscle in single fibre in vitro preparations decreases as velocity at which the fibre shortens decreases. Conversely, eccentric actions of muscle generate far greater forces than concentric muscle actions, since force increases as the velocity at which the fibre lengthens increases. Traditional resistance exercise consists of repetitions of

concentric and eccentric phases of movement in conjunction with a brief isometric contraction in between these phases (Hortobagyi et al. 2001). The maximum force generated during the concentric phase is the limiting load during one whole repetition cycle. Hence the eccentric phase in traditional resistance exercise is under-loaded. This is important because concentric contractions have been found to generate 40-50% less force than eccentric contractions at 1-RM (Dudley et al. 1991; Hather et al. 1991).

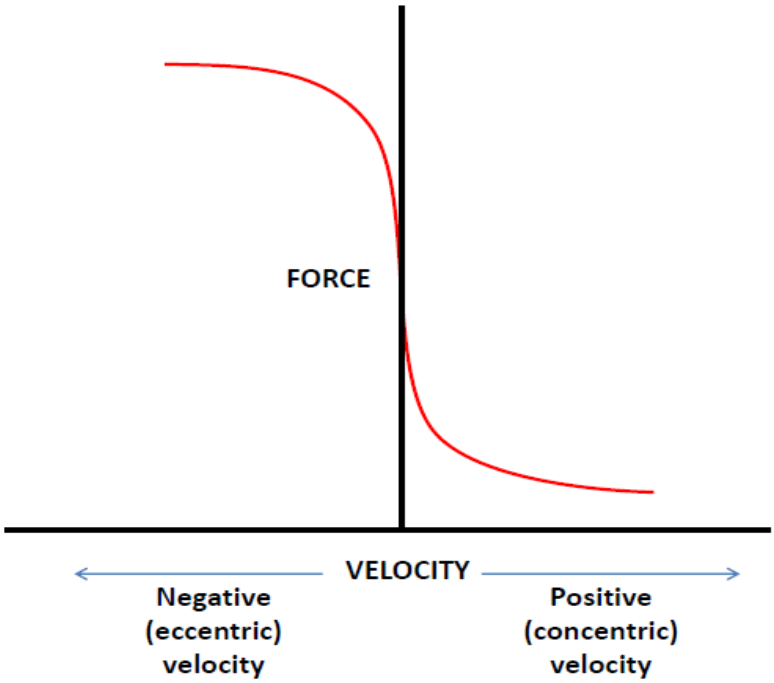

Figure 1-2: Schematic graph of force generated at positive (concentric) and negative (eccentric) velocities. The force generated by the muscle increases with increasing velocities of negative or lengthening actions, and decreases with increasing velocities of shortening actions.

Neuromuscular responses to eccentric exercise

Since a given cross-sectional area of muscle produces greater force during a lengthening than during a shortening contraction (Katz 1939; Hill 1938), a lower number of muscle fibres require activation to accomplish an eccentric contraction than a concentric contraction to generate the same force, resulting in lower rate of firing of motoneurons (Bigland-Ritchie and Woods 1976).

Nardone, Romano, and Schieppati (1989), investigating motor unit recruitment in the triceps surae using intramuscular electrodes, suggested that lengthening contractions selectively activate high-threshold motor units. The mechanism is believed to originate in inhibitory and facilitatory pathways within the spinal cord (Wakeling, Uehli, and Rozitis 2006). However, the literature thus far is equivocal on this matter. A phosphocreatine/creatine (PCr/Cr) depletion study by Beltman et al. (2004) did not detect preferential recruitment of specific motor units during eccentric contractions. Furthermore, Coburn et al. (2006), using mechanomyography on the quadriceps muscle during a bout of isokinetic eccentric contractions performed at an angular velocity of $300^{o}.s^{-1}$, suggested that torque modulation in this experiment was more likely due to alterations in motor unit firing rate.

Literature suggests that there is perhaps a difference in the execution of concentric and eccentric contractions at the cortical level. Transcranial magnetic stimulation of the brain suggests the involvement of cortical centres during lengthening contractions (Sekiguchi et al. 2007), and movement-related cortical potentials derived from electroencephalogram (EEG) are found to be greater during eccentric contractions (Fang et al. 2004). This helps explain the

difficulties encountered in coordination during eccentric exercise. As an example, some research volunteers struggle to successfully perform eccentric cycling on a bicycle ergometer (Mueller et al. 2009).

Metabolic demand of eccentric contractions

Negative work is perceptibly less energy expensive than positive work. Benedict (1928) noted that caloric expenditure in women while ascending stairs was five times greater than during descent. Abbott, Bigland, and Ritchie (1952), using two opposing cycle ergometers, found that the ratio of the biological cost of concentric versus eccentric cycling was 3.7. This ratio was greater at higher cadences, suggesting that at higher rates of muscle-lengthening, eccentric work has a 4-fold lower energy cost, which is explained by the force-velocity relationship (Hill 1938; Katz 1939). Since cycling at higher cadences results in greater lengthening velocities, this causes a greater negative power output at similar torques. Bigland-Ritchie and Woods (1976), in an effort to expand on these findings, measured oxygen consumption, power and integrated electromyography (EMG) in subjects cycling on a motor-driven bicycle ergometer. They found that EMG activity was halved during eccentric cycling for the same power output of concentric cycling. Further, while a close linear relationship between oxygen consumption and mechanical power output during concentric exercise had been established previously (Fischer 1931), Bigland-Ritchie and Woods (1976) detected a 6-fold drop in oxygen consumption during eccentric cycling for the same mechanical power output as concentric cycling. This reduced metabolic cost of negative work has since been confirmed in both

young, active subjects (Seliger et al. 1968; Moore, Young, and Phillips 2012) and in old, sedentary subjects (LaStayo et al. 2003).

Using surface EMG, Molinari et al. (2006) showed that eccentric contractions elicited fewer electrical signs of muscle fatigue than concentric contractions in 20 subjects, despite greater work output and torque generation during the former. Further, Tesch et al. (1990) found that when measuring torque and EMG activity during maximal concentric versus eccentric exercise for 3 bouts of 32 contractions, these parameters were maintained through lengthening contractions significantly better than during concentric contractions. A reduced mechano-chemical strain, in the form of a lower ATP hydrolysis rate, has been demonstrated during eccentric actions versus concentric actions by Ryschon et al. (1997) using ^{31}P-nuclear magnetic resonance spectroscopy in combination with a quantitative dynamometer. Later, Horstmann et al. (2001) made 64 subjects perform 1 minute (min) of maximal isokinetic eccentric and concentric contractions of the knee and ankle joints, and reported greater torque production, lower fatigue levels, and reduced lactate accumulation and ammonia production during eccentric contractions.

Since eccentric contractions clearly impart a lower metabolic cost and cause less muscle fatigue than concentric contractions, and because eccentric exercise at low-moderate intensities is not associated with muscle soreness (Paulsen et al. 2012), it has clinical applications in conditions characterised by cardiovascular and respiratory compromise. Cardiopulmonary response to low-moderate intensity eccentric versus concentric exercise was measured in older subjects by Vallejo et al. (2006). They found that during three bouts of contractions at 65%

1-RM, eccentric contractions resulted in significantly lower heart rate, systolic blood pressure, cardiac index and expired ventilation than concentric contractions. Similar findings have been reported by others (Hortobagyi and DeVita 2000; Okamoto, Masuhara, and Ikuta 2006), and this has been exploited in exercise regimens in the elderly (Chung, Dean, and Ross 1999), coronary artery disease (Meyer et al. 2003); and in the management of chronic obstructive pulmonary disease (Rooyackers, Berkeljon, and Folgering 2003; Rocha Vieira et al. 2011) and cancer (Lastayo et al. 2010; LaStayo et al. 2011).

Muscle soreness and functional decline

Delayed onset muscle soreness (DOMS) following high-load eccentric exercise typically peaks at 24 to 48h post-exercise, and subsides in ~5-7 days (Clarkson, Nosaka, and Braun 1992; Ebbeling and Clarkson 1989; Whitehead et al. 2006). It is commonly reported following maximal load eccentric exercise, and is minimal with low-load eccentric training (Lastayo et al. 1999). DOMS is suggested to occur due to nociceptor sensitisation by histamine, prostaglandins, bradykinin, free radicals and tissue breakdown products (Proske and Morgan 2001; Clarkson and Hubal 2002). Further, disruption of the extracellular matrix (ECM) has been suggested to stimulate an inflammatory response that is responsible for soreness (Stauber et al. 1990). However, a clear association between histological findings and soreness has proved difficult to identify given a paucity of longitudinal data. Inflammatory change in muscle is commonly associated with DOMS symptoms (Child et al. 1999; Fielding et al. 1993; Hellsten et al. 1997; O'Reilly et al. 1987; Round, Jones, and Cambridge 1987), though some studies in humans have reported soreness and raised passive

tension without accompanying inflammation (Crameri et al. 2007; Malm et al. 2000; Paulsen, Crameri, et al. 2010; Paulsen, Egner, et al. 2010).

Reduction in force-generation capacity following eccentric exercise ranges from 15-70% of pre-exercise levels, and typically persists for ~7-8 days (Hyldahl and Hubal 2014). Maximal intensity eccentric exercise has been reported to cause force loss of ~50% (Paulsen, Crameri, et al. 2010; Paulsen, Egner, et al. 2010; Hellsten et al. 1997; Child et al. 1999; Lauritzen et al. 2009; Beaton et al. 2002; Beaton, Tarnopolsky, and Phillips 2002). In contrast, moderate-low intensity eccentric exercise in the form of downhill running (Malm et al. 2004; Feasson et al. 2002) and eccentric cycling (Friden, Sjostrom, and Ekblom 1983) produces a force loss of ~16-24%. Extreme degrees of force loss have been reported in studies exercising the elbow flexors eccentrically (Lauritzen et al. 2009; Paulsen, Egner, et al. 2010; Sayers and Clarkson 2001), perhaps because the lower limbs are more accustomed to eccentric loading, and are therefore more habituated to this stimulus. Indeed, Sayers and Clarkson (2001) detected a 70% force loss in the elbow flexors immediately after exercise. These subjects recovered between 33-47 days post-exercise, as opposed to ~1 week recovery noted following maximal eccentric knee extension protocols (Paulsen et al. 2012).

1.2.3.2 Skeletal muscle adaptions to eccentric training

Architectural adaptations to eccentric training

Maximal eccentric and concentric contractions have been shown to increase both fascicle length and pennation angle in the vastus lateralis muscle in humans (Blazevich et al. 2007). Reeves et al. (2009), comparing traditional resistance

training to maximal eccentric training in humans, found the latter increased fascicle length to a larger degree than concentric training. In contrast, increases in pennation angle were greater following the conventional training regimen, while muscle thickness improved to a similar extent after both.

Increase in fascicle length is suggested to be due to the addition of sarcomeres in series following resistance training. This is based on findings in animal models where muscle fibres respond to chronic (Williams et al. 1988) and intermittent stretching (Holly et al. 1980) with the serial addition of sarcomeres. Since larger loads are overcome during eccentric training, this imparts a more profound stretch on muscle fibres, which acts as a potent stimulus for the addition of sarcomeres in series. Potier, Alexander, and Seynnes (2009) found that fascicle length increased by 34% following high intensity eccentric training of the biceps femoris. Hence while both concentric and eccentric training regimens alter the architectural characteristics of muscle, eccentric exercise at high loads seems to increase fascicle length more profoundly. This has applications in situations where shortening of muscle is a factor, such as rehabilitation and in certain sports.

Muscle size and strength adaptations to concentric vs eccentric training

It has been suggested that eccentric overload during low-moderate intensity resistance training results in greater muscle hypertrophy than concentric training alone (Friedmann et al. 2004; Hortobagyi, Barrier, et al. 1996). Special devices, such as bicycle ergometers, have been used for exercise at moderate-low intensities over long durations (Bigland-Ritchie and Woods 1976). Subjects have

to resist the drive of the ergometer, which imposes a lengthening contraction upon the thigh muscles. LaStayo et al. (2000) used a similar device to train 14 young subjects, divided equally into eccentric and concentric groups, over 8 weeks using progressively ramped exercise. Isometric strength was found to improve by 36%, and fibre-cross sectional area by 54% in the eccentric group alone. In another study in elderly subjects using a similar protocol, LaStayo et al. (2003) reported a 60% increase in both isometric strength and fibre cross-sectional area of the vastus lateralis in the eccentric group, while improvements in the concentric group were not statistically significant.

Early studies found no difference in strength gains and muscle hypertrophy following concentric versus eccentric training at high intensities (Higbie et al. 1996; Johnson 1972; Jones and Rutherford 1987; Komi and Buskirk 1972). Indeed, some authors proposed that concentric contractions were better at achieving strength gains than eccentric contractions (Mayhew et al. 1995; Raue et al. 2005). A meta-analysis by Roig et al. (2009) canvassed 1,954 studies before including clinical trials fulfilling specific criteria, namely: adult participants; 18-65 years of age; minimum 4-week training duration; 2 or more training sessions per week; reported outcome measures including one of either muscle mass or muscle strength. They included studies using a variety of methods to measure changes in muscle hypertrophy. These included muscle girth measurement, dual X-ray absorptiometry (DEXA), magnetic resonance imaging (MRI) and computerised tomography (CT). Eccentric training performed at high intensities was found to improve total and eccentric strength more significantly than concentric training, though strength gains from eccentric exercise were more

pronounced when training and testing velocities were the same. Concentric training demonstrated a trend towards greater gains in concentric strength alone, however this was not significant.

Eccentric training at high intensities in Roig et al. (2009) was found to be more effective at increasing muscle girth than concentric training. Muscle girth measurements are obtained by measuring the circumference of the limb under investigation, under tension, using a measuring tape; the reviewers acknowledged its low reproducibility as a measure of muscle hypertrophy. Of the four studies that used the most reliable imaging techniques (MRI and CT), three (Higbie et al. 1996; Seger, Arvidsson, and Thorstensson 1998; Vikne et al. 2006) found that high load eccentric training promoted greater increases in muscle mass. The fourth study (Blazevich et al. 2007) found no difference between the two, despite similar populations and interventional methods. Overall, Roig et al. (2009) identified a trend towards increase in muscle cross-sectional area (CSA) measured using MRI/CT following high-intensity eccentric training, which they attributed to the greater work done during eccentric contractions. However, more recent high-load training studies have detected no differences in muscle hypertrophy or strength gains when comparing high load concentric and eccentric exercise training modalities in young volunteers (Farup et al. 2014; Franchi et al. 2014).

Neuromuscular adaptations to eccentric training

Data on the neuromuscular adaptations to chronic eccentric training is limited. Maximal intensity eccentric training over 12 weeks has been shown to increase EMG activity 7-fold more during eccentric strength testing, when compared to

the increase in EMG activity during concentric strength testing following concentric training (Hortobagyi, Hill, et al. 1996). However, eccentric training was shown to increase EMG activity during concentric strength testing to the same extent that concentric training increased EMG activity during eccentric strength testing, indicating that enhanced adaptations were mode-specific. While eccentric training has been found to improve concentric muscle power and performance of the stretch shortening cycle (SSC) of muscle to a greater degree than traditional or concentric training modalities (Elmer et al. 2012; Gross et al. 2010; Liu et al. 2013; Yarrow et al. 2008), these studies have been undertaken using protocols of varying intensity and duration. There is little data available on the impact of eccentric training on the rate of force development during contraction; and these results are inconclusive as to whether eccentric training has a positive or negative effect on this neuromuscular adaptation (Blazevich et al. 2008; Miller et al. 2006).

Metabolic adaptations to eccentric training

Very few studies have examined metabolic adaptations to eccentric training. Some evidence suggests that training comprising lengthening contractions might have positive effects on lipid profiles and insulin sensitivity. More studies are required to buttress these conclusions, given the paucity of data, and the underlying mechanisms of metabolic health benefits of eccentric training remain unexplored. Zeppetzauer et al. (2013) compared carbohydrate and metabolic adaptations to concentric training in the form of upward hiking and eccentric training in the form of downward hiking, using a cross-over design in 45 young, sedentary volunteers. They found that glucose tolerance and low-density

lipoprotein (LDL) cholesterol levels were improved using both modalities, but that a more significant improvement occurred following eccentric training. Paschalis et al. (2011) trained 20 healthy young female individuals over 8 weeks, using a high-intensity protocol. The volunteers performed either maximal isokinetic concentric exercise or maximal isokinetic eccentric exercise of both legs for one session a week. The investigators found that the eccentric training protocol alone improved resting blood lipid profiles and insulin sensitivity at the end of the 8-week protocol. In order to determine whether these apparent benefits of eccentric training might be demonstrated in the elderly, recently, Chen et al. (2017) recruited healthy men aged 60-76 years, who performed progressive resistance training in the form of either 30-60 high intensity concentric, or eccentric contractions per week. They found that there were greater decreases in fasting triacylglycerols, total, and low-density lipoprotein cholesterols following eccentric training, and high-density lipoprotein cholesterol (HDL) increased only after eccentric training.

1.3 Mechanisms underlying muscle hypertrophic response to resistance exercise and resistance exercise training

1.3.1 Muscle protein turnover and the hypertrophic response to resistance training

1.3.1.1 Muscle protein balance

The process of muscle protein metabolism is the result of an equilibrium between muscle protein synthesis (MPS) and muscle protein breakdown (MPB). In the fasted state, typically, MPB rates (0.03–0.07%.h^{-1}) exceed MPS rates (0.03-0.07%.h^{-1}) (Smith, Villareal, and Mittendorfer 2007; Phillips et al. 1997;

Atherton and Smith 2012), resulting in a negative protein balance, and therefore a net loss in muscle protein. However this catabolic state undergoes a reversal after dietary protein intake, following which MPS rates exceed those of MPB, once again rendering muscle protein balance positive by increasing MPS nearly twofold (Rennie et al. 1982). As a result of this cycle, muscle proteins lost in the post-absorptive or fasted state are regained. This plastic nature of muscle is perhaps best exemplified by its response to exercise. Muscle loading during exercise promotes muscle protein turnover in the form of increases in both MPS and MPB. Since exercise elevates MPB to a greater degree than MPS, this results in a negative net protein balance (Kumar, Atherton, et al. 2009), such that in the fasted state, exercise is catabolic. However dietary protein intake alongside exercise, by providing amino acid substrates for MPS, ensures that increase in muscle protein synthetic rates are sustained for up to 24h post-exercise (Miller et al. 2005; Cuthbertson et al. 2006). The maintenance of this positive balance results in the remodelling associated with adaptations to exercise, such as hypertrophy and mitochondrial biogenesis (Perry et al. 2010; Egan et al. 2013).

1.3.1.2 MPS response to resistance exercise

Physiological adaptations to training depend on the mode of exercise being performed. Resistance exercise training continues to be the best method by which muscle hypertrophy can be achieved. It is widely accepted that repeated increases in MPS following episodic bouts of acute resistance exercise over a period of chronic training results in an overall net positive protein balance such that MPS exceeds MPB (Wilkinson et al. 2014), culminating in increase in muscle fibre size, and consequently, increases in whole muscle CSA (Ahtiainen

et al. 2003; Hulmi et al. 2009). A number of studies have aimed to determine the ideal method by which to optimise anabolic responses to resistance exercise training, by investigating the effects of (i) intensity of exercise, (ii) frequency of repetition and (iii) contraction mode. Following a bout of resistance exercise, MPS has been demonstrated to increase to up to 4-fold above baseline (Kumar, Selby, et al. 2009). A sigmoidal dose-response to variation in resistance exercise intensity has been demonstrated by Kumar, Atherton, et al. (2009), such that MPS was found to plateau when exercise intensity was greater than 60% of 1-RM. This was maintained even when the number of contractions performed at a lower intensity of 40% 1-RM was increased to match the volume of work done. However, this does not necessarily mean that exercise intensities at and above this 60% 1-RM "ceiling" are required to increase MPS. Indeed, a single bout of high-volume, but low intensity resistance exercise to failure has been found to increase both the degree and duration of the consequent MPS response to a greater extent than a high intensity bout at low volume (Burd et al. 2010). It is worth noting, however, that such an approach required a greater volume of work to be performed during the low intensity (30% 1-RM) exercise than during the high intensity (1-RM) exercise bout. These findings have been corroborated by data from a recent training study comparing muscle hypertrophy following high versus low intensity resistance exercise training (Mitchell, Churchward-Venne, et al. 2012), suggesting that mechanical loading with heavier weights is not necessarily likely to induce a greater anabolic response, either in terms of skeletal muscle protein turnover or muscle hypertrophy.

Following a single session of resistance exercise, the time course of MPS increase has been found to depend on nutritional and training status (Tipton et al. 1999), as well as the intensity and volume of exercise performed. Immediately post-resistance exercise, typically a latent period is observed during which MPS does not rise above post-absorptive levels, perhaps as a result of blunting of MPS response during exercise secondary to the inhibitory effect of ATP turnover on MPS rates. Indeed, rodent studies have demonstrated a suppression in MPS during muscle contraction in accordance with the contractile duty cycle (Rose et al. 2009). While similar studies examining MPS during the course of exercise in humans do not exist, it has been suggested that it is likely that inhibition of MPS also occurs during resistance exercise in humans (Atherton, Phillips, and Wilkinson 2015). In support of this position, proponents have highlighted that while increases in MPS have been demonstrated as soon as an hour after moderate intensity resistance exercise (Blazevich et al. 2007), muscle protein synthetic rates remain at baseline for as long as 3h following a bout of high-intensity eccentric contractions (Cuthbertson et al. 2006). Hence, it is possible that the energy demands of a specific exercise bout determines suppression of MPS during exercise and also the latency period that proceeds it.

Following this latency period, MPS increases from 45 to 150min after the completion of the resistance exercise bout, remaining elevated up to 4h post-exercise (Kumar, Atherton, et al. 2009). It is worth noting however, that if protein nutrition is not provided during this period, this results in a negative net protein balance. Despite resistance exercise acting as a strong stimulus for MPS, the parallel increases in MPB render it catabolic in the fasted state (Phillips et al.

1997). In combination with dietary protein intake, however, positive net protein balance is restorable, and has been demonstrated to be sustained for up to 48h in the untrained state (Cuthbertson et al. 2006; Miller et al. 2005).

1.3.1.3 MPB response to resistance exercise

MPB overall is examined far less than MPS in the literature, mainly because of the complexity of the techniques involved in its measurement (Kumar, Atherton, et al. 2009; Biolo et al. 1992; Zhang et al. 1996). However, a number of studies have investigated the effect of resistance exercise on MPB. It is apparent that MPB following resistance exercise follows a similar temporal pattern to MPS. Immediately after exercise there is a sharp, albeit transient rise in MPB of up to 50%, and this might be sustained for up to 24h (Biolo et al. 1995; Phillips et al. 1997). At rest, and in the post-absorptive state, MPB is much higher than MPS, resulting in a net negative protein balance. Even though resistance exercise acts as a profound stimulus for MPS, this rapid rise in MPB is what renders it catabolic (Biolo et al. 1995; Phillips et al. 1997). It is worth noting however, that this increase in MPB post-resistance exercise is blunted in the trained state, suggesting that diminishment in MPB forms an important component of skeletal muscle adaptation to resistance exercise training (Phillips et al. 1999). Interestingly, in contrast to its effect on post-exercise increases in MPS, whether or not nutrition has an impact on post-exercise increase in MPB is inconclusive (Tipton et al. 1999; Biolo et al. 1997; Rasmussen et al. 2000; Borsheim et al. 2002; Glynn et al. 2010).

1.3.1.4 Chronic resistance training and muscle protein metabolism

Due to the availability of stable isotope tracer techniques, much is known about the muscle protein metabolic response to acute resistance exercise. Unfortunately, there is less data on the impact of chronic resistance exercise training on muscle protein metabolism. What is known is that muscle protein turnover in response to resistance exercise changes during the course of an extended period of training. As an example, the post-exercise MPS response to a single bout of resistance exercise has been shown to be approximately 50% greater in sedentary individuals versus those who are highly trained (Phillips et al. 1999). In addition, following 8 weeks of resistance exercise training, the MPS response in the trained leg of volunteers has been found to be significantly lower than in the contralateral untrained leg (Phillips et al. 2002; Kim, Staron, and Phillips 2005). Also, there is a blunting of MPS response in successive bouts of resistance exercise (Wilkinson et al. 2014; Tang et al. 2008). Hence, it is apparent that muscle hypertrophy does not occur linearly. Indeed, hypertrophy in response to chronic resistance exercise training occurs for the most part in the initial weeks of regimens (Seynnes, de Boer, and Narici 2007; Tesch et al. 2004; DeFreitas et al. 2011), at which point there is a plateauing of increase in muscle mass gains even with progressively increased overloading during training (DeFreitas et al. 2011).

1.3.2 Exercise metabolism and molecular mechanisms regulating muscle hypertrophy

As mentioned earlier (Section 1.3.1.2), the repetition of bouts of muscle contraction as part of regular exercise training of different modalities acts as a

strong stimulus for adaptation. Over an extended period, there is remarkable plasticity and malleability in skeletal muscle in terms of functional adaptation and remodelling as a result of contractile activity (Fluck and Hoppeler 2003; Coffey and Hawley 2007). Skeletal muscle adaptation to training is reflected in alterations in contractile function and protein (Adams et al. 1993; Widrick et al. 2002), regulation of metabolism (Green et al. 1992), mitochondrial function (Spina et al. 1996), transcriptional events (Pilegaard, Saltin, and Neufer 2003), and intracellular signalling (Benziane et al. 2008). The molecular mechanisms that are widely acknowledged to drive skeletal muscle adaptation to exercise training consist of gradual changes in enzymatic activity and protein content. These alterations are reflective of the activation and/or inhibition of certain signalling pathways that modulate transcription, translation and exercise-induced gene expression.

Post-exercise, transient changes occur in transcriptional response in genes involved in myogenic regulation, carbohydrate metabolism, mitochondrial metabolism and oxidative phosphorylation, the mobilisation, oxidation, and transport of lipid, among others (Pilegaard, Saltin, and Neufer 2003; Coffey et al. 2006; Mahoney et al. 2005; Louis et al. 2007). Furthermore, the repetitive increases in mRNA over the recovery period following acute exercise has been shown to drive the synthesis of corresponding proteins, resulting in gradual remodelling in the structure of muscle and long-term adjustments in muscle function (Perry et al. 2010) (Figure 1-3). It has been suggested that these changes act towards enhancing delivery of substrate, contractile function, and the mitochondrial respiratory capacity of muscle during exercise (Egan and Zierath

2013). The overall effect of such changes has been suggested to optimise performance in consequent exercise sessions, ensure homeostasis despite metabolic alterations, and hence improve resistance to fatigue (Holloszy and Coyle 1984; Booth and Thomason 1991).

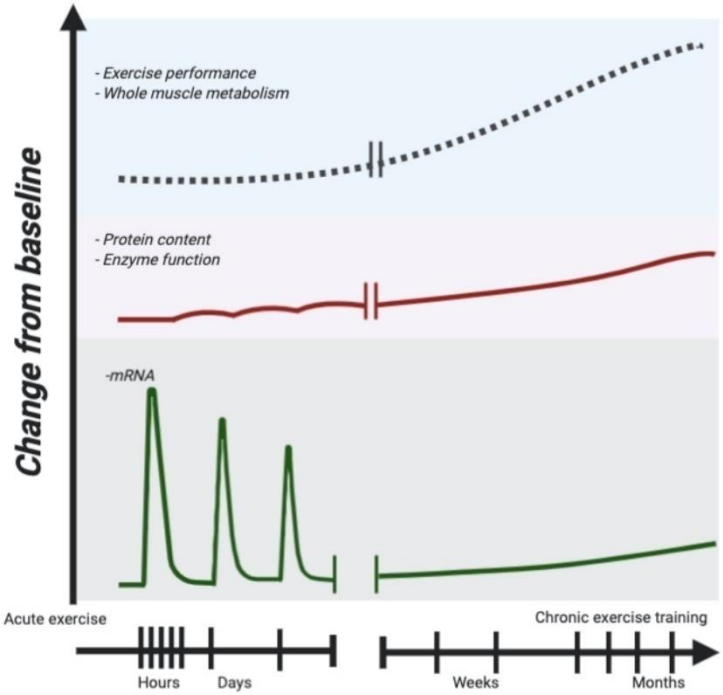

Figure 1-3: Molecular processes underpinning muscular adaptation to exercise and training. The figure depicts mRNA expression changes (lower section) and protein content (middle section) with time following repetitive bouts of acute exercise and hence, chronic exercise training. Phenotypic adaptations to training occur as a result of repeated stimulation from exercise bouts. Each causes a short-lived elevation in expression of specific genes during recovery. Greater changes in expression typically occur up to 12 hours post-bout, returning to baseline within a day. Changes in translation and an increased rate of muscle protein synthesis post-exercise cause a modest alteration in protein content in the same direction when exercise bouts are repeated. Protein accumulation occurs gradually as a response to pulsed, repetitive rises in mRNA expression. Adaptation to chronic training is hence a result of individual acute exercise bouts culminating in improved thresholds of function. Changes in enzyme function and protein content in turn impact upon muscle metabolism at the substrate level, allowing for improved exercise performance (top section of graph). Adapted from Egan and Zierath (2013).

1.3.2.1 Metabolic response to acute exercise and in post-exercise recovery

When acute exercise is performed, different metabolic pathways contribute to the provision of energy, based on the intensity and power output of the bout. The rate at which ATP is required is determined by absolute power output. Relative exercise intensity determines to what degree carbohydrate, lipid, and fuel stores in circulation and in muscle contribute to energy provision. Indirect calorimetry in combination with isotope tracer techniques have allowed for an understanding of substrate utilisation during exercise (Romijn et al. 1993; van Loon et al. 2001). The proportion in which fuel sources are utilised from substrates in extramuscular and intramuscular stores is regulated quantitatively and over time so that the metabolic demands of exercise can be met.

Skeletal muscle, at low to moderate exercise intensities, is primarily fuelled by (i) glucose, acquired from hepatic glycogenolysis and gluconeogenesis, or by oral ingestion, and (ii) free fatty acids (FFAs) derived by their liberation through adipose tissue lipolysis (McArdle, Katch, and Katch 2006; McArdle WD 2009). In terms of extramuscular sources of fuel, use of circulating FFAs by muscle reduces moderately with increasing intensity, and rises until close to maximal intensities (van Loon et al. 2001). Alongside this, absolute rates of carbohydrate oxidation increase such that energy provision at high intensities is largely derived from muscle glycogen. Prolonged exercise (>60min) at fixed intensities results in an increase in contribution of energy from fat oxidation. The contribution of muscle glycogen thus declines, substituted by a progressive rise in plasma FFA oxidation (Romijn et al. 1993).

The main carbohydrate source during moderate-high intensities of exercise is muscle glycogen. Relative exercise intensity is directly proportional to the rate at which muscle glycogen is broken down (Hargreaves 2000). When muscle glycogen levels are high initially, it is broken down at higher rates. However, with continuation of exercise, rate of breakdown of glycogen parallels the rate of decline of glycogen concentration. In contrast, intramuscular triglycerides (IMTGs) are a mere ~1-2% of the whole-body fat stores, but with prolonged exercise at moderate intensity are a key fuel source. However, their contribution is lower at low or higher exercise intensities (Romijn et al. 1993; van Loon et al. 2001). During resistance exercise, as with endurance exercise, muscle glycogen and IMTG are sources of fuel provision (Koopman et al. 2006), though it is relatively less clear as to how carbohydrate and fat are utilised.

A number of amino acids such as alanine, aspartate, glutamine, glutamate as well as branched-chain amino acids play a part in skeletal muscle energy production (Gibala 2006). With prolonged exercise, however, the remaining amino acids contribute to gluconeogenesis (Ahlborg et al. 1974). It is generally assumed in calculations of substrate utilisation during exercise that protein contribution is constant. During moderate exercise intensities, it has been estimated that protein contributes 5% to 15% to energy provision. However, in states of energy compromise, this contribution increases (Wagenmakers et al. 1991; Horton et al. 1998). The energy for this is acquired by degradation of intramuscular protein and branched-chain amino acid (BCAA) oxidation via branched-chain α-keto acid dehydrogenase complex (Wagenmakers et al. 1991).

Once exercise is stopped, there is a drop in metabolic rate, which however still remains slightly increased, i.e. <10% up to 24h. The degree to which energy is used post-exercise is related to duration, intensity and the modality of the bout (Borsheim and Bahr 2003). The recovery period consists of two stages. The first involves a return to homeostasis at the myocellular level immediately after exercise for some hours. The second involves contributions to exercise adaptations at the cellular level. In addition, homeostatic recovery involves oxygen store replenishment, oxidation of lactate and its removal, phosphocreatine (PCr) and ATP re-synthesis, fuel store replenishment and fluid balance restitution (Borsheim and Bahr 2003). Fat oxidation and a sparing of carbohydrate sources also occurs (Kiens and Richter 1998), as opposed to the dependence on carbohydrate metabolism during exercise. The first allows for fuel provision to be met, while the second permits re-synthesis of muscle glycogen. This extended rise in fat oxidation post-exercise is an important process in terms of the effects of exercise on whole-body lipid metabolism (Horton et al. 1998). Other processes play a part in homeostatic recovery, such as a re-esterification of lipids from formerly liberated FFAs (Wolfe et al. 1990), increased rates of myofibrillar protein synthesis (Wilkinson et al. 2008), and re-synthesis of glycogen and IMTG (Kiens and Richter 1998; Schenk and Horowitz 2007). Muscle glycogen is replenished to pre-exercise levels around 24 to 48h post-exercise (Jentjens and Jeukendrup 2003).

1.3.2.2 Gene transcriptional response to resistance exercise and resistance training

The nature of the exercise stimulus determines the type of proteins being synthesised over the recovery period (McGlory, Devries, and Phillips 2017). It is known that prolonged low load stimulation of skeletal muscle by aerobic or endurance exercise training causes an elevation in expression of mitochondrial genes (Perry et al. 2010), proteins (Egan et al. 2013; Holloszy 1967; Perry et al. 2010), and eventually improved mitochondrial content (Gibala et al. 2006), resulting in an oxidative phenotype and enhanced fatigue resistance (Egan and Zierath 2013). While resistance exercise training has also been shown to stimulate the transcription of genes and new muscle protein accretion (Mitchell, Churchward-Venne, et al. 2012; Nader et al. 2014), these have largely been linked to the myofibrillar protein fraction (Nader et al. 2014; Wilkinson et al. 2008). Nevertheless, early in exercise training, especially in the training naïve, a significant increase in gene expression common to both endurance and resistance exercise has been observed (Fluck and Hoppeler 2003; Vissing and Schjerling 2014). Sophistication and specificity in the muscle transcriptome, and hence the protein synthetic response, only seems to occur with sustained training, thereby resulting in divergent oxidative and hypertrophic phenotypes following endurance and resistance exercise training respectively (Fluck and Hoppeler 2003; Mahoney and Tarnopolsky 2005).

In contrast to our more thorough understanding of transcriptional responses to endurance exercise training, knowledge with respect to resistance exercise and resistance exercise training is less complete (Egan and Zierath 2013; McGlory,

Devries, and Phillips 2017). Nevertheless, in order to be able to investigate changes in gene transcription underlying skeletal muscle adaptations following resistance exercise and resistance exercise training on a genome-wide level, microarray technology has been adopted extensively. Gordon et al. (2012) investigated the effect of 12-weeks of maximal intensity resistance training in one arm on the activation of immune response in skeletal muscle in healthy young men and women following an acute bout of resistance exercise, while the other arm was not trained. A week after the final session of the training protocol, participants performed a bout of maximal resistance exercise in both arms at the same training intensity. Using microarrays, they found that resistance training blunted the post-exercise upregulation of immune activation and oxidative stress response, and the downregulation of glucose metabolism and oxidative phosphorylation. Furthermore, they found that training had enhanced the post-exercise transcriptional up-regulation of cytoskeletal and extracellular matrix development. The investigators also used microarrays to determine sex differences in the skeletal muscle transcriptome (Liu et al. 2010) at rest and following the bout of maximal resistance exercise performed after 12 weeks of training. At rest, they detected a greater abundance of genes associated with fatty acid oxidation, gene transcription and gene translation events in female muscle. Following the exercise bout, male muscle was found to undergo a longer period of transcriptional change compared to female muscle. Sex specificity was highlighted for several signalling pathways in females, notably the activation of notch signalling and transforming growth factor 1-beta (TGF1b) signalling in females.

Raue et al. (2012) investigated the effects of acute resistance exercise, progressive resistance training, and age on the skeletal muscle transcriptome, in order to acquire insight into the molecular underpinnings of sarcopenia. They recruited two cohorts of young and old adults for this purpose, who took part in two studies (Study A, n=28; Study B, n=36). The 12-week training protocol comprised 36 sessions of 3 sets of 10 bilateral knee extensions at 70-75% of one-repetition maximum. Vastus laterals muscle biopsies were taken before and 4h after the first and last training sessions. It was found that 661 genes were differentially expressed in response to the 1st and 36th training bouts that correlated with increases in muscle strength and size observed as a result of progressive resistance training. The gene response to resistance exercise response was most profound in fast-twitch (Myosin heavy chain-IIA) muscle fibres. They also found that muscle in the young cohorts was more responsive to resistance exercise, and that age affected basal muscle gene expression.

Damas et al. (2018) investigated how training status might affect the global skeletal muscle transcriptome at rest and following acute resistance exercise. They recruited 9 young men who underwent resistance exercise training for 10 weeks. Muscle biopsies were taken before and 24h after resistance exercise in the untrained and trained states, following which microarray analysis was carried out. They found that in the untrained state there was an upregulation of gene transcription related to inflammation, stress, muscle damage, structural remodelling, protein turnover and translation capacity following exercise. In contrast, trained muscle demonstrated changes in gene transcripts related to energy metabolism, including an up-regulation of antioxidant and immune

system-related genes, and those related to extracellular matrix and cytoskeletal structure, muscle growth, development and contraction.

While microarray analysis has been used successfully in the above studies, notably, investigators have either compared pre-training muscle samples with those obtained after several weeks of resistance exercise training or following an acute bout of resistance exercise. Hence, less is known about the transcriptional events that contribute to skeletal muscle adaptation to resistance exercise over the course of training. Murton et al. (2014) therefore, aimed to determine the time course of transcriptional events in skeletal muscle following resistance exercise in exercise-naïve subjects in the form of 2 sessions of 5 bouts of 30 sets of maximal isokinetic concentric knee extensions, performed 48h apart. Muscle samples from the vastus lateralis were obtained at baseline and 24h following each exercise bout. After the first bout of resistance exercise a wide inter-subject variability in skeletal muscle transcriptome was observed. However, following the second bout of exercise, a more consistent response was detected, consisting of a differential expression in genes associated with ubiquitination, inhibition of oxidative metabolism, and immune activation. From these findings, it is apparent that transcriptional response at the outset of resistance exercise training is transient and needs to be measured at multiple time points for a clearer understanding of skeletal muscle adaptation to emerge, which is addressed in Chapter 3 and Chapter 4.

Transcriptional response to concentric versus eccentric exercise

Chen et al. (2003) were the first to investigate the effect of eccentric exercise on gene expression in skeletal muscle. They recruited 3 male volunteers who

performed 300 shortening contractions in one leg in the form of rising from a chair, and 300 shortening and 300 lengthening contractions in the form of rising from and a subsequent controlled descent to a seated position. They found that there was a greater response in gene expression linked to inflammation (chemokine (C-C motif) ligand 2, delta CCAAT/enhancer-binding protein, and Interleukin-1 receptor) and vascular remodelling (tenascin C and lipocortin II) in the eccentrically exercised leg.

Building on these findings, the same research group (Kostek et al. 2007) examined muscle gene expression over the 24h following a bout of eccentric and concentric contractions in human muscle, using temporal gene expression profiling. They recruited 5 males who performed a bout of concentric contractions in one leg and eccentric contractions in the contralateral leg, in the form of stepping onto a box with one leg and stepping down with the contralateral leg respectively. Muscle samples were taken before exercise and at 3, 6 and 24h post-exercise. They found that 51 transcripts were differentially expressed between the exercise modalities and suggested that eccentric and concentric contractions activated distinct molecular signalling pathways as early as 3h following exercise.

Most recently, Deane et al. (2019) found that of the significantly differentially expressed genes in skeletal muscle of young individuals (n=8) 5h following high load eccentric versus high load concentric exercise, 590 were upregulated, and 204 were downregulated following the former, while 227 were upregulated and 63 were downregulated after the latter. In older volunteers (n=8), this difference in magnitude of gene response between modes of contraction was less

pronounced: 724 genes were upregulated, and 198 were downregulated following eccentric exercise, while 665 genes were upregulated and 328 were downregulated after concentric exercise. However, the profiles of the genes involved in response to concentric and eccentric exercise were highly similar in the young, and were only moderately contraction-specific in the old volunteers. Hence, the muscle transcriptional response in this study was similar between concentric and eccentric exercise, perhaps because these subjects were exercise naïve.

Notably, no studies have examined the time course of molecular changes in these responses to eccentric and concentric exercise chronically, which will be addressed in Chapter 3.

1.3.2.3 Signalling pathways regulating muscle protein metabolism

The regulation of skeletal muscle protein metabolism in response to exercise involves the transduction of signals towards the apparatus that regulates mRNA translation by the activation of cellular pathways. A number of signalling pathways have been associated with the muscle protein metabolic response to exercise (Figure 1-4).

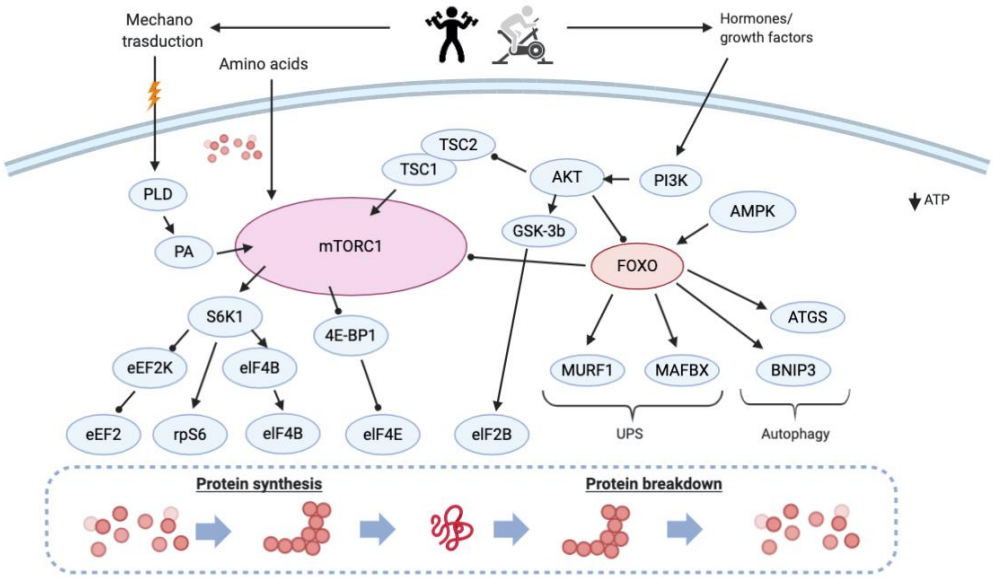

Figure 1-4: Intracellular signalling pathways thought to regulate muscle protein synthesis and muscle protein breakdown. Lines terminating in arrowheads indicate activation; lines terminating in circles indicate inhibition. Abbreviations: AKT, serine threonine kinase 1; AMPK, AMP-activated protein kinase; ATGS, autophagy-related genes; BNIP3, BCL-2/E1B-19kD-interacting protein-3; EEF2, eukaryotic elongation factor 2; EEF2K, eukaryotic initiation factor 2 kinase; EIF2B/4A/4B/4E, eukaryotic initiation factor 2B/4A/4B/4E; 4EBP1, eukaryotic initiation factor 4E-binding protein-1; FOXO, forkhead box-containing proteins O; GSK, glycogen synthase kinase; MAFBX, muscle atrophy F-box; mTORC1, mechanistic target of rapamycin complex 1; MURF1, muscle ring finger protein 1; PLD, phospholipase D; PA, phosphatidic acid; PI3K, phosphoinositide 3-kinase; RPS6, ribosomal protein S6; S6K1, ribosomal protein S6 kinase 1; TSC1/2, tumor suppressor complex 1/2. Adapted from Atherton, Phillips, and Wilkinson (2015).

The mechanistic or mammalian target of rapamycin complex 1 (mTORC1) is often considered to be the main driver of this response (Goodman et al. 2011) (Figure 1-5). This is because the pharmacological inhibition of mTORC1 by rapamycin was found to inhibit resistance exercise training-induced stimulation of MPS (Drummond et al. 2009), lending credence to its importance, previously espoused based on animal models (Bodine et al. 2001). mTORC1 activation is linked to phosphorylation of a number of mRNA "translation factors" such as 4E-binding protein (4EBP1), ribosomal protein S6 kinase (P70S6K1), and eukaryotic initiation factors 4G/A/B (EIF4G/A/B), resulting in the formation of the eukaryotic initiation factor 3F (EIF3F) scaffold, and hence the 48S pre-initiation complex. Alongside this, activation of eukaryotic initiation factor 2B (EIF2B) transfers Met-tRNAi to the ribosome while the 48S pre-initiation complex is being formed, allowing for increased efficiency of translation. Altogether these processes result in the formation of the polyribosome and promotion of mRNA translation (Proud 2009).

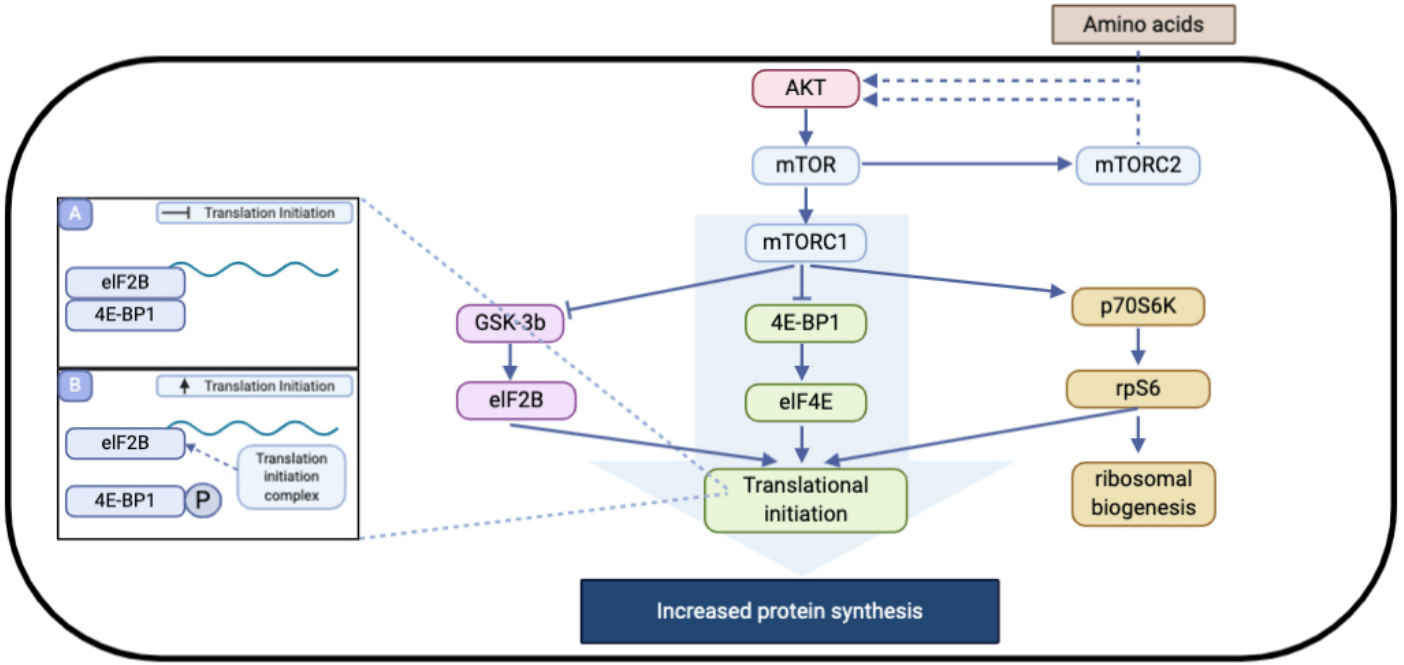

Figure 1-5: The Akt-mTOR signalling pathway involved in translation initiation and increased protein synthesis. During (A), eukaryotic translation initiation factor 4E (eIF4E)-binding protein 1 (4E-BP1) binds to eukaryotic translation initiation factor 4E (eIF4E), hence prohibiting the binding of other proteins involved in translational initiation, effectively inhibiting it. During (B), phosphorylation and hence disinhibition of 4E-BP1 by mTORC1 drives eukaryotic translation initiation factor 4G (eIF4G) and eukaryotic translation initiation factor 4A (eIF4A) recruitment to eIF4E, resulting in the propagation of translation initiation signalling.

However, while mTORC1 activation and the activation of its substrates is an established feature of the regulation of MPS response to exercise, the mechanistic events that occur proximal to its activation remain unclear. In essence, signals that are triggered upstream by exercise result in the activation of signalling pathways like mTORC1 that regulate MPS. However, what exactly these triggers are is a matter of debate. This is because exercise acts as a trigger for a number of events including mechanotransduction (Goodman et al. 2011), endocrine processes (Hansen et al. 2001) and auto/paracrine processes (Bamman et al. 2001).

Early work in animal (Bodine et al. 2001) and cell culture (Rommel et al. 2001) models focused on the role of insulin-like growth factor (IGF1) which is released from the muscle and liver. It was thought that increased activity and expression of IGF and its splice variants activated the insulin-mediated PI3K-serine threonine kinase 1 (Akt)-mTORC1 signalling pathway, thus increasing MPS. However, data from recent studies has led to a re-evaluation of the importance of this pathway. Investigators West et al. (2009) created “high” and “low” hormone environments in healthy young men by varying muscle recruitment volume. They found that “low” or systemic concentrations of anabolic hormones, including IGF1 did not have an impact on acute MPS responses to exercise. Further, 14 days of growth hormone administration has been found not to affect MPS despite increases in serum GH, insulin-like growth factor (IGF1) and IGF1 mRNA expression (Doessing et al. 2010).

The process of conversion of mechanical signals into molecular signals as a response to cellular movement is called mechanotransduction (Figure 1-6). A

number of candidates “mechanosensors” have received attention as activators of intracellular signalling pathways in response to resistance exercise. One of these is protein tyrosine kinase 2 (FAK) protein, which is located in the sarcolemma. There is a notable increase in FAK following muscle loading (Gordon, Fluck, and Booth 2001). Indeed, serum response factor (SRF), the transcription factor for FAK, has been suggested to be associated with the expression of muscle protein itself as demonstrated in stretch-induced hypertrophy in roosters (Fluck et al. 1999).

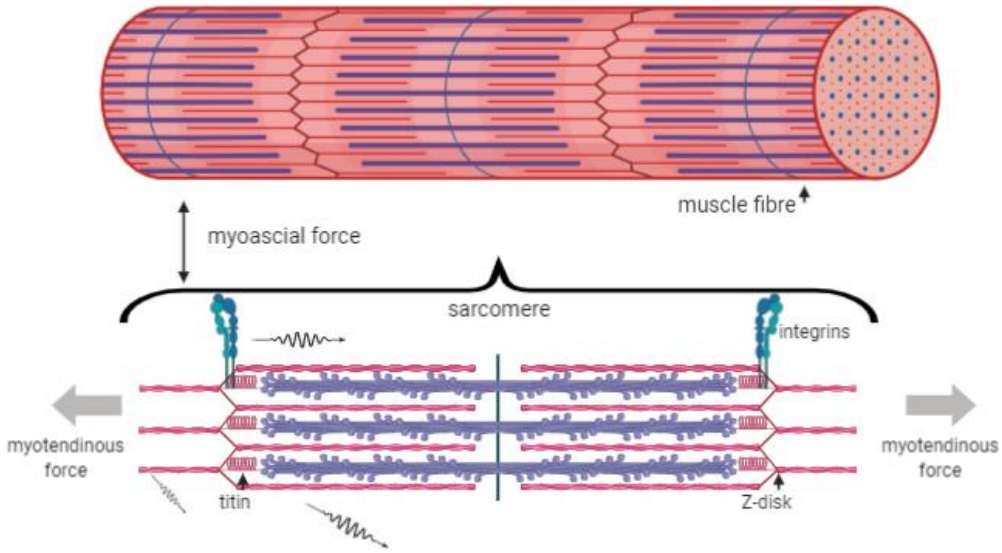

Figure 1-6: Mechanotransduction. Cascade of mechanotransduction signalling in skeletal muscle activated by application of mechanical force to muscle cells (adapted from (Durieux et al. 2007)).

Another sensor of interest is mitogen activated protein kinase (MAPK). MAPK appears to be more related to intensity, and perhaps muscle tension. MAP kinases take part in cell differentiation integrating extracellular mechanical and metabolic cues towards the regulation of downstream gene expression (Mauro

et al. 2002). MAPK phosphorylation has been shown in isolated animal muscle to respond differently to concentric versus eccentric contraction modes (Wretman et al. 2001), and to correlate with peak tension development in rats (Martineau and Gardiner 2001). It is possible, therefore, that the rate of peak tension development, rather than mechanical loading, is more contributory to intracellular signalling processes. Although these intracellular signalling processes control the activity of transcription factors and the expression of genes, the downstream activation of transcription factors, which are said to be responsible for the remodelling of skeletal muscle with hypertrophy, is unexplored.

The molecular regulation of resistance exercise-induced increase in MPB has been difficult to determine, due to very few studies investigating these pathways, and widespread variations in findings among these studies (Murton and Greenhaff 2013; Murton, Constantin, and Greenhaff 2008). In addition, the fact that there are a number of candidate proteolytic systems, including calcium-mediated pathways (Delgado et al. 2001; Jackson, Green, and Miller 1997), caspase-mediated pathways (Fan et al. 2005; Workeneh et al. 2006) and lysosomal degradation (Bechet et al. 2005; Lecker et al. 2004; Sandri 2011) confuses the matter. Nevertheless, skeletal muscle protein breakdown is thought primarily to be dependent on the ubiquitin-proteosome pathway activity, that occurs through muscle-specific E3 ubiquitin ligases, namely (i) muscle atrophy F box (atrogin-1/MAFbx) and (ii) muscle RING finger 1 (MuRF1). These are key regulators of skeletal muscle protein breakdown in catabolic conditions (Sandri 2008). As per this model, activation, and hence dephosphorylation of

forkhead box protein O (FOXO) transcription factors results in up-regulation of MAFbx/atrogin-1 and MuRF1 transcripts (Sandri et al. 2004). The activity of FOXO is mainly modulated by post-translational modification, in that Akt phosphorylation drives the export of FOXOs from the nucleus to the cytoplasm. Hence, Akt is seen as being situated critically at the junction between muscle protein synthesis and degradation.

While this pathway is widely considered to be key to muscle atrophy, it is less clear as to its role vis-à-vis protein breakdown during skeletal muscle hypertrophic adaptation to resistance exercise training, or post-exercise transient changes in muscle protein turnover. Studies that have examined the response in genes related to skeletal muscle breakdown following a bout of resistance exercise have shown MuRF1 mRNA expression to be increased 2-4h post-exercise, while atrogin-1/MAFbx mRNA expression has been found to be increased earlier, and decreased later (>12h) in the recovery period (Coffey et al. 2006; Louis et al. 2007), as well as following a repeat bout of exercise (Murton et al. 2014). While this pattern of expression of proteolytic genes does correspond to the biphasic response of skeletal muscle protein breakdown post-resistance exercise (Phillips et al. 1997), a clear link between these two sets of processes has not been established in humans. Notably, differences in molecular regulation between concentric and eccentric contractions has been observed (Nedergaard et al. 2007), such that there is an increased expression of the ubiquitin-proteosome system components related to structure following the latter, including proteasome subunit alpha 1 (PSMA1), MuRF2, MuRF3 and ubiquitin splice forms I and II.

1.3.2.4 Ribosomal biogenesis and skeletal muscle hypertrophy to resistance exercise

MPS regulation is a coordinated process that requires ribosomes for the translation of mRNA, and a sufficient density of myonuclei for ribosome and mRNA transcription (reviewed extensively by Brook et al. (2019)). Hence MPS regulation depends both on translational efficiency - in terms of ribosomal activity - as well as translational capacity - in terms of ribosome number (Millward et al. 1973).

Ribosomes are composed of RNA in the form of ribosomal RNA (rRNA). The transcription of rRNA is carried out by RNA polymerase 1 (POL1) in the form of a 47S pre-rRNA. This undergoes subsequent cleavage into 28S, 18S, 5.8S to form a mature ribosome by assembling with ribonuclear proteins (Chaillou, Kirby, and McCarthy 2014). Transcription intermediary factors 1A (TIF-1A), Transcription intermediary factors 1A (TIF1B) and upstream binding factor (UBF) are pivotal in the formation of a pre-initiation complex with POL1 at their rDNA (ribosomal DNA) promoter. TIF1A and UBF transcriptional regulation occurs via a number of signalling proteins, such as AMPK, mTORC1, MAPK and S6K1 (reviewed in Kusnadi et al. (2015)). This allows for regulation of ribosomal biogenesis to be impacted by several pathways including nutrients, hormones and contractile activity.

A regulator of key import in ribosomal biogenesis is c-MYC proto-oncogene (c-MYC), which is a protein that participates in the regulation of cell growth and most facets of ribosome formation (Brook et al. 2019). Direct upregulation of several proteins that contribute to transcriptional control of rDNA, such as TIF-

1A, TIF1B, UBF and Pol1, is driven by c-MYC (Brook et al. 2019). In addition, c-MYC drives transcription of POL1 through rDNA chromatin structure remodelling, and by direct interaction with the SL1 (Selective factor 1) complex, allowing for stabilisation at the promoter of POL1 recruitment (van Riggelen, Yetil, and Felsher 2010). It has been shown that changes in MPS from one day to the next that eventually contribute to the maintenance of muscle mass in healthy individuals occur mainly through transient elevations in translational efficiency, in the absence of substantial change in RNA content (Chesley et al. 1992). It has therefore been suggested that ribosome number needs to be maintained to a sufficient degree to keep up with muscle protein synthetic requirements of nutritional intake and regular activity (Brook et al. 2019). Indeed, pathways associated with ribosomal biogenesis are closely linked to those that control translational efficiency such as the mTORC1 pathway (Mayer and Grummt 2006). Further, biomarkers associated with increased ribosomal biogenesis, such as raised c-MYC or 47S pre-RNA, have been detected in response to muscle loading over a chronic period, and with nutritional changes (Stec et al. 2016).

Animal models of overload-induced hypertrophy have demonstrated a rapid early increase in ribosomal biogenesis (Marino et al. 2008; Miyazaki et al. 2011; McCarthy et al. 2011; West et al. 2016). In humans, stimulation of muscle contraction has also been shown to cause a rapid increase in expression of markers of rDNA transcription. As an example, there is increased expression of 45S-pre-RNA 24h post-resistance exercise (Stec et al. 2016). In addition, RNA content has been demonstrated to be increased following 2 bouts of acute

resistance exercise (Bickel et al. 2005). This response has been shown to be sustained at the 3-week and 6-week time points of a resistance exercise training protocol (Brook et al. 2015).

Studies have also used k-means cluster analysis to demonstrate the importance of ribosome biogenesis in muscle hypertrophy. In a study by Stec et al. (2016), healthy adults (n=42) aged 60-75 years were divided into 3 responder groups based on degree of hypertrophy of type II muscle fibres following a 4-week resistance exercise training program: (i) no change (-7%); (ii) moderate increase (22%); and (iii) extreme increase (83%). At the start of the training protocol all groups showed similar RNA content. However, at the end of training, in (i), no increase in concentration of RNA was detected; in (ii), a trend (+9%) towards an increase was detected; and in (iii), and a significant increase was shown. These findings were reproduced in young volunteers who were clustered based on alterations in thickness of the vastus lateralis (Mobley et al., 2018). Furthermore, age-associated deficits in long-term MPS, and consequent hypertrophy have also been shown to be associated with poor ribosomal biogenesis and c-MYC induction (Brook et al. 2016).

Overall, the above studies indicate that in those with the most hypertrophic change following training, enhanced translational capacity appears to be a feature. Whether or not this is a prerequisite for, or limitation to hypertrophy is not currently known with clarity. In a study comprising a large sample (n=66) of individuals of mixed gender and ages (Kim et al. 2007), following the first exercise bout, total RNA content was found to be increased only in responders who demonstrated extreme hypertrophic response, which would suggest that

those who are able to increase ribosomal capacity have greater hypertrophic potential. However, after 16 weeks of training, non-responders and moderate responders demonstrated similar RNA content increases, suggesting that growth of muscle is multifaceted and is unlikely to be dependent solely on enhancement of translational capacity.

1.3.2.5 The role of satellite cells in resistance exercise-induced muscle hypertrophy

Satellite cells are skeletal muscle specific stem cells are located between the sarcolemma and basal lamina of muscle fibres in a quiescent state. They can be stimulated to divide in response to external stimuli such as exercise and muscle damage (Zammit 2008). On activation, a satellite cell can fuse with an existing myofibre, as a result of which a myonucleus is added to the fibre syncytium. While it is clear that satellite cells contribute to muscle regeneration, whether or not they have a role to play in hypertrophy in adult skeletal muscle is widely debated (O'Connor et al. 2007). That satellite cells contribute to muscle hypertrophy relies on the notion of a myonuclear domain: a theoretical concept referring to the amount of cytoplasm associated with a single nucleus. Proponents argue that each myofibre consists of many myonuclear domains (Allen, Roy, and Edgerton 1999). Satellite cells act as a source of myonuclei at a rate commensurate with the maintenance of a stable myonuclear domain size during skeletal muscle growth in normal conditions. Therefore, they increase the overall number of myonuclei, and hence the genetic material required for production of protein without altering protein synthetic kinetics for each nucleus significantly. Muscle fibres have been shown to be unable to grow beyond 17%

during hypertrophy without the addition of more nuclei through the satellite cell cycle (Kadi et al. 2004). It has been suggested, therefore, that while muscle fibres can undergo hypertrophy without a corresponding increase in myonuclear number (McCarthy and Esser 2007), this occurs only up to a limit, at which point satellite cell activation may be necessary for the continuation of muscle growth. Further, Bruusgaard et al. (2010) have shown that the addition of muscle fibres via satellite cell activation appears to be protected from apoptotic processes during muscle atrophy, perhaps contributing to the maintenance of the myonuclear domain post-disuse. In addition, up-regulation of a number of myogenic regulatory factors (MRFs), serving as proxies for satellite cell activation, differentiation and proliferation has been demonstrated in the hours following an acute bout of resistance exercise (McKay et al. 2008). However, in several experimental models that featured depletion of satellite cells (McCarthy et al. 2011) and inhibition of myostatin (Wang and McPherron 2012), hypertrophy of skeletal muscle fibres has been demonstrated without satellite cell incorporation. Indeed, where there is a 90% depletion of satellite cell population, muscle hypertrophy in response to overload has been demonstrated to be normal, while regeneration in response to acute muscle injury has been found to be impaired, indicating that the role played by satellite cells differs substantially between these two paradigms of muscle growth (McCarthy et al. 2011).

1.4 Anabolic response to feeding and exercise in ageing and obesity

1.4.1 Blunting of anabolic response in ageing

In early studies, age-related loss of muscle mass in the elderly, known as sarcopenia (Mitchell, Williams, et al. 2012), was often ascribed to reduced basal rates of MPS (Yarasheski, Zachwieja, and Bier 1993; Rooyackers et al. 1996; Welle et al. 1993), and high basal rates of MPB (Trappe et al. 2004), or a combination of the two resulting in an overall negative protein balance. In these studies, rates of basal MPS were found to be higher by 20-30%, and markers of MPB by ~50% in the elderly compared to the young. However, these observed differences were too large to convincingly align with the observed rate of muscle wasting observed in the elderly, which had been found in longitudinal studies to occur at a rate of 0.5-1.5% per year between 50-80 years of age (Hughes et al. 2001). Furthermore, there was a general lack of replication of these findings by other investigators (Volpi et al. 2001; Volpi et al. 1998). These inconsistencies in the data, in combination with data from rodent models (Mosoni et al. 1995) and humans (Pannemans et al. 1998) that have shown that protein nutrition requirements might be greater as age advances, led to the proposition that the mechanisms underpinning decline in muscle mass with age were related to an inefficiency in the elderly to utilise amino acids towards MPS relative to the young.

Cuthbertson et al. (2005) investigated whether age-related muscle wasting was a result of diminished metabolic response to amino acid ingestion. They measured muscle myofibrillar and sarcoplasmic muscle protein synthetic rates

in 20 young and 24 old men of similar BMI, as the rate of incorporation of [^{13}C] leucine from a primed, constant infusion of [1-^{13}C] α-ketoisocaproic acid. Muscle samples were taken 3h following the ingestion of 0-40g of essential amino acids in order to determine dose-response relationships. A diminished muscle protein synthetic response to essential amino acid ingestion was noted in the elderly. Moreover, there was reduced activation of anabolic signalling; that is, an adverse phosphorylation state of mTOR, p70s6 kinase and eIF4BP-1 in the elderly relative to the young. That the anabolic response to amino acid ingestion in elderly muscle is blunted has been confirmed by other investigators since (Katsanos et al. 2005, 2006; Paddon-Jones et al. 2004). Similarly, studies also indicate that the anabolic response to exercise is blunted in the elderly relative to young volunteers. Kumar, Selby, et al. (2009) investigated the myofibrillar protein synthetic response to resistance exercise at intensities ranging from 20-90% of 1 repetition maximum (1-RM) in healthy, young and old men. Muscle samples were obtained before, immediately after and 1, 2- and 4h post-exercise, and myofibrillar protein synthetic rate was measured via the incorporation of [1,2-(13)C] leucine. While there was a sigmoidal dose-response relationship between MPS 1-2h post-exercise, and exercise intensity, this was blunted in the older men.

However, it is unclear what contributes to anabolic resistance per se. A possible driver of age-related muscle loss in the elderly could be reduced habitual physical activity. Inactivity in the form of two weeks of unilateral lower limb immobilisation has been shown to cause anabolic resistance in young adults (de Boer et al. 2007; Glover et al. 2008). Since aging is associated with reduced

habitual physical activity (Frisard et al. 2007; Ivey et al. 2000), this may contribute to reduced anabolic sensitivity to protein nutrition in the elderly. This is supported by the fact that physical activity has been demonstrated to render the muscle more anabolically responsive to amino acid ingestion in young males (Burd et al. 2011). Another possible mechanism could be metabolic inflexibility (Storlien, Oakes, and Kelley 2004) of elderly muscle, which is explained in more detail in Section 1.4.2.

1.4.2 Blunting of anabolic response in obesity

Obesity is defined as excessive fat accumulation; it poses a significant health risk, and is also a significant risk factor in a number of chronic conditions including diabetes, cardiovascular disease and cancer (Pi-Sunyer 1999). Studies have examined the anabolic response to feeding and exercise in obesity in both the young and old. Early studies in young volunteers demonstrated both elevated (Nair et al. 1983) as well as lowered (Guillet et al. 2009) muscle protein synthetic rates in obesity compared to healthy weight controls in the post-absorptive state, while more recently, basal MPS has been shown to be similar in both young (Beals, Sukiennik, Nallabelli, Emmons, van Vliet, Young, Ulanov, Li, Paluska, De Lisio, et al. 2016; Beals, Skinner, McKenna, Poozhikunnel, Farooqi, van Vliet, Martinez, Ulanov, Li, Paluska, et al. 2018) and older (Murton et al. 2015) volunteers. Murton et al. (2015) sought to determine whether obesity worsened the blunting in anabolic response to protein nutrition noted in the elderly (Cuthbertson et al. 2005), and whether it impacted upon muscle mass and strength. For this, the investigators recruited 11 obese and 15 healthy-weight men between the ages of 55-75 years and determined muscle protein synthetic

and leg protein breakdown rates under postprandial conditions using a hyperinsulinemic hyperaminoacidemic-euglycemic clamp. Administration of insulin and amino acids stimulated an increase in muscle protein synthetic rates in healthy-weight volunteers alone. However, the lowered muscle protein synthetic response in the obese was offset by a reduction in leg protein breakdown, which was not noted in the lean subjects. Obesity was also associated with greater leg fat mass and changes in gene transcripts, consistent with muscle de-conditioning. Further, no differences were noted in leg lean mass between obese and lean volunteers, or in strength or work done during isometric and isokinetic leg function tests between the two groups.

Recently, Beals and colleagues (2016) aimed to examine the effects of ingestion of protein in the form of 6 ounces of lean pork on muscle protein synthetic rates in young obese, young overweight, and young healthy-weight males (n=10 for each group) using stable isotope labelled amino acids (in this case L-[ring-13C6] phenylalanine with the primed continuous method). Following lean pork ingestion, they demonstrated a significant increase in the myofibrillar protein synthetic response in the healthy-weight group, but not in overweight and obese groups, which was in agreement with findings from previous studies in young volunteers (Guillet et al. 2009; Chevalier et al. 2015). Similar to Murton et al. (2015), leg lean mass was found to be greater in the obese.

Smeuninx et al. (2017) aimed to determine the myofibrillar protein synthetic response to moderate protein ingestion in the form of a milk protein isolate drink in 10 older lean, 7 older obese, and 18 young lean male volunteers using a stable amino acid isotope tracer infusion approach. They found that post-prandial rates

of myofibrillar protein synthesis rose by approximately 81% in young lean, 38% in older lean and only 9% in older obese volunteers. Hence, in line with Murton et al. (2015), they found that obesity worsened the already impaired myofibrillar protein synthetic response to moderate doses of protein ingestion in older individuals. Further, also similar to Murton et al. (2015), leg muscle fibre area was in fact greater in the old obese versus the old lean group.

Most recently, Beals and colleagues (2018) aimed to examine the impact of obesity on the anabolic response to both combined feeding and resistance exercise in the young. For this they recruited 9 healthy-weight and 9 obese young adults who received infusions of L-[ring-$^{13}C_6$] phenylalanine. Blood samples were taken in the basal and fed states, as were muscle biopsy samples from one leg that performed exercise, and from the contralateral non-exercise leg. Volunteers performed a bout of exercise consisting of unilateral leg extensions, and immediately after, consumed 36g of protein in the form of lean pork. Pork ingestion stimulated MPS in both legs in the two groups, and this response was greater in the exercise leg than in the non-exercise leg. However, the MPS response in the exercise leg was significantly lower in the obese group compared to the healthy weight group. Furthermore, this was associated with deranged anabolic signalling, in that while pork ingestion increased p70S6K phosphorylation in the fed state in both legs in both groups, this response was diminished in the exercise leg in the obese group compared to the young.

Overall, research thus far indicates that muscle protein synthetic responses to protein nutrition are impaired in the obese. However, while anabolic response is adversely affected, leg lean mass in both the young and old, and leg muscle fibre

area in old volunteers is paradoxically greater in the obese, suggesting that the metabolic health of muscle is impaired in obesity rather than its size or function. One possible explanation for this could be that higher body weight in obesity results in a greater leg workload, which acts as a more profound stimulus for hypertrophy, increasing muscle mass in the legs more significantly relative to healthy-weight controls.

The mechanisms that contribute to the blunted anabolic response in the obese are unclear. Skeletal muscle is known to display a remarkable degree of metabolic flexibility when challenged by changes such as alteration in diet composition or energy deprivation (Storlien, Oakes, and Kelley 2004). In lean persons, in insulin-stimulated conditions, muscle switches from mainly fat oxidation and increased fatty acid uptake to inhibiting lipid breakdown and increasing glucose uptake, oxidation and storage (Kelley and Mandarino 2000). However, obese individuals manifest greater fat oxidation rates in muscle, and are relatively insulin resistant, rendering them metabolically inflexible (Kelley et al. 1999). It is possible that this metabolic inflexibility of muscle in the obese transiently restricts the efficiency of mitochondrial ATP resynthesis towards increasing MPS due to the greater oxygen consumption associated with fat oxidation. As mentioned earlier, metabolic inflexibility of muscle is possibly a feature in the elderly as well, given the increase in fat mass that is associated with increasing age (Kuk et al. 2009), and the fact that muscle in the elderly has been shown to have reduced mitochondrial content (Carter, Chen, and Hood 2015). It is therefore possible that diminished muscle metabolic quality and resilience of muscle contributes to the anabolic resistance to protein feeding in

the elderly (Cuthbertson et al. 2005). This in turn might be exacerbated in obesity, resulting in the worsening of the anabolic response noted in the elderly obese compared to normal-weight controls (Murton et al. 2015; Smeuninx et al. 2017). This will be addressed in Chapter 5.

It has also been proposed that inflammation might play a role in the blunted anabolic response in the elderly obese. This is because a chronic low-grade systemic inflammatory state has been associated with both obesity (Ouchi et al. 2003; Ouchi et al. 2011; Esposito et al. 2002; Hotamisligil, Shargill, and Spiegelman 1993; Kern et al. 1995; Wood et al. 2005; Ziccardi et al. 2002), as well as ageing (Bruunsgaard et al. 1999; Bruunsgaard et al. 2000; Cohen et al. 1997; Ferrucci et al. 2005; Harris et al. 1999; Kania et al. 1995; Paik et al. 2013; Paolisso et al. 1998; Puzianowska-Kuznicka et al. 2016). However, it is unlikely that inflammation is what drives muscle anabolic blunting in these individuals since muscle inflammatory cytokines were not shown to be elevated in the anabolically resistant obese elderly volunteers relative to healthy-weight controls in the aforementioned study by Murton et al. (2015).

1.5 The influence of inflammation on muscle mass and metabolic regulation

A heightened inflammatory state has been associated with alterations in muscle protein turnover and mass in both animals (Combaret et al. 2002; Frost et al. 2007; Garcia-Martinez, Lopez-Soriano, and Argiles 1993; Goodman 1994; Haddad et al. 2005; Zamir et al. 1992; Zamir et al. 1991) and humans (Puthucheary et al. 2013; Puzianowska-Kuznicka et al. 2016; Schaap et al. 2009; Schaap et al. 2006). However, whether inflammation influences muscle mass

and metabolic regulation is controversial. While conditions featuring a heightened state of inflammation have been shown to feature widespread and rapid alterations in muscle mass (Hasselgren et al. 1987) and muscle metabolism (Mizock 2001) in both rodents and humans, studies have as yet been unable to pinpoint the mechanistic drivers of these changes in states of overt inflammatory burden. Furthermore, whether non-overt inflammation, such as resistance exercise-induced muscle inflammation, or the low-grade chronic systemic inflammation observed in ageing (see Section 1.4.1) and obesity (see Section 1.4.2) has any effect at all on muscle mass regulation is controversial.

1.5.1 The influence of overt inflammation on muscle mass loss and muscle metabolism in the critically ill

Sepsis is a potentially life-threatening condition caused by the body's response to infection, characterised by widespread, uncontrolled systemic inflammation. It is a significant cause of morbidity and mortality in ICUs globally (Bone et al. 1992). During sepsis, a massive, uncontrolled systemic response occurs in response to the presence of microbial antigens in the circulation (Cohen 2002), and the organs most frequently afflicted include the genitourinary tract, the lungs and the abdominal cavity (Angus et al. 2001). Muscle undergoes rapid wasting (Puthucheary et al. 2013). Indeed, in critically ill patients, the degree of muscle wasting in sepsis is an independent predictor of mortality (Ali et al. 2008). Muscle wasting has been found to be the main driver of physical disability in patients who survive critical illness (Herridge et al. 2003). This is found to persist in these patients following discharge (Pfoh et al. 2016), associating closely with mortality rates (Dinglas et al. 2017). Hence, the National Institute for Health and

Clinical Excellence (NICE) guidelines list both physical disability and muscle wasting as public health issues. Muscle wasting induced by sepsis, and consequent weakness hence clearly has a detrimental impact on both recovery trajectories as well as survival rates.

1.5.1.1 Molecular mechanisms underpinning inflammation-induced muscle mass loss in endotoxemia

There has been a very limited number of clinical trials in patient volunteers investigating the mechanisms underlying muscle mass loss in the context of inflammation (reviewed in detail in Crossland et al. (2019); Figure 1-7), in particular because it is very difficult to obtain baseline measurements from these volunteers. However, some investigators have circumvented this experimental hurdle by conducting acute studies in which endotoxins are administered to healthy volunteers. These experiments demonstrated an acute attenuation of MPS as well as MPB, alongside amino acid efflux, indicating that endotoxin administration did not affect muscle protein balance in these volunteers (Vesali et al. 2009). However, it is difficult to reconcile the findings of these studies in healthy human volunteers with the observed dramatic and rapid muscle mass loss in patients in inflammatory states (Debigare, Cote, and Maltais 2001; Puthucheary et al. 2013). The few studies that have investigated the effect of endotoxin infusion on muscle protein balance in the post-prandial state further confuse the issue. In these studies, amino acid administration has been shown to aid the reversal of muscle protein loss in the post-prandial state versus the post-absorptive state. However, muscle protein synthetic and muscle protein

breakdown rates were found to be no different between interventions (Rittig et al. 2016).

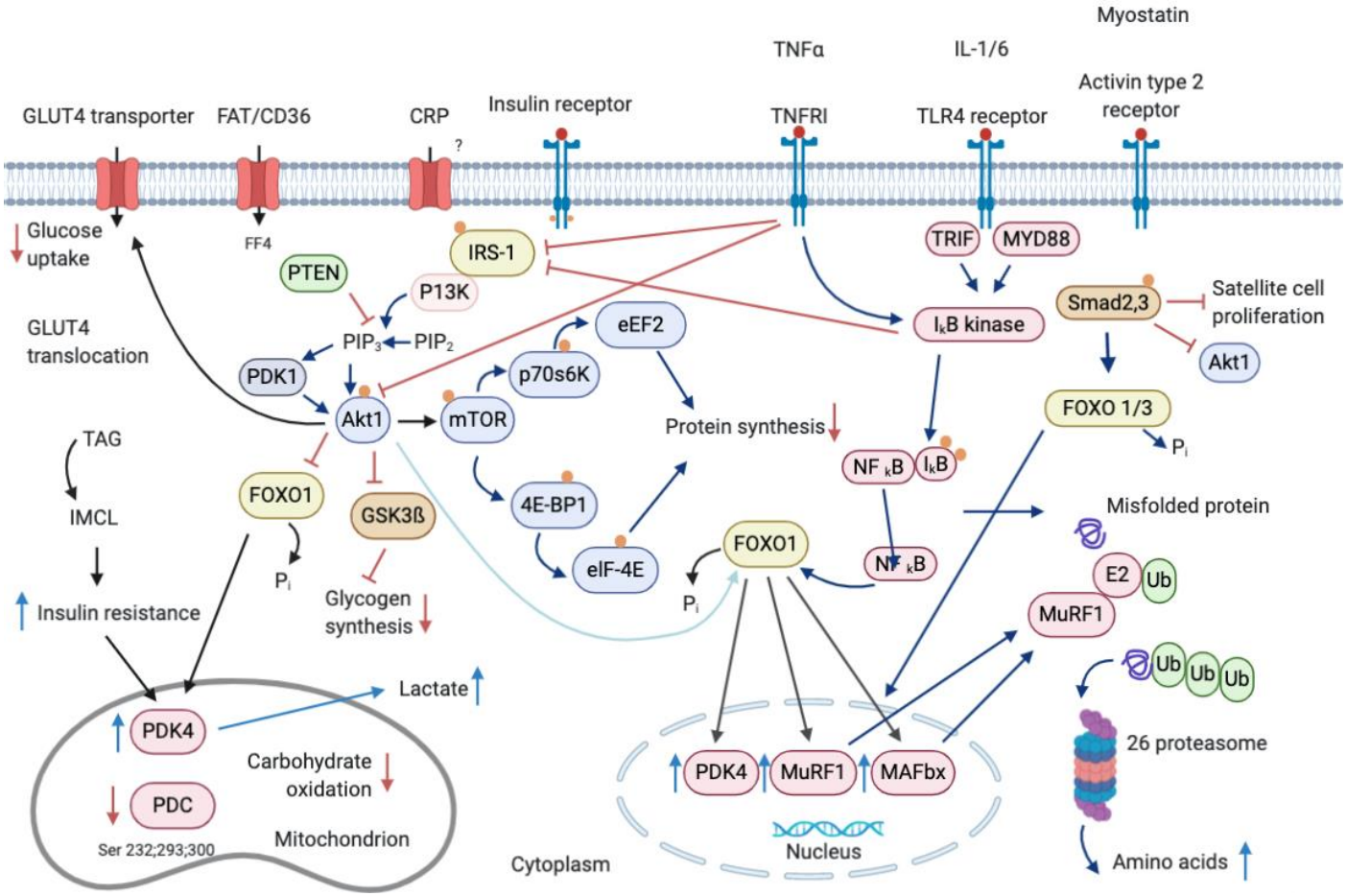

Figure 1-7: Integration of molecular pathways linked to muscle protein synthesis, muscle protein breakdown and muscle metabolism in muscle mass loss. This depiction is theoretical as such an integration has not been demonstrated in humans. Adapted from Crossland et al. (2019).

Abbreviations: GLUT4, Glucose transporter type 4; TAG, triacylglycerol; IMCL, intramyocellular lipid; PDK4, pyruvate dehydrogenase kinase 4; PDC, pyruvate dehydrogenase complex; FAT, fatty acid translocase; CD36, cluster of differentiation 36; FF4, long-chain fatty acid receptor FF4; PTEN, phosphatase and tensin homolog; PDK1, pyruvate dehydrogenase kinase 1; FOXO1, forkhead box protein O 1 ; Pi, inorganic phosphate; CRP, C-reactive protein; PIP3, phosphatidylinositol 3,4,5 trisphosphate ; PIP2, phosphatidylinositol 4,5-bisphosphate ; IRS-1, insulin receptor substrate 1; PI3K, phosphatidylinositol-3-kinase; AKT1, serine threonine kinase 1; GSK3B, glycogen synthase kinase 3 beta; mTOR, mammalian target of rapamycin; p70s6K, ribosomal protein S6 kinase beta-1; Eef2, eukaryotic elongation factor 2; MuRF1, muscle RING-finger protein-1; MAFbx, muscle atrophy F-box; IL-1/6, interleukin 1/6; TLR4, toll like receptor 4; TRIF, TIR-domain-containing adapter-inducing interferon-β; MYD88, myeloid differentiation primary response 88; IkB, IKK complex; NFkB, nuclear factor-kappa B ; E2, ubiquitin conjugating enzyme; Ub, ubiquitin.

Puthucheary et al. (2013) used stable isotope tracers to measure temporal changes in MPS and leg protein breakdown 24h post-ICU admission. It was found that, in the post-absorptive state, MPS in patients was reduced on day 1 compared to healthy controls. However, by day 7 MPS had risen regardless of nutritional status to rates that were similar to the post-prandial state in healthy controls. In addition, leg protein breakdown was elevated in patients throughout the trial. Further, and in line with these findings, the investigators showed that muscle wasting in the first week of critical illness, measured as muscle CSA

(ultrasound) and muscle protein-to-DNA ratio, occurred early and at a rapid rate. Wasting was found to be worse in patients with multi-organ failure versus single organ failure. The findings from this study of atrophy and negative muscle protein balance after ICU admission are in line with studies that showed that within 6-8h of admission to ICU, a significant rise in muscle mRNA expression of cytokines, and a profound inactivation of those proteins that regulate translation initiation factor activation and MPS relative to sex and age-matched healthy controls (Constantin et al. 2011a) occurred. Also in line with the pattern of elevated leg protein breakdown demonstrated by Puthucheary et al. (2013): an inhibition of the Akt/mTOR/S6K1 signalling cascade was accompanied by increased expression levels of muscle-specific E3-ligase (MAFbx and MuRF1) and 20S proteasome mRNA and protein expression relative to control (Constantin et al. 2011a). In catabolic conditions, the development of muscle atrophy has been linked to Akt/FOXO signalling, possibly via activation of the ubiquitin/proteasome pathway (Crossland et al. 2008). In animal models, upregulation of skeletal muscle MAFbx and MuRF1 mRNA expression has been demonstrated hours following the onset of endotoxaemia (Dehoux et al. 2003; Wray et al. 2003). This appears to be due to reduced Akt phosphorylation resulting in an activation of FOXO transcription factors and elevated expression of downstream FOXO targets, including MAFbx and MuRF1 (Crossland et al. 2008). These molecular events suggest that the ubiquitin/proteasome pathway might be the main driver of MPB early on.

Hence, in the early stages of ICU admission, it is apparent that significant acute changes occur in muscle CSA, protein turnover and the molecular events linked

to its regulation. However, bed rest also contributes to muscle wasting in these conditions, and the drivers through which it exerts this influence are different from those of inflammation in humans. In humans, bed rest leads to pronounced inhibition of MPS which in turn leads to muscle atrophy; whilst in ICU patients with heightened systemic inflammation, MPS is suppressed and MPB is elevated, leading to rapid muscle atrophy (Puthucheary et al. 2013). The importance of the role of cytokines has led to the development of anti-cytokine therapies to attenuate the systemic inflammation in these states, which have included antibodies against the tumor necrosis factor (TNF alpha), soluble TNF alpha receptors and interleukin-1 (IL-1) beta receptor antagonists. While TNF alpha blockade has been demonstrated to have a positive impact on survival in shock in animals (Beutler, Milsark, and Cerami 1985; Tracey et al. 1987), trials subsequently in patients with sepsis did not reveal any benefits (Reinhart and Karzai 2001). Similarly, IL-1 beta inhibition by a recombinant receptor antagonist has been found to reduce animal mortality in shock in animal models (Ohlsson et al. 1990), but these findings could not be replicated in human trials (Opal et al. 1997) highlighting the need for human based studies to further our knowledge on this topic.

1.5.1.2 Inflammation-induced insulin resistance in muscle

Muscle atrophy is stimulated by several catabolic conditions frequently linked to insulin resistance, including inflammation (Hasselgren et al. 1987), and it has also been suggested that there exists between muscle atrophy and insulin signalling a certain degree of molecular cross-talk in inflammatory states (Crossland et al. 2019). Indeed, in severe sepsis, endogenous glucose production,

glucose oxidation, and whole-body glucose disposal are known to be perturbed (Chambrier et al. 2000), which in turn are also associated with greater rates of mortality (Falciglia et al. 2009).

Most of the studies examining the molecular and cellular mechanisms associated with inflammation-induced insulin resistance have been carried out in animal models. Elevated levels of lactate in blood is a common feature of sepsis (Crossland et al. 2019). Based on animal studies, it has been suggested that this might be due to an impairment in pyruvate dehydrogenase complex (PDC) activity, resulting in the curbing of utilisation of pyruvate in the mitochondria, stemming from an upregulation of pyruvate dehydrogenase kinase (PDK) by sepsis (Vary 1999; Alamdari et al. 2008). TNF alpha has been suggested to play a role in insulin resistance (Plomgaard et al. 2005), perhaps via inhibition of insulin signalling substrate 1 (IRS-1) (Hotamisligil et al. 1996; del Aguila, Claffey, and Kirwan 1999). Hence, it is possible that elevated cytokines might contribute to alterations in carbohydrate metabolism and states of inflammation, which has been suggested to partially be caused by disturbances in Akt signalling (Crossland et al. 2008). Indeed, it has been found in vivo that drugs that suppress muscle inflammation also attenuate carbohydrate metabolism dysregulation in muscle (Cree et al. 2010; Crossland et al. 2017; Crossland et al. 2010).

In addition, increases in circulating C-reactive protein (CRP) have been associated with parameters such as body fat, fasting insulin levels and several conditions featuring metabolic dysregulation (Festa et al. 2000). It has in fact been suggested that CRP might play a role in disturbances in substrate metabolism (Crossland et al. 2019). CRP has been shown to bind to low density

and very low density lipoproteins (De Beer et al. 1982), and to affect macrophage phenotype (Dong and Wright 1996), which has been implicated in insulin resistance modulation. However, a direct association between circulating levels of CRP and insulin resistance has not been established.

1.5.2 Non-overt inflammation and the hypertrophic response to concentric versus eccentric exercise

It is well established in human studies that high load eccentric exercise results in greater plasma levels of pro-inflammatory cytokines than high load concentric exercise (Paulsen et al. 2012). Further, a gene transcript profiling study has shown that a single bout of high-load eccentric exercise induces greater inflammatory gene responses in human quadriceps muscle than concentric exercise (Chen et al. 2003), likely due to the greater forces generated by lengthening actions (Katz 1939). High load eccentric training has been reported by some to produce greater muscle hypertrophy than high load concentric training (Hather et al. 1991), and whether the greater degree of inflammation following the former is contributory has been examined in acute studies. Animal and in vitro studies have explored the link between eccentric exercise-induced inflammation and the mechanisms underlying muscle growth, although chronic exercise intervention studies are missing. These studies demonstrated an increase in the expression of pro-inflammatory cytokines interleukin-6 (IL-6), TNF alpha and IL-1 beta in rodent muscle up to the fifth day after a bout of eccentric exercise (Peake et al. 2005), accompanied from 24 to 96h post-exercise by an increased expression of myoblast determination protein 1 (MyoD) and myogenic factor 5 (MYF5), myogenin and myogenic factor 6 (MYF6) which are

myogenic regulatory factors (MRFs) (Armand et al. 2003). Furthermore, IL-6 deficiency has been shown to promote satellite cell-mediated hypertrophy in isolated cells (Serrano, Baeza-Raja, Perdiguero, Jardí, et al. 2008). In humans it has been demonstrated that a bout of 300 maximal unilateral lengthening contractions increased satellite cell expression 24h post-exercise, which peaked at 72h (McKay et al. 2009). This was associated with an increase in muscle IL-6 mRNA expression, which correlated strongly with an increase in serum IL-6 concentration ($r^2 = 0.89$, $p<0.01$).

One way of understanding how eccentric exercise-induced inflammation impacts upon the mechanisms regulating muscle hypertrophy is by comparing molecular responses to high load concentric versus high load eccentric exercise. In subjects who performed an acute bout of concentric exercise in one leg, and eccentric exercise in the contralateral leg, Kostek et al. (2007) demonstrated that 51 genes associated with protein synthesis, stress response/early growth, and sarcolemmal structure were differentially expressed between the two limbs within 24h of the bout. However, no chronic studies thus far have examined the muscle mRNA response to concentric versus eccentric chronically, and this will be addressed in Chapter 3.

Studies investigating the effect of a combination of non-steroidal anti-inflammatory drug (NSAID) administration and eccentric exercise on myogenesis and muscle mass regulation have also examined the influence of inflammation on exercise-induced muscle growth, since the chief therapeutic use of NSAIDs is the reduction of pain by alleviating inflammatory response. NSAIDs are widely used medications (Warner et al. 2002) that act by

competitive inhibition of isoforms of the cyclooxygenase (COX) enzyme (Scholer et al. 1986), effectively blocking prostaglandin production. Prostaglandins contribute to inflammation, pain and changes in body temperature (Figure 1-8).

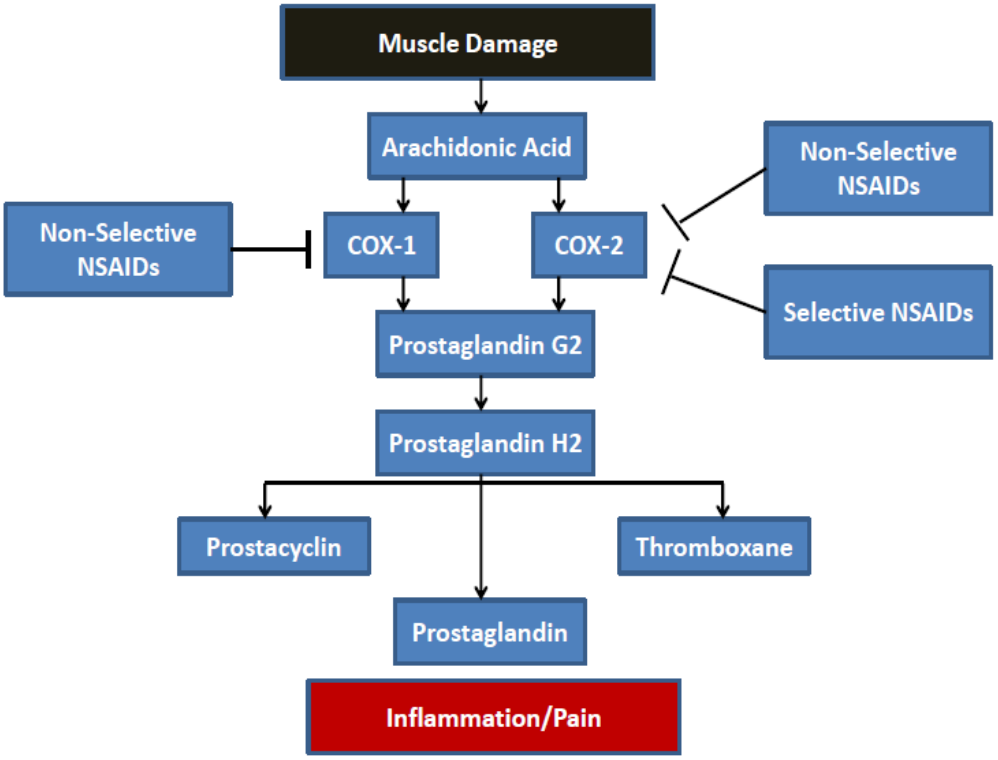

Figure 1-8: Schematic of COX inflammatory pathway and probable mechanism of action of non-steroidal anti-inflammatory drugs.

Mikkelsen et al. (2009b) recruited 8 young male endurance athletes, who were made to perform a bout of maximal lengthening contractions in each leg, during which one leg received an infusion of the NSAID indomethacin, and the contralateral leg received a placebo infusion. They found that on the seventh day post-exercise, the number of Pax7(+) cells per myofibre was raised significantly (by 96%) in the leg infused with placebo, but not in the leg infused with indomethacin, suggesting a positive impact of inflammation on satellite cell

proliferation. In contrast, Mackey et al. (2016) found that ingestion of 1200mg/day of the NSAID ibuprofen daily for two weeks before a bout of electrically evoked eccentric contractions resulted in a potentiation of satellite cell activation (ActiveNotch1^{+} satellite cells) during the first week of recovery post-exercise, suggesting that satellite cell activation and hence regeneration of human skeletal muscle was expedited by the anti-inflammatory effect of NSAIDs. However, it is important to note that the electrically evoked eccentric contraction protocol in the study by Mackey et al. (2016) caused extensive muscle fibre damage, which may explain the divergence in findings from Mikkelsen et al. (2009a). In another study, Trappe et al. (2002) recruited 24 young male volunteers who performed a bout of 10-14 sets of 10 supramaximal eccentric knee extensions. Sets were separated by a minute of rest. Subjects then consumed either 1200mg/day of ibuprofen, 4000mg/day of paracetamol or a placebo, each drug divided into three doses per day. At 24 hours post-exercise, MPS was found to be significantly greater (~76%) in the placebo group. The authors concluded that consumption of the non-selective COX-1 inhibitor suppressed MPS by inhibiting prostaglandin F2 alpha (PGF2alpha), which has been shown to be a potent regulator of MPS in many studies (Horsley and Pavlath 2003; Palmer 1990; Rodemann and Goldberg 1982). It is worth noting that other acute studies are not wholly in agreement with these findings. In the aforementioned study by Mikkelsen et al. (2011), muscle samples obtained at 24-48h post-exercise showed no significant differences in either myofibrillar FSR (fractional synthetic rate) or muscle collagen FSR between the NSAID-infused leg versus the leg infused with placebo.

It is unclear whether early inflammatory responses to high-load eccentric exercise persist in more chronic eccentric exercise intervention studies, and hence contribute to greater muscle hypertrophy with chronic training. A limited number of studies have examined the impact of chronic NSAID administration on muscular adaptation to sustained eccentric exercise training. Krentz et al. (2008) conducted a double-blind study during which 18 young volunteers (males, n=12; females, n=6) were randomly assigned a 400mg dose of ibuprofen following combined concentric-eccentric resistance exercise of the elbow flexors in one arm, and placebo following exercise of the other arm on the subsequent day. Subjects trained 5 days a week, performing 6 sets of 4-10 repetitions per session. No significant difference in muscle thickness was noted between the arms after 6 weeks. Trappe et al. (2011), also utilising a double-blind design, investigated the effect of chronic NSAID ingestion on skeletal muscle adaptations to progressive resistance training in elderly male (n=24) and female (n=12) volunteers. Participants were randomly assigned to one of three groups, consuming either placebo (age: 67 ± 2 years; n = 12), acetaminophen (age: 64 ± 1 years; n = 11; 4000mg/day), and ibuprofen (age: 64 ± 1 years; n = 13; 1200mg/day). Subjects underwent a 12-week progressive resistance training program, consisting of three non-consecutive training sessions per week. Contrary to the findings of Krentz et al. (2008), Trappe et al. (2011) detected a near 50% increase in quadriceps muscle volume above placebo after 12 weeks of training in the two NSAID groups. The findings of Trappe et al. (2011) run contrary to MPS data from their own acute studies (Trappe et al. 2002), surprisingly finding that chronic NSAID administration augmented muscle hypertrophy above placebo. It is possible that the muscle hypertrophy noted in

the NSAID group by Trappe et al. (2011) might have been due to an inhibition of low-grade systemic inflammation associated with ageing (Schaap et al. 2009; Schaap et al. 2006). Whether chronic NSAID ingestion might influence inflammation and hence regulation of muscle mass will be addressed in Chapter 4 in the context of high-intensity resistance exercise training.

1.6 Aims of this thesis

The overall aim of this thesis was to investigate muscle mass and metabolic regulation as a result of resistance exercise, obesity and pharmacological intervention. Three studies were designed in order to achieve this and are described in the following experimental chapters.

Chapter 3. High-load eccentric training has been suggested to produce greater muscle hypertrophy than concentric training, possibly because of the greater forces generated during the former, though studies overall are equivocal on the matter. It has also been suggested that the greater muscle inflammation induced by eccentric exercise stimulates myogenic and/or hypertrophic processes acutely, which with chronic training result in these purported greater skeletal muscle adaptations. However, whether exercise-induced inflammation *per se* has an influence on muscle mass regulation is unknown, with divergent responses to inflammation noted in acute versus chronic exercise intervention studies. This study therefore quantified the temporal impact of 12 weeks of combined maximal concentric-eccentric training versus maximal concentric training on muscle CSA, volume and targeted mRNA expression, in young, healthy, recreationally trained male volunteers, in order to determine whether any differences in muscle CSA, muscle volume and strength gains could be

ascribed to greater work done and/or associated with greater exercise-induced changes in expression of genes involved in inflammation, myogenesis and other cellular functions thought to regulate muscle mass and fuel metabolism. No study thus far has examined either the time course of skeletal muscle adaptations, or the molecular processes underlying these adaptations during combined concentric-eccentric or concentric training.

Chapter 4. Sportspersons often use non-steroidal anti-inflammatory drugs to reduce soreness associated with rigorous training, perhaps to increase training work output and therefore increase skeletal muscle mass adaptations. However, it is not known whether NSAIDs in fact do increase work output during training. Studies have also investigated whether there is a direct physiological effect of NSAID administration on skeletal muscle adaptations in response to resistance exercise and resistance exercise training. However, while acute studies in the young suggest that NSAIDs suppress myogenic and hypertrophic responses to exercise, chronic studies in the elderly have demonstrated an increase in muscle volume and strength in response to combined NSAID ingestion and progressive resistance training. No studies thus far have examined the effect of chronic NSAID ingestion on resistance training-induced skeletal muscle adaptations and the molecular processes that underpin these adaptations in the young. This study therefore quantified the temporal effect of 12 weeks of combined chronic NSAID administration and high-intensity resistance training on muscle CSA, muscle volume and targeted mRNA expression in young, healthy recreationally trained male volunteers, and examined whether ingestion of NSAIDs improved work output during training.

Chapter 5. The mechanistic basis for the blunting of MPS in obese elderly volunteers is unclear given anabolic signalling in response to feeding in these individuals has been shown to be no different from normal-weight controls. Further, it unlikely that inflammation plays a role since the blunted anabolic response in the elderly obese is not accompanied by greater muscle inflammation than healthy-weight controls. There is a need for intervention in these individuals to ameliorate this anabolic resistance, given ageing is associated with a decline in muscle mass and strength, the impact of which, in terms of disability, mortality and health outcomes, are worsened with obesity. It is possible that the metabolic inflexibility and low metabolic quality/resilience of muscle in the elderly obese transiently limits the rate of increase in muscle mitochondrial ATP resynthesis towards increasing MPS due to the greater oxygen consumption associated with fat oxidation. This study therefore aimed to determine whether forcibly increasing carbohydrate oxidation using a pharmacological intervention resolved the anabolic response to protein nutrition, and combined exercise and protein nutrition in obese older volunteers compared to control intervention. No study thus far has tested whether anabolic resistance in the elderly obese might be resolved by increasing the efficiency of mitochondrial ATP resynthesis in a state of lipid-induced metabolic inflexibility.

CHAPTER 2. General Methods

2.1 Purpose

This chapter describes in detail the methods common to experimental chapters. Where a method is specific to an experimental chapter, it will be described in its own methods section.

2.2 Overview

As a whole, this work addresses a number of research questions through different study methodologies. Experiments described in Chapters 3 and 4 investigated changes in quadriceps muscle CSA, muscle volume, strength, and vastus lateralis muscle mRNA abundance in response to different modalities of resistance exercise training, as well as the combination of NSAIDs and resistance exercise training on the same parameters in young individuals. Resistance exercise was performed using an isokinetic dynamometer. Isometric strength was measured using a fixed dynamometer with a force cell. Temporal change in muscle CSA and muscle volume was determined using 3-Tesla (3T) MRI. Vastus lateralis muscle samples were obtained in the resting state to determine temporal change in targeted mRNA abundance using RT-PCR micro-fluidic cards. Muscle protein expression was measured in the experiment in Chapter 4 alone, and is therefore described in its own methods (Section 4.2.9).

The experiment described in Chapter 5 investigated the acute muscle protein synthetic response to protein feeding and protein feeding in combination with resistance exercise in older individuals in an effort to determine whether pharmacologically increasing carbohydrate oxidation acutely via oral administration of dichloroacetate (DCA) could modulate this response. Dual

energy X-Ray absorptiometry (DEXA) was used to determine body composition, 3T MRI to determine thigh muscle volume, and Magnetic Resonance Spectroscopy (MRS) was used to determine intramyocellular and extramyocellular lipid content of the vastus lateralis. Primed constant infusions of stable isotope amino acid, or "tracer", alongside vastus lateralis muscle sampling were used to determine muscle protein synthetic rates. Resistance exercise was performed on an isokinetic dynamometer. Indirect calorimetry was used to determined rates of substrate oxidation, and arterialised venous sampling was used to measure plasma glucose and serum insulin serially. While vastus lateralis muscle sampling and MR measurements are described in detail here, being common to all experimental chapters, the rest of the methods used in Chapter 5 are described in its own methods (Section 5.2).

2.3 Participant Enrolment

2.3.1 Recruitment

All studies were approved by the University of Nottingham Ethics Committee and were conducted in accordance with the Helsinki Declaration. Males alone were recruited because of the effect of gender on MPS (Yarasheski, Zachwieja, and Bier 1993). For the experiments described in Chapters 3 and 4, healthy male volunteers aged 18-35 years were recruited. For Chapter 5, healthy male volunteers aged 60 years and above were recruited. Volunteers were recruited using advertisements at the University of Nottingham and in the community by the use of posters and research awareness events. If individuals informed us that they might be interested in taking part, they were provided a study information sheet, which outlined in layman's terms the general aims of the study, the

experimental protocol, inclusion and exclusion criteria and what each study day entailed.

2.3.2 Screening and consent

The experimental protocol was verbally explained at the first point of contact - at screening - after which written, informed consent for participation in the study was obtained. However, before participation could be confirmed, all potential participants completed a health questionnaire (Appendix A), and underwent a clinical examination comprising a medical history, blood pressure and heart rate measurements, an electrocardiogram (ECG), height and weight measurements to determine body mass index (BMI), and blood tests for full blood count, clotting panel, urea, electrolytes, and liver function.

2.3.3 General exclusion criteria

For experiments in Chapters 3 and 4, volunteers were not recruited if they presented with frailty, active malignancy, and any medical condition that rendered their participation unsafe or unsuitable for the experiments, including a history of cardiac, pulmonary, liver, kidney, neurological, vascular or autoimmune disease, clotting disorders, myopathy, uncontrolled hypertension (BP>160/90 mmHg), metabolic disorders including diabetes, uncontrolled thyroid disease, anaemia, alcohol abuse or visually obvious muscle wasting or skeletal deformity.

For the experiment in Chapter 5, volunteers were unable to participate if they had type 2 diabetes, a previous diagnosis of cancer, unresolved hypertension, cardiovascular disease, ongoing musculoskeletal complaints, frailty

(underweight and self-report of exhaustion, weakness, slowness, and low physical activity levels), or any other chronic condition. Furthermore, subjects were not allowed to participate if they were trying to lose weight.

For experiments in all chapters, volunteers were unable to participate if they were on anti-inflammatory medications, anti-depressant medications or corticosteroids. In addition, volunteers were only recruited if they were non-smokers and did not consume more than 21 units of alcohol per week due the effects of smoking (Petersen et al. 2007) and alcohol (Parr et al. 2014) on MPS.

A total of 8, 17, and 9 healthy volunteers were studied to complete the protocols detailed in Chapters 3, 4, and 5 respectively. Volunteers in Chapter 3 also formed the control group in Chapter 4, which addressed a separate experimental aim.

2.4 Resistance exercise and muscle functional measurements

In the experiments described in all chapters, participants performed lower limb resistance exercise on an isokinetic dynamometer (HUMAC Norm, CSMi Solutions, MA, US; Fig. 1). Participants had to perform maximal isokinetic concentric contractions in experiments in Chapters 3, 4 and 5, and maximal isokinetic eccentric contractions for experiments in Chapter 4. In addition, isometric strength was tested as part of experiments. For experiments in Chapters 3 and 4 this was measured using an isometric knee extension unit (Medical Engineering Unit, University of Nottingham, UK). For Chapter 5, it was measured using the isokinetic dynamometer.

2.4.1 Performance of resistance exercise

Before starting exercise or testing, the isokinetic dynamometer was calibrated using instructions from the HUMAC Norm manual. For all measurements, participants were seated securely using buckled straps on the chair of the dynamometer, with the knee of the exercising leg flexed at 90°. The lever arm was attached to the ankle with sufficient cushioning to avoid discomfort, and the chair was adjusted such that the actuator axis of the dynamometer was aligned to the lateral femoral epicondyle (Figure 2-1). Range of motion was determined and fixed for the specific bout that was to be performed. Participants were then asked to grab firmly on to handlebars on either side of the chair of the dynamometer before beginning exercise or testing, during which they received real-time visual feedback on their performance, and verbal encouragement throughout to ensure maximum effort.

In order to perform maximal isokinetic concentric contractions for experiments in Chapters 3, 4 and 5, for each contraction, participants were asked to forcibly extend their knee against the lever arm as hard as possible to move it. Throughout this upward movement, a continuous downward resistance was imparted by the dynamometer to the raising of the lever arm, and at a pre-determined speed. This allowed for the contraction to be performed at maximal intensity. The volunteer was asked to sustain this force from 90° of knee flexion to full extension, before releasing the knee back to 90° of flexion.

In order to perform maximal isokinetic eccentric contractions in Chapter 3, participants were first asked to extend the knee without resistance so as to raise the lever arm from 90° of knee flexion to full extension. At full extension, a

downward force was imparted by the dynamometer upon the lever arm at a pre-determined velocity, which participants had to resist throughout range of motion, i.e. from full knee extension to 90° of knee flexion, which once again allowed for the contraction to be performed at maximal intensity.

Contractions were performed at an angular velocity of 90°.s^{-1} in all experiments, as at this speed of contraction all muscle fibres of the quadriceps muscle group are recruited (Tesch et al. 1990).

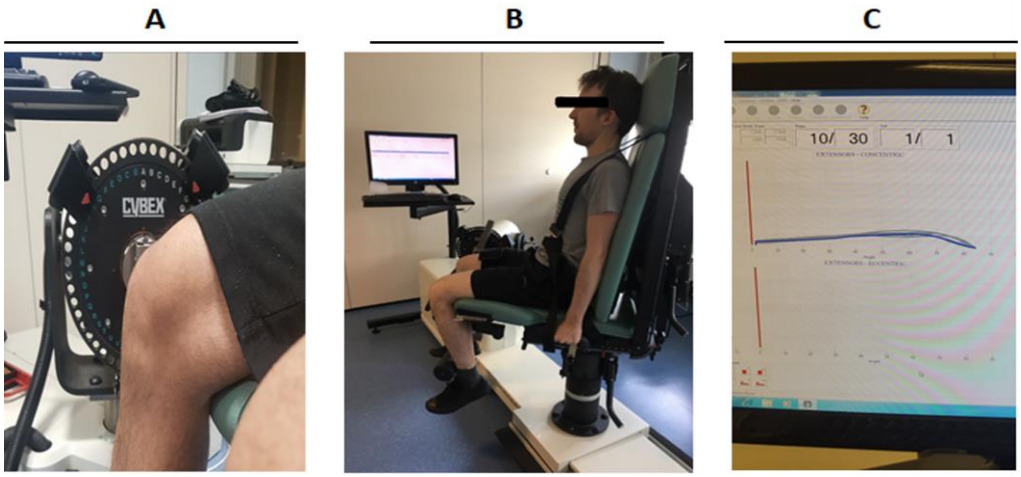

Figure 2-1: A participant on the CYBEX isokinetic dynamometer. (A) The actuator axis is aligned to the lateral femoral condyle of the participant. (B) During the bout the participant is securely seated on the chair with the exercising (right) leg attached at the ankle to the lever arm of the dynamometer. (C) The screen provides visual feedback to the participant as each contraction of the quadriceps in performed.

2.4.2 Isometric strength testing

In experiments in Chapters 3 and 4, isometric strength of the knee extensors was measured using an isometric knee extension unit (Medical Engineering Unit, University of Nottingham, UK), which comprised a fixed dynamometer that measured muscle strength using a force cell. To carry out measurements, the force cell was fixed at both ends to an examination table, and interfaced with an amplifier that recorded force when tension was transmitted through the force cell. The force generated was displayed on a screen. Participants were asked to lie down with knees flexed at 90° from the edge of the examination table. Following calibration of the dynamometer, one end of the force cell was connected to a stabilisation strap attached to the participant's ankle, while the other was securely fastened to the table. Participants were asked to perform a single static maximal voluntary contraction of the knee extensors by pushing the leg away from the table as hard as possible for 5s, and the reading from the screen was noted. After resetting the dynamometer to zero, and following 2min of rest, participants were asked to repeat the contraction, and isometric strength was determined as the highest value of three such attempts.

To measure isometric strength of the knee extensors on the isokinetic dynamometer for experiments in Chapter 5, after being seated securely for testing as described in Section 2.4.1, participants were made to perform a static maximal voluntary contraction by pushing their leg as hard as possible against the lever arm for 5s. The lever arm did not move as it was rendered fixed by the isokinetic dynamometer. After 2min of rest, the participant was asked to repeat

the contraction. Isometric strength was then determined as the highest value of three such attempts.

2.5 MRI and MRS

2.5.1 Overview and principles

Based on the principle of nuclear magnetic resonance, magnetic resonance imaging produces high quality images of internal body structures. It was used to determine quadriceps cross-sectional area (CSA) and volume in experiments in Chapters 3 and 4, and volume of the thigh muscle group in the experiment Chapter 5.

Nuclei in atoms that have an odd number of protons or neutrons possess a charge and an intrinsic spin that can produce an associated magnetic moment. Since the body is composed of ~50-60% water, the hydrogen atom is the most abundant isotope in the body, and contains the simplest nuclei comprising a single proton. MRI images hence depend on how these hydrogen nuclei behave within a magnetic field. While in normal conditions these nuclei are aligned randomly, under the influence of a magnetic field they experience a torque, which results in their attempting to re-align in a parallel or anti-parallel fashion with the field. As a result, the nuclei precess about the magnetic field axis characteristically, and at a frequency called the Larmor frequency. The variation of this frequency is proportional to the strength of the magnetic field. On application of a radio frequency pulse set at the Larmor frequency, some nuclei absorb the energy thus produced, moving from a low to high energy state, causing an alteration in the magnetisation alignment. The nuclei eventually relax into their original

alignment and lower energy state, but in the process radiate the absorbed energy at the Larmor frequency. The detection of these changes is used to produce an image of the area being scanned. Gradients in the magnetic field cause differently located nuclei to precess at different speeds, allowing for the recording of spatial information.

2.5.2 General pre-scan preparation and protocol

In all experiments, before the participants entered the MRI scanner, they were informed of the procedures involved. They were asked to complete an MRI safety form to assess whether the scan could be performed safely, and to remove all metallic objects from their body. During the scan they were provided ear plugs/defenders, and were also given an alarm button to alert scanner operators as to any issues or discomfort they might experience. In the scanner, they were placed on a movable bed in the supine position, with their heads resting on a pillow, and sandbags placed to stabilise the area to be scanned.

2.5.3 MRI

In experiments in chapters 3 and 4, axial plane scans of the thigh were taken using a 3T MRI scanner (GE 3T 750 Discovery) to determine quadriceps muscle CSA and volume. A T1-Fast Spin Echo protocol was used (repetition time 600ms, echo time 15ms, number of excitations 2, field of view 22mm, slice thickness 10mm, no gap between slices). Mid-thigh muscle CSA was measured in order to determine change at the level of the mid-thigh region. Axial plane scans along the entire length of the quadriceps muscle were obtained to determine anatomical cross-sectional area (ACSA). The ACSA data reported in

the results represents the peak value ($ACSA_{peak}$) measured at mid-femur length. The method of obtaining 48 axial scans from each leg was the same at all time-points. Using these axial scans, the contours of the whole quadriceps muscle group for each MRI scan were digitized using Osirix image analysis software (Pixmeo, Geneva, Switzerland) (Figure 2-2) Subsequently, quadriceps muscle volume was calculated as follows:

$$Volume\ Quads\ (cm^3) = \Sigma \text{ACSA} \times \text{slice thickness (Franchi et al. 2014)}$$

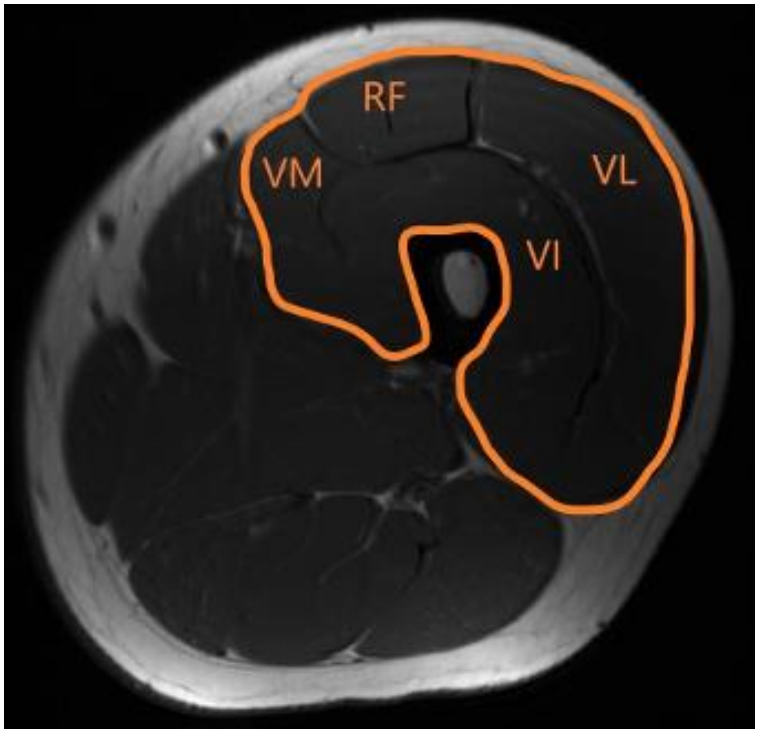

Figure 2-2: Magnetic Resonance Imaging scan. Image of the left thigh at 50% of femur length from a representative participant. The contours that comprise the quadriceps muscle are outlined, and the four heads of the quadriceps - the vastus lateralis, rectus femoris, vastus medialis, and vastus intermedius – are identified.

For the experiment in Chapter 5, axial plane scans of the thigh were taken using a 3T MRI scanner (Phillips Ingenia) to measure thigh muscle volume. A T1-weighted Spin Echo sequence was used to get the best contrast between the different muscle groups. Axial images were acquired using a field of view of 200x200x74.7mm at a resolution of 1x1x1mm with a 0.1mm gap between slices. The total acquisition time was 3min for a total of 68 slices. Thigh muscle volume was quantified by a semi-automated method. For this, first MRIConvert software was used to convert the obtained DICOM images from the axial scans to NIFTI format. The newly formatted images were then loaded onto 3D slicer software and the region of interest was identified. Next, the software was used to segment the muscle. For this, first, where the MR image was corrupted by bias field, correction was applied by adjusting the number of iterations and changing the convergence threshold. Next, fiducial points were set in the muscle tissue, and parameters applied for segmentation. Lastly, to separate subcutaneous and inter-myofibrillar fat, the segmented volume was imported into a Segment Editor for smoothing, before thigh muscle volume was quantified from the segments.

2.5.4 MRS

There is widespread interest in analysis of lipid content of skeletal muscle by ^{1}H magnetic resonance spectroscopy (Schick et al. 1993; Boesch et al. 2006; Boesch 2007). An in vivo non-invasive method for quantification of the intramyocellular lipid (IMCL) and the extramyocellular lipid (EMCL) content in human volunteers using ^{1}H magnetic resonance spectroscopy (MRS) has been developed previously (Ren, Sherry, and Malloy 2010). A novel modelling of the lipid strand orientation in the extracellular compartment of muscle has also been

presented (Khuu et al. 2009) and was the basis of the method used in the present study; in that while IMCL is often assumed to be composed of spherical droplets having a spectra independent to muscle fibre orientation, the EMCL can be modelled as a long strand of fat aligned with the muscle fibres. This induces an asymmetric spectrum due to susceptibility effects in the measure spectra.

Pre-scan preparation was carried out as described in Section 2.5.2. A single spectroscopic voxel was placed on the vastus lateralis at the mid-point of a line connecting the greater trochanter of the femur to the insertion of the muscle into the quadriceps tendon. The voxel size was 15x15x50mm, the longer dimension positioned in the same direction as the muscle had been measured. The acquisition technique of the spectra was STEAM (STimulated Echo Acquisition Mode: see http://mriquestions.com/steam.html for more details), and was obtained using a flip angle of 90 and a repetition time of 2s. A total of 5 different echo times was acquired (40, 60, 80, 100 and 160ms) and was repeated 8 times to gain SNR (signal-to-noise ratio) in the spectra (acquisition time of 6min 40s). A single acquisition was also performed without water saturation for quantification purposes (acquisition time of 2min). Spectra were exported using the Philips format (SPAR/SDAT), loaded and processed using an in-house software written by Olivier Mougin. First, non-water saturated spectra (NWS) and water saturated spectra (WS) were selected. Next, the zero order phase was adjusted, and if required, phase per spectra and first order phase were adjusted as well. Following this, the start and end of the frequency range was optimised, and this range was applied to zoom in on the specified area. The EMCL frequency was then changed to fit the first peak. Subsequently, the IMCL

frequency was adjusted to fit the second peak. If visible, the third peak was adjusted as well. Where the peaks were overlapped, the spectrum was readjusted. Once appropriate adjustments were made, results were able to be acquired as PNG images as well as raw data.

2.6 Muscle Sampling

2.6.1 Bergstrom percutaneous muscle biopsy technique

In the experiments described in chapters 3 and 4, muscle samples were obtained from the vastus lateralis using the Bergstrom percutaneous muscle biopsy technique (Bergstrom 1975), using a Bergstrom biopsy needle (Figure 2-3). This technique provides an easily reproducible method of acquiring muscle samples. When performed correctly, it is quick, causes relatively little trauma and is well-tolerated (Constantin-Teodosiu et al. 1996; Murton et al. 2014). It does not require general anaesthesia, scarring is minimal and there is no need for repeated biopsies (Goldberger, Henry, and Randall 1978; Edwards, Young, and Wiles 1980; Edwards, Round, and Jones 1983). Suction is applied to the biopsy needle while the sample is being cut, which increases the likelihood of sampling adequate amounts of tissue (Greig, Askanazi, and Kinney 1985; Tarnopolsky et al. 2011).

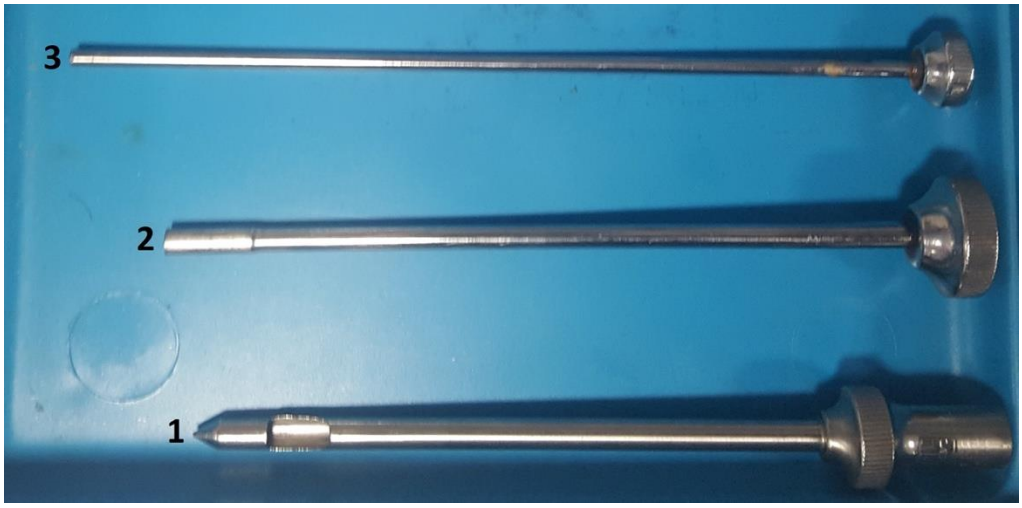

Figure 2-3: The Bergstrom needle. (1) Hollow outer needle with a sharp tip and a small opening; (2) inner cutting cylinder with a sharp edge and; (3) stylet.

Volunteer consent for the muscle biopsy procedure was obtained. A point roughly 10-15cm superior to the middle of upper border of the patella was noted. The location on the leg from which muscle was to be obtained was identified as a region 4-5cm lateral to this point. The subject was asked to tense the quadriceps muscle to confirm the location. The area was then cleaned with betadine solution, and 1ml of 1% lidocaine was injected beneath the skin. Next, 5-10ml (depending on the weight of the volunteer) of 1% lidocaine was injected into the muscle while aspirating between injections to avoid infiltration of local anaesthetic into the blood vessels in the area. Following a minute of waiting for the lidocaine to act, a size 11 scalpel was used to make a 2cm incision in the anaesthetised area. Firm pressure was then applied immediately to the incision site using gauze. A size 5 Bergstrom needle (size corresponding to the diameter of the window located above the tip of the needle) was then introduced into the incision site, and firm pressure was applied until a “give” sensation was felt, indicating the passage of the needle through the tough fascia – a layer of

connective tissue atop the muscle. The inner cutting cylinder was subsequently alternatingly released from and pushed into the needle to “cut” adjacent muscle, while the aforementioned suction was applied atop the needle so as increase the amount of sample obtained (Figure 2-4). The Bergstrom needle was then removed entirely, and pressure was once again applied to reduce bleeding. Once bleeding was minimised, the edges of incision site were approximated using steristrips. Where serial biopsies were taken, a distance of 2.5cm was ensured between biopsy sites. It has been previously demonstrated that serial needle muscle biopsy sampling, where repeated biopsies are taken 2.5cm distal to each other, does not elicit confounding changes in the muscle transcriptome when analyzed by microarray technologies (Murton et al. 2014).

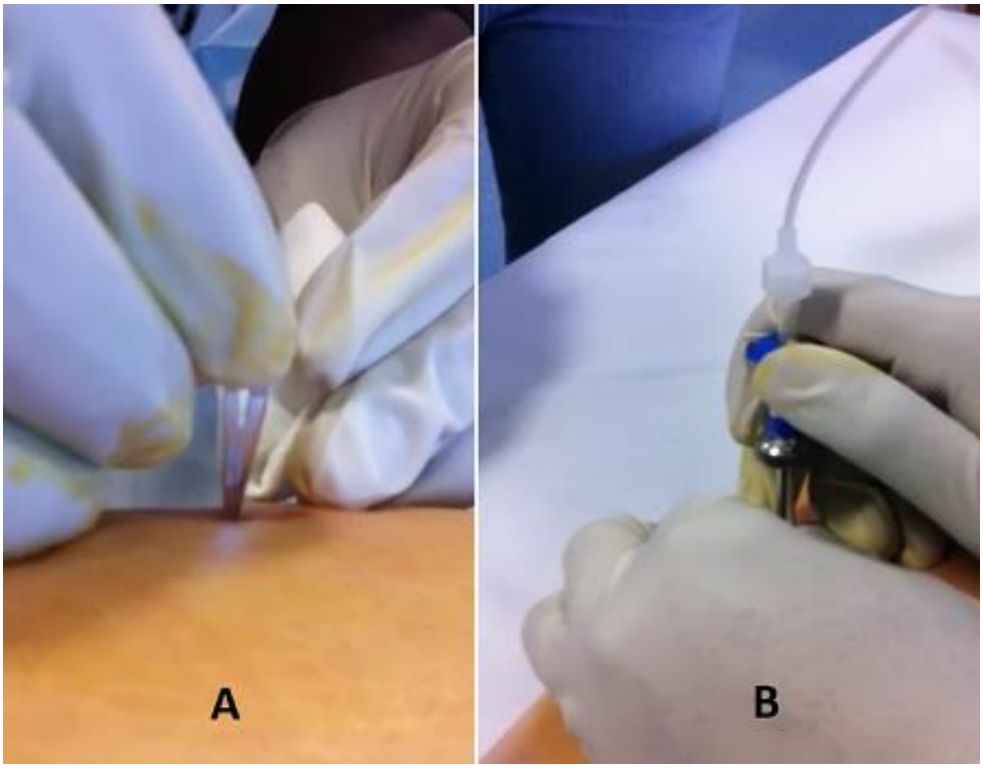

Figure 2-4: Bergstrom percutaneous muscle biopsy technique. Following administration of local anaesthetic (1% lidocaine) into the identified biopsy site, (A) a 1cm incision is made in the skin. After adequate compression is applied, the modified Bergstrom biopsy needle is then inserted into the incision site (B); cuts are then made by alternately releasing and pushing in the cutting cylinder while suction is applied.

2.6.2 Minimally invasive percutaneous biopsy technique

In the experiment described in Chapter 5, muscle biopsies were obtained from the vastus lateralis muscle using the minimally invasive percutaneous micro-needle or Bard biopsy technique as described by Hayot et al. (2005). This technique uses the same method of preparation as the previously described Bergstrom biopsy technique. However, it is performed using a spring-loaded

Bard® Magnum® reusable core biopsy system (MG1522) with a 12-gauge needle (MN1210) (Figure 2-5).



Figure 2-5: The Bard® Magnum® core biopsy system (MG1522) with a 12 gauge needle (MN1210). It was used to perform minimally invasive muscle biopsies.

Following the same method of location of the biopsy site, anaesthetisation, but after making a smaller incision (<1cm), the Bard needle was inserted into the incision site and repeated cuts were made using the retracting needle. This incision is smaller as it is sufficient for the passage of the Bard needle through the skin. A total of 6 passes were made through the same incision to obtain 30-60mg (wet weight) of muscle tissue, which was dabbed dry on a non-woven gauze to absorb any blood, and then rapidly frozen in nitrogen. The main advantage of this technique is that repeated samples can be taken with a smaller needle, rendering the procedure less invasive than the Bergstrom technique.

2.7 Muscle mRNA expression measurements

2.7.1 Overview

In Chapters 3 and 4, targeted muscle mRNA expression was quantified using real-time polymerase chain reaction (qPCR). Due to its high sensitivity and higher sample throughput compared to traditional PCR, it is one of the most commonly used techniques for mRNA expression quantification. It combines the detection and amplification processes, building on the principles of PCR, resulting in gene amplification following each thermal cycle being determined in “real-time”. The target RNA is first isolated, and then reverse-transcribed into a cDNA template. The thusly formed cDNA template binds in a 5` to 3` direction to its complimentary DNA oligonucleotides. The cDNA is then heated so the cDNA strand can separate, and then cooled to allow annealing of probes and primers to the cDNA template in the presence of DNA polymerase and deoxyribonucleoside triphosphates (dATP, dCTP, dGTP, dTTP). Further steps of heating and cooling therefore allow for exponential amplification of the target amplicon (Figure 2-6).

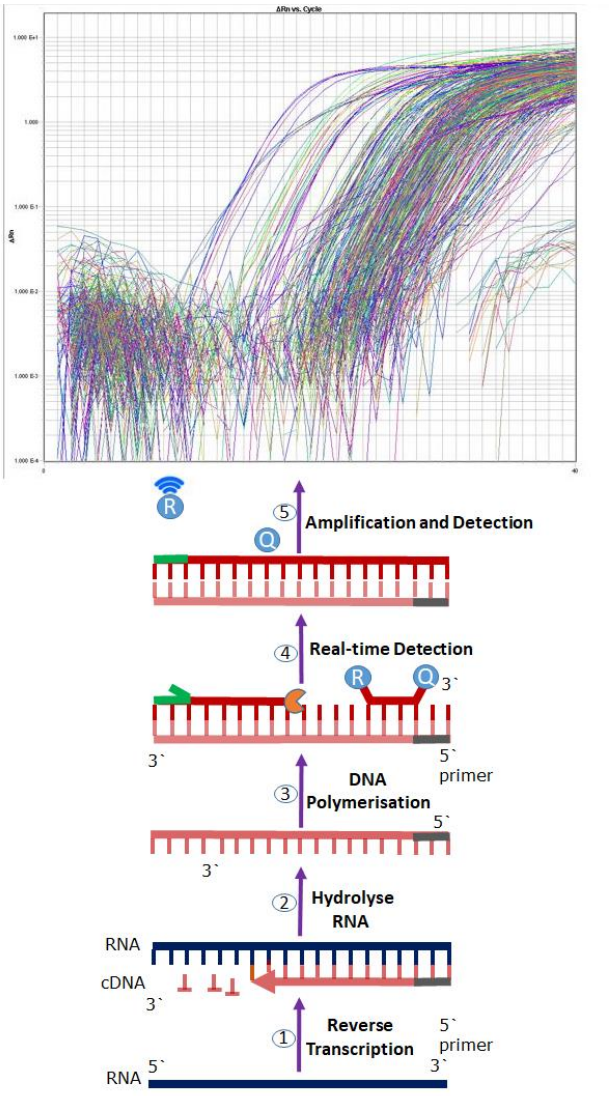

*Figure 2-6: Schematic representation of steps involved in qPCR. (1) Isolation and reverse transcription of the target RNA into a cDNA template using reverse transcriptase. (2) RNA hydrolysis using RNAse. (3) Subsequent binding of newly formed cDNA template to its complimentary DNA oligonucleotides in a 5` to 3` direction in the presence of DNA polymerase and deoxyribonucleoside triphosphates (dATP, dCTP, dGTP, dTTP), and resultant liberation of promoter dye. (4) Real time product quantification due to increase in intensity of reporter dye after each thermal stage.*Muscle mRNA extraction

Total RNA was extracted from ~30mg of snap-frozen wet muscle by homogenising in TRIzol® (1ml, Invitrogen, Paisley, UK) and glycogen (10µg/µl; Sigma-Aldrich, Dorset, UK) for ~30s using a polytron homogenizer. Homogenates were then incubated for 5min at room temperature following which Chlorophorm (200µl) was added and vortexed briefly before further incubation at room temperature for 3min. The homogenates were then centrifuged at 12,000g for 15min at 4°C. These centrifuged samples thus comprised three layers. The clear upper aqueous layer containing RNA was transferred to a new Eppendorf tube. Using iso-propanol, isolated RNA was precipitated (500µl, Sigma-Aldrich, Dorset, UK) and incubated for 20min at 4°C. After incubation the samples were centrifuged for 15min at 12,000g at 4°C to pellet the isolated RNA, the supernatant removed, and the isolated RNA pellet air-dried. Once dry, the pellet was washed in 1ml of 75% ethanol, briefly vortexed and then centrifuged at 7,500rpm for 10min at 4°C. Supernatant was then removed, the pellet was air-dried and then dissolved in 50µl of RNase-free water.

Using a NanoDrop® (NanoDrop® Technologies Inc, Wilmingtion, USA), RNA concentration was determined spectrophotometrically by measurement of nucleic acid absorbance at 260nm and 280nm, and calculated using Beer-Lambert law (which predicts a linear change in absorbance with concentration). Using the 260:280 ratios, RNA purity was determined. A value within a range of 1.8-2.0 was considered highly purified RNA.

2.7.3 First strand cDNA synthesis

After spectrophotometric quantification, 1µg of total RNA was incubated in 1µl dNTPs (Promega U1240) and 2µl random hexamers (Promega C1181). The final volume was made of 13µl dNTPs (Promega U1240) using RNase-free water. In order to denature the RNA secondary structure, samples were heated for 5min at 65°C in a PCR thermocycler, and then immediately placed on ice. By adding 4µl Enzyme buffer, 1µl DTT, 1µl RNase inhibitor (Promega N2511) and 1µl SuperScript^{TM} III reverse transcriptase (Invitrogen Ltd, Paisley, UK), first strand cDNA was created from the RNA and then processed in a PCR thermocycler using heating stages of 25°C for 5min, 50°C for 60min and lastly 70°C for 15min. Samples were then stored at -80°C until further analysis.

2.7.4 Gene expression array cards

DNA amplification quantification following each thermal cycle can be measured by a variety of different probes with different chemistries. We used Taqman® Gene Expression Array Cards, which contained Taqman® probes. This custom designed low-density RT-PCR microfluidic array card approach was used to measure the relative abundance of multiple mRNA genes from the muscle samples. The low-density RT-PCR microfluidic array card finally designed (384 format; Applied Biosystems, Foster City, CA, USA) consisted of 93 unique gene targets (see Appendix B for definitions) and was used to measure the relative mRNA abundance from multiple pathways involved in cell growth and regulation, DNA damage, stress responses, energy metabolism, inflammation, extracellular matrix and muscle differentiation and signalling.

Taqman probes consist of a 3’ minor groove binder (MGB) non-fluorescent quencher (NFQ) short oligonucleotide, and a 5’ reporter dye (6-carboxyl-fluorescein; FAM™). The 3’ quencher and the 5’ reporter are covalently attached to the short oligonucleotide. The presence of a 3`quencher probe in close proximity to the fluorescent 5`reporter dye in the Taqman probe prevents the excitation of the latter, as the energy for excitation is transferred to the 3’ quencher probe by Fluorescent Resonance Energy Transfer (FRET). But after the thermal separation of cDNA, the Taqman® probe containing the 5’ reporter and 3’ quencher anneals to the cDNA strand. In the polymerization phase, the attached 5’ reporter and 3’ quencher are cleaved by DNA polymerase, which through its exonuclease activity liberates the quencher and reporter probes from the oligonucleotide. This allows the reporter probe to be detected spectrophotometrically in response to excitation by a light source following each thermal cycle, allowing for determination of DNA amplification after every cycle. In the early thermal cycles, the end products cannot be quantified, as the intensity of the reporter dye is lower than background fluorescence levels. Once the intensity exceeds this background fluorescence (Ct threshold), quantification can occur until the plateau phase. If a specific sequence of DNA is expressed to a high degree, amplification of that sequence is noted in earlier thermal cycles.

2.7.5 Quantification of mRNA expression

ABI PRISM 7900HT sequence detection system was used to quantify mRNA expression, and data analysed using SDS 2.1 software (Applied Biosystems, USA). Data were further analysed using RQ Manager software (Applied Biosystems, USA), where the threshold level was normalised across all plates

before Ct values were calculated for each gene target and sample. By the comparative Ct method, relative mRNA expression is quantified in relation to the housekeeping gene. Hence choosing a suitable housekeeping gene which does not change with the intervention is key to interpreting gene expression data. Housekeeping genes that are ordinarily chosen for studies are those which are universally expressed and transcribed at a relatively constant rate e.g. (GAPDH; Glyceraldehyde 3-phosphate dehydrogenase), 18s and HMBS (hydroxymethylbilane synthase). For the experiments in Chapters 3 and 4 we used HMBS. Using the $2^{\Delta\Delta Ct}$ method, relative mRNA expression levels were quantified using the participant's baseline sample as the calibrator, and HMBS as the housekeeping gene (endogenous control), from the following formula:

$$2^{\Delta\Delta Ct} = \Delta Ct_{target\ gene\ baseline} - \Delta Ct_{target\ gene\ baseline} - \Delta Ct_{target\ gene\ post-intervention},$$

whereby: $\Delta Ct = Ct_{target\ gene} - Ct_{housekeeping\ gene}$

2.7.6 Ingenuity Pathway Analysis

Muscle mRNA abundance was analysed using Ingenuity Pathway Analysis (IPA) software (Redwood City, CA, USA). IPA is a commercial web-based software application that allows for integration, analysis and understanding of data obtained from gene expression, SNP and miRNA microarrays; and proteomics, metabolomics and RNA sequencing experiments. It can be implemented towards small-scale experimental analysis that generates chemical and gene lists. Interpretation and analysis of data by IPA is based on a manually curated and comprehensive database (Ingenuity Knowledge Base,

https://analysis.ingenuity.com/). Regulators, mechanisms, relationships, pathways and functions pertinent to changes noted in an analysed dataset are identified by powerful algorithms. IPA analysis therefore enables the understanding of experimental results in the context of biological systems. (https://www.qiagen.com/ie/products/life-science-research/research-applications/gene-expression-analysis/analysis/ingenuity-pathway-analysis/)

2.7.6.1 Workflow for IPA analysis

Analysis of mRNA abundance by IPA was performed as follows:

1) First, fold change from mean baseline was calculated from the aforementioned Ct values at each timepoint, and for both legs in Chapter 3 and both groups in Chapter 4 to determine differential expression of mRNAs.
2) Subsequently, statistical significance (p-value) was determined from the fold change data for each timepoint using a paired sample t-test. Fold change and statistical significance of all genes for each timepoint in Chapter 3 and Chapter 4 are shown in Appendix C and Appendix D, respectively.
3) Data filtering was set with a fold change cut-off of 1.5 and p-value cut-off of $p<0.05$ to select for the most significantly changed genes. These genes were then used as the input for the subsequent core IPA analysis.
4) The overall outcome of IPA (e.g. upstream regulator analysis, cellular function, activation status) was predicted by calculating a regulation Z-score and an overlap p-value, which were based on: (1) the number of regulated target genes' function; (2) the magnitude of expression change;

(3) the direction of expression change; and (4) their concordance with the IPA database, which is constructed from a curated literature database.

5) A second p-value called the "p-value of overlap" was calculated by IPA to identify significantly enriched function pathways from the submitted list of significantly changed genes. These p-values were generated from the right-tailed Fisher's Exact Test, and a significance threshold of $p<0.05$ was used to assess the statistical significance of the function pathways.

In order to control for any enrichment of false positive results when undertaking multiple comparisons (type II errors) IPA utilises Bonferroni's corrected p-value set at < 0.05.

2.8 Statistical Analysis

Data are presented throughout the thesis as mean ± SEM, unless otherwise stated. Statistical analysis for Chapter 5 is described in its own methods (Section 5.2.9). For experiments in Chapters 3 and 4, comparisons of mean values of muscle CSA, muscle volume, isometric strength and training work output over time (i) between exercise regimens, i.e. ECC + CON versus CON; or (ii) between treatments, i.e. PLA vs NSAID, were performed using a repeated measures two-way ANOVA (time and exercise regimen or treatment as independent factors) with Tukey's or Sidak's post hoc test analysis where appropriate. To identify a significant difference between the means of two groups in terms of total training work output over the duration of the protocol, a paired sample t-test was used. Significance was set at $p<0.05$. Analysis was performed using GraphPad Prism 7.03 (GraphPad Software Inc., California, USA).

CHAPTER 3. The effect of twelve weeks of unilateral combined maximal concentric-eccentric training versus unilateral maximal concentric training on the time course of change in muscle cross-sectional area, muscle volume, strength and targeted muscle gene networks in healthy, young male volunteers

3.1 Introduction

High load eccentric exercise training has been reported to increase muscle hypertrophy, Type IIx muscle fibre cross-sectional area (CSA) and strength gains over 10 to 12 weeks when compared to concentric exercise training (Higbie et al. 1996; Vikne et al. 2006), which has been at least partially attributed to the greater work done during eccentric contractions (Roig et al. 2009). However, these reports are equivocal, reflected by reports of no differences in muscle hypertrophy or strength gains when comparing high load concentric and eccentric exercise training modalities (Farup et al. 2014; Franchi et al. 2014).

High load eccentric exercise is also known to cause short-term muscle force loss and DOMS compared to high load concentric training (Paulsen et al. 2012), both of which diminish with repeated bouts of exercise (McHugh 2003). Muscle inflammation is commonly reported to accompany high load eccentric exercise (Hyldahl and Hubal 2014), though is not commonly measured. This has resulted in combined high load concentric and eccentric training regimens being adopted, aimed at improving tolerability and volunteer compliance over high load eccentric exercise training alone. Nineteen weeks of this combined high load concentric and eccentric training has been reported to induce an 11% greater increase in strength gains and 5% greater increase in muscle fibre area compared with high load concentric training (Hather et al. 1991; Dudley et al. 1991). However, thus far no study has examined the temporal response to these two different training modalities. Furthermore, studies comparing muscle adaptations to concentric versus eccentric training have used different training protocols, varying in training loads and number of repetitions, methods of

assessment of architectural and functional gains, and have involved both trained and untrained volunteers (Roig et al. 2009; Higbie et al. 1996). It is therefore difficult to draw conclusions on the comparative efficacy of the two modalities at inducing muscle training related adaptations.

The mechanisms that underpin the purported greater changes in muscle adaptation to eccentric exercise training are not well understood. Proposals such as greater neural activation and differing recruitment strategies, greater work done, and increased post-exercise MPS have not been definitive (Higbie et al. 1996; Seger and Thorstensson 2005; Cuthbertson et al. 2006; Moore et al. 2005; Franchi et al. 2015).

Application of gene transcript profiling in a study showed that 300 concentric and 300 eccentric contractions (in the form of rising from and controlled lowering to a seated position) induced greater inflammatory mRNA responses in human quadriceps muscle after 4-8h compared with 300 concentric contractions alone (Chen et al. 2003). Furthermore, these inflammation-related mRNA responses were maintained 6h following a second bout of eccentric exercise (Hubal et al. 2008). It has been suggested that there is a potentiating effect of inflammation on muscle satellite cell proliferation (Mikkelsen et al. 2009b), and that a greater degree of muscle inflammation resulting from high load eccentric training may be responsible for greater muscle mass and strength gains when compared with concentric training (Mikkelsen et al. 2011). In keeping with this, an increase in the serum concentration of the pro-inflammatory cytokine IL-6 has been shown to occur in rodent muscle after maximal electrically evoked eccentric contractions, which was accompanied by

increased expression of MRFs between 24 to 96h following contraction (Armand et al. 2003). IL-6 has also been demonstrated to stimulate satellite cell-mediated hypertrophy in isolated cells (Serrano, Baeza-Raja, Perdiguero, Jardi, et al. 2008), and local NSAID infusion following acute maximal eccentric exercise in healthy volunteers has been shown to decrease muscle satellite cell proliferation (Mikkelsen et al. 2009b). McKay et al. (2009) showed that a bout of maximal lengthening contraction increased satellite cell number 24h following exercise, which peaked at 72h post-exercise. Furthermore, this was associated with an increase in muscle IL-6 mRNA expression which correlated strongly with an increase in serum IL-6 concentration ($r^2 = 0.89$, $p<0.01$). These findings suggest that increased expression of myogenic growth factors and satellite cell proliferation 24 to 72h after maximal eccentric exercise occurs as a consequence of inflammation induced by this form of contraction. However, these studies examined acute responses to acute exercise. Contrary to these acute observations, Krentz et al. (2008) showed in young volunteers performing high-load combined concentric-eccentric elbow flexor exercise training for 6 weeks (5x per week), that concurrent daily ingestion of the NSAID ibuprofen had no effect on muscle hypertrophy and strength above that of a placebo group. Furthermore, contrary to the premise that inflammation induces myogenic and hypertrophic responses, 6 weeks of NSAID administration alongside progressive resistance training in older volunteers was reported to increase muscle volume and strength above a group consuming placebo during training (Trappe et al. 2011).

3.1.1 Aims and hypothesis

Overall, the mechanisms responsible for purported greater muscle mass and strength gains observed following combined high load concentric and eccentric training represents gaps in our understanding. Importantly, no time-course data are available regarding muscle molecular adaptations to combined high load concentric and eccentric exercise training compared to concentric exercise alone. The aims of the present study therefore were to test the hypothesis that 12 weeks of unilateral combined maximal isokinetic concentric and eccentric resistance training would produce greater gains in muscle CSA, muscle volume and isometric strength compared to maximal isokinetic concentric exercise training performed by the contralateral limb in healthy, young, male recreationally active volunteers. It was also hypothesised that greater gains in muscle CSA and volume in the limb performing combined concentric and eccentric training would (i) be due to greater work being done over the 12 weeks of training, and (ii) be associated with greater exercise-induced changes in the expression of genes involved in inflammation, myogenesis and other cellular functions though to regulate muscle mass and fuel metabolism. Time course of change in muscle CSA and volume during training was measured using MRI, in conjunction with measurement of mRNA expression using RT-PCR microfluidic, low-density array cards focused on 93 targeted gene transcripts, and subsequent bioinformatics analysis (Ingenuity Pathway Analysis).

3.2 Methods

3.2.1 Participants

Figure 3-1 schematically depicts the present study protocol. Eight healthy, non-smoking, non-vegetarian males (age: 24.3 ± 1.7years, BMI: 23.5 ± 2.5 $kg.m^{-2}$) were recruited to the study. Volunteers had to have participated in resistance exercise training or sports such as squash, football and basketball on at least 2-3 days per week for a minimum of two years to minimise neural responses to resistance exercise (Moritani 1993). Ethical approval and participant consent were obtained (Ethics Reference No: I 07 2011), and screening was carried out as described in General Methods (Section 2.3). Participants were asked to refrain from taking anabolic supplements, i.e. protein and creatine, for the duration of the study, but were allowed to continue with their regular training regimens apart from lower limb resistance training.

3.2.2 Exercise training protocol

An exercise training protocol consisting of 5 bouts of maximal isokinetic concentric knee extensions has been shown to be well-tolerated (Jones et al. 2004). However, preliminary work had shown that 5 sets of 30 maximal eccentric isokinetic contractions of the knee extensors caused considerable discomfort and muscle force loss, which would very much deter volunteers from complying with a 12-week training program. Hence, a protocol was adopted that comprised a combination of maximal isokinetic concentric and eccentric contractions performed on an isokinetic dynamometer as described in General Methods (Section 2.4.1).

Participants trained 3 times per week for 12 weeks and were not in an overnight fasted state for their training sessions. During each session, participants performed 3 bouts of 30 maximal isokinetic eccentric contractions of the knee extensors with their dominant leg, interspersed with 2 bouts of 30 maximal isokinetic concentric extensions (ECC + CON) with the same leg. Additionally, participants performed 5 bouts of 30 maximal isokinetic concentric contractions of the knee extensors with the non-dominant leg (CON). All contractions were performed at an angular velocity of $90°.s^{-1}$, with 3min of rest between bouts. Participants received real-time visual feedback and verbal encouragement through each bout.

A within-subject bilateral internally-controlled design was used because it has been shown to minimise the variability of responses to training (Hubal et al. 2005; Moore, Young, and Phillips 2012), and ensures that any hypertrophic adaptation is the result of localised responses to exercise (West et al. 2010). Such a design has been used in a number of acute and chronic exercise training studies that have compared responses to eccentric and concentric exercise intervention (Moore et al. 2005; Cuthbertson et al. 2006; Kostek et al. 2007; Kumar, Selby, et al. 2009; Franchi et al. 2015).

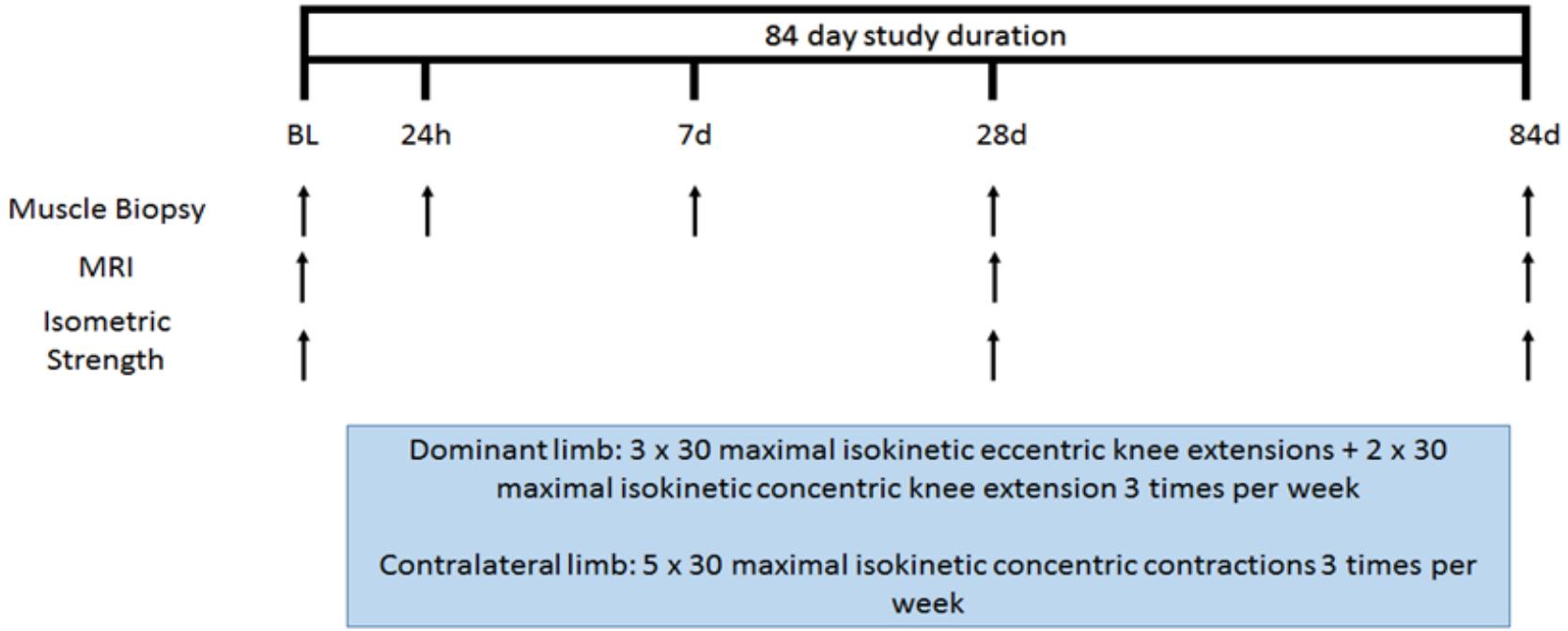

Figure 3-1: Study protocol. Participants performed 3x 30 maximal isokinetic eccentric and 2 x 30 maximal isokinetic concentric knee extensions 3 times per week for 12 weeks in the dominant limb and 5 x 30 maximal isokinetic concentric contractions 3 times per week for 12 weeks in the contra-lateral limb. Muscle biopsies were taken at baseline (BL), 24 hours (24h), 7 days (7d), 28 days (28d) and 84 days (84d). Isometric strength was measured at BL, 28d and 84d. MRI scans of both thighs were taken at BL, 28d and 84d.

3.2.3 Muscle function during training

3.2.3.1 Total work output during exercise training

Total work output per week of training by each limb was calculated as the work done during the 450 maximal contractions performed each week. Total work output for each limb over the 12-week exercise training protocol was calculated as the sum of work done from all exercise sessions by each limb.

3.2.3.2 Isometric knee extensor strength

Isometric strength of the knee extensors was determined as described in General Methods (Section 2.4.2), using an isometric knee extension unit (Medical Engineering Unit, University of Nottingham, UK). Measurements were made at baseline (BL), 28 days (28d) and 84 days (84d) (Figure 3-1). All measurements were made at the same time of day, at least 72h after the last training session.

3.2.4 MRI mid-thigh muscle CSA and thigh muscle volume measurements

Quadriceps muscle cross-sectional area (CSA) and volume measurements of both legs were performed at BL, 28d and 84d time-points (Figure 3-1) using 3T MRI as described in General Methods (Section 2.5.3).

3.2.5 Muscle sample collection

Vastus lateralis muscle biopsies were obtained from both limbs in the resting state at BL, 24h, and a minimum of 72h from the previous exercise session at 7d, 28d and 84d (Figure 3-1). Participants attended the laboratory in a fasted state, having abstained from alcohol and strenuous exercise for 48h prior.

Muscle samples were obtained using the Bergstrom percutaneous needle biopsy technique (Bergstrom 1975) as described in General Methods (Section 2.6.1). Samples were immediately snap frozen and stored in liquid nitrogen for further analysis.

3.2.6 Muscle mRNA expression

Muscle mRNA extraction was performed from muscle samples obtained at BL, 24h, 7d, 28d and 84d as described in General Methods (Section 2.7.2) (i.e. from 76 biopsies in total due to tissue availability). First strand cDNA was synthesised from 1µg of total RNA as described in General Methods (Section 2.7.3). TaqMan low density arrays were performed using an ABI PRISM 7900HT sequence detection system, and data analysed using SDS 2.1 software (Applied Biosystems, USA) as described in General Methods (Section 2.7.4). Muscle mRNA expression was quantified using qPCR as described in General Methods (Section 2.7.5). Data were further analysed using RQ Manager software (Applied Biosystems, USA), where the threshold level was normalised across all plates before Ct values were calculated for each gene target and sample. Relative quantification of mRNAs of interest was measured using the $2^{-\Delta\Delta Ct}$ method with hydroxymethylbilane synthase (HMBS) as the endogenous control, with the mean of the baseline sample used as the calibrator. Mean Ct values for HMBS were no different between CON and ECC + CON legs (data not shown). A total of 93 transcripts were targeted for analysis for the experiments in Chapters 3 and 4 (see Appendix B for definitions). As limited muscle gene array data exist pertaining to chronic eccentric training (Friedmann-Bette et al. 2010), muscle transcripts identified as being altered following acute eccentric exercise in array

studies involving healthy male participants were selected for inclusion (Chen et al. 2003; Higbie et al. 1996). In addition, muscle transcripts seen to be differentially expressed in healthy male participants over the course of 10 weeks of concentric resistance training (Murton et al. 2014) were selected. Based on these studies, approximately 50 genes were chosen that were linked to cell growth and regulation, DNA damage, stress responses, energy metabolism, inflammation, extracellular matrix and muscle differentiation and signalling. Additionally, mRNA expression of myogenic transcription/regulatory factors such as MyoD, myogenin (MYOG), myostatin (MSTN) and MYF5 were selected for inclusion. Further genes of interest known to be differentially regulated in muscle inflammatory states and linked to muscle mass regulation were also included (Crossland et al. 2008; Greenhaff et al. 2008; Mallinson et al. 2009), namely serine-threonine protein kinase 1 (AKT1), forkhead box protein O 1 (FOXO1), protein tyrosine kinase 2 (PTK2), pyruvate dehydrogenase kinase 4 (PDK4), DNA (cytosine-5)-methyltransferase (Met1A), cathepsin-L, MAFbx, MuRF1 and proteasomal alpha subunit 1 (PSMA1).

3.2.6.1 Ingenuity Pathway Analysis

Muscle mRNA abundance was analysed using Ingenuity Pathway Analysis software as described in General Methods (Section 2.7.6).

3.2.7 Statistical analysis

Statistical analysis for comparisons between CON and ECC + CON was carried out as described in General Methods (Section 0). The statistical analysis

underpinning Ingenuity Pathway Analysis is also described in General Methods (Section 0).

3.3 Results

3.3.1 Baseline characteristics of limbs

Table 3-1 shows isometric strength, muscle CSA and muscle volume in CON and ECC + CON legs at baseline. There was no difference in these parameters between legs at baseline.

Table 3-1: Baseline characteristics of limbs. Isometric strength and mid-thigh quadriceps muscle cross-sectional area (CSA) and quadriceps muscle volume are shown for the leg trained using the concentric exercise protocol (CON) and the leg trained using the combined concentric and eccentric exercise protocol (ECC + CON). All values expressed as mean ± SEM.

	CON	ECC + CON
Isometric Strength (N)	51.6 ± 6.6	56.5 ± 7.6
Quadriceps CSA (cm^2)	88.9 ± 4.7	89.3 ± 4.3
Quadriceps Volume (cm^3)	2520.8 ± 137.4	2483.4 ± 144.4

3.3.2 Muscle function during training

3.3.2.1 Total work output

Figure 3-2A shows the total amount of work done per week of training in both CON and ECC + CON groups. Total work output per week during training was

greater in ECC + CON compared to CON, such that there was a treatment effect ($p<0.001$). Figure 3-2B shows the total amount of work done through the entire training protocol, which was greater in ECC + CON than CON (16.8 ± 2.3%, $p<0.001$).

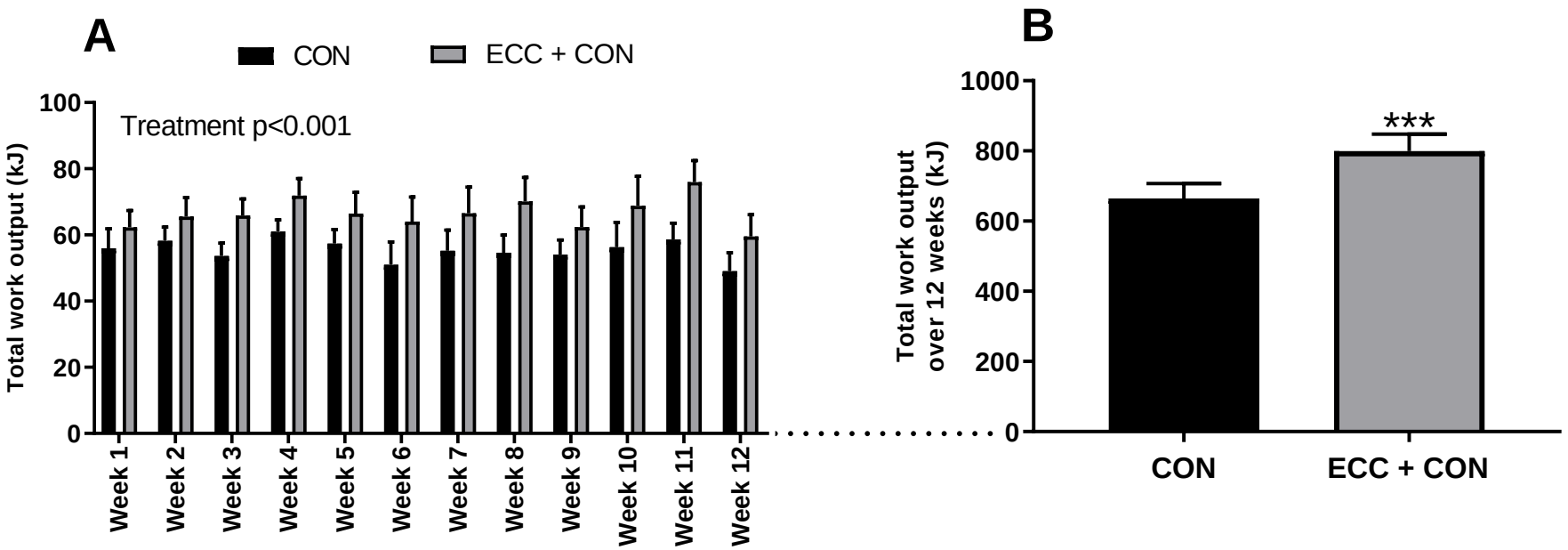

*Figure 3-2: (A) Total work output (kJ) per week, (B) total work output (kJ) over 12 weeks in concentric (CON) and combined concentric + eccentric (ECC + CON) trained legs. Values expressed as mean ± SEM. For (B) ***p<0.001 compared to CON.*

3.3.2.2 Strength

Isometric strength of the knee extensors was increased over time, such that $F(1.5, 18.1) = 7.4$, $p<0.01$. Isometric strength increased on average above baseline in both legs at 28d, but this was not statistically significant (13.9 ± 27.4% in CON; 11.8 ± 17.5% in ECC + CON). Isometric strength did increase significantly above baseline at 84d in both legs (44.8 ± 14.1% in CON, $p<0.05$; 36.9 ± 12.7% in ECC + CON, $p<0.05$). However, there was no difference in change in isometric strength from baseline between legs at any time-point. Graphs depicting absolute values for isometric strength in CON and ECC + CON are shown in Figure 3-3.

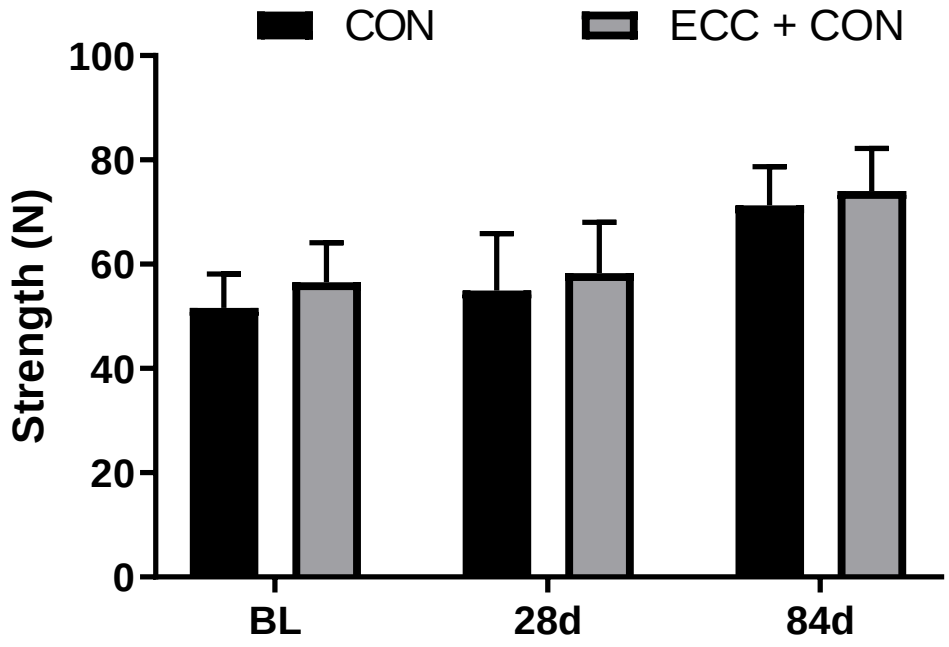

Figure 3-3. Isometric strength (N) in concentric (CON) and combined concentric + eccentric (ECC + CON) trained legs at baseline (BL), 28 days (28d) and 84 days (84d) of training. Values expressed as mean ± SEM.

3.3.3 Muscle CSA and muscle volume

Figure 3-4A shows percentage change in quadriceps muscle CSA from baseline at 28d and 84d in both CON and ECC + CON legs (graphs depicting absolute values shown in Appendix E). Quadriceps muscle CSA was increased above baseline in both legs by 28d (4.3 ± 0.9% in CON, $p<0.001$; 4.0 ± 0.7% in ECC + CON, $p<0.001$) and 84d (3.9 ± 0.8% in CON, $p<0.001$; 4.0 ± 1.1% in ECC + CON, $p<0.001$). There was no increase in muscle CSA in either the CON leg or the ECC + CON leg from 28d to 84d. No difference was seen in change in muscle CSA with exercise training at any time point when comparing CON and ECC + CON legs.

Figure 3-4B shows percentage change in quadriceps muscle volume from baseline at 28d and 84d in both CON and ECC + CON limbs (graphs depicting absolute values shown in Appendix E). Quadriceps muscle volume was also increased above baseline in both legs at 28d (3.1 ± 0.8% in CON, $p<0.001$; 3.0 ± 0.4% in ECC + CON, $p<0.001$) and 84d (3.8 ± 0.8% in CON, $p<0.001$; 3.4 ± 0.7% in ECC + CON, $p<0.001$). Similar to muscle CSA, there was no increase in muscle volume in either the CON leg or the ECC + CON leg from 28d to 84d. No difference was seen in change in muscle volume with exercise training between CON and ECC + CON legs at any time point.

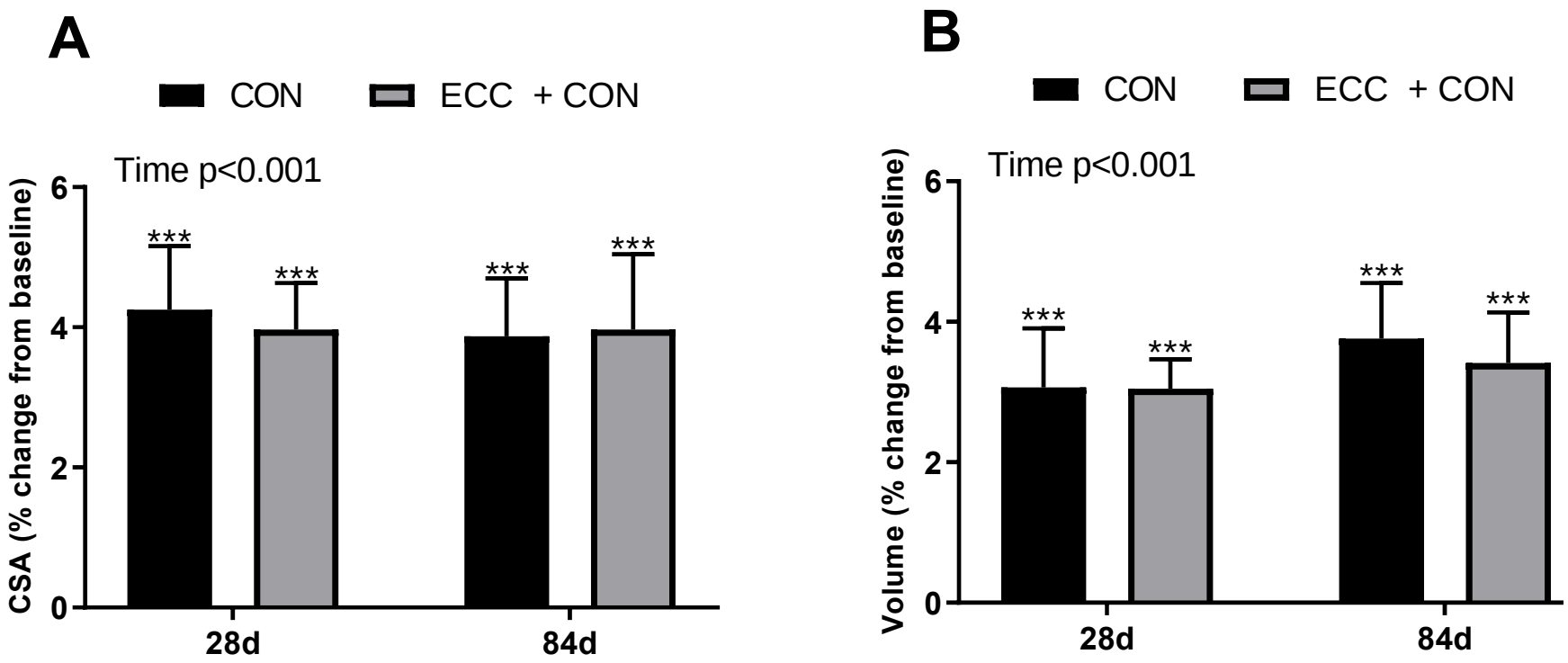

*Figure 3-4: (A) Percentage change from baseline in mid-thigh quadriceps muscle cross-sectional area (%), and (B) quadriceps muscle volume (%) in concentric (CON) and combined concentric + eccentric (ECC + CON) trained legs at 28 days (28d) and 84 days (84d) of training. Values expressed as mean ± SEM. ***p<0.001 compared to baseline.*

3.3.4 Muscle mRNA expression

Heat maps were generated (GraphPad Prism version 7.03) to depict muscle mRNA expression relative to baseline (log_2) in both CON and ECC + CON legs at each time point for each volunteer.

Figure 3-5 illustrates the change in mRNA abundance from mean baseline in both CON and ECC + CON legs at 24h (Figure 3-5A) and 7d (Figure 3-5B). An increase in mRNA abundance in response to training was apparent in both legs at 24h (Figure 3-5A) and 7d (Figure 3-5B). There was also a noticeable pattern of divergence between legs, such that there was generally a greater increase in abundance of muscle mRNA transcripts in the ECC + CON leg compared to the CON leg at 24h (Figure 3-5A) and 7d (Figure 3-5B)

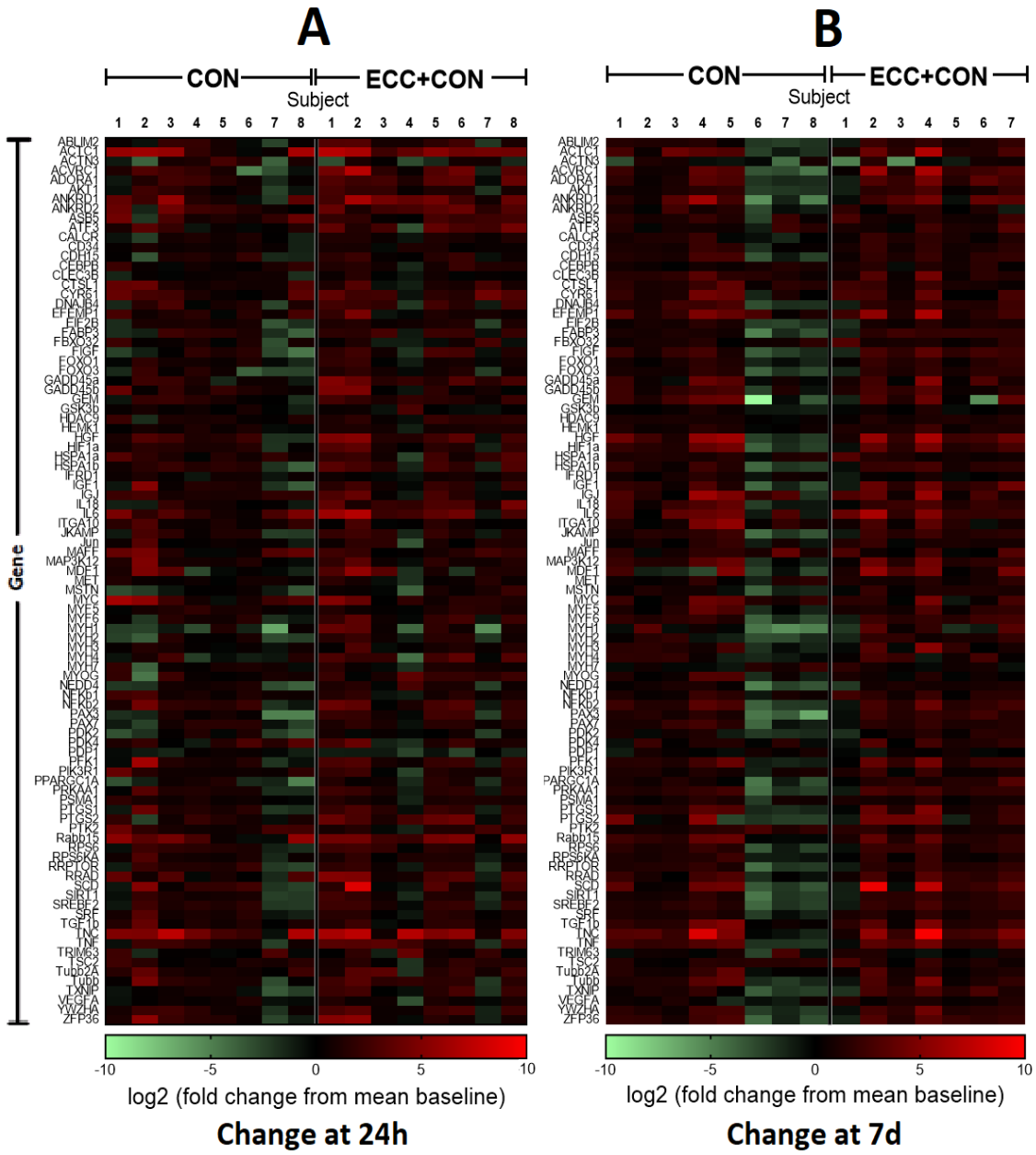

Figure 3-5: Data reflecting change in vastus lateralis mRNA abundance at 24 hours (A) and 7 days (B) in concentric (CON; n=8) and combined concentric + eccentric (ECC + CON; n=8 at 24 hours, n=7 at 7 days) trained legs for each participant. Change is depicted relative to mean baseline in the form of colours (heat map). Values are log_2 of fold change from mean baseline. Individual gene abbreviations shown on y-axis (see Appendix B for definitions). Red indicates a greater abundance and green indicates less abundance relative to baseline.

Figure 3-6 illustrates the change in mRNA abundance from mean baseline in both CON and ECC + CON legs at 28d (Figure 3-6A) and 84d (Figure 3-6B). The early mRNA abundance change noted at 24h and 7d (Figure 3-5) in both legs had waned at 28d (Figure 3-6A) and 84d (Figure 3-6B) The divergence in response between legs had also waned at 28d (Figure 3-6A) and 84d (Figure 3-6B).

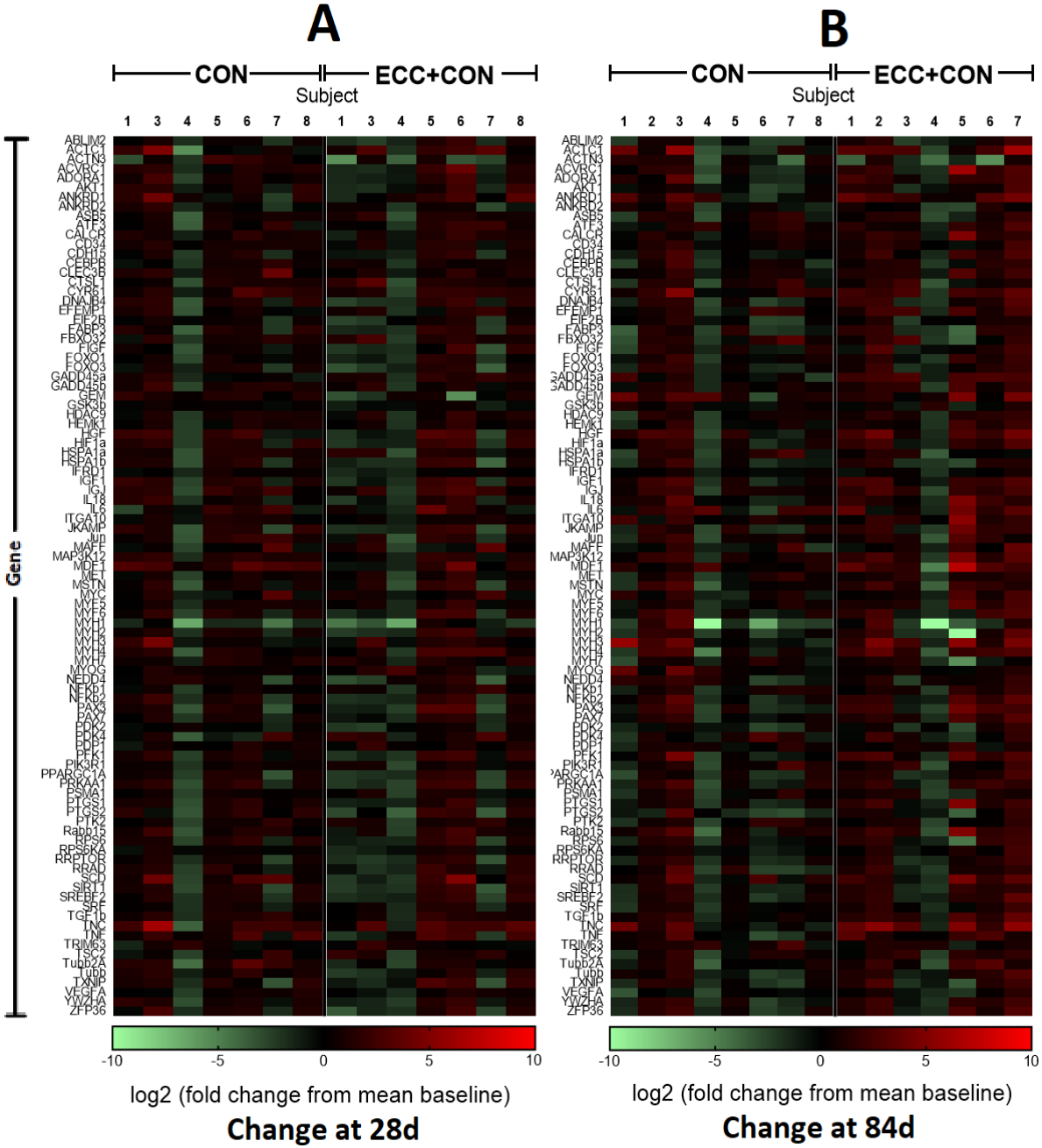

Figure 3-6: Data reflecting change in vastus lateralis mRNA abundance at 28 days (A) and 84 days (B) in concentric (CON; n=8) and combined concentric + eccentric (ECC + CON; n=8 at 28 days, n=7 at 84 days) trained legs for each participant. Change is depicted relative to mean baseline in the form of colours (heat map). Values are log_2 of fold change from mean baseline. Individual gene abbreviations shown on y-axis (see Appendix B for definitions). Red indicates a greater abundance and green indicates less abundance relative to baseline.

3.3.4.1 Ingenuity Pathway Analysis

Figure 3-7 depicts those cellular functions detected by IPA as being most altered from baseline in the CON leg and ECC + CON leg at 24h, 7d, 28d and 84d, based on collective changes in mRNA abundance from baseline at these time points. The x-axis displays cellular functions most affected, while the y-axis displays the -log of the p-values. The p-value associated with each cellular function is a measurement of the likelihood that the association between a set of focus transcripts and a given function is due to random chance. The -log of p-value was calculated using Fisher's exact test (right-tailed).

Overall, IPA detected altered cellular functions [–log (p value) > 5.0; Figure 3-7] associated with organismal injuries and abnormalities, cellular development, skeletal and muscular development and function, tissue development, carbohydrate metabolism, skeletal and muscular disorders and lipid metabolism. Figure 3-7 also illustrates that in the main the largest magnitude of response to exercise training occurred at 24h and 7d in both limbs, which was invariably greatest in the ECC + CON trained leg and particularly at 7d.

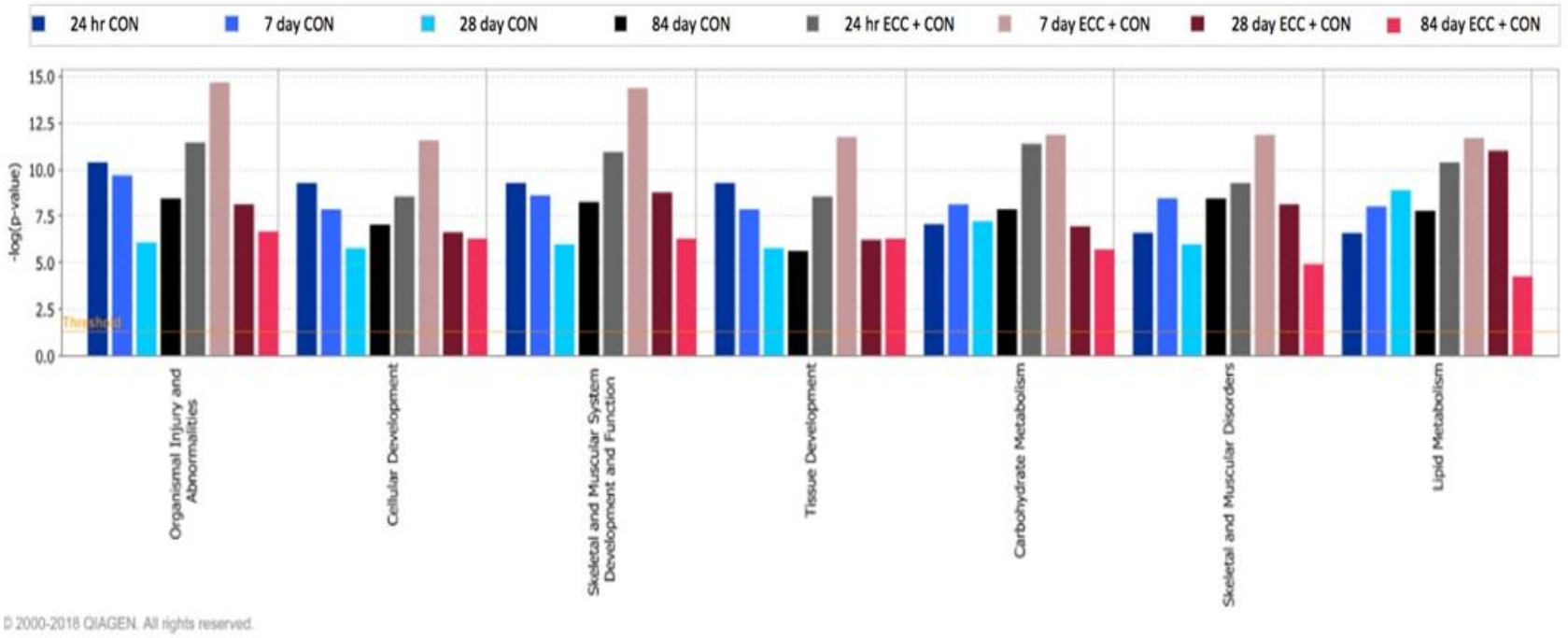

Figure 3-7: Cellular functions identified by IPA as being altered from baseline in vastus lateralis muscle in concentric (CON) and combined concentric + eccentric (ECC + CON) trained legs at 24 hours, 7 days, 28 days and 84 days based on mRNA expression data generated using the low-density microarray cards. The x-axis displays cellular functions most affected by training, whereas the y-axis displays the –log of the p value. The –log of the p value was calculated by Fisher's exact test right-tailed ($p < 0.05$). The threshold line corresponds to a p value of 0.05.

Figure 3-8 to Figure 3-13 are included by way of example to illuminate precisely how any cellular function identified as being altered in Figure 3-7, namely skeletal and muscular system development and function, tissue development and cellular development were influenced by training, and to also highlight the clear differences in response between limbs at 24h and 7d. Each figure depicts the most differentially regulated mRNAs from baseline (outer ring), and the cellular events predicted to result from these collective mRNA changes in CON and ECC + CON legs at 24h and 7d. In choosing which functions to depict, those were taken into account that changed most in each group relative to baseline at these time points, and for which the most network predictions were made with high confidence. The function "organismal injuries and abnormalities" is not shown as the mRNAs associated with this function and the predictions from their collective change were identical to "skeletal and muscular development and function" and would be redundant to include. Schematics for the rest of the cellular functions are shown in Appendix F.

In the case of skeletal and muscular system development and function at 24h (Figure 3-8), 16 mRNAs were identified as being altered in abundance relative to baseline in the CON leg (Figure 3-8A). The most upregulated mRNAs from baseline were tenascin C (TNC) and IL-6, whilst MSTN, paired box gene 3 (PAX3) and peroxisome proliferator-activated receptor gamma coactivator 1 alpha (PPARGC1A) were the most downregulated transcripts. Based on the collective changes in mRNA abundance from baseline, IPA predicted amongst a number of things activation of migration of muscle cells, and inhibition of muscle formation and quantity of muscle with highest confidence at 24h. In the

ECC + CON leg at 24h, 18 mRNAs were identified as being altered in abundance relative to baseline (Figure 3-8B). In contrast to the CON leg however, all mRNAs were increased in abundance. In common with the CON leg, IPA predicted activation of migration of muscle cells with high confidence. Proliferation of myoblasts and muscle cells, and formation of muscle were also predicted to be activated with high confidence. This latter prediction was in direct contrast to the CON leg, where formation of muscle was predicted to be inhibited with high confidence. In addition to this, inhibition of muscle function was predicted in the ECC + CON leg at 24h, which was not predicted for the CON leg.

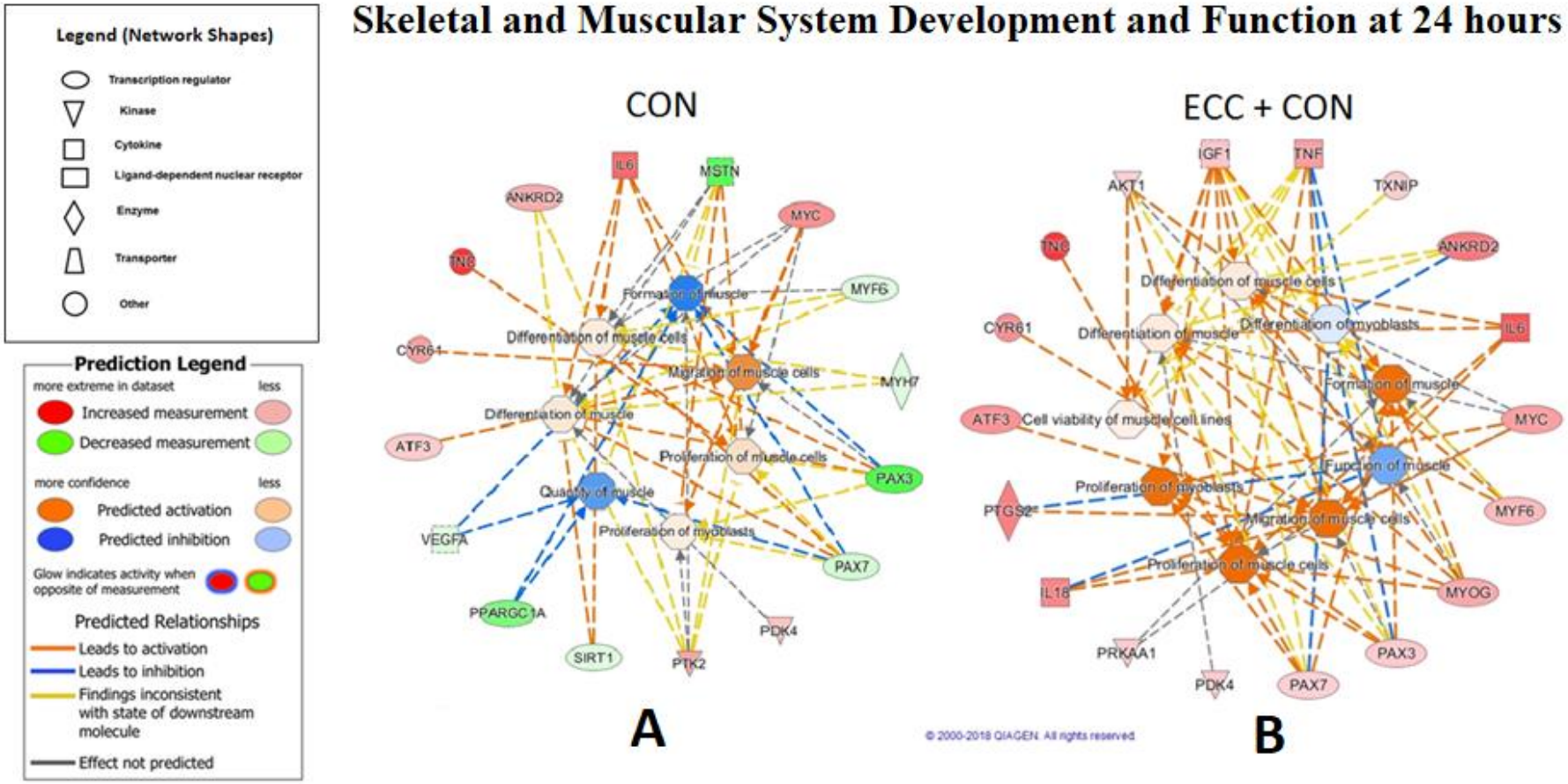

Figure 3-8: Differentially regulated muscle mRNAs (outer ring) associated with skeletal and muscular system development and function in (A) concentric (CON) and (B) combined eccentric + concentric (ECC + CON) trained legs at 24 hours of training compared to baseline, and the cellular events predicted by Ingenuity Pathway Analysis to result from the collective changes in mRNA abundance.

Abbreviations: IL-6, interleukin 6; MSTN, myostatin; MYC, MYC proto-oncogene; MYF6, myogenic factor 6; MYH7, myosin heavy chain 7; PAX3, paired box 3; PAX7, paired box 7; PDK4, pyruvate dehydrogenase kinase 4; PTK2, protein tyrosine kinase 2; SIRT1, sirtuin 1; PPARGC1A, peroxisome proliferator-activated receptor gamma coactivator 1 alpha; VEGFA, vascular endothelial growth factor A; ATF3, activating transcription factor 3; CYR61, cysteine-rich angiogenic inducer 61; TNC, tenascin C; ANKRD2, ankyrin repeat domain 2; AKT1, serine-threonine protein kinase1; PRKAA1, protein kinase AMP-activated catalytic subunit alpha 1; IGF1, insulin growth factor 1; TNF, tumour necrosis factor; TXNIP, thioredoxin-interacting protein; IL-18, interleukin 18; PTGS2, prostaglandin-endoperoxide synthase 2.

Figure 3-9 relates to skeletal and muscular system development and function at 7d. Fifteen mRNAs were differentially regulated from baseline in the CON leg (Figure 3-9A), which were in the main the same transcripts that were changed in abundance at 24h. Not surprisingly therefore, similar to 24h, IPA predicted activation of migration of muscle cells, and inhibition of muscle formation with highest confidence at 7d. Twenty-four mRNAs were found to be differentially expressed from baseline at 7d in the ECC + CON leg, and similar to 24h all were increased in expression (Figure 3-9B). Also, in keeping with 24h, IPA predicted activation of migration of muscle cells, proliferation of myoblasts and muscle cells, formation of muscle, and inhibition of muscle function with highest confidence at 7d in the ECC + CON leg. In addition to this, differences between legs in the cellular events predicted at 24h were still evident at 7d, i.e. formation of muscle was predicted to be inhibited in CON but activated in ECC + CON,

whilst the predicted inhibition of muscle function in ECC + CON was not seen in CON.

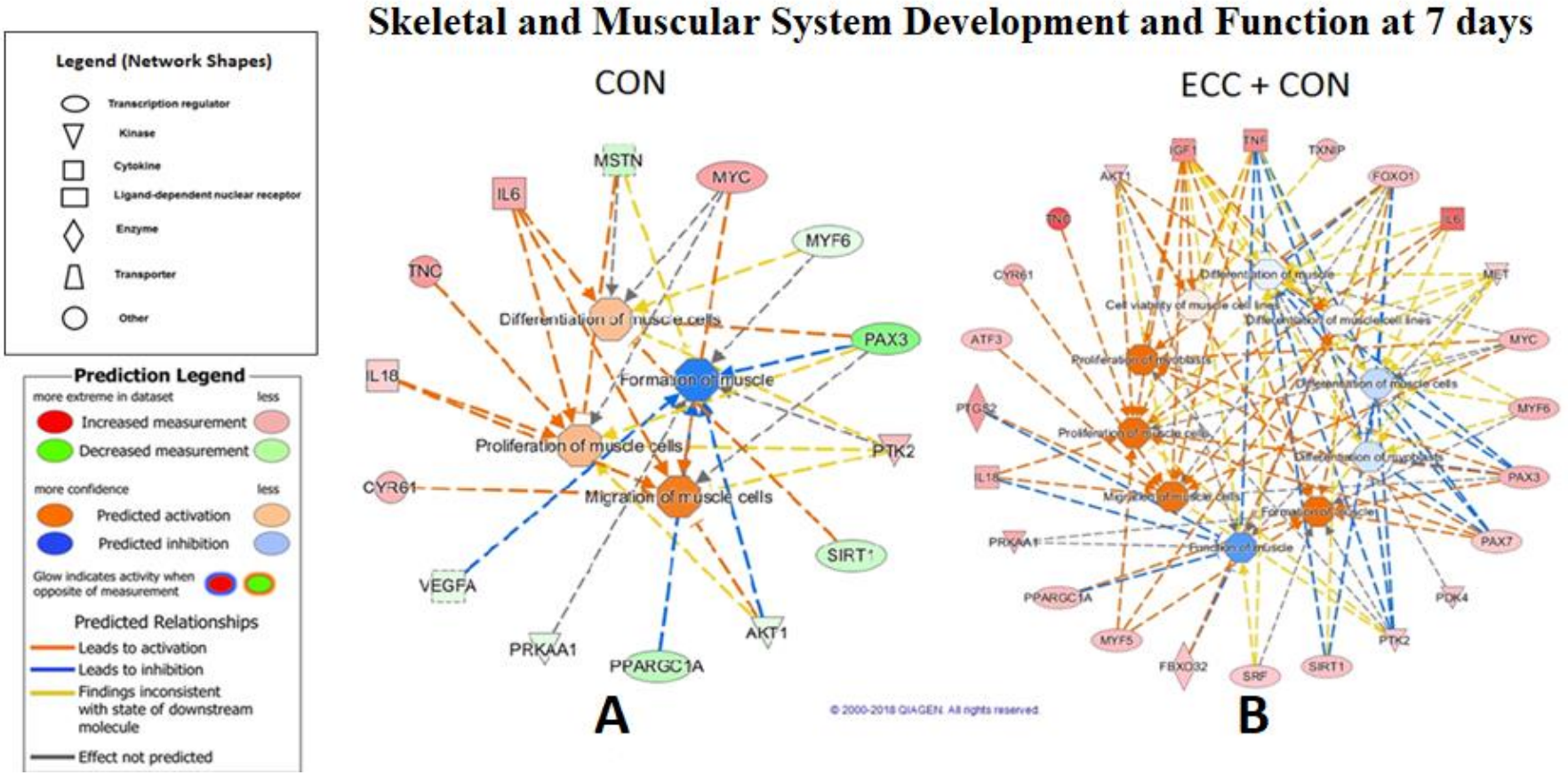

Figure 3-9*: Differentially regulated muscle mRNAs (outer ring) associated with skeletal and muscular system development and function in (A) concentric (CON) and (B) combined eccentric + concentric (ECC + CON) trained legs at 7 days of training compared to baseline, and cellular events predicted by Ingenuity Pathway Analysis to result from the collective changes in mRNA abundance.*

Abbreviations: MYC, MYC proto-oncogene; MYF6, myogenic factor 6; PAX3, paired box 3; PTK2, protein tyrosine kinase 2; SIRT1, sirtuin 1; AKT1, serine-threonine protein kinase1; PPARGC1A, peroxisome proliferator-activated receptor gamma coactivator 1 alpha; PRKAA1, protein kinase AMP-activated catalytic subunit alpha 1; VEGFA, vascular endothelial growth factor A; CYR61, cysteine-rich angiogenic inducer 61; IL-18, interleukin 18; TNC, tenascin C; IL-6, interleukin 6; MSTN, myostatin; TXNIP, thioredoxin-interacting protein; FOXO1, forkhead box protein O 1; PAX7, paired box 7; PDK4, pyruvate dehydrogenase kinase 4; SRF, serum response factor; FBXO32, F-box protein 32; MYF5, myogenic factor 5; PPARGC1A, peroxisome proliferator-activated receptor gamma coactivator 1 alpha; PTGS2, prostaglandin-endoperoxide synthase 2; ATF3, activating transcription factor 3; IGF1, insulin growth factor 1; TNF, tumour necrosis factor.

In the case of tissue development at 24h (Figure 3-10), 21 mRNAs were identified as being altered in abundance relative to baseline in the CON leg (Figure 3-10A). The most upregulated mRNAs from baseline were TNC and IL-6, whilst MSTN, PAX3, PPARGC1A and vascular endothelial growth factor D (VEGFD) were the most downregulated transcripts. Based on the collective changes in mRNA abundance from baseline, IPA predicted amongst a number of things inhibition of muscle formation with highest confidence at 24h. In the ECC + CON leg at 24h, 19 mRNAs were identified as being altered in abundance relative to baseline (Figure 3-10B). IL-6 and TNC were once again the most upregulated transcripts. Once again, in contrast to the CON leg, all mRNAs were increased in abundance. Also, in contrast to the CON leg, based on the collective

changes in mRNA abundance from baseline, IPA predicted with high confidence activation of formation of muscle, proliferation of connective tissue cells, proliferation of muscle cells and proliferation of myoblasts.

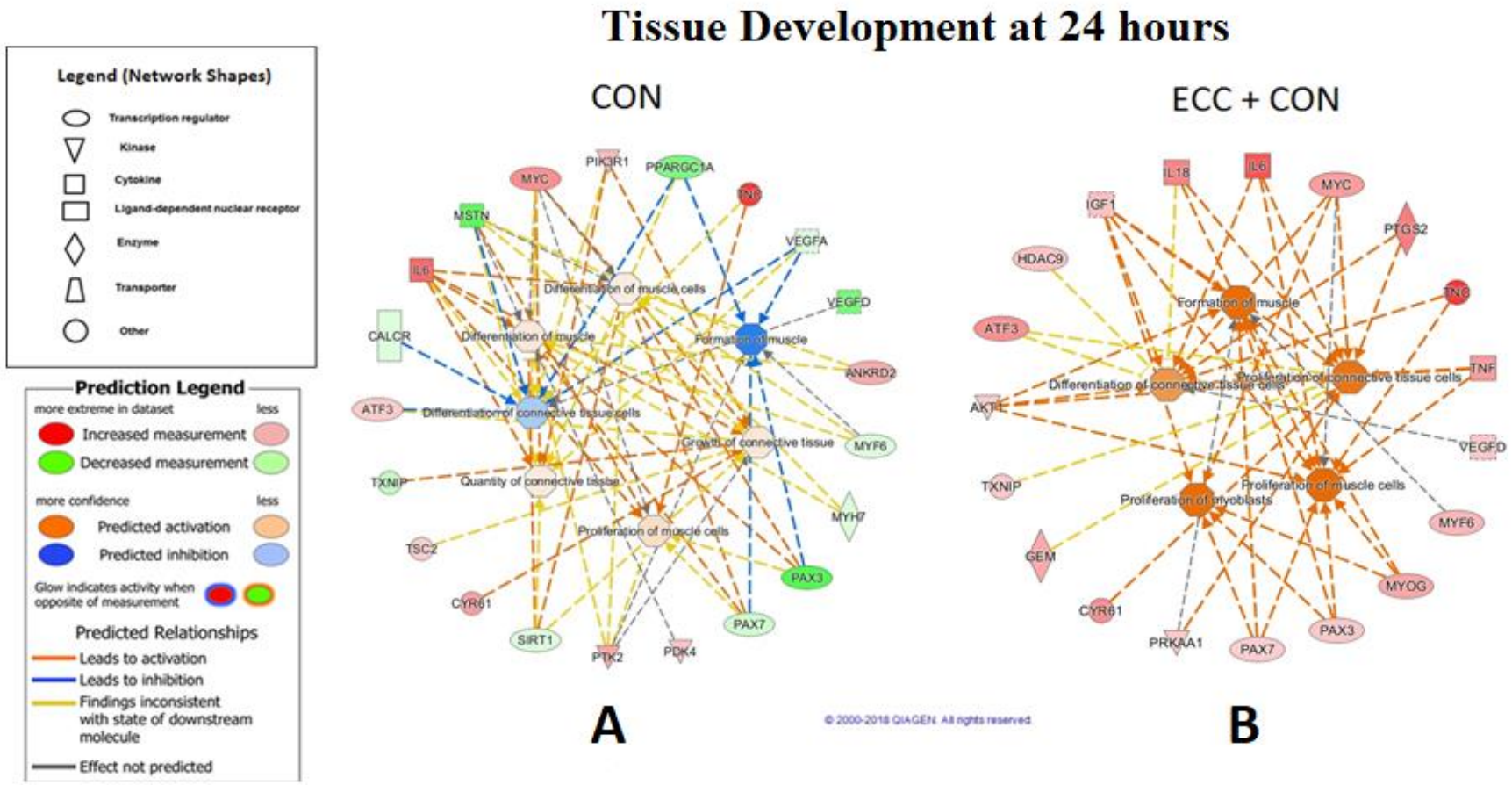

Figure 3-10*: Differentially regulated muscle mRNAs (outer ring) associated with tissue development in (A) concentric (CON) and (B) combined eccentric + concentric (ECC + CON) trained legs at 24 hours of training compared to baseline, and the cellular events predicted by Ingenuity Pathway Analysis to result from the collective changes in mRNA abundance.*

Abbreviations: PIK3R1, phosphoinositide-3-kinase regulatory subunit 1; PPARGC1A, peroxisome proliferator-activated receptor gamma coactivator 1 alpha; PRKAA1; TNC, tenascin C; VEGFA, vascular endothelial growth factor A; VEGFD, vascular endothelial growth factor D; ANKRD2, ankyrin repeat domain 2; MYF6, myogenic factor 6; MYH7, myosin heavy chain 7; PAX3, paired box 3; PAX7, paired box 7; PTK2, protein tyrosine kinase 2; SIRT1, sirtuin 1; CYR61, cysteine-rich angiogenic inducer 61; TSC2, Tuberous Sclerosis Complex Subunit 2; TXNIP, thioredoxin-interacting protein; ATF3, activating transcription factor 3; CALCR, calcitonin receptor; IL-6, interleukin 6; MSTN, myostatin; MYC, c-myc proto-oncogene; PTGS2, prostaglandin-endoperoxide synthase 2; TNF, tumour necrosis factor; MYOG, myogenin; PRKAA1, protein kinase AMP-activated catalytic subunit alpha 1; GEM, GTP-binding protein; AKT1, serine-threonine protein kinase1; HDAC9, Histone Deacetylase 9; IGF1, insulin growth factor 1; IL-18, interleukin 18.

Figure 3-11 relates to tissue development at 7d. Thirteen mRNAs were differentially regulated from baseline in the CON leg (Figure 3-11A). MYC proto-oncogene (MYC), IL-6 and TNC were the most upregulated transcripts, while PAX3 was the most downregulated transcript. Based on the collective changes in mRNA abundance from baseline, inhibition of formation of muscle was predicted by IPA with highest confidence. In contrast to the CON leg, twenty-eight mRNAs were found to be differentially expressed from baseline at 7d in the ECC + CON leg, and all were increased in expression (Figure 3-11B). Also, in contrast to the CON leg, IPA predicted activation of formation of muscle, growth of connective tissue, proliferation of connective tissue cells,

proliferation of migration of muscle cells, proliferation of myoblasts, regeneration of muscle and differentiation of connective tissue with high confidence.

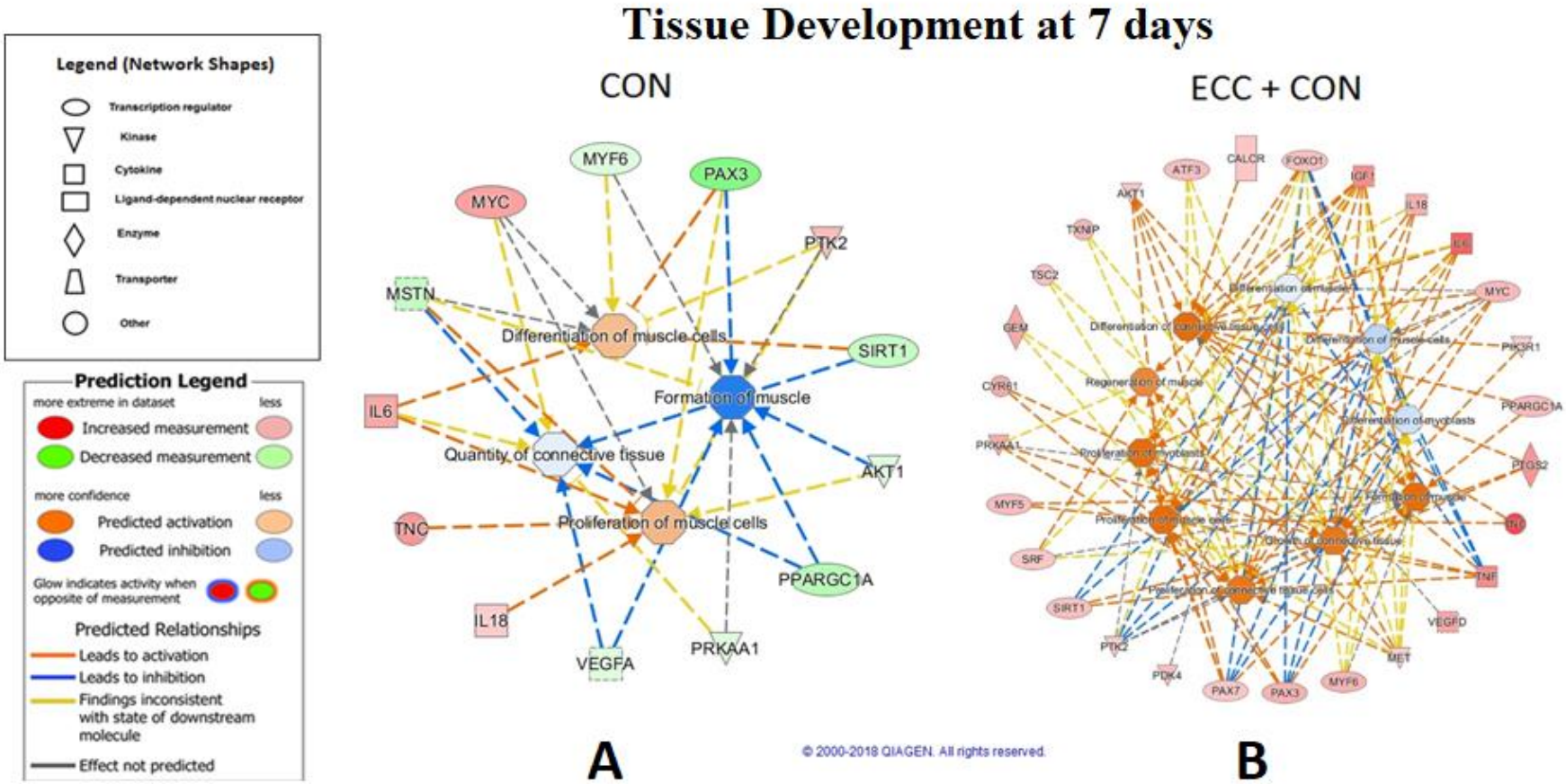

Figure 3-11: Differentially regulated muscle mRNAs (outer ring) associated with tissue development in (a) concentric (CON) and (b) combined eccentric + concentric (ECC + CON) trained legs at 7 days of training compared to baseline, and cellular events predicted by Ingenuity Pathway Analysis to result from the collective changes in mRNA abundance.

Abbreviations: MYF6, myogenic factor 6; PAX3, paired box 3; PTK2, protein tyrosine kinase 2; SIRT1, sirtuin 1; AKT1, serine-threonine protein kinase1; PPARGC1A, peroxisome proliferator-activated receptor gamma coactivator 1 alpha; PRKAA1, protein kinase AMP-activated catalytic subunit alpha 1; VEGFA, vascular endothelial growth factor A; IL-18, interleukin 18; TNC, tenascin C; IL-6, interleukin 6; MSTN, myostatin; MYC, MYC proto-oncogene; CALCR, calcitonin receptor; FOXO1, forkhead box protein O 1; IGF1, insulin growth factor 1; PIK3R1, phosphoinositide-3-kinase regulatory subunit 1; PTGS2, prostaglandin-endoperoxide synthase 2; TNF, tumour necrosis factor; VEGFD, vascular endothelial growth factor D; MET, MET proto-oncogene; PAX7, paired box 7; PDK4, pyruvate dehydrogenase kinase 4; SRF, serum response factor; MYF5, myogenic factor 5; CYR61, cysteine-rich angiogenic inducer 61; GEM, GTP-binding protein; TSC2, Tuberous Sclerosis Complex Subunit 2; TXNIP, thioredoxin-interacting protein; ATF3, activating transcription factor 3.

In the case of cellular development at 24h (Figure 3-12), 16 mRNAs were identified as being altered in abundance relative to baseline in the CON leg (Figure 3-12A). The most upregulated mRNAs from baseline were TNC and IL-6, whilst MSTN, PAX3, PPARGC1A and VEGFD were the most downregulated transcripts. Based on the collective changes in mRNA abundance from baseline, IPA did not make any network predictions with high confidence. In the ECC + CON leg at 24h, 17 mRNAs were identified as being altered in abundance relative to baseline (Figure 3-12B). Once again, in contrast to the CON leg, all mRNAs were increased in abundance. Also in contrast to the CON leg, IPA

predicted activation of proliferation of muscle cells and proliferation of myoblasts with highest confidence.

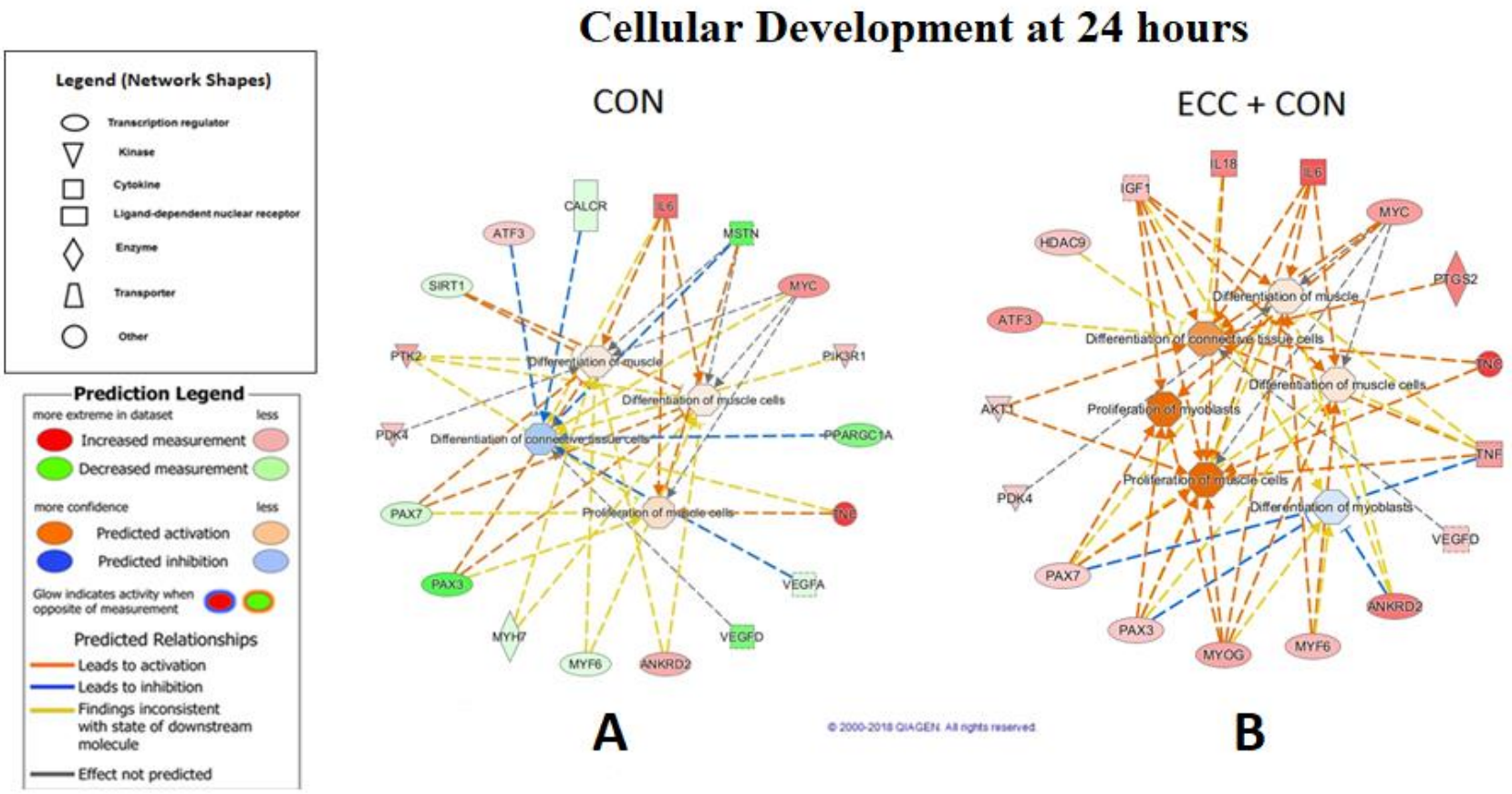

Figure 3-12: Differentially regulated muscle mRNAs (outer ring) associated with cellular development in (A) concentric (CON) and (B) combined eccentric + concentric (ECC + CON) trained legs at 24 hours of training compared to baseline, and the cellular events predicted by Ingenuity Pathway Analysis to result from the collective changes in mRNA abundance.

Abbreviations: IL-6, interleukin 6; MSTN, myostatin; MYC, MYC proto-oncogene; PIK3R1, phosphoinositide-3-kinase regulatory subunit 1; PPARGC1A, peroxisome proliferator-activated receptor gamma coactivator 1 alpha; TNC, tenascin C; VEGFA, vascular endothelial growth factor A; VEGFD, vascular endothelial growth factor D; ANKRD2, ankyrin repeat domain 2; MYF6, myogenic factor 6; MYH7, myosin heavy chain 7; PAX3, paired box 3; PDK4, pyruvate dehydrogenase kinase 4; PTK2, protein tyrosine kinase 2; SIRT1, sirtuin 1; ATF3, activating transcription factor 3; IL-18, interleukin 18; PTGS2, prostaglandin-endoperoxide synthase 2; TNF, tumour necrosis factor; MYOG, myogenin; PAX7, paired box gene 7; AKT1, serine-threonine protein kinase1; HDAC9, Histone Deacetylase 9; IGF1, insulin growth factor 1.

Figure 3-13 relates to cellular development at 7d. Thirteen mRNAs were differentially regulated from baseline in the CON leg (Figure 3-13A). MYC, IL-6, TNC and PTK2 were the most upregulated transcripts, while PAX3 was the most downregulated transcript. Based on collective changes in mRNA abundance, IPA predicted an inhibition of differentiation of connective tissue cells, and activation of development of connective tissue cells with high confidence at 7d. In contrast to the CON leg, 24 mRNAs were found to be differentially expressed from baseline at 7d in the ECC + CON leg, and similar to 24h all were increased in expression (Figure 3-13B). Also similar to 24h, IPA predicted activation of proliferation of myoblasts and muscle cells, but in addition also predicted activation of differentiation of connective tissue cells, all with high confidence at 7d in the ECC + CON leg.

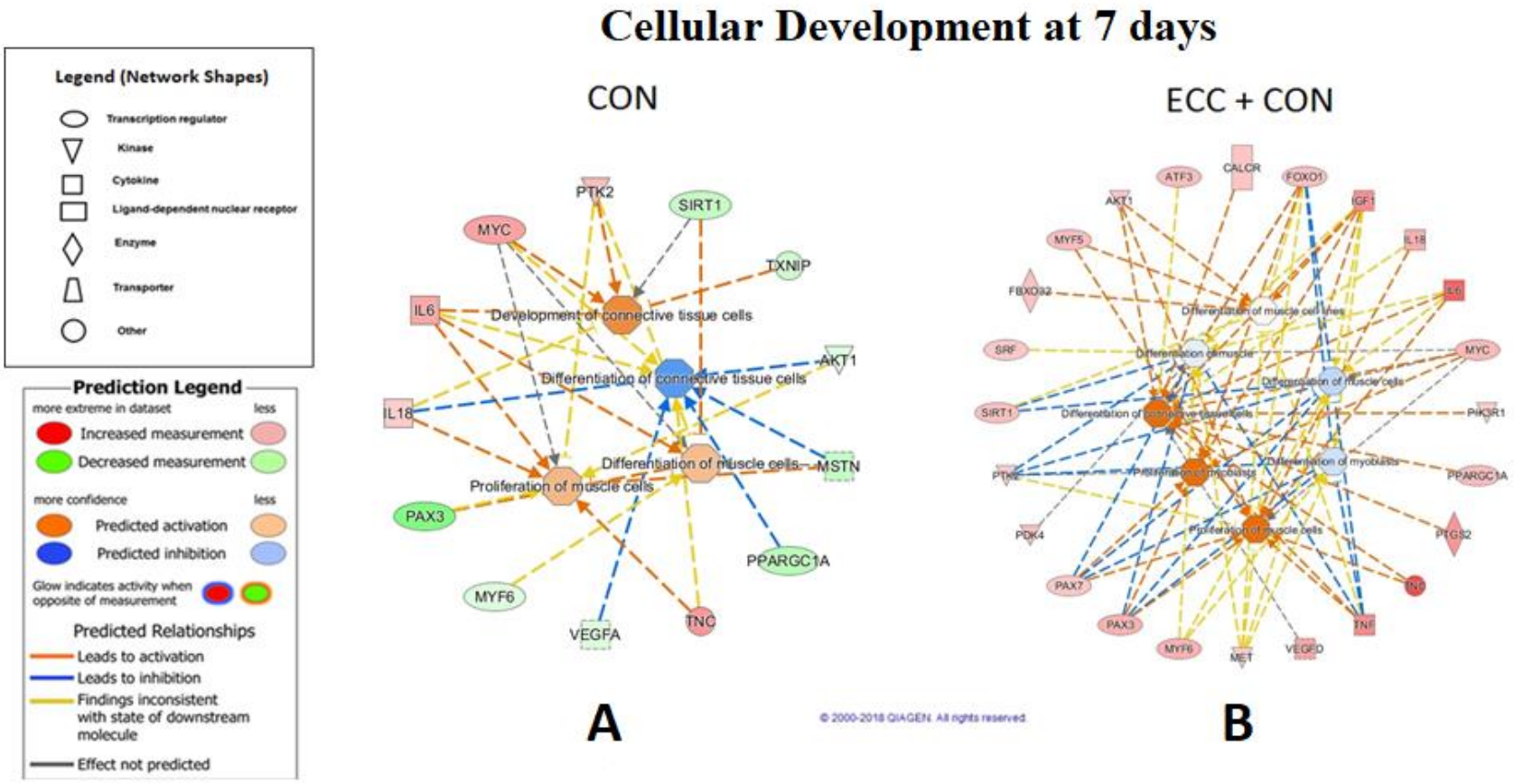

Figure 3-13: Differentially regulated muscle mRNAs (outer ring) associated with cellular development in (A) concentric (CON) and (B) combined eccentric + concentric (ECC + CON) trained legs at 7 days of training compared to baseline, and the cellular events predicted by Ingenuity Pathway Analysis to result from the collective changes in mRNA abundance.

Abbreviations: PTK2, protein tyrosine kinase 2; SIRT1, sirtuin 1; TXNIP, thioredoxin-interacting protein; AKT1, serine-threonine protein kinase1; MSTN, myostatin; PPARGC1A, peroxisome proliferator-activated receptor gamma coactivator 1 alpha; TNC, tenascin C; VEGFA, vascular endothelial growth factor A; MYF6, myogenic factor 6; PAX3, paired box 3; IL-18, interleukin 18; IL-6, interleukin 6; MYC, MYC proto-oncogene; CALCR, calcitonin receptor; FOXO1, forkhead box protein O 1; IGF1, insulin growth factor 1; PIK3R1, phosphoinositide-3-kinase regulatory subunit 1; PPARGC1A, peroxisome proliferator-activated receptor gamma coactivator 1 alpha; PTGS2, prostaglandin-endoperoxide synthase 2; TNF, tumour necrosis factor; VEGFD, vascular endothelial growth factor D; MET, MET proto-oncogene; MYF6, myogenic factor 6; PAX7, paired box 7; PDK4, pyruvate dehydrogenase kinase 4; SIRT1, sirtuin 1; SRF, serum response factor; FBXO32, F-box protein 32; MYF5, myogenic factor 5; ATF3, activating transcription factor 3.

Table 3-2 provides a summary of the gene networks predicted to be activated or inhibited with high confidence for all functions detected as altered by IPA in Figure 3-7.

Table 3-2: A summary of those events associated with cellular functions predicted by IPA to be most altered based on muscle mRNA abundance change from baseline in concentric (CON) and combined concentric + eccentric (ECC + CON) trained legs at 24 hours (24h) and 7 days (7d). Activation of an event predicted with high confidence indicated by (+). Inhibition of an event predicted with high confidence indicated by (-).

	24 h		7 d	
	CON	ECC + CON	CON	ECC + CON
Skeletal and muscular system development and function	(-) Formation of muscle (-) Quantity of muscle (+) Migration of muscle cells	(+) Formation of muscle (+) Migration of muscle cells (+) Proliferation of muscle cells (+) Proliferation of myoblasts	(+) Migration of muscle cells (-) Formation of muscle	(+) Proliferation of myoblasts (+) Proliferation of muscle cells (+) Migration of muscle cells (-) Formation of muscle
Organismal injuries and abnormalities	(+) Atrophy of muscle (+) Cell death of muscle cells	No strong prediction	(+) Atrophy of muscle (+) Apoptosis of muscle cells (+) Cell death of muscle cells	No strong prediction
Lipid metabolism	(+) Secretion of lipid (+) Synthesis of lipid (+) Concentration of lipid	No strong prediction	(+) Concentration of lipid	(+) Secretion of lipid (+) Synthesis of fatty acid

	24 h		7 d	
	CON	ECC + CON	CON	ECC + CON
Tissue development	(-) Formation of muscle	(+) Formation of muscle (+) Proliferation of connective tissue cells (+) Proliferation of muscle cells Proliferation of myoblasts	(-) Formation of muscle	(+) Differentiation of connective tissue cells (+) Proliferation of myoblasts (+) Regeneration of muscle (+) Proliferation of muscle cells (+) Proliferation of connective tissue cells (+) Formation of muscle
Carbohydrate metabolism	No strong prediction	No strong prediction	No strong prediction	(+) Transport of carbohydrate
Skeletal and muscular disorders	(+) Atrophy of muscle (+) Cell death of muscle cells	No strong prediction	(+) Atrophy of muscle (+) Apoptosis of muscle cells (+) Cell death of muscle cells	No strong prediction

	24 h		7 d	
	CON	ECC + CON	CON	ECC + CON
Cellular development	No strong prediction	(+) Proliferation of myoblasts (+) Proliferation of muscle cells	(+) Development of connective tissue cells (-) Differentiation of connective tissue cells	(+) Differentiation of connective tissue cells (+) Proliferation of myoblasts (+) Proliferation of muscle cells

3.4 Discussion

A major finding of the present study was that the greater amount of work done during 84 days of unilateral combined concentric and eccentric maximal voluntary isokinetic exercise training in young, trained males did not result in differential temporal responses or gains in muscle CSA, muscle volume or strength when compared with the contralateral leg which performed only maximal voluntary concentric exercise training. Of further note, mRNA abundance associated with a number of cellular functions was altered from baseline in both limbs throughout the course of training, with changes in abundance being most marked at 24h and 7d in the limb that performed combined concentric and eccentric training. However, the early changes in mRNA abundance had waned in both limbs by 28d and 84d of training. It is therefore concluded that initial mRNA abundance responses to maximal load exercise training are unlikely to reflect chronic training-induced muscle hypertrophy, suggesting some caution when extrapolating chronic muscle adaptive responses from acute exercise intervention studies. Moreover, neither the greater amount of work done over the course of 84 days of high load combined concentric and eccentric training, nor marked differences in mRNA responses during the early phase of training impacted on muscle CSA, volume or strength gains compared to concentric training alone.

A ~4% increase in muscle CSA was detected in both limbs (Figure 3-4). This magnitude of change is similar to other studies comparing hypertrophic responses to 10-12 weeks of maximal concentric resistance training versus maximal eccentric resistance training (Friedmann-Bette et al. 2010; Farup et al.

2014). However, some studies have shown that eccentric training produces greater gains in muscle mass than concentric training at low-moderate intensities (Friedmann-Bette et al. 2010; Hortobagyi, Barrier, et al. 1996) and high intensities (Higbie et al. 1996; Vikne et al. 2006). A meta-analysis by Roig et al. (2009) considered a number of studies that utilised a variety of exercise protocols, to compare muscle mass and strength gains following concentric versus eccentric training. The authors reported that eccentric training performed at high intensities may have resulted in greater muscle mass gains than concentric training. Of note however, in those studies where workloads were matched between concentric and eccentric training protocols, no difference was noted in muscle mass gains between the two modalities. The authors concluded therefore that the greater muscle forces generated during eccentric training were responsible for the differential gains in muscle mass. Moore et al. (2005) tested this proposition by having young male volunteers perform lengthening contractions of the biceps brachii with one arm and shortening contractions in the contralateral arm two times per week for nine weeks, but with work output matched between arms. In keeping with the conclusion of Roig et al. (2009) a similar increase in muscle CSA was observed in each limb at the end of the training period. Conversely, more recent studies have reported that when workloads were unmatched between concentric and eccentric training at 80% of 1-RM, still no differences in gains in vastus lateralis muscle volume (Franchi et al. 2014), or vastus lateralis muscle thickness and fascicle length (Franchi et al. 2018) were demonstrable over 8-10 weeks of training. In the present study it was shown that despite the ECC + CON leg performing more work per week (Figure 3-2A) and across the whole training period (Figure 3-2B), no differential

temporal gains in muscle CSA (Figure 3-4A), volume (Figure 3-4B) or isometric strength over the CON leg were observed. This observation is supported by Cadore et al. (2014) who reported no difference in the increase in vastus lateralis muscle thickness, determined using ultrasound, over the course of 6-weeks isokinetic eccentric exercise training of the quadriceps compared to another group of volunteers who performed isokinetic concentric exercise, despite work output being ~40% greater over the course of training in the eccentric exercise trained group. Indeed, several studies have reported no differences in muscle mass adaptation when comparing maximal eccentric vs maximal concentric exercise training (Farup et al. 2014; Smith and Rutherford 1995; Blazevich et al. 2007), and recent studies have shown similar hypertrophic responses following concentric training versus eccentric training at 80% of 1-RM when workloads were not matched between the two training modalities (Franchi et al. 2014).

Many studies to date have focused on acute muscle responses to eccentric exercise to provide insight on chronic muscle adaptation. However, based on the collective results of this study, it would be misleading to conclude that early mRNA responses to eccentric exercise training will reflect chronic training-induced muscle hypertrophy. This is illustrated by Figure 3-7 where, with perhaps the exception of lipid metabolism, the heightened effect of combined eccentric and concentric exercise on a number of cellular functions altered by exercise was found to be transitory. Moreover, Figure 3-8 to Figure 3-13 clearly demonstrate mRNA responses in the first week of training were very different when comparing exercise modalities, with wholesale differences in mRNA abundance changes from baseline and cellular predictions evident between

limbs. It remains unclear why the magnitude of mRNA responses declined from 28 days onwards in the ECC + CON limb despite training workloads remaining different between limbs.

Acute exercise intervention studies have reported greater muscle damage and inflammatory responses to be associated with high load eccentric exercise, which has been proposed to stimulate myogenesis and cellular processes involved in muscle growth. For example, utilising gene transcript profiling Chen et al. (2003) reported a greater muscle inflammatory response 4-8h following 300 eccentric contractions of one leg when compared with 300 concentric contractions in the contralateral leg. Similarly, Kostek et al. (2007) identified 51 differentially expressed genes associated with protein synthesis, stress response/early growth, and sarcolemmal structure when comparing eccentrically and concentrically exercised limbs over 24h following exercise, albeit in n=3 volunteers. In keeping with this, local NSAID infusion following acute maximal eccentric exercise in healthy volunteers was reported to decrease muscle satellite cell proliferation (Mikkelsen et al. 2009b). On the other hand, Deane et al. (2019) found the muscle transcriptional response to unilateral concentric and eccentric resistance exercise in young volunteers was highly similar following 5h of recovery, which may have been attributable to the volunteers being exercise naïve. The point being made here is that studies to date have invariably only documented the acute muscle responses to eccentric and concentric exercise. In the context of this point, although “organismal injury and abnormalities” was identified by IPA as a cellular function significantly altered by training in the present study, which in keeping with the literature was most altered in the ECC

+ CON limb at 24h and 7d of training compared to the CON limb (Figure 3-7), the magnitude of this insult had waned in both limbs by 28 days of training, and to an extent that the response between limbs was similar at 28 and 84 days (Figure 3-7). In short, the heightened effect of eccentric exercise on the muscle injury was short lived and may therefore explain why eccentric training did not produce greater gains in muscle CSA and volume in the present study.

As an aside, studies investigating the impact of resistance exercise induced-inflammation *per se* on myogenesis and other processes linked to regulation of muscle mass appear to be equivocal. Using robust experimental designs involving local and systemic NSAID administration, it has been reported that the presence of inflammatory mediators in the first eight days of post-eccentric exercise recovery stimulated satellite cell proliferation (greater Pax7+ cells per myofiber; (Mikkelsen et al. 2009b)), whilst others have suggested the blunting of inflammation by NSAID consumption potentiated satellite cell activation (ActiveNotch1^{+} satellite cells) during the first week of recovery from electrically evoked eccentric contractions (Mackey et al. 2016). Importantly, few studies have investigated the impact of chronic resistance exercise-induced inflammation on muscle adaptation. Trappe et al. (2011) reported 6-weeks of progressive resistance exercise training produced a ~25–50% greater increase in muscle volume in elderly volunteers consuming either acetaminophen or ibuprofen during the training period. The authors suggested that NSAID ingestion, by blocking inflammation, positively impacted on muscle hypertrophy. This may indeed be the case, given a number of cellular functions linked to muscle injury and growth were found to be significantly altered

throughout the 12 weeks of exercise training in the present study, irrespective of whether concentric or eccentric exercise was performed (Figure 3-7). However, such a conclusion must be contextualised, given these participants were young, healthy males. Aging is associated with a low grade inflammatory state (Schaap et al. 2009; Schaap et al. 2006). Hence, the muscle hypertrophy noted by Trappe et al. (2011) in elderly volunteers in response to a combination of NSAID ingestion and progressive resistance training might have been due to an inhibition of this low grade inflammation, rather than solely of inflammation induced by resistance training.

The temporal effects of concentric versus combined concentric and eccentric chronic training on muscle CSA, muscle volume and strength were examined. It was observed that muscle CSA (Figure 3-4A) and volume (Figure 3-4B) increased by ~4% by 28d with both training modalities, but perhaps surprisingly was unchanged thereafter despite work done during training being maintained. In keeping with this observation, Ogasawara et al. (2013) reported that progressive resistance exercise training of the triceps brachii increased its CSA by 0.25% per day during the initial 6 weeks of training, which then waned between 9-24 weeks of training. Similarly, DeFreitas et al. (2011) demonstrated the magnitude of increase in muscle CSA from baseline was greatest in the initial 4 weeks (6.7%) of high intensity resistance training of the quadriceps compared to the increase recorded over 8 weeks (9.6%). A potential mechanism underpinning this phenomenon was provided by Brook et al. (2015), who by quantifying chronic changes in MPS from deuterated water tracer incorporation, demonstrated an increase in quadriceps MPS over the initial three weeks of

training, which then waned from 3-6 weeks despite a progressive increase in training workload in this time. Hence, hypertrophic remodelling was most active during the early stages of training. This is in line with evidence indicating that chronic resistance training blunts the MPS response to acute resistance training (Phillips et al. 2002; Phillips et al. 1999). Therefore, it is likely that the MPS response to resistance training in our study reached a plateau in both CON and ECC + CON legs between 28d and 84d and supports the concept that muscle adaptations to resistance exercise do not occur in a linear fashion.

Clearly it is important to identify why greater force generation during eccentric training did not translate to greater gains in muscle CSA and volume in the present study. One possible explanation is that the MPS response to resistance exercise reaches an upper ceiling at submaximal exercise intensities. Kumar, Selby, et al. (2009) reported that the MPS response to resistance exercise was dose-dependent and plateaued at an intensity of 75-80% of 1-RM, suggesting that MPS was maximised in both CON and ECC + CON legs in the present study. Alternatively, it is known that the energy cost of eccentric exercise is considerably less than concentric exercise, despite muscle mechanical force generation being greater (Hoppeler 2014a). This being the case, it is entirely feasible that increases in muscle metabolic stressors known to be linked to muscle protein synthetic and hypertrophic responses, such as the cellular energy charge, the cellular redox state, and muscle lactate and pH (Egan et al. 2013), would be less during eccentric compared with concentric exercise.

To conclude, this study found that while combined maximal, isokinetic concentric and eccentric training produced greater work output during a 12-week

training regimen compared to maximal, isokinetic concentric training, this did not translate to more rapid or greater increases in quadriceps muscle CSA, volume or strength. Furthermore, change in muscle mRNA abundance associated with a number of cellular functions was observed with both training modalities, but was most marked at 24 hours and 7 days in the limb that performed combined concentric and eccentric training. However, the early changes in mRNA abundance waned in both limbs by 28 days of training, and it is unclear why this occurred in the face of training workloads being sustained and different between limbs. Thus, neither the greater amount of mechanical work done during combined eccentric and concentric training, nor marked differences in mRNA responses during the early phase of training, was associated with differential gains in muscle CSA, volume or strength compared to concentric training alone. This may be explained by a non-linear relationship existing between mechanical force generation and muscle protein synthetic responses to exercise and/or the blunted metabolic stress of eccentric exercise diminishing hypertrophic stimulation.

CHAPTER 4. The effect of twelve weeks of combined chronic non-steroidal anti-inflammatory drug ingestion and unilateral maximal concentric training on the time course of change in muscle cross-sectional area, muscle volume, strength, targeted muscle gene networks and expression of inflammatory protein targets in healthy, young male volunteers

4.1 Introduction

Non-steroidal anti-inflammatory drugs (NSAIDs) are a class of medication widely used for their anti-inflammatory and analgesic effects, available both by prescription and over-the-counter (OTC) (Baum, Kennedy, and Forbes 1985). Early studies on the prevalence of NSAID use in the general population showed that they were used by over 30 million people every day (Singh 2000; Baum, Kennedy, and Forbes 1985). More recent work has shown that around 98 million NSAID prescriptions were filled in 2012 in the United States (Nash 2012), where over 30 billion doses of both prescription and OTC NSAIDs are consumed annually (Cryer et al. 2016). NSAIDs act by competitive inhibition of the cyclooxygenase (COX) enzyme (Scholer et al. 1986), effectively blocking the production of prostaglandins (Figure 1-8) that contribute to inflammation, pain and changes in body temperature. Both COX-1 and COX-2 catalyse the synthesis of prostaglandins, including prostaglandin D2, prostaglandin E2, prostaglandin F2alpha and prostaglandin I2 (Figure 1-8) (Markworth et al. 2014). NSAIDs are hence administered for their anti-inflammatory effects in conditions like osteoarthritis (Schnitzer 2002), rheumatoid arthritis and polymyositis (Kean and Buchanan 2005; Zochling et al. 2006). They are also taken to reduce pain in a variety of conditions and situations, such as maxillofacial pain in dentistry, temporomandibular joint pain (Ong and Seymour 2003), kidney stones, gallstones, post-operative/post-traumatic pain, sprains, strains and contusions (Simon 1997; Lipton et al. 1998) and dysmenorrhea (Connolly 2003).

NSAIDs are also used by individuals who perform sport at a variety of levels. In a recent study in Irish collegiate student-athletes, of the 149 individuals who completed an anonymous questionnaire online, 94% reported using NSAIDs previously and 63.8% had used NSAIDs before or after practice and/or competition (O'Connor et al. 2019). From a sample of 129 athletes at the University of Birmingham, 68% reported using NSAIDs over the prior 12 months (Rudgard, Hirsch, and Cox 2019). This was a diverse group, of whom 84.4% were triathletes, 70.9% were runners and 52.5% were cyclists. One of the earliest studies on the use of NSAIDs in endurance sport showed that 34.6% of participants at the Jungfrau-Marathon had detectable levels of NSAIDs in their urine (Mahler 2001). At the Brazil Iron Man triathlon in 2008, nearly 60% of respondents to a questionnaire reported taking NSAIDs (Gorski et al. 2011). Most recently, among participants in a mountain ultramarathon event, 48.3% reported taking NSAIDs either before, during or immediately after the event (Martinez et al. 2017). At the 2016 London marathon, 45.9% of respondents to a questionnaire planned to take an NSAID during the race, and of a cohort that provided data at the starting line, 34.1% had taken an NSAID on the morning of the competition.

Widespread use of NSAIDs is also reported in elite athletes at the Olympics and in football. Interviews and questionnaires as part of sample collection protocols for doping control have been used to ascertain the extent of NSAID use at the Olympics. Nearly a third of all Olympic athletes in the late 1990s to the 2000s reported NSAID use in the three days before data was collected from them, and in certain sports this was reported to be as high as 50% (Corrigan and Kazlauskas

2003; Huang, Johnson, and Pipe 2006; Suzic Lazic et al. 2011; Thuyne and Delbeke 2008; Tsitsimpikou, Jamurtas, et al. 2009; Tsitsimpikou, Tsiokanos, et al. 2009). Further, it has been estimated that the degree of use of NSAIDs in Olympic athletes is 6-10 times higher than in an age-matched population (Berglund 2001). Indeed, elite athletes have been reported to be 3.6 times more likely to use NSAIDs than age-matched controls (Alaranta et al. 2006). Data on the use of medications in youth and adult elite footballers are published by FIFA Medical Assessment and Research Centre (F-MARC). Their findings show that the most commonly used medications were painkillers, and that a fifth of youth players, and a third of adult players reported using them before each match, whether they were playing or otherwise (Tscholl et al. 2009; Tscholl, Junge, and Dvorak 2008; Tscholl and Dvorak 2012).

NSAIDs are not included on the World Anti-Doping Agency list of performance-enhancing medications, and many are available without prescription, making them easily accessible to individuals who participate in sport. However, both the purpose, and the extent to which these medications are used is concerning. At first glance this might reflect the obvious cost of being involved in competitive sporting endeavours. However, evidence suggests that they are perhaps misused and abused. In the aforementioned study in collegiate athletes (O'Connor et al. 2019), 13.9% of respondents consumed NSAIDs over the recommended dosage, which in turn was 22.9% higher for OTC NSAIDs. Of those amateur athletes in the study at the University of Birmingham who took NSAIDs (Rudgard, Hirsch, and Cox 2019), only a quarter were advised to do so by a pharmacist or doctor. Between 45-65% of participants in city marathons in

Bonn and Berlin reported taking NSAIDs prior to racing (Kuster et al. 2013), even though only 11% had pain going in (Brune et al. 2009). Further, despite a wide awareness campaign directed towards team physicians at the 2014 FIFA (Fédération Internationale de Football Association) World Cup, the incidence of NSAID use did not reduce (Vaso et al. 2015). This is troubling because there is extensive literature documenting the adverse effects of NSAID ingestion on the cardiovascular, gastrointestinal and musculo-skeletal systems, particularly with prolonged use (Bhala et al. 2013; Warner et al. 2002; Warner et al. 1999; McGettigan and Henry 2013; McGettigan and Henry 2011; Bjorkman 1998).

The majority of respondents in studies examining the reasons for NSAID use/misuse in both amateur and elite sport report taking these medications to reduce pain and inflammation during training and competition and to hasten recovery from force loss and functional decline associated with DOMS (O'Connor et al. 2019; Rudgard, Hirsch, and Cox 2019; Tscholl et al. 2009). Acutely, these purported benefits are assumed to improve performance during training and competition (O'Connor et al. 2019; Gorski et al. 2011), despite the fact that the evidence on the effectiveness of NSAIDs on both ameliorating perceived muscle soreness and aiding recovery from functional decline is equivocal (Connolly, Sayers, and McHugh 2003).

Chronic NSAID use to relieve pain during training is perhaps undertaken with a view towards indirectly enhancing muscle strength and size adaptations by allowing for more work to be performed. Indeed, the notion of "pushing through the pain" of strenuous training by using NSAIDs is so common among individuals who perform regular rigorous exercise as to have received attention

of recent in the media (Reynolds 2017), and is apparent from the extent to which these medications have been reported to be used towards pain relief during training. Over 50% of elite triathlete respondents in the aforementioned study by Gorski et al. (2011) reported consuming NSAIDs in the three months prior to an event to alleviate soreness during training. At the amateur level, NSAID use before practice to prevent pain was reported by two-fifths of respondents in the aforementioned study in Irish collegiate student-athletes (O'Connor et al. 2019), and ~50% of respondents in the University of Birmingham study (Rudgard, Hirsch, and Cox 2019). Some reviewers have even suggested that the reduction of perception of effort by relieving pain is a performance-enhancing effect of NSAIDs during both endurance and resistance exercise sessions (Holgado et al. 2018; Lundberg and Howatson 2018). However, no studies have examined whether work done during training sessions is increased by chronic NSAID ingestion.

Studies have also investigated whether there is a direct physiological effect of NSAID administration on resistance exercise-induced muscular adaptations via mechanisms underlying myogenesis and hypertrophy. The first human study to investigate the impact of NSAIDs on anabolic response to high intensity exercise found that MPS was suppressed by administration of 1200mg of the NSAID ibuprofen in young, healthy, men (Trappe et al. 2002). Investigators have since demonstrated that NSAIDs inhibit satellite cell proliferation for up to 8 days after a bout of maximal eccentric exercise (Mackey et al. 2007; Mikkelsen et al. 2009b), and blunt anabolic signalling in the hours following a bout of unaccustomed high intensity resistance exercise (Markworth et al. 2014).

However, data from chronic studies suggests that NSAID administration might be beneficial to training-induced muscle adaptations in the elderly, in whom age-related loss of muscle mass is a significant concern (Mitchell, Williams, et al. 2012). Petersen et al. (2011) demonstrated that consumption of 1200mg of ibuprofen per day by elderly osteoarthritis patients alongside 12 weeks of progressive resistance training, while not affecting muscle hypertrophy, improved muscle strength above placebo. Trappe et al. (2011), utilising a double-blind design, randomly assigned doses of either 4000mg/day of paracetamol, 1200mg/day of ibuprofen or placebo to healthy elderly volunteers (24 males, 12 females) who concurrently underwent a 12-week progressive resistance training program comprising three sessions a week. The investigators found that chronic NSAID ingestion alongside resistance training resulted in a ~47% greater increase in quadriceps muscle volume compared to placebo. It might be proposed that this was due to NSAID-mediated suppression of low-grade systemic inflammation associated with ageing (Schaap et al. 2009). However, whether or not inflammation exerts an influence on muscle mass regulation is far from agreed on in the literature. In young volunteers in Chapter 3, gene networks associated with muscle injury and inflammation (Figure 3-7) were altered in the first week of resistance training. Novel insight might be gleaned by examining whether chronic NSAID ingestion impacts upon skeletal muscle adaptations to training in the young by influencing this training-induced initial inflammatory response in these individuals.

4.1.1 Aims and hypothesis

The aim of this study was to determine whether daily ingestion of 75mg of the NSAID diclofenac by young, recreationally active male volunteers over 84 days of unilateral maximal concentric training of the quadriceps would (i) increase training work output above consumption of placebo intervention and (ii) impact upon resistance training-induced increases in muscle CSA, volume, and strength by influencing the initial inflammatory response in muscle observed during high intensity resistance training in the young. Time course of change in muscle CSA, volume and mRNA expression were measured as described in the experiment in Chapter 3. Inflammatory protein expression in muscle was measured to confirm muscle inflammation in the young during resistance training, and to examine how NSAIDs influence exercise-induced muscle inflammation during chronic training. By designing this study, it was hoped to unravel new insights into the impact of combined chronic NSAID ingestion and resistance exercise training on muscle hypertrophy and strength gains in the young. Further, no study thus far has examined the time course of muscle molecular adaptations to combined chronic NSAID ingestion and high intensity resistance training.

4.2 Methods

4.2.1 Participants

Figure 4-1 schematically depicts the overall study protocol. Seventeen healthy, non-smoking, non-vegetarian males (age: 24.5 ± 1.1years; BMI: 24.2 ± 0.7kg.m^{-2}) were recruited to this study. Participants were determined to be recreationally active using the same criteria as described in Chapter 3 (Section 3.2.1). Ethical approval and participant consent were obtained (Ethics Reference No: I 07

2011), and screening was carried out as described in General Methods (Section 2.3). Restrictions on supplementation and training outside the study protocol were advised as described in Chapter 3 (Section 3.2.1).

4.2.2 Exercise training protocol

A training protocol was implemented, modelled on a rehabilitation protocol consisting of a bout of 5 sets of maximal isokinetic concentric knee extensions previously shown by our laboratory to be well-tolerated (Figure 4-1) (Jones et al. 2004). Participants trained 3 times per week for 12 weeks and were not in an overnight fasted state for their training sessions. During each session, participants performed 5 bouts of 30 maximal isokinetic concentric contractions of the knee extensors in the non-dominant leg, on an isokinetic dynamometer as described in General Methods (Section 2.4.1), at the same angular velocity and with the same rest period between bouts as experiments in Chapter 3 (Section 3.2.2), while receiving real-time visual feedback and verbal encouragement.

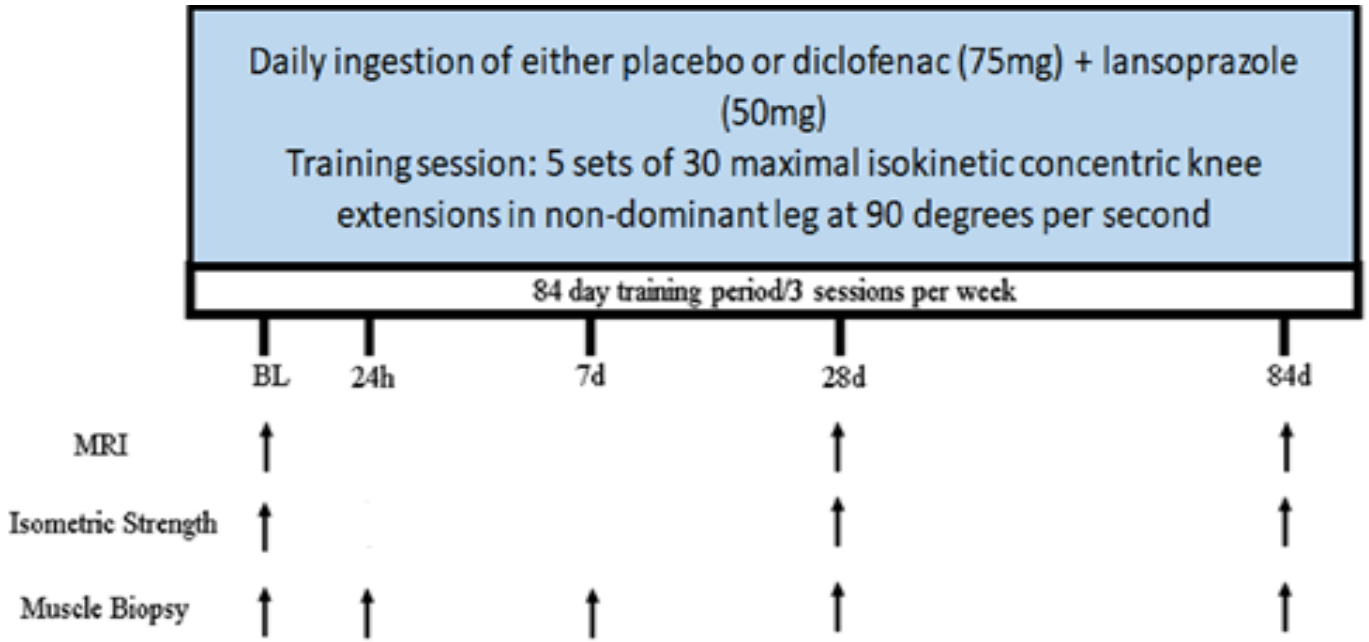

Figure 4-1: Study protocol. Subjects performed 5x30 maximal isokinetic concentric knee extensions 3 times per week for 12 weeks in the non-dominant limb with either daily ingestion of placebo or the NSAID diclofenac. Muscle biopsies were taken at baseline (BL), 24 hours (24h), 7 days (7d), 28 days (28d) and 84 days (84d). Isometric strength was measured at BL, 28d and 84d. MRI of the thigh was performed at BL, 28d and 84d.

4.2.3 NSAID intervention

This study was a randomised, placebo-controlled, double-blind 12-week experiment (Figure 4-1). Participants were randomly assigned to either a control intervention group who were given placebo in the form of lactose capsules (PLA; n=8, 24.3 ± 1.7years, BMI 23.5 ± 0.9kg.m^{-2}) or an NSAID intervention group who were administered diclofenac capsules (NSAID; 75mg diclofenac + 15mg lansoprazole; n=9, 24.8 ± 1.4years, BMI 24.8 ± 0.9kg.m^{-2}). Both treatments were ingested daily for 12 weeks alongside the training protocol. The experiment in this chapter, and Chapter 3, were performed concurrently, such that the eight volunteers who participated in the experiments in the latter formed the control treatment group (PLA) of the present study.

Both diclofenac and placebo capsules were obtained from the NUH Pharmacy Department – Clinical Trials Services. Volunteers were randomised into two groups using an online randomisation tool (http://www.randomization.com/). NUH Pharmacy Department determined which of the two groups was NSAID and PLA, and blinded the investigators to the treatment, which was achieved by providing both diclofenac and placebo capsules in the same brown bottles, rendering them indistinguishable.

Diclofenac, chemically known as 2-(2,6-dichloranilino) phenylacetic acid, has a strong preference for inhibition of COX-2 iso-enzyme as opposed to COX-1 (Warner et al. 1999), thus causing fewer incidences of gastric adverse effects than noted with aspirin, naproxen and other NSAIDs (Henry et al. 1996). However, participants were still advised to take the tablets with food, and in combination with a proton-pump inhibitor, lansoprazole, so as to prevent chronic

stomach irritation from the NSAID. Tablets were given to the subjects every 4 weeks. They were asked to return the bottles so compliance of drug consumption could be determined by the number of pills remaining after the 4-week period. Potential side effects of the diclofenac consumption were monitored by monthly blood sampling for hepatic (alanine aminotransferase, ALT) measures.

4.2.4 Muscle function during training

4.2.4.1 Total work output during isokinetic knee extensor exercise training

Total work output per week was calculated as the work done during all 450 maximal contractions performed each week. Total output over 12 weeks was calculated as sum of the work done from all exercise sessions during the protocol.

4.2.4.2 Isometric knee extensor strength

Isometric strength (best of 3 attempts) of the knee extensor muscles was determined as described in General Methods (Section 2.4.2), at baseline (BL), 28 days (28d) and 84 days (84d) (Figure 4-1). All measurements were made at the same time of day, at least 72h after the last training session.

4.2.5 MRI mid-thigh muscle CSA and thigh muscle volume measurements

Quadriceps muscle CSA and volume measurements were performed at BL, 28d and 84d time-points (Figure 4-1). This was determined from axial plane scans of the thigh using 3T MRI as described in General Methods (Section 2.5.3).

4.2.6 Muscle sample collection

Vastus lateralis muscle biopsies were obtained from the non-dominant limb in the resting state using the Bergstrom technique (Bergstrom 1975) as described in General Methods (Section 2.6.1) at BL, 24h, and a minimum of 72h from the previous exercise session at 7d, 28d and 84d (Figure 4-1).

4.2.7 Muscle mRNA expression

Muscle mRNA extraction was performed from muscle samples obtained at BL, 24h, 7d, 28d and 84d as described in General Methods (Section 2.7.2) (i.e. from 78 biopsies in total due to tissue availability). First strand cDNA was synthesised from 1µg of total RNA as described in General Methods (Section 2.7.3). TaqMan low density arrays were performed using an ABI PRISM 7900HT sequence detection system, and data analysed using SDS 2.1 software (Applied Biosystems, USA) as described General Methods (Section 2.7.4). Muscle mRNA expression was quantified using real time PCR as described in General Methods (Section 2.7.5). Data were further analysed using RQ Manager software (Applied Biosystems, USA), where the threshold level was normalised across all plates before Ct values were calculated for each gene target and sample. Relative quantification of mRNAs of interest was measured using the $2^{-\Delta\Delta Ct}$ method with M (HMBS) as the endogenous control, with the mean of the baseline sample used as the calibrator. Mean Ct values for HMBS were no different between PLA and NSAID groups (data not shown). A total of 93 muscle mRNA transcripts were targeted for quantification, and the rationale for their selection is described in Chapter 3 (3.2.6).

4.2.8 Ingenuity Pathway Analysis

Muscle mRNA abundance was analysed using Ingenuity Pathway Analysis software as described in General Methods (Section 2.7.6).

4.2.9 Muscle protein expression measurements

Western blotting was used to quantify target protein expression of inflammatory proteins IL-6, TNF alpha precursor and mature TNF alpha from muscle samples obtained at BL, 24h, 7d, 28d and 84d. Also known as immunoblotting, it is a well-established and widely used analytical technique for the detection of specific proteins, and the phosphorylation status of proteins in tissue extract. It is based on the formation of a protein-antibody complex via the specific binding of a primary antibody to a membrane-bound target protein separated by its molecular weight. The primary antibody is generally detected using a specific secondary antibody.

Protein was extracted from approximately 30mg snap-frozen tissue using a method modified from Blough, Dineen, and Esser (1999). Homogenisation of samples was carried out in 50mM Tris buffer pH 7.5 and protease and phosphatase inhibitors (Sigma-Aldrich, UK) were added. Samples were then centrifuged and the supernatant with cytosolic protein fraction collected. The Bradford assay was used to measure protein content. Protein samples were run on a 4–12% Bis-tris acrylamide gel (Invitrogen, Paisley, UK) at 200V for 1h. They were then transferred to a polyvinylidene difluoride membrane for 1h 45min. This transfer was checked with Ponceau S staining, and then blocked in 5% BSA-TBS-Tween. Probing of membranes was carried out with PDK4

primary antibody (1:500; Abcam, Cambridge, UK) overnight at 4°C. TBS-Tween was used to wash the membrane, which was then incubated with the secondary antibody HRP-linked anti-rabbit IgG (H+L) (DyLight® 680 Conjugate, Cell Signaling, USA). Incubation of the membranes was then carried out with ECL chemiluminescence detection reagent (Pierce, Cramlington, UK), and this was followed by exposure to X-ray film. Duoscan T1200, Agfa, Mortsel, Belgium was used to scan the blot. Genetools from Syngene (Cambridge, UK) was used to identify bands. Density volume was adjusted by the subtraction of the local background and then normalised with actin (1:1000; Sigma-Aldrich, UK).

4.2.10 Statistical analysis

Statistical analysis for comparisons between PLA and NSAID was carried out as described in General Methods (Section 0). The statistical analysis underpinning Ingenuity Pathway Analysis is described in General Methods (Section 0).

4.3 Results

4.3.1 Participant characteristics at baseline

Table 4-1 shows the baseline characteristics of participants in both the PLA and NSAID groups. Both groups were well matched with respect to age, height, weight, muscle CSA/volume and strength at baseline. None of the subjects taking the NSAID presented with any side effects associated with this medication (i.e. stomach irritation/pain or abnormal ALT levels) during the course of the study.

Table 4-1: Participant characteristics at baseline. All values expressed as mean ± SEM. Age, height, body mass, body mass index (BMI), isometric strength, quadriceps muscle cross-sectional area (CSA) and quadriceps muscle volume are shown for the group consuming placebo (PLA; n=8) and the group consuming NSAIDs (NSAID; n=9).

Variables	PLA	NSAID
Age (years)	24.3 ± 1.7	24.8 ± 1.4
Height (m)	1.83	1.80
Body mass (kg)	78.7 ± 3.0	80.6 ± 3.2
BMI (kg.m-^{2})	23.5 ± 0.9	24.8 ± 0.9
Isometric strength (kg)	51.6 ± 6.6	50.2 ± 6.5
Quadriceps muscle CSA (cm^{2})	88.9 ± 4.7	85.5 ± 2.8
Quadriceps muscle volume (cm^{3})	2530.8 ± 137.4	2397.6 ± 99.5

4.3.2 Muscle function during training

4.3.2.1 Total Work Output

Figure 4-2A shows the total amount of work done per week of training in both PLA and NSAID groups. Total work output per week during training was no different between groups. Figure 4-2B shows the total amount of work done over the entire training protocol. There was no difference between NSAID and PLA in the total amount of work done over the 12 weeks of training.

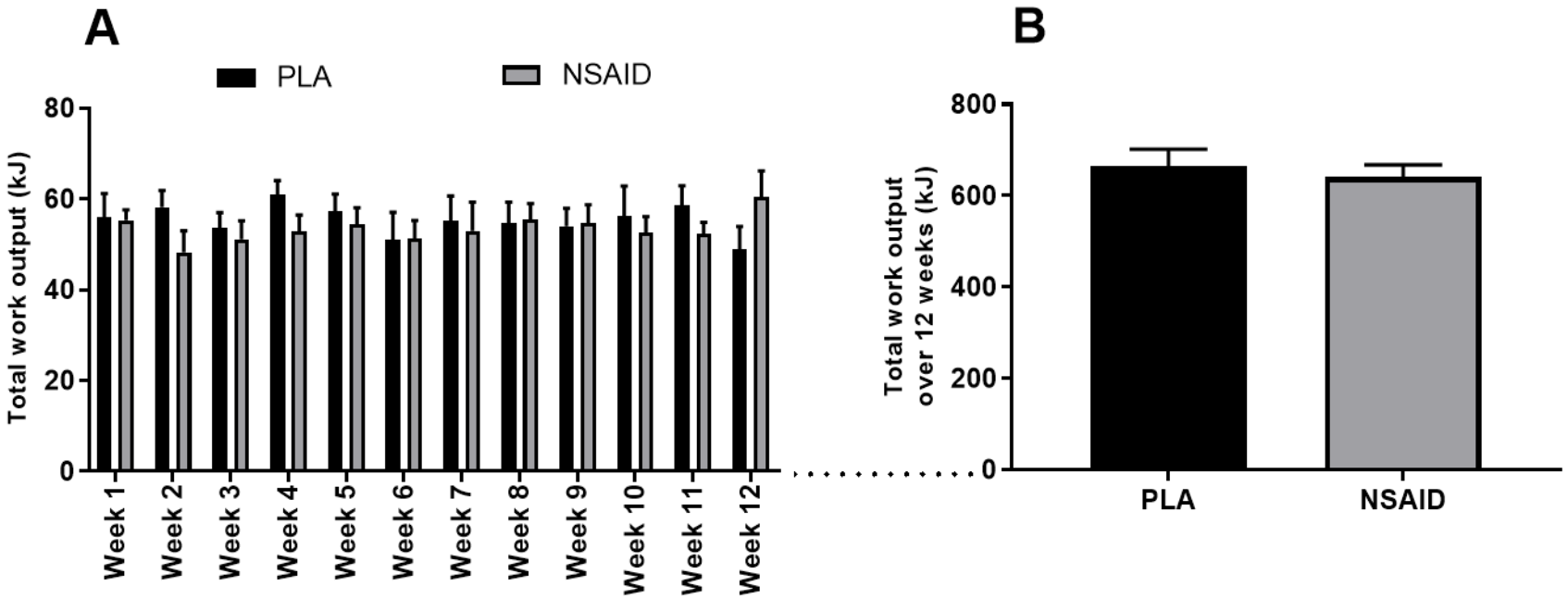

Figure 4-2. (A) Total work output (kJ) per week, (B) total work output (kJ) over 12 weeks in the group consuming placebo (PLA, n=8) and the group consuming diclofenac (NSAID; n=9). Values expressed as mean ± SEM.

4.3.2.2 Strength

Isometric strength of the knee extensors increased over time, such that F (1.7, 23.6) = 9.6, p<0.01. Isometric strength increased on average above baseline in both groups at 28d, but this was not statistically significant (13.9 ± 27.4% in PLA; 31.3 ± 13.7% in NSAID). By the end of training, isometric strength had increased from baseline in both groups by ~40-50% on average (PLA 44.7 ± 14.1%, NSAID 50.8 ± 17.4%). However, this increase was significant in the PLA group alone (p<0.05). Graphs depicting absolute values for isometric strength are shown in Figure 4-3.

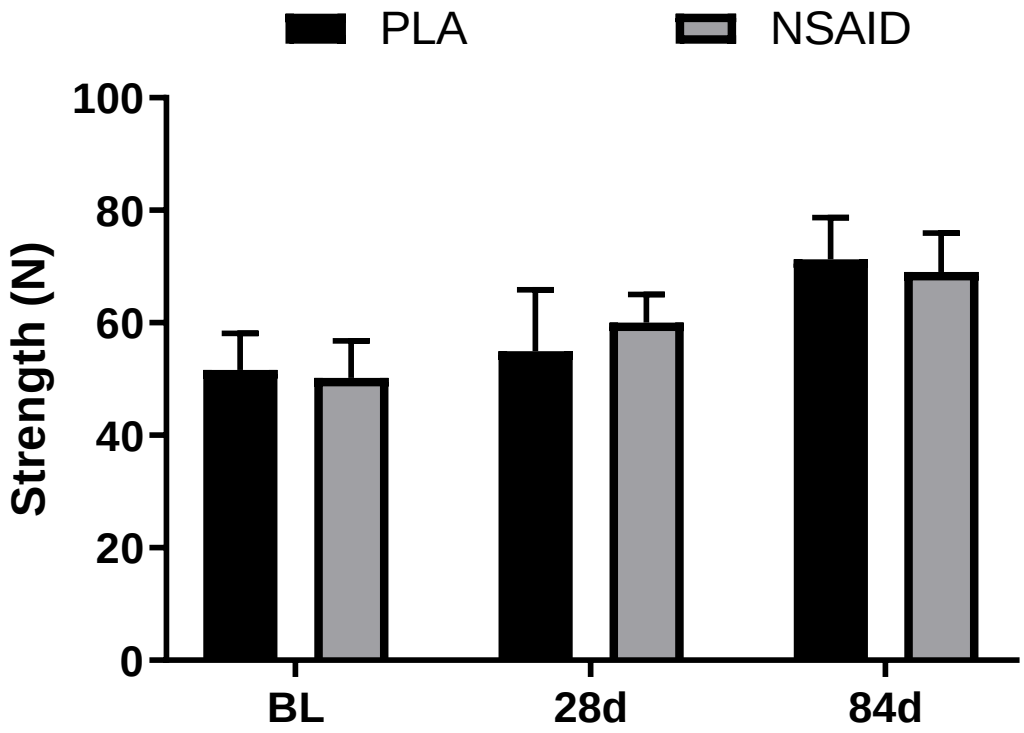

Figure 4-3. Isometric strength (N) in the group consuming placebo (PLA; n=8) and the group consuming diclofenac (NSAID; n=9) at baseline (BL), 28 days (28d) and 84 days (84d). Values represented as mean ± SEM. Baseline set at 0.

4.3.3 Muscle CSA and muscle volume

Figure 4-4A shows percentage change in quadriceps muscle CSA from baseline at 28d and 84d in both NSAID and PLA groups (graphs depicting absolute values shown in Appendix G). Quadriceps muscle CSA increased above baseline at 28d in both the PLA group (4.3 ± 0.9%, $p<0.01$) and the NSAID group (4.2 ± 1.1%, $p<0.05$). Muscle CSA was also increased from baseline in the PLA group at 84d (3.9 ± 0.8%, $p<0.01$), but not above 28d. However, muscle CSA continued to increase after 28d in the NSAID group such that the increase in CSA from baseline at 84d was 7.7 ± 1.6%. This increase was greater than baseline ($p<0.01$), 28d ($p<0.05$) and the PLA group at 84 days ($p<0.05$).

Figure 4-4B shows percentage change in quadriceps muscle volume from baseline at 28d and 84d in both NSAID and PLA groups (graphs depicting absolute values shown in Appendix G). Quadriceps muscle volume increased above baseline at 28d in both the PLA group (3.1 ± 0.8%, $p<0.05$) and the NSAID group (4.4 ± 1.2%, $p<0.05$). Muscle volume was increased from baseline in the PLA group (3.8 ± 0.8%, $p<0.01$), but not above 28d. Similar to muscle CSA, muscle volume continued to increase after 28d in the NSAID group such that the increase in volume from baseline at 84d was 8.3 ± 2.0%. This increase was significantly greater than both baseline ($p<0.01$), 28d ($p<0.05$) and the PLA group at 84 days ($p<0.05$).

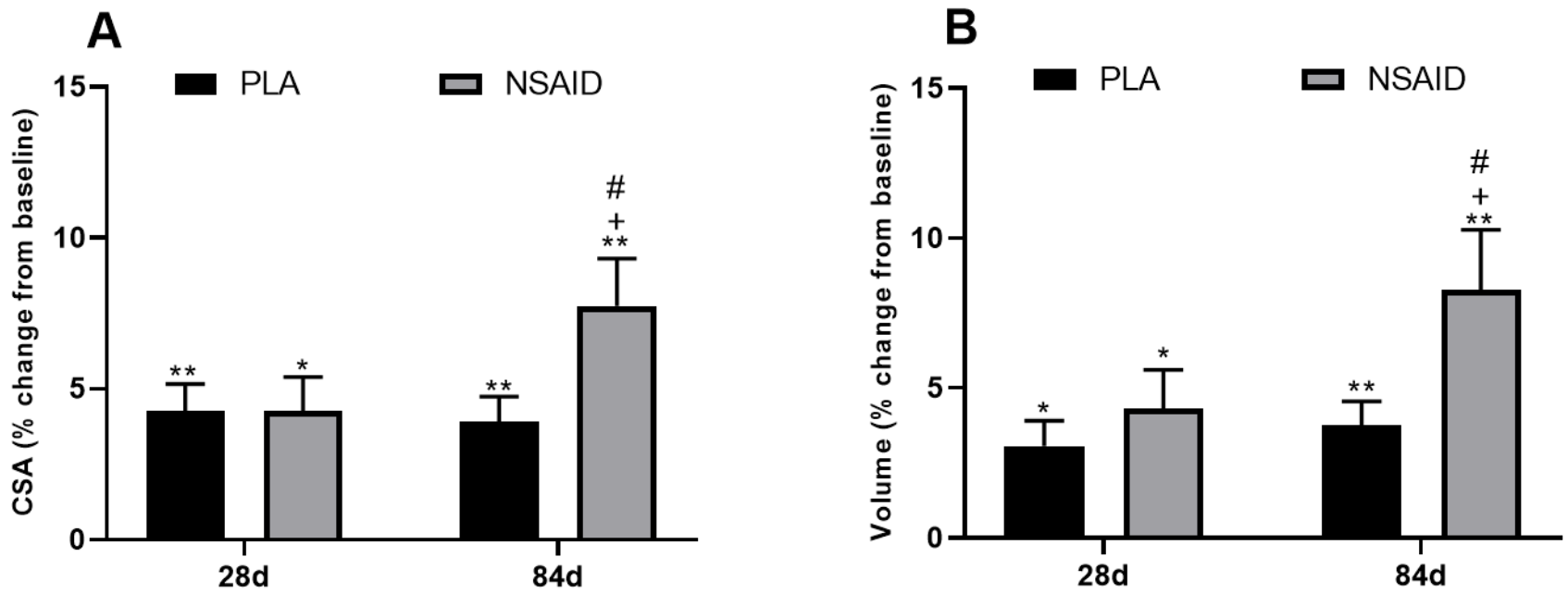

*Figure 4-4.(A) Percentage change from baseline in mid-thigh quadriceps muscle cross-sectional area (%), and (B) quadriceps muscle volume (%) in the group consuming placebo (PLA; n=8) and the group consuming diclofenac (NSAID; n=9) from baseline (BL) at 28 days (28d) and 84 days (84d) of training. Values expressed as mean ± SEM. Baseline set at 0. *p<0.05 compared to baseline, **p<0.01 compared to baseline, +p<0.05 compared to day 28, #p<0.05 NSAID vs PLA.*

4.3.4 Muscle mRNA expression

4.3.4.1 Ingenuity Pathway Analysis

Figure 4-5 depicts those cellular functions most altered from baseline in the PLA (Figure 4-5A) and NSAID (Figure 4-5B) groups at 24h, 7d, 28d and 84d. The x-axis displays cellular functions most affected, while the y-axis displays the -log of the p-values. The p-value associated with each cellular function is a measurement of the likelihood that the association between a set of focus transcripts and a given function is due to random chance. The -log of p-value was calculated using Fisher's exact test (right-tailed).

Overall, in the PLA group (Figure 4-5A), IPA detected that the most altered cellular functions [–log (p value) > 4.0], in descending order of magnitude, were those associated with skeletal and muscular development and function, organismal injuries and abnormalities, lipid metabolism, carbohydrate metabolism, skeletal and muscular disorders and tissue development. Figure 4-5 also illustrates that in the main, in the PLA group, the largest magnitude of response to exercise training occurred at 24h and 7d. However, this early response to training had waned by 28d and 84d for all cellular functions.

In the NSAID group (Figure 4-5B), IPA detected that the most altered cellular functions [–log (p value) > 7.5] were the same as those in the PLA group, albeit in a different descending order of magnitude. Unlike the PLA group, however, the large response to training at 24h and 7d was, in the main, maintained in magnitude at 28d and 84d in the NSAID group for all cellular functions. For

lipid metabolism alone, the response at 28d and 84d was greater than at 24h and 7d.

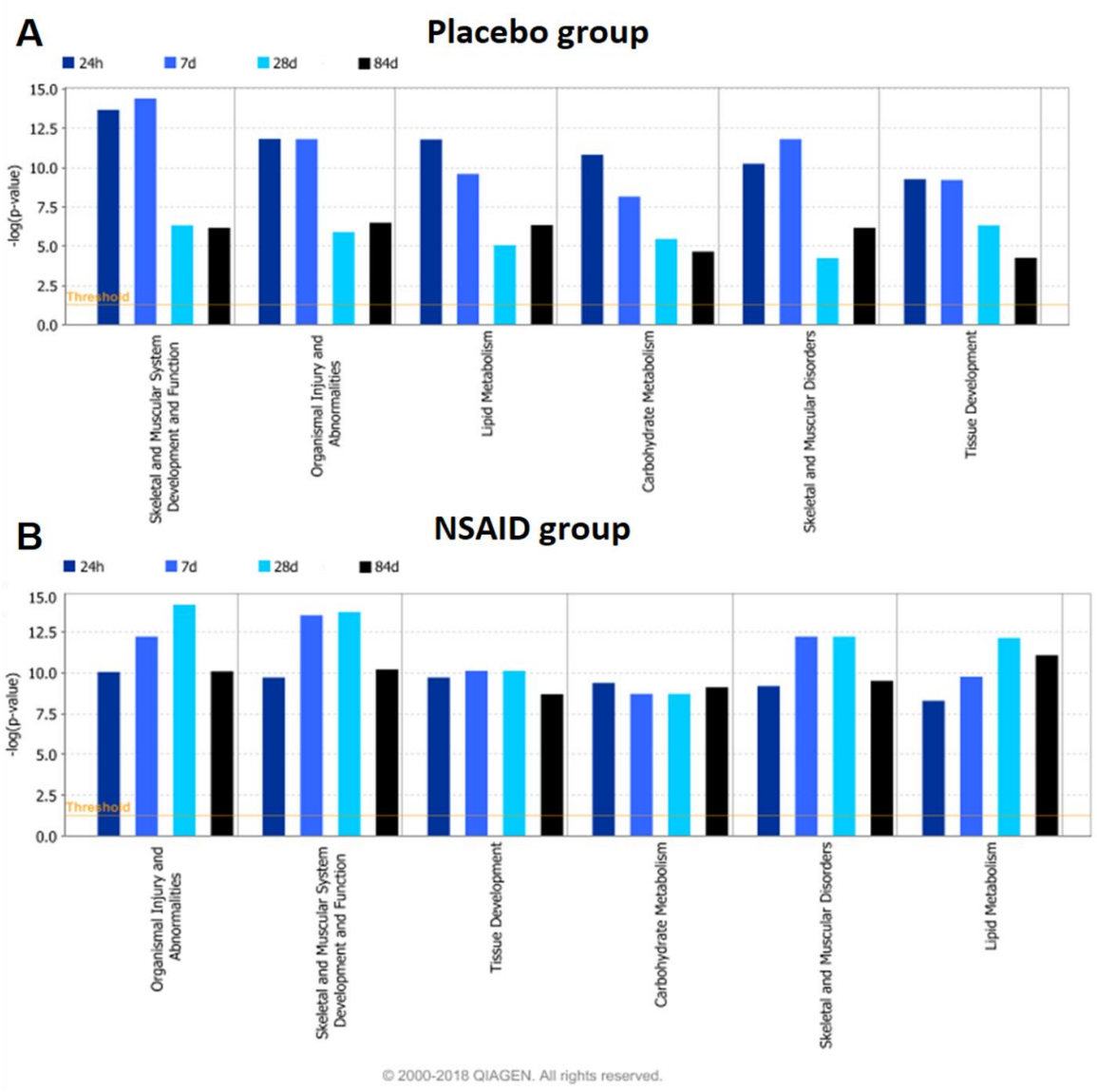

Figure 4-5: Cellular functions identified by IPA as being altered from baseline (n = 8) in vastus lateralis muscle in (A) the group consuming placebo (n=8), and in (B) the group consuming diclofenac (n=9) at 24 hours (24h), 7 days (7d), 28 days (28d) and 84 days (84d) based on mRNA expression data generated using the low-density microarray cards. The x-axis displays cellular functions most affected by training, whereas the y-axis displays the -log of the p value. The p-value associated with each cellular function is a measurement of the likelihood that the association between a set of focus transcripts and a given function is due to random chance. The –log of the p value was calculated by Fisher's exact test right-tailed ($p<0.05$). The threshold line corresponds to a p value of 0.05.

Figure 4-6 to Figure 4-21 are included by way of example to illuminate precisely how any cellular function identified as being altered in Figure 4-5 was influenced by resistance exercise training (PLA) and training plus NSAID ingestion (NSAID), and to also highlight the differences in response between NSAID and PLA at 28d and 84d. Each figure depicts the most differentially regulated mRNAs from baseline (outer ring), and the cellular events predicted to result from these collective mRNA changes in NSAID and PLA groups at a specific time-point. The functions selected to be shown here, for each group, included those associated with skeletal and muscular system development and function, organismal injuries and abnormalities, lipid metabolism and tissue development. In choosing which functions to depict, those were taken into account that changed most in each group relative to baseline, and for which the most network predictions were made with high confidence. Schematics for the rest of the cellular functions that changed in each group are shown in Appendix H.

Figure 4-6 relates to skeletal and muscular development and function in the PLA group at (A) 24h and (B) 7d. Fifteen mRNAs were identified as being altered in abundance relative to baseline at 24h (Figure 4-6A). The most upregulated mRNAs from baseline were MYC and IL-6, while MSTN, PAX3 and peroxisome PPARGC1A were the most downregulated transcripts. Based on the collective changes in mRNA abundance from baseline, IPA did not make any network predictions with high confidence. At 7d (Figure 4-6B), 13 mRNAs were identified as being altered in abundance relative to baseline. The most upregulated mRNAs from baseline were MYC and IL-6, while PAX3 was the most downregulated transcript. Based on the collective changes in mRNA

abundance from baseline, in contrast to 24h, IPA predicted with high confidence activation of differentiation of muscle and inhibition of function of muscle.

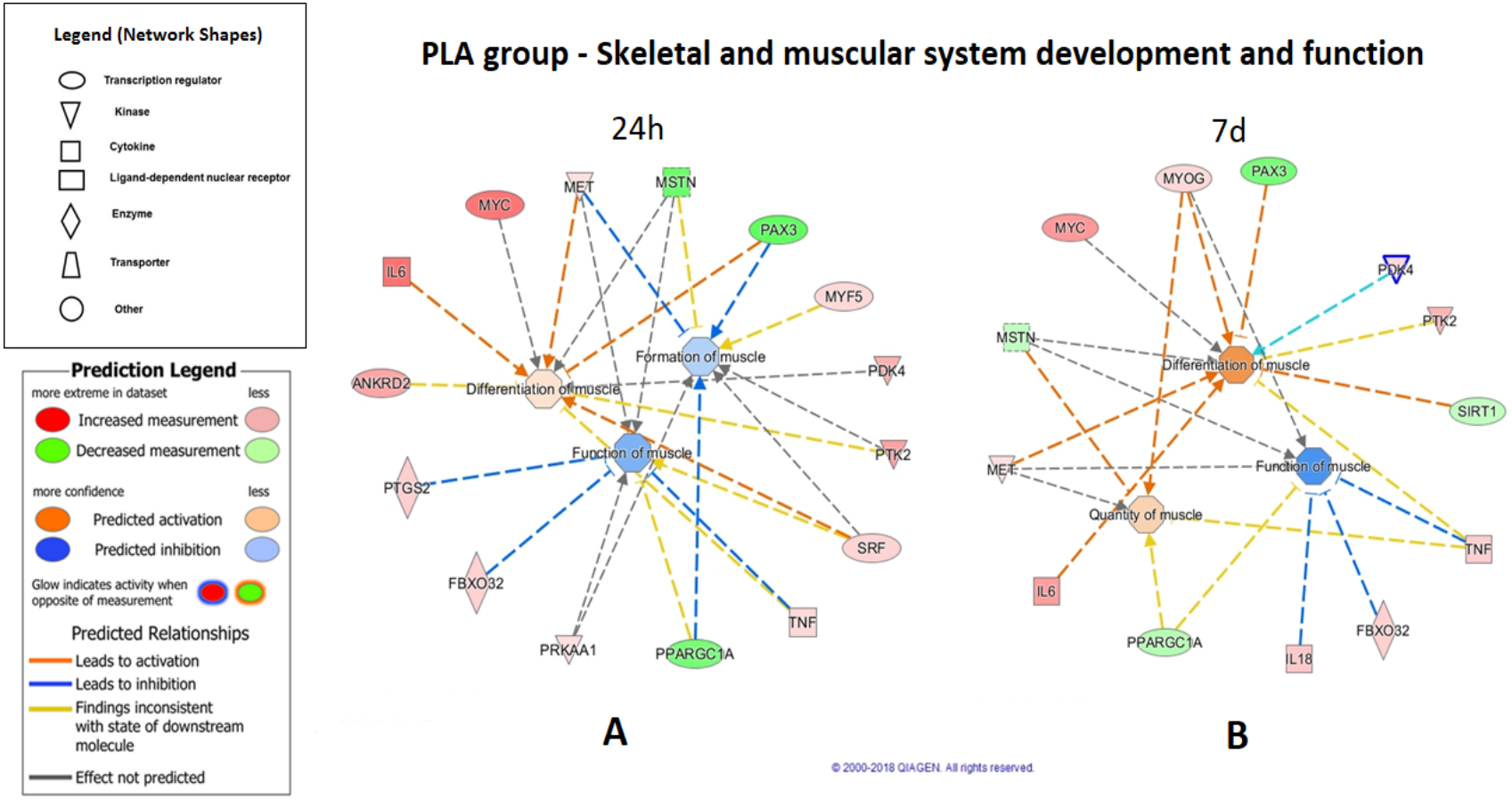

Figure 4-6: Differentially regulated muscle mRNAs (outer ring) associated with skeletal and muscular system development and function in the group consuming placebo (PLA) at (A) 24 hours (24h) and (B) 7 days (7d) compared to baseline, and the cellular events predicted by Ingenuity Pathway Analysis to result from the collective changes in mRNA abundance.

Abbreviations: ANKRD2, ankyrin repeat domain 2; IL-6, interleukin 6; MSTN, myostatin; MYC, MYC proto-oncogene; MET, MET proto-oncogene, PAX3, paired box gene 3; MYF5, myogenic factor 5; PDK4, pyruvate dehydrogenase kinase 4; PTK2, protein tyrosine kinase 2, SRF, serum response factor; TNF, tumour necrosis factor; PPARGC1A, peroxisome proliferator-activated receptor gamma coactivator 1 alpha; PRKAA1, protein kinase AMP-activated catalytic subunit alpha 1; FBXO32, F-box protein 32; PTGS2, prostaglandin-endoperoxide synthase 2; SIRT1, sirtuin 1.

Figure 4-7 relates to skeletal and muscular development and function in the PLA group at 84d. Schematics for predicted cellular events related to skeletal and muscular development and function were not generated by IPA for this group at 28d. While the degree of change of mRNA abundance from baseline at this time-point was sufficient to pass the threshold for this cellular function to be deemed altered (Figure 4-5), the number of genes associated with the function that changed was insufficient to pass the threshold for a reliable network prediction. Four mRNAs were identified as being altered in abundance relative to baseline, of which vascular endothelial growth factor A (VEGFA), AKT1 and PPARGC1A were downregulated while MYOG was upregulated. Based on collective changes in abundance of these mRNAs, IPA predicted with high confidence an inhibition of formation of muscle.

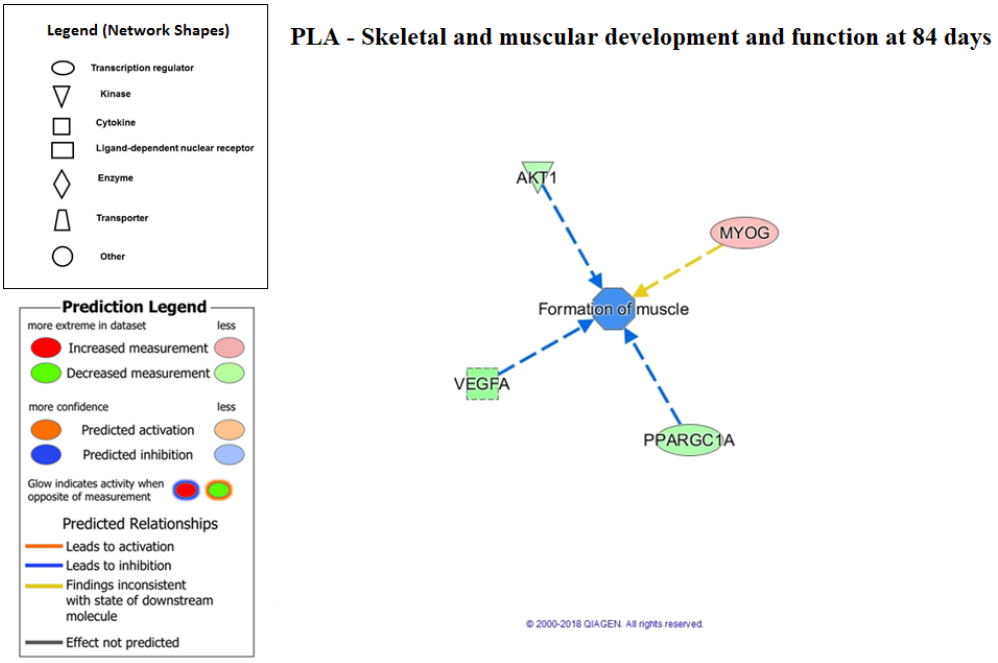

Figure 4-7: Differentially regulated muscle mRNAs (outer ring) associated with skeletal and muscular system development and function in the group consuming placebo (PLA) at 28 days (28d) compared to baseline, and the cellular events predicted by Ingenuity Pathway Analysis to result from the collective changes in mRNA abundance. Abbreviations: AKT1, serine-threonine protein kinase 1; MYOG, myogenin; VEGFA, vascular endothelial growth factor A; PPARGC1A, peroxisome proliferator-activated receptor gamma coactivator 1 alpha.

Figure 4-8 relates to skeletal and muscular development and function in the NSAID group at (A) 24h and (B) 7d. Twelve mRNAs were identified as being altered in abundance relative to baseline at 24h (Figure 4-8A). The most upregulated mRNAs from baseline were TNF and MYF6 while MSTN and myosin heavy chain 7 (MYH7) were the most downregulated transcripts. Based on the collective changes in mRNA abundance from baseline, IPA predicted with a high degree of confidence the activation of formation of muscle. At 7d (Figure 4-8B), 14 mRNAs were identified as being altered in abundance relative

to baseline. TNF was the most upregulated transcript, while tripartite motif containing 63 (TRIM63) was the only downregulated transcript. Similar to 24h, IPA predicted activation of formation of muscle with high confidence.

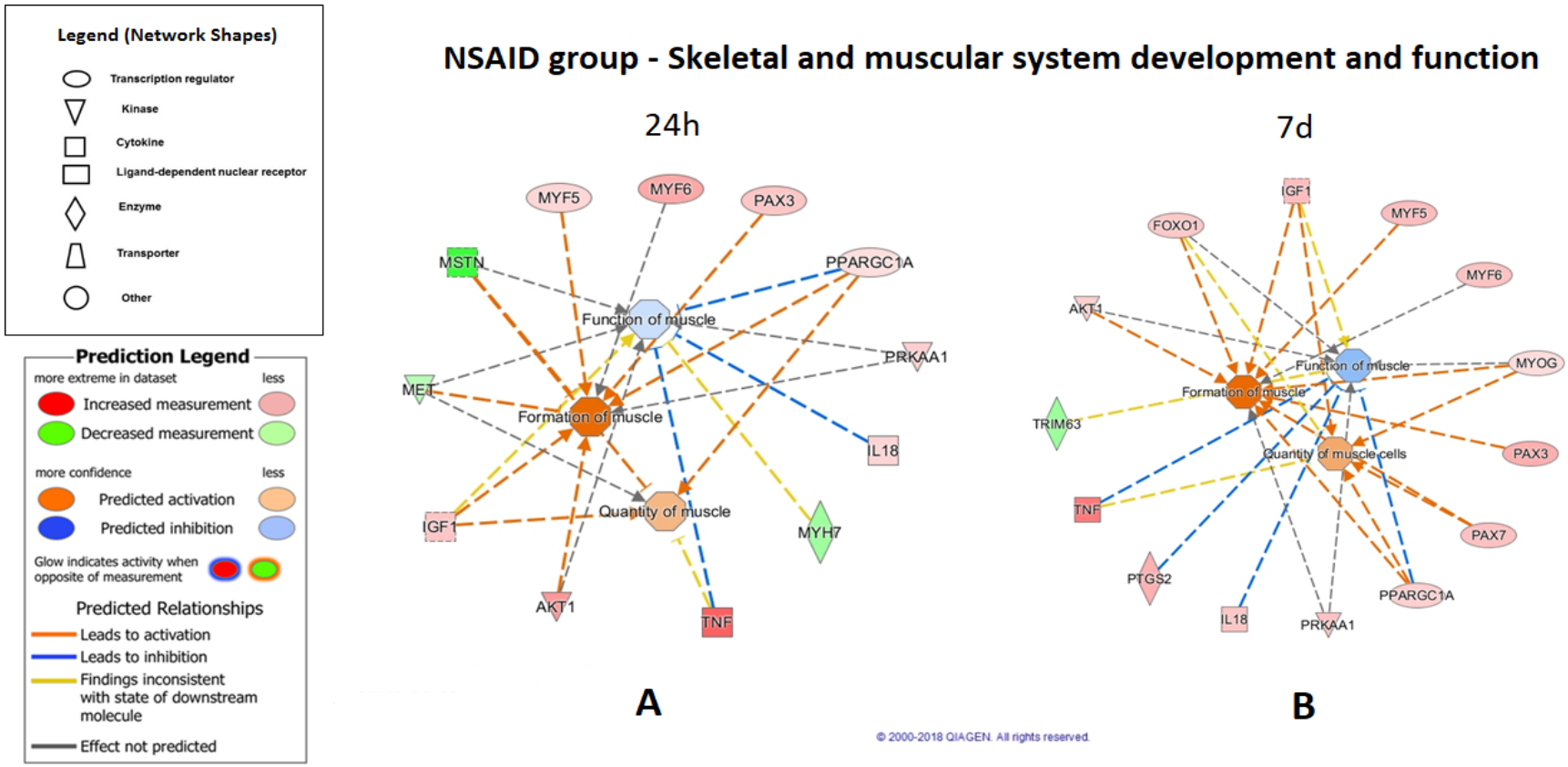

Figure 4-8: Differentially regulated muscle mRNAs (outer ring) associated with skeletal and muscular system development and function in the group consuming diclofenac (NSAID) at (A) 24 hours (24h) and (B) 7 days (7d) compared to baseline, and the cellular events predicted by Ingenuity Pathway Analysis to result from the collective changes in mRNA abundance.

Abbreviations: MYF5, myogenic factor 5; myogenic factor 6; PAX3, paired box gene 3; PAX7, paired box gene 7; PTGS2, prostaglandin-endoperoxide synthase 2; PPARGC1A, peroxisome proliferator-activated receptor gamma coactivator 1 alpha; PRKAA1, protein kinase AMP-activated catalytic subunit alpha 1, IL-18, interleukin 18; MHY7, myosin heavy chain 7; TNF, tumour necrosis factor; AKT1; serine-threonine protein kinase 1; IGF1, insulin-like growth factor 1; MET, MET proto-oncogene; MSTN, myostatin; MYOG, myogenin; TRIM 3, tripartite motif containing 63; FOXO1, forkhead box protein O 1.

Unlike the PLA group, schematics for predicted cellular events related to skeletal and muscular development and function were generated by IPA at both 28d and 84d for the NSAID group, as a sufficient number of genes changed at both these time-points for network predictions to be made. Figure 4-9 relates to skeletal and muscular development and function in the NSAID group at (A) 28d and (B) 84d. Twenty mRNAs were identified as being altered in abundance relative to baseline at 28d (Figure 4-9A). The most upregulated mRNAs from baseline were TNF, IGF1, IL-6 and prostaglandin-endoperoxide synthase 2 (PTGS2) while TRIM63 was the most downregulated transcript. Based on the collective changes in mRNA abundance from baseline, IPA predicted activation of formation of muscle with the highest confidence. At 84d (Figure 4-9B), 13 mRNAs were identified as being altered in abundance relative to baseline. The most upregulated mRNAs from baseline were IGF1, PTGS2 and TNF, while TRIM63 was the only downregulated transcript. Based on the collective changes in mRNA abundance from baseline IPA predicted activation of formation of muscle with the highest confidence.

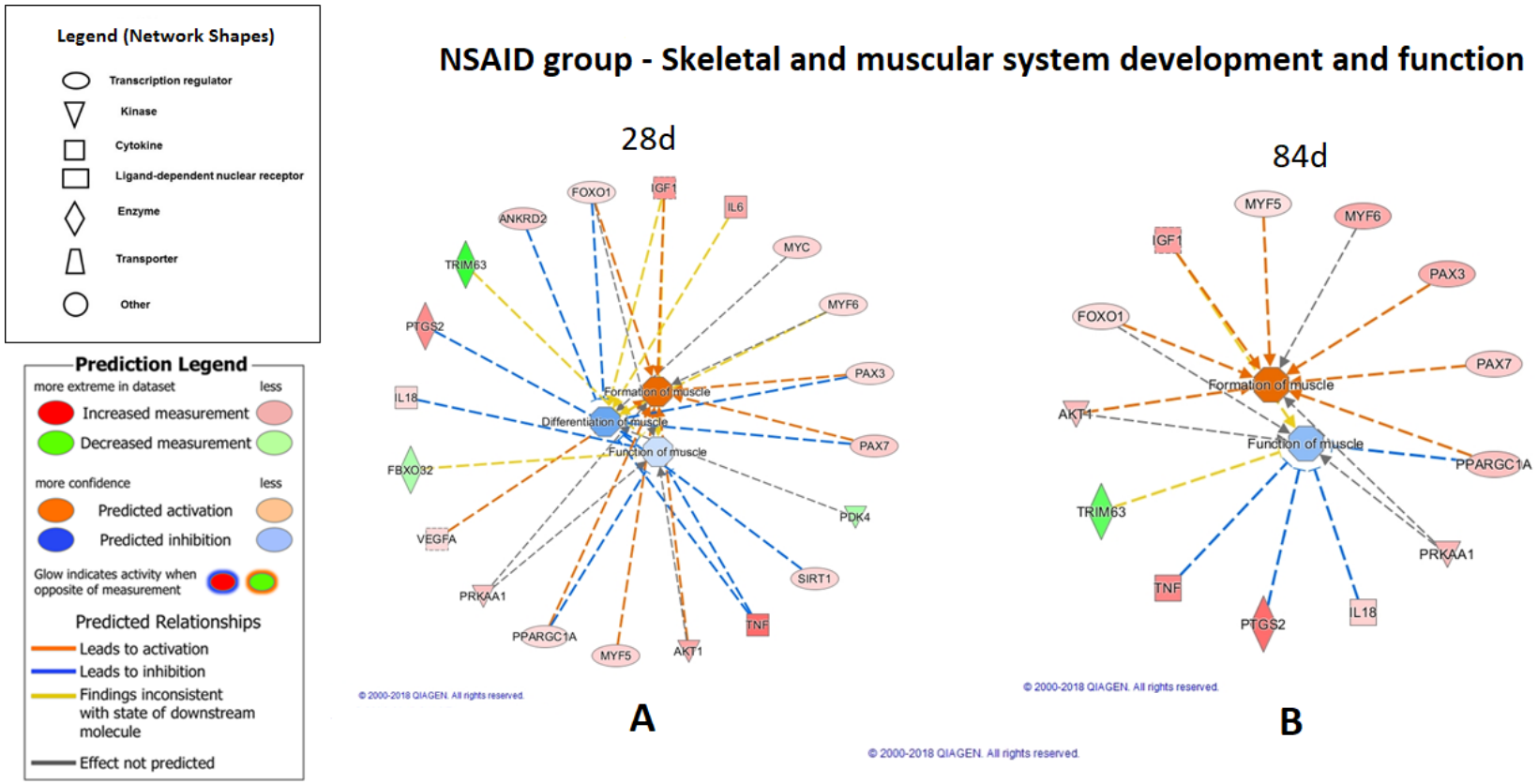

Figure 4-9: Differentially regulated muscle mRNAs (outer ring) associated with skeletal and muscular system development and function in the group consuming diclofenac (NSAID) at (A) 28 days (28d) and (B) 84 days (84d) compared to baseline, and the cellular events predicted by Ingenuity Pathway Analysis to result from the collective changes in mRNA abundance.

Abbreviations: ANKRD2, ankyrin repeat domain 2; IGF1, insulin-like growth factor 1; IL-6, interleukin 6; MYC, MYC proto-oncogene; MYF6, myogenic factor 6; PAX3, paired box gene 3; PAX7, paired box gene 7; PDK4, pyruvate dehydrogenase kinase 4; SIRT1, sirtuin 1; TNF, tumour necrosis factor; AKT1, serine-threonine protein kinase 1; MYF5, myogenic factor 5; PTGS2, prostaglandin-endoperoxide synthase 2; PPARGC1A, peroxisome proliferator-activated receptor gamma coactivator 1 alpha; PRKAA1, protein kinase AMP-activated catalytic subunit alpha 1; VEGFA, vascular endothelial growth factor A; FBXO32, F-box only protein 32; IL-18, interleukin 18; TRIM 3, tripartite motif containing 63; FOXO1, forkhead box protein O 1.

Figure 4-10 relates to organismal injuries and abnormalities in the PLA group at (A) 24h and (B) 7d. Eight mRNAs were identified as being altered in abundance relative to baseline at 24h (Figure 4-10A). IL-6 was the most upregulated transcript, while PPARGC1A and MSTN were the most downregulated transcripts. Based on the collective changes in mRNA abundance from baseline, IPA predicted activation of atrophy of muscle with a high degree of confidence. At 7d (Figure 4-10B), in contrast, 29 mRNAs were identified as being altered in abundance relative to baseline. The most upregulated mRNAs from baseline were EGF-containing fibulin-like extracellular matrix protein 1 (EFEMP1), integrin subunit alpha 10 (ITGA10), joining chain of multimeric IgA And IgM (JCHAIN), MYC, and TNC while myosin heavy chain 1 (MYH1), neural precursor cell expressed developmentally down-regulated protein 4 (NEDD4) and PAX3 were the most downregulated transcripts. Based on the collective

changes in mRNA abundance from baseline, IPA predicted activation of atrophy of skeletal muscle and tumorigenesis of tissue with the highest confidence.

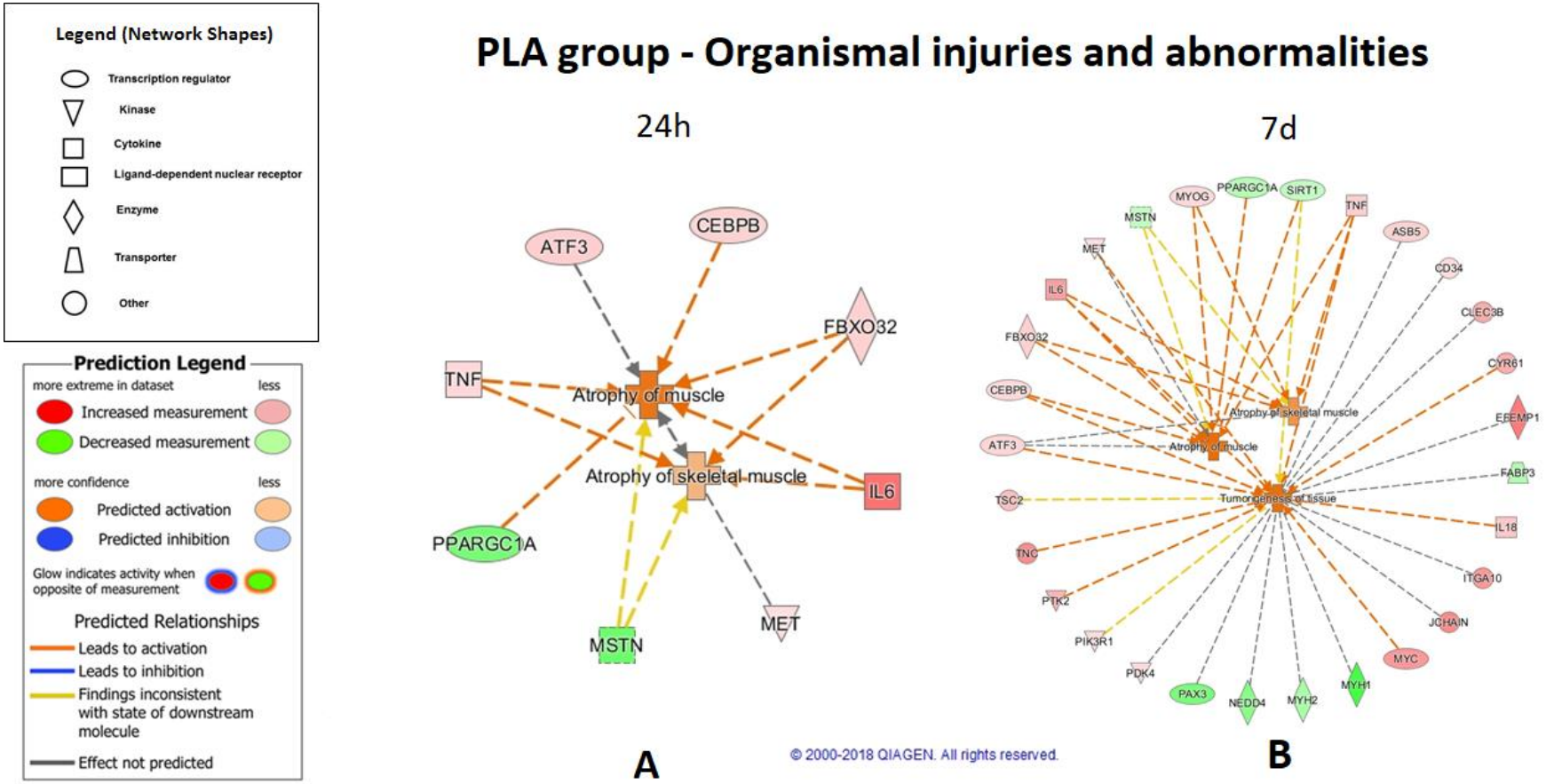

Figure 4-10: Differentially regulated muscle mRNAs (outer ring) associated with organismal injuries and abnormalities in the group consuming placebo (PLA) at (A) 24 hours (24h) and (B) 7 days (7d) compared to baseline, and the cellular events predicted by Ingenuity Pathway Analysis to result from the collective changes in mRNA abundance.

Abbreviations: CEBPB, CCAAT enhancer binding protein beta; FBXO32, F-box protein32; TNF, tumour necrosis factor; ATF3, activating transcription factor 3; MSTN, myostatin, PPARGC1A, peroxisome proliferator-activated receptor gamma coactivator 1 alpha; MET, MET proto-oncogene; SIRT1, sirtuin 1; ASB5, ankyrin repeat and SOCS box containing 5; CD34, hematopoietic progenitor cell antigen CD34; CLEC3B, C-type lectin domain family 3 member B, CYR61, cysteine-rich angiogenic inducer 61; EFEMP1, EGF-containing fibulin-like extracellular matrix protein 1; FABP3, fatty acid binding protein 3; IL-18, interleukin 18; ITGA10 (integrin subunit alpha 10); JCHAIN, joining chain of multimeric IgA And IgM; MYC, MYC proto-oncogene; MYH1, myosin heavy chain 1; MYH2; myosin heavy chain 2; NEDD4, neural precursor cell expressed developmentally down-regulated protein 4; PAX3, paired box gene 3; PDK4, pyruvate dehydrogenase kinase 4; PIK3R1, phosphoinositide-3-kinase regulatory subunit 1; PTK2, protein tyrosine kinase 2; TNC, tenascin C; TSC2, tuberous sclerosis complex 2; MYOG, myogenin.

Figure 4-11 relates to organismal injuries and abnormalities in the PLA group at (A) 28d and (B) 84d. Eleven mRNAs were identified as being altered in abundance relative to baseline at 28d (Figure 4-11A). Myosin heavy chain 1 (MYH1) was the only downregulated transcript. Based on the collective changes in mRNA abundance from baseline, IPA predicted with a high degree of confidence the activation of tumorigenesis of tissue. At 84d (Figure 4-11B), 5 mRNAs were identified as being altered in abundance relative to baseline. The most upregulated transcript was MYOG, while AKT1 and PPARGC1A were

downregulated. Based on the collective changes in mRNA abundance from baseline IPA predicted activation of atrophy of muscle with highest confidence.

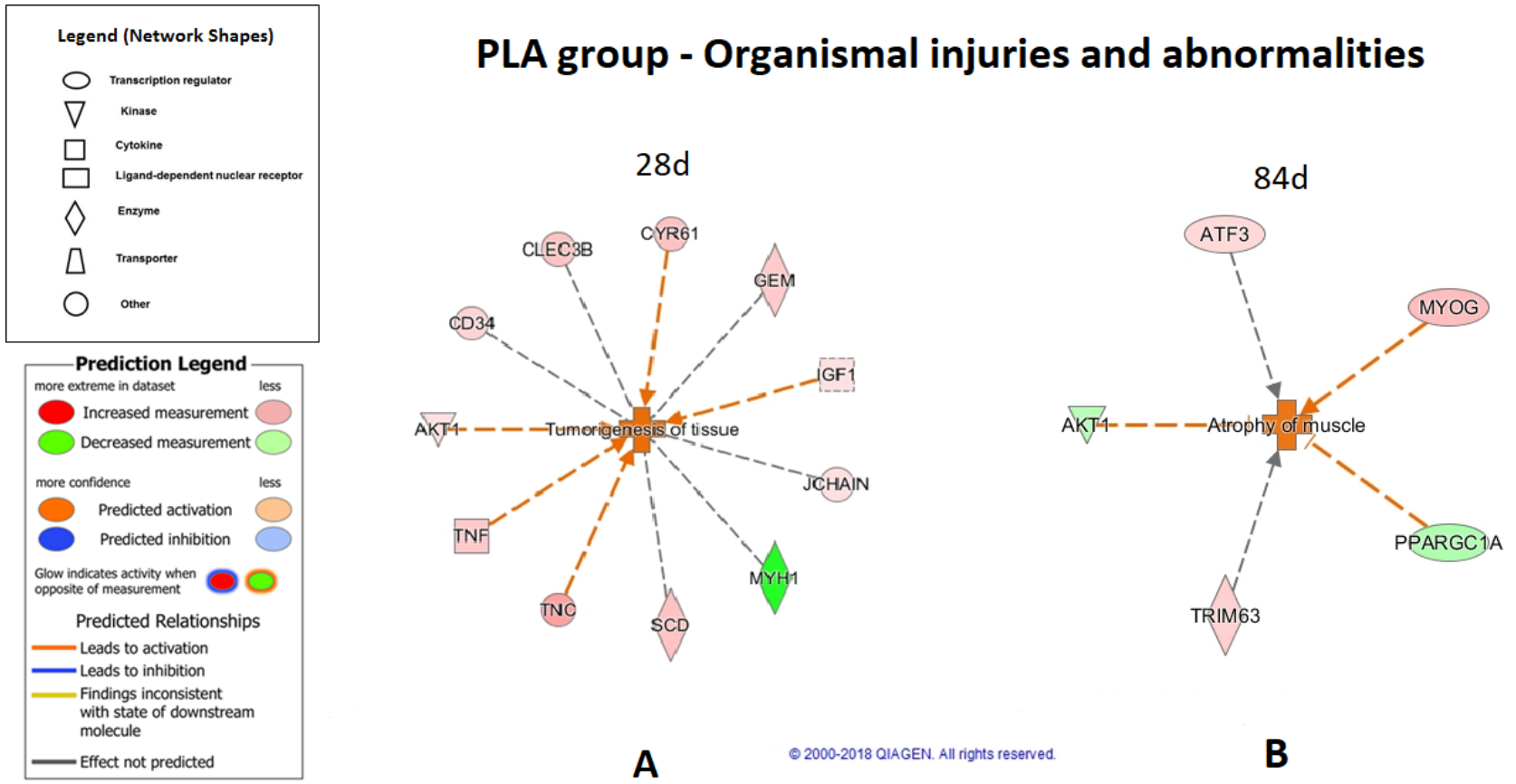

Figure 4-11: Differentially regulated muscle mRNAs (outer ring) associated with organismal injuries and abnormalities in the group consuming placebo (PLA) at (A) 28 days (28d) and (B) 84 days (84d) compared to baseline, and the cellular events predicted by Ingenuity Pathway Analysis to result from the collective changes in mRNA abundance.

Abbreviations: CYR61, cysteine-rich angiogenic inducer 61; GEM, GTP-binding protein; IGF1, insulin-like growth factor 1; JCHAIN ,joining chain of multimeric IgA and IgM); MYH1, myosin heavy chain 1; SCD, stearoyl-CoA desaturase; TNC, tenascin C; TNF, tumour necrosis factor; AKT1, serine-threonine protein kinase 1; CD34, hematopoietic progenitor cell antigen CD34; CLEC3B, C-type lectin domain family 3 member B; ATF3, activating transcription factor 3; MYOG, myogenin; PPARGC1A, peroxisome proliferator-activated receptor gamma coactivator 1 alpha; TRIM63, tripartite motif containing 63.

Figure 4-12 relates to organismal injuries and abnormalities in the NSAID group at (A) 24h and (B) 7d. Twelve mRNAs were identified as being altered in abundance relative to baseline at 24h (Figure 4-12A). The most upregulated mRNAs from baseline were TNF and IL-6, while MSTN was the most downregulated transcript. Based on the collective changes in mRNA abundance from baseline, IPA predicted activation of wasting with high confidence. It also predicted the inhibition of fibrosis and atrophy of muscle comprehensively. At 7d (Figure 4-12B), 12 mRNAs were identified as being altered in abundance relative to baseline. The most upregulated transcripts were TNF, IL-6 and MYC, while TRIM63 was the only downregulated transcript. In contrast to 24h, IPA did not make any comprehensive predictions based on the collective changes in mRNA abundance from baseline at this time-point.

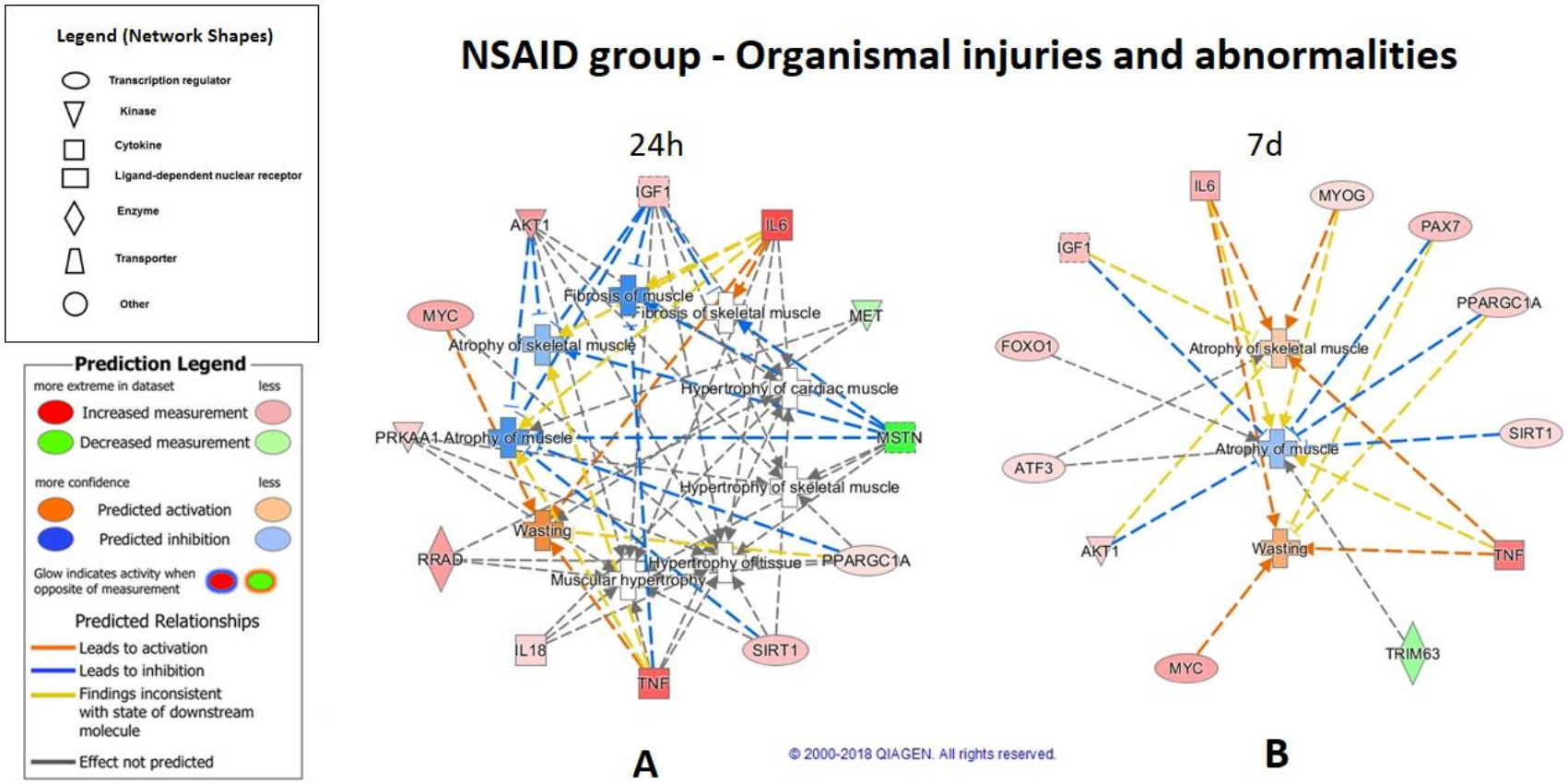

Figure 4-12: Differentially regulated muscle mRNAs (outer ring) associated with organismal injuries and abnormalities in the group consuming diclofenac (NSAID) at (A) 24 hours (24h) and (B) 7 days (7d) compared to baseline, and the cellular events predicted by Ingenuity Pathway Analysis to result from the collective changes in mRNA abundance.

Abbreviations: IL-6, interleukin 6; MET, MET proto-oncogene; MSTN, myostatin; PPARGC1A, peroxisome proliferator-activated receptor gamma coactivator 1 alpha; SIRT1, sirtuin 1; TNF, tumour necrosis factor; IL-18, interleukin 18; RRAD, Ras related glycolysis inhibitor and calcium channel regulator; PRKAA1, protein kinase AMP-activated catalytic subunit alpha 1; MYC, MYC proto-oncogene; AKT1, serine-threonine protein kinase 1; IGF1, insulin-like growth factor 1; MYOG, myogenin; PAX7, paired box gene 7; TRIM 3, tripartite motif containing 63; FOXO1, forkhead box protein O 1.

Figure 4-13 relates to organismal injuries and abnormalities in the NSAID group at (A) 28d and (B) 84d. Twenty-one mRNAs were identified as being altered in abundance relative to baseline at 28d (Figure 4-13A). The most upregulated mRNAs from baseline were TNF, actin alpha cardiac muscle 1 (ACTC1), ankyrin repeat domain 1(ANKRD1), PTGS2 and stearoyl-Coenzyme A desaturase 1(SCD), while TRIM63 and F-box protein 32 (FBXO32) were the most downregulated transcripts. Based on the collective changes in mRNA abundance from baseline, IPA predicted with a high degree of confidence the activation of hypertrophy, and inhibition of atrophy of muscle. In contrast to 28d, 9 mRNAs were identified as being altered in abundance relative to baseline at 84d (Figure 4-13B). TRIM63 was the only downregulated transcript. Based on the collective changes in mRNA abundance from baseline, IPA again predicted inhibition of atrophy of muscle with high confidence.

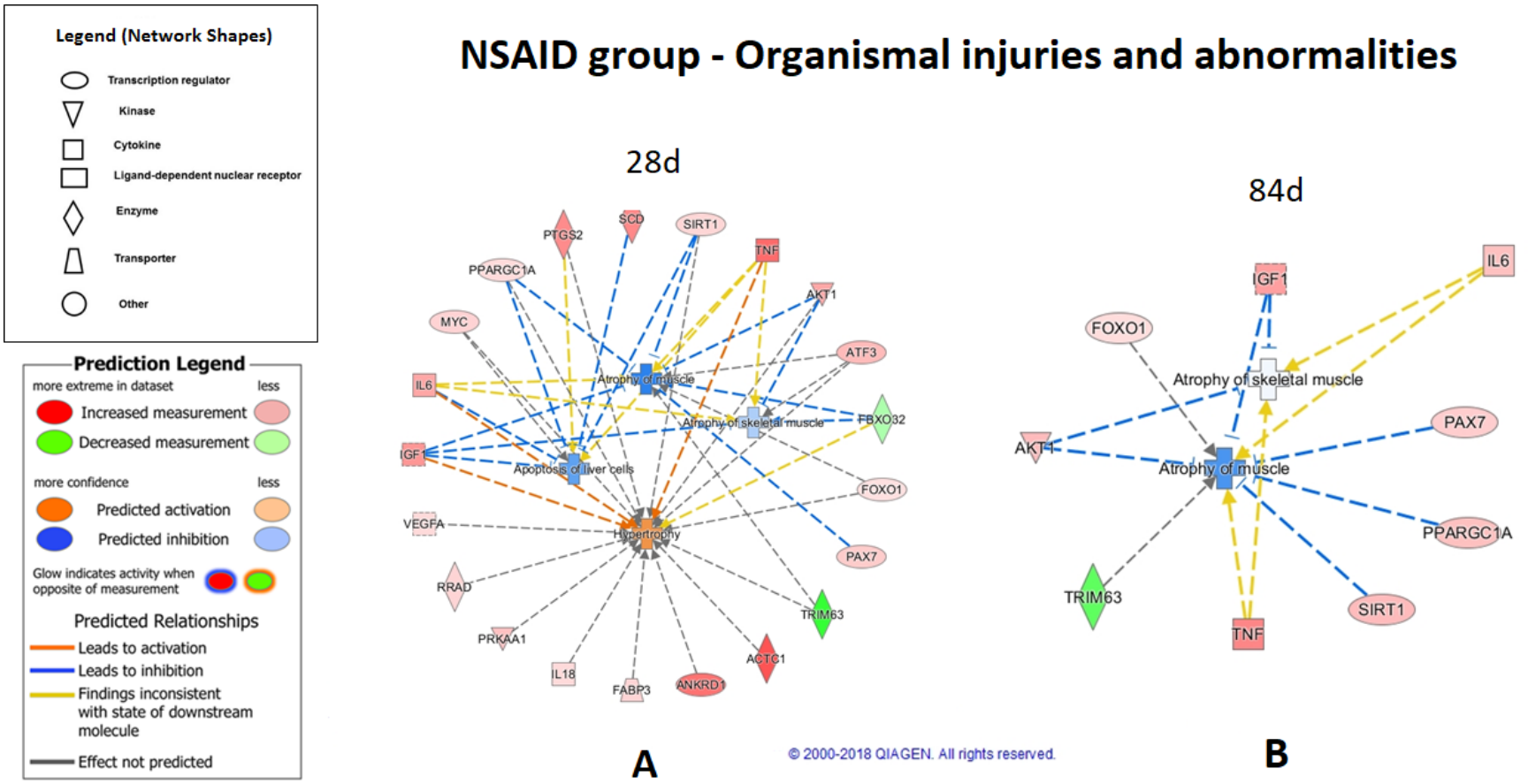

Figure 4-13: Differentially regulated muscle mRNAs (outer ring) associated with organismal injuries and abnormalities in group consuming diclofenac (NSAID) at (A) 28 days (28d) and (B) 84 days (84d) compared to baseline, and the cellular events predicted by Ingenuity Pathway Analysis to result from the collective changes in mRNA abundance.

Abbreviations: SIRT1, sirtuin 1; TNF, tumour necrosis factor; AKT1, serine-threonine protein kinase 1; ATF3, activating transcription factor 3; FBXO32, F-box protein32; FOXO1, forkhead box protein O 1; PAX7, paired box gene 7; TRIM 3, tripartite motif containing 63; ACTC1, actin alpha cardiac muscle 1; ANKRD1, ankyrin repeat domain 1; FABP3, fatty acid binding protein 3; IL-18, interleukin 18; PRKAA1, protein kinase AMP-activated catalytic subunit alpha 1; RRAD, Ras related glycolysis inhibitor and calcium channel regulator; VEGFA, vascular endothelial growth factor A; IGF1, insulin-like growth factor 1; IL-6, interleukin 6; MYC, MYC proto-oncogene; PPARGC1A, peroxisome proliferator-activated receptor gamma coactivator 1 alpha; PTGS2, prostaglandin-endoperoxide synthase 2; SCD, stearoyl-CoA desaturase.

Figure 4-14 relates to lipid metabolism in the PLA group at (A) 24h and (B) 7d. Seventeen mRNAs were identified as being altered in abundance relative to baseline at 24h (Figure 4-14A). The most upregulated mRNAs from baseline were IL-6, TNC and MYC, while PPARGC1A, MSTN and PDK2 were the most downregulated transcripts. Based on the collective change in mRNA abundance from baseline, IPA predicted the activation of several cellular events including concentration of lipid, release of fatty acid, release of lipid, secretion of lipid, synthesis of fatty acid, synthesis of lipid and concentration of fatty acid with the highest confidence. At 7d (Figure 4-14B), 17 mRNAs were identified as being altered in abundance relative to baseline. The most upregulated mRNAs from baseline were IL-6, TNC and MYC, while FABP3, PPARGC1A and SIRT1 were the most downregulated transcripts. Based on the collective changes in mRNA abundance from baseline, IPA predicted activation of synthesis of fatty

acid and concentration of lipid with the highest confidence. Notably, inhibition of oxidation of fatty acid was also predicted comprehensively.

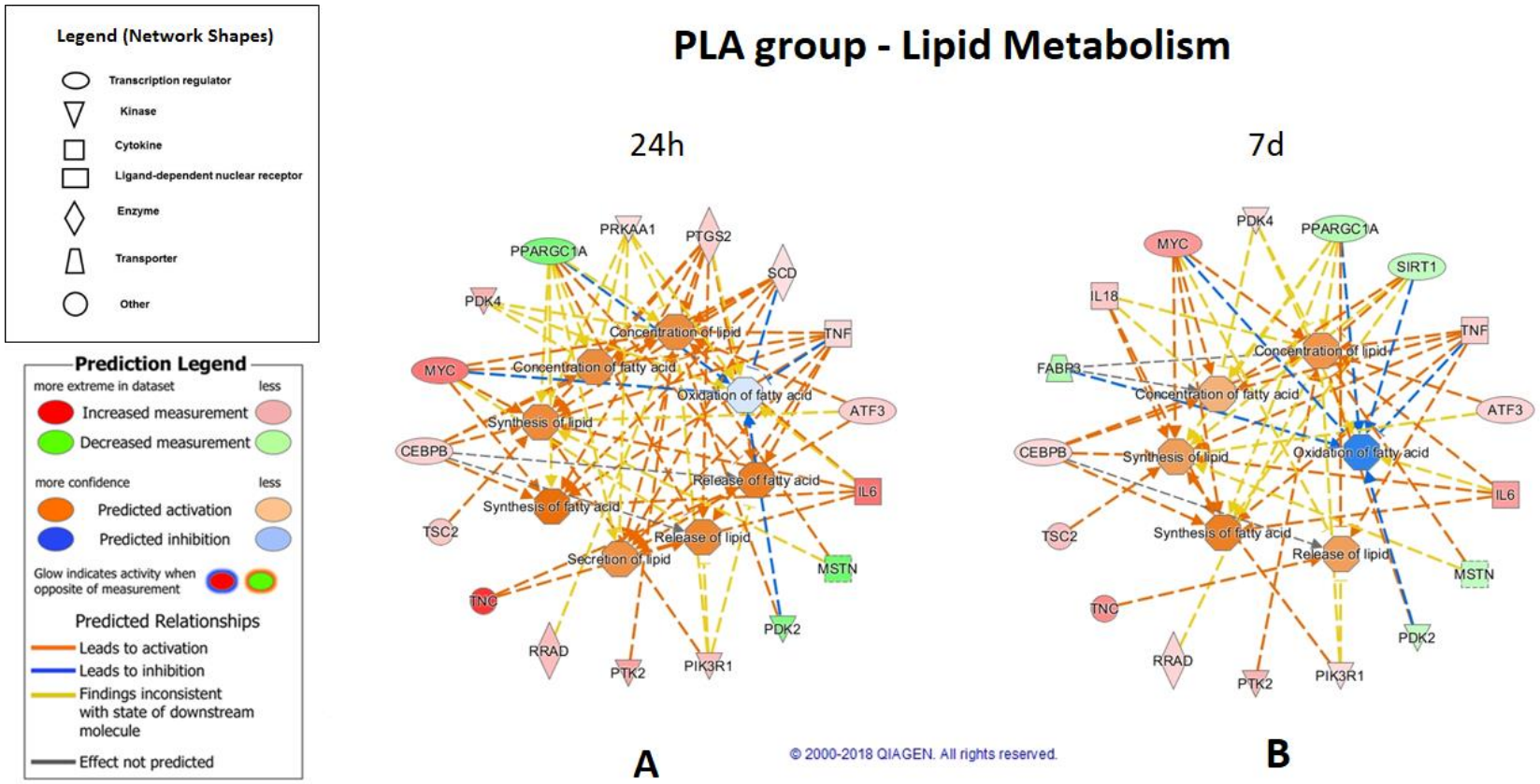

Figure 4-14: Differentially regulated muscle mRNAs (outer ring) associated with lipid metabolism in the group consuming placebo (PLA) at (A) 24 hours (24h) and (B) 7 days (7d) compared to baseline, and the cellular events predicted by Ingenuity Pathway Analysis to result from the collective changes in mRNA abundance.

Abbreviations: PRKAA1, protein kinase AMP-activated catalytic subunit alpha 1; PTGS2, prostaglandin-endoperoxide synthase 2; SCD, stearoyl-CoA desaturase; TNF, tumour necrosis factor; ATF3, activating transcription factor 3; IL-6, interleukin 6; IL-18, interleukin 18; MSTN, myostatin; PDK2, pyruvate dehydrogenase kinase 2; PIK3R1, phosphoinositide-3-kinase regulatory subunit 1; PTK2, protein tyrosine kinase 2; RRAD, Ras related glycolysis inhibitor and calcium channel regulator; TNC, tenascin C; TSC2, tuberous sclerosis complex 2; CEBPB, CCAAT enhancer binding protein beta; MYC, MYC proto-oncogene; PDK4, pyruvate dehydrogenase kinase 4; PPARGC1A, peroxisome proliferator-activated receptor gamma coactivator 1 alpha, SIRT1, sirtuin 1.

Figure 4-15 relates to lipid metabolism in the PLA group at (A) 28d and (B) 84d. Four mRNAs were identified as being altered in abundance relative to baseline at 28d (Figure 4-15A). These were IGF1, SCD, TNF and AKT1, all of which were upregulated. Based on the collective changes in mRNA abundance from baseline, IPA predicted activation of concentration of fatty acid, accumulation of lipid and secretion of lipid with the highest confidence. At 84d (Figure 4-15B), 8 mRNAs were identified as being altered in abundance relative to baseline. In contrast to 28d, most of the differentially expressed transcripts at this time point were downregulated, including FABP3, PPARGC1A, PTGS2, PDK2, VEGFA and AKT1. ATF3 and SCD were the upregulated transcripts. Based on the collective changes in mRNA abundance from baseline, in contrast to 28d, IPA predicted inhibition of fatty acid metabolism, oxidation of fatty acid, and synthesis of lipid with the highest confidence.

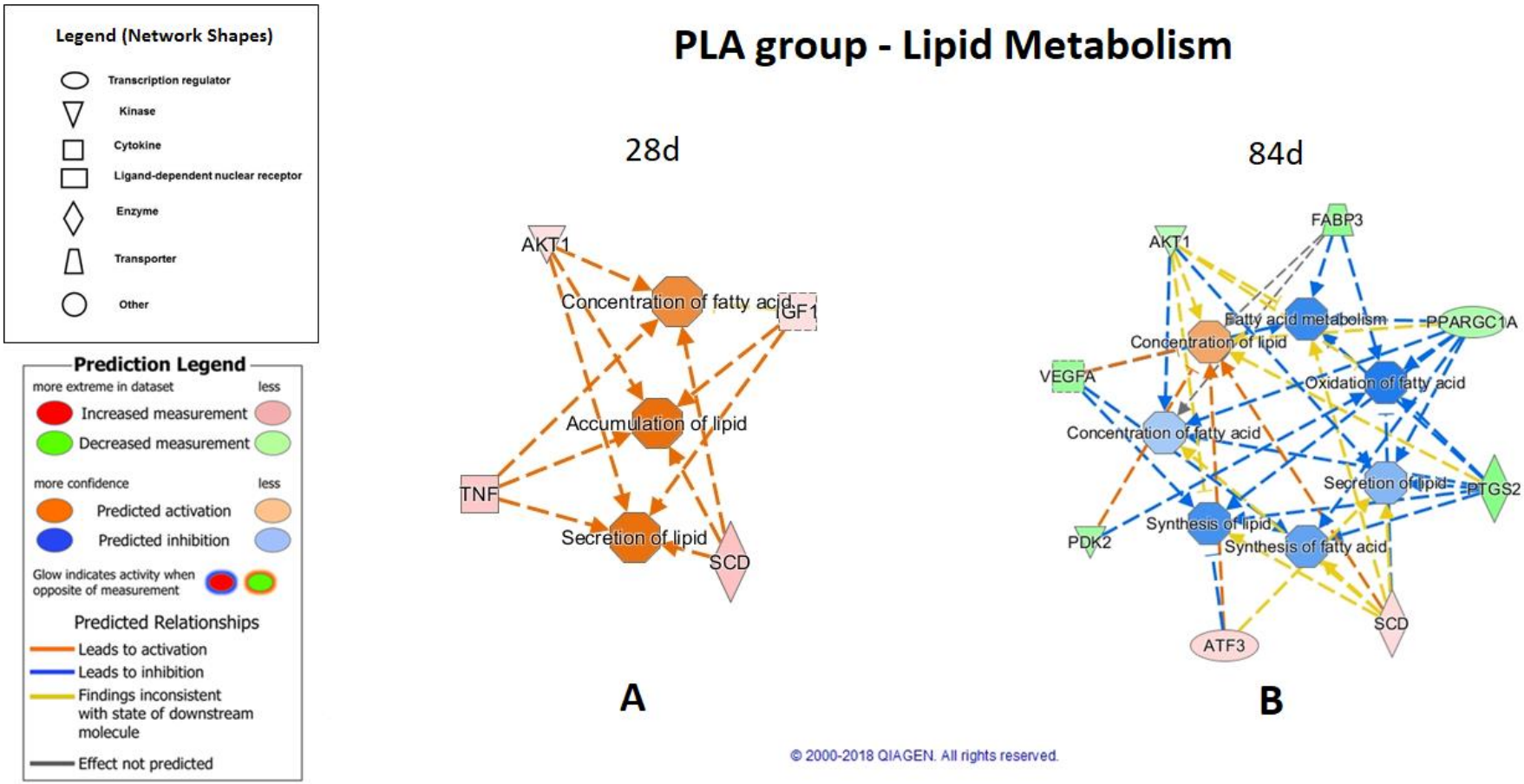

Figure 4-15: Differentially regulated muscle mRNAs (outer ring) associated with lipid metabolism in the group consuming placebo (PLA) at (A) 28 days (28d) and (B) 84 days (84d) compared to baseline, and the cellular events predicted by Ingenuity Pathway Analysis to result from the collective changes in mRNA abundance.

Abbreviations: AKT1, serine-threonine protein kinase 1; insulin-like growth factor 1; SCD, stearoyl-CoA desaturase; TNF, tumour necrosis factor; FABP3, fatty acid binding protein 3; PPARGC1A, peroxisome proliferator-activated receptor gamma coactivator 1 alpha; PTGS2, prostaglandin-endoperoxide synthase 2; ATF3, activating transcription factor 3; PDK2, pyruvate dehydrogenase kinase 2; VEGFA, vascular endothelial growth factor A.

Figure 4-16 relates to lipid metabolism in the NSAID group at (A) 24h and (B) 7d. Thirteen mRNAs were identified as being altered in abundance relative to baseline at 24h (Figure 4-16A). The most upregulated mRNAs were TNF and IL-6, while MSTN was the most downregulated transcript. Based on the collective changes in mRNA abundance from baseline, IPA predicted a number of cellular events including concentration of lipid, fatty acid metabolism, secretion of lipid, synthesis of fatty acid, synthesis of lipid and transport of lipid with highest confidence. At 7d (Figure 4-16B), 15 mRNAs were identified as being altered in abundance relative to baseline. The most upregulated transcripts were MYC, SCD and TNF. Based on the collective changes in mRNA abundance from baseline IPA predicted activation of fatty acid metabolism, secretion of lipid, synthesis of fatty acid and synthesis of lipid with highest confidence.

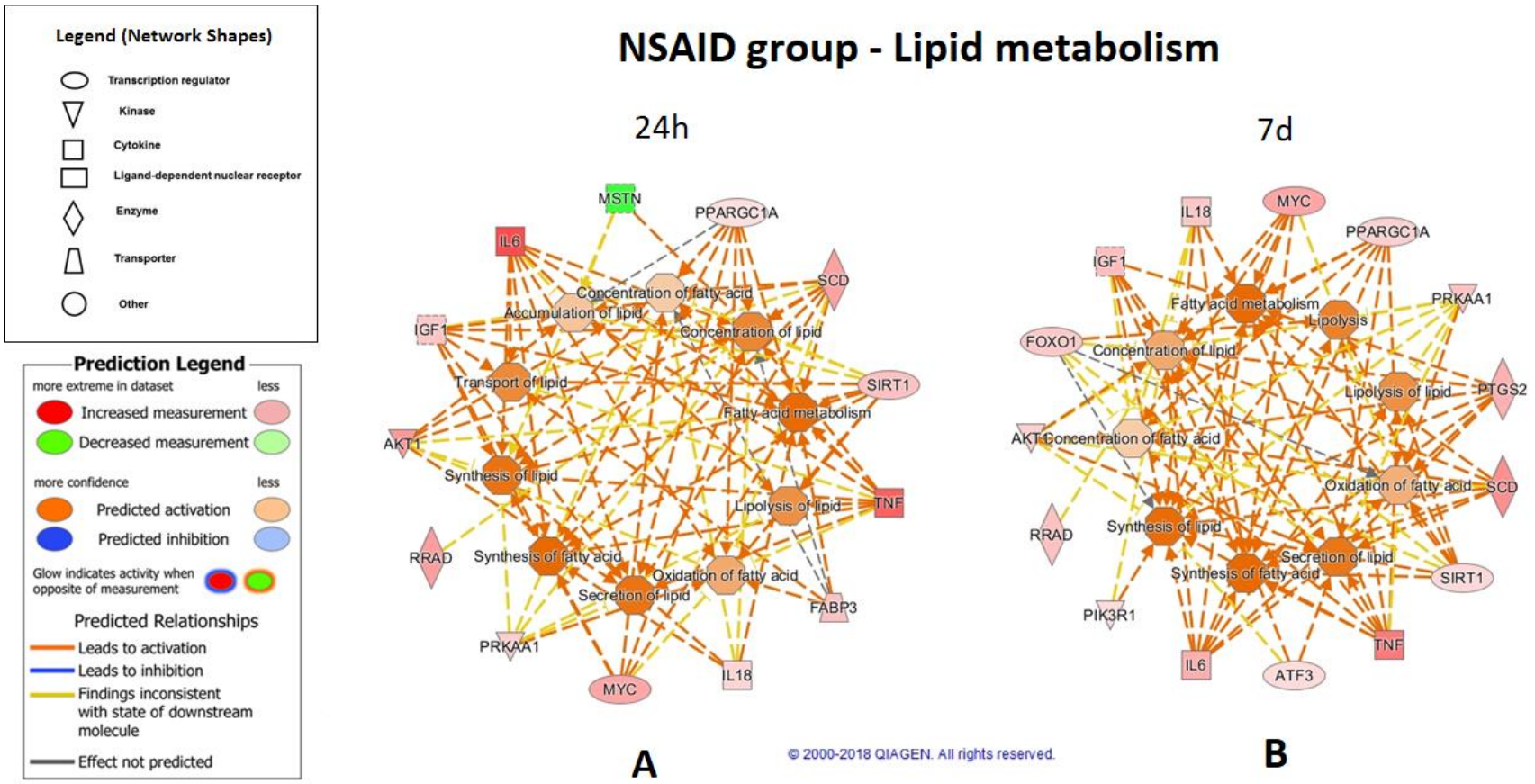

Figure 4-16: Differentially regulated muscle mRNAs (outer ring) associated with lipid metabolism in the group consuming diclofenac (NSAID) at (A) 24 hours (24h) and (B) 7 days (7d) compared to baseline, and the cellular events predicted by Ingenuity Pathway Analysis to result from the collective changes in mRNA abundance.

Abbreviations: MSTN, myostatin; PPARGC1A, peroxisome proliferator-activated receptor gamma coactivator 1 alpha; SCD, stearoyl-CoA desaturase; SIRT1, sirtuin 1; TNF, tumour necrosis factor; FABP3, fatty acid binding protein 3; IL-18, interleukin 18; MYC, MYC proto-oncogene; PRKAA1, protein kinase AMP-activated catalytic subunit alpha 1; RRAD, Ras related glycolysis inhibitor and calcium channel regulator; AKT1, serine-threonine protein kinase 1; IGF1, insulin-like growth factor 1; IL-6, interleukin 6; PTGS2, prostaglandin-endoperoxide synthase 2; ATF3, activating transcription factor 3; PIK3R1, phosphoinositide-3-kinase regulatory subunit 1; FOXO1, forkhead box protein O 1.

Figure 4-17 relates to lipid metabolism in the NSAID group at (A) 28d and (B) 84d. Seventeen mRNAs were identified as being altered in abundance relative to baseline at 28d (Figure 4-17A), unlike the PLA group at the same time-point when only four were altered (Figure 4-15A). The most upregulated mRNAs from baseline were SCD, TNF, PTGS2, AKT1, IGF1 and IL-6, while PDK4 was the only downregulated transcript. Based on the collective changes in mRNA abundance from baseline, IPA predicted activation of several cellular events including fatty acid metabolism, secretion of lipid, synthesis of fatty acid and synthesis of lipid comprehensively. At 84d (Figure 4-17B), 14 mRNAs were identified as being altered in abundance relative to baseline. The most upregulated mRNAs were PTGS2, SCD, TNF and IGF1. Based on the collective changes in mRNA abundance from baseline, similar to 28d, IPA predicted the activation of a number of cellular events with highest confidence, including fatty

acid metabolism, lipolysis of lipid, oxidation of fatty acid, secretion of lipid, synthesis of fatty acid, synthesis of lipid and transport of lipid.

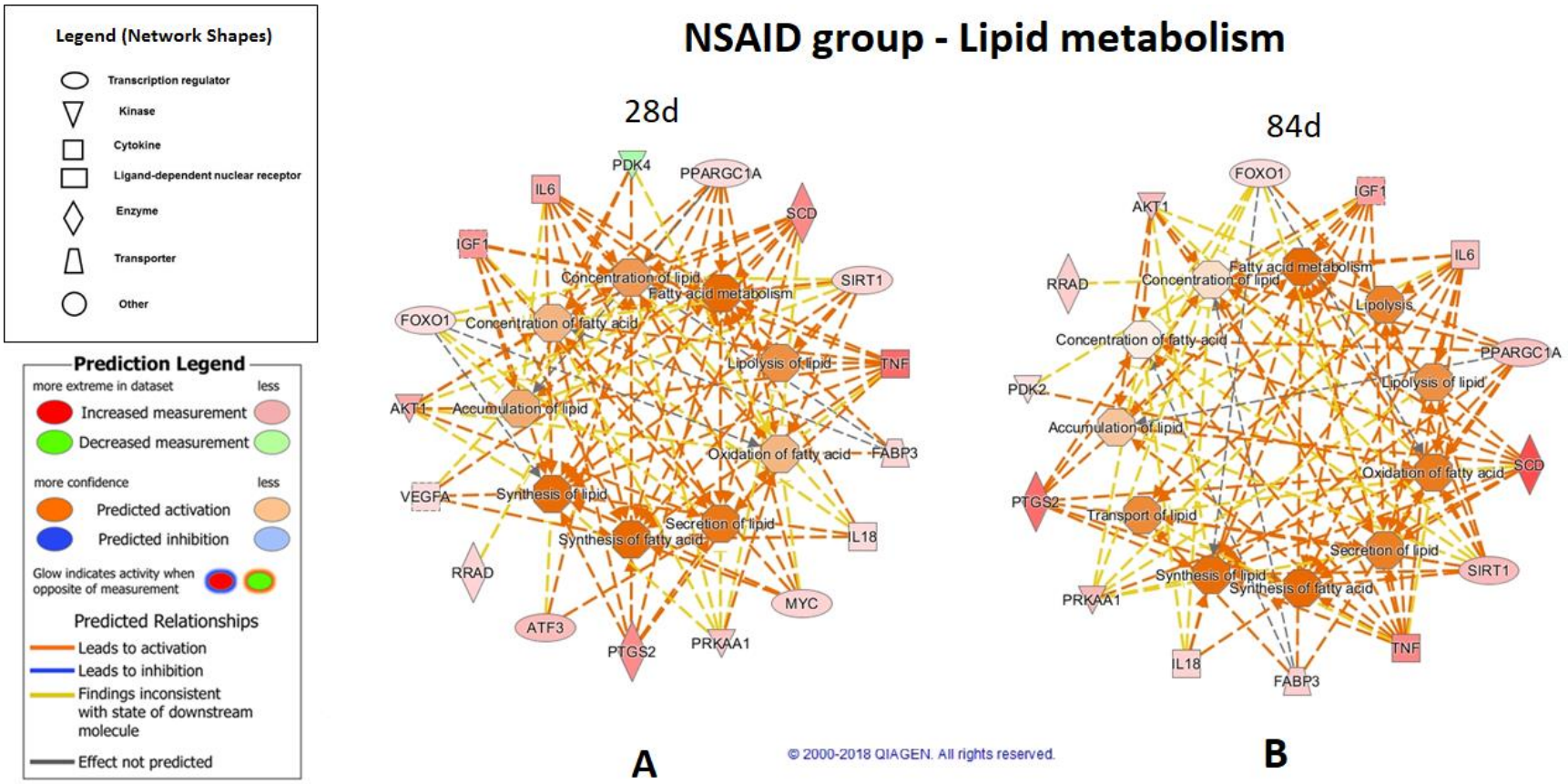

Figure 4-17: Differentially regulated muscle mRNAs (outer ring) associated with lipid metabolism in the group consuming diclofenac (NSAID) at (A) 28 days (28d) and (B) 84 days (84d) compared to baseline, and the cellular events predicted by Ingenuity Pathway Analysis to result from the collective changes in mRNA abundance.

Abbreviations: PPARGC1A, peroxisome proliferator-activated receptor gamma coactivator 1 alpha; SCD, stearoyl-CoA desaturase; SIRT1, sirtuin 1; TNF, tumour necrosis factor; FABP3, fatty acid binding protein 3; IL-18, interleukin 18; MYC, MYC proto-oncogene; PRKAA1, protein kinase AMP-activated catalytic subunit alpha 1; PTGS2, prostaglandin-endoperoxide synthase 2; ATF3, activating transcription factor 3; RRAD, Ras related glycolysis inhibitor and calcium channel regulator; vascular endothelial growth factor A, VEGFA; AKT1, serine-threonine protein kinase 1; FOXO1, forkhead box protein O 1; IGF1, insulin-like growth factor 1; IL-6, interleukin 6; PDK4, pyruvate dehydrogenase kinase 4; PPARGC1A, peroxisome proliferator-activated receptor gamma coactivator 1 alpha..

Figure 4-18 relates to tissue development in the PLA group at (A) 24h and (B) 7d. Twenty mRNAs were identified as being altered in abundance relative to baseline at 24h (Figure 4-18A). The most upregulated mRNAs from baseline were IL-6 and MYC, while PPARGC1A, MSTN and PAX3 were the most downregulated transcripts. Based on the collective changes in mRNA abundance from baseline, IPA predicted activation of growth of connective tissue with highest confidence. At 7d (Figure 4-18B), 16 mRNAs were identified as being altered in abundance relative to baseline. The most upregulated mRNAs from baseline were once again, IL-6 and MYC, while PAX3 was the most downregulated transcript. Based on the collective changes in mRNA abundance from baseline, IPA predicted activation of differentiation of muscle with high confidence.

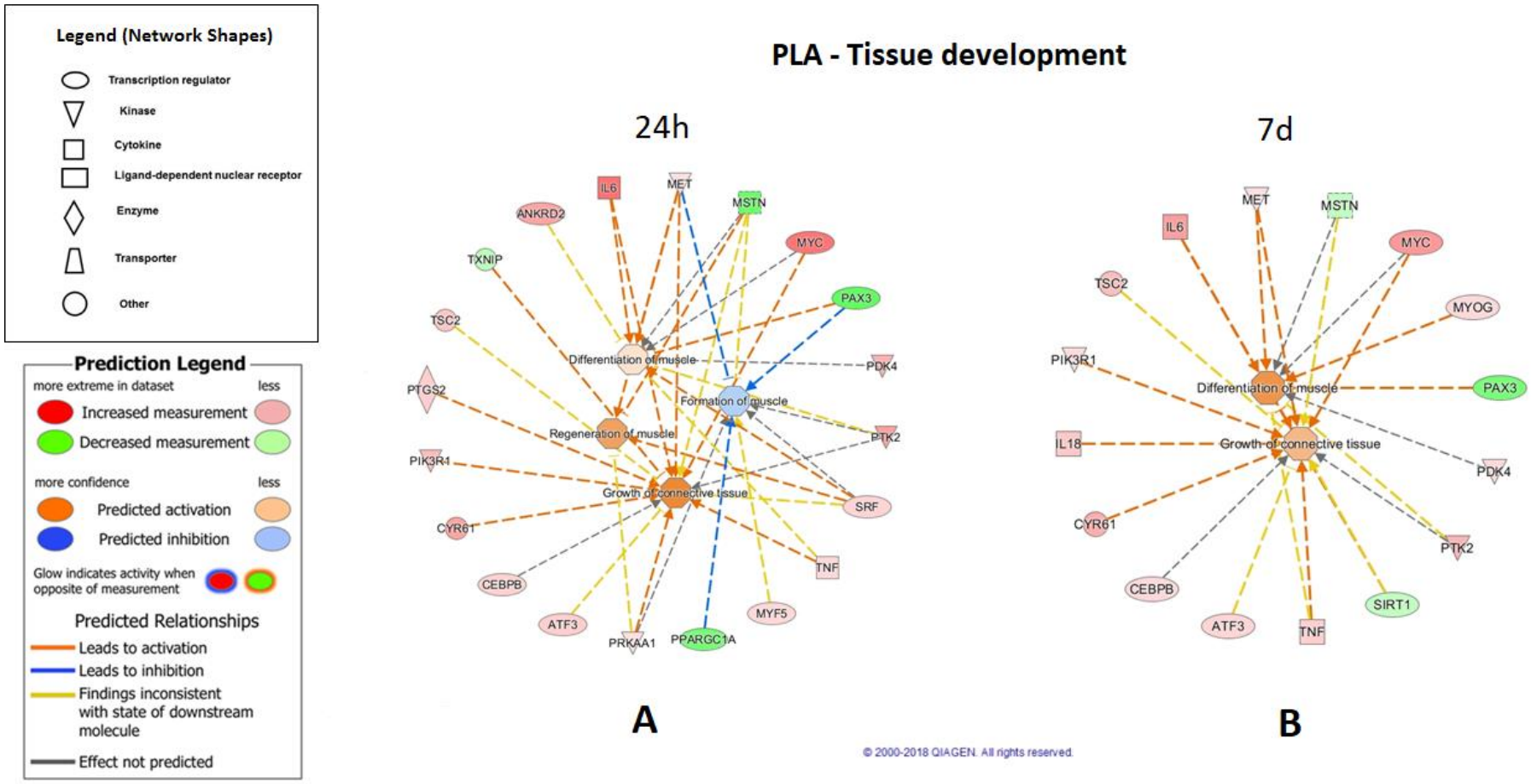

Figure 4-18: Differentially regulated muscle mRNAs (outer ring) associated with tissue development in the group consuming placebo (PLA) at (A) 24 hours (24h) and (B) 7 days (7d) compared to baseline, and the cellular events predicted by Ingenuity Pathway Analysis to result from the collective changes in mRNA abundance.

Abbreviations: MET, MET proto-oncogene; MSTN, myostatin; MYC, MYC proto-oncogene; PAX3, paired box 3 gene; PDK4, pyruvate dehydrogenase kinase 4; PTK2, protein tyrosine kinase 2; SRF, serum response factor; TNF, tumour necrosis factor; MYF5, myogenic factor 5; PPARGC1A, peroxisome proliferator-activated receptor gamma coactivator 1 alpha; PRKAA1, protein kinase AMP-activated catalytic subunit alpha 1; ATF3, activating transcription factor 3; CEBPB, CCAAT enhancer binding protein beta; CYR61, cysteine-rich angiogenic inducer 61; PIK3R1, phosphoinositide-3-kinase regulatory subunit 1; PTGS2, prostaglandin-endoperoxide synthase 2, TSC2, tuberous sclerosis complex 2; TXNIP, thioredoxin interacting protein; ANKRD2, ankyrin repeat domain 2; IL-6, interleukin 6; MYOG, myogenin; SIRT1, sirtuin 1; IL-18, interleukin 18.

Figure 4-19 relates to tissue development in the NSAID group at (A) 24h and (B) 7d. Seventeen mRNAs were identified as being altered in abundance relative to baseline at 24h (Figure 4-19A). The most upregulated mRNAs from baseline were TNF and IL-6, while MSTN was the most downregulated transcript. Based on the collective changes in mRNA abundance from baseline, IPA predicted activation of formation of muscle and quantity of connective tissue with highest confidence. At 7d (Figure 4-19B), 25 mRNAs were identified as being altered in abundance relative to baseline. The most upregulated mRNAs were TNF and TNC. Based on the collective changes in mRNA abundance from baseline IPA predicted activation of formation of muscle, proliferation of connective tissue cells, quantity of connective tissue and development of connective tissue cells with highest confidence.

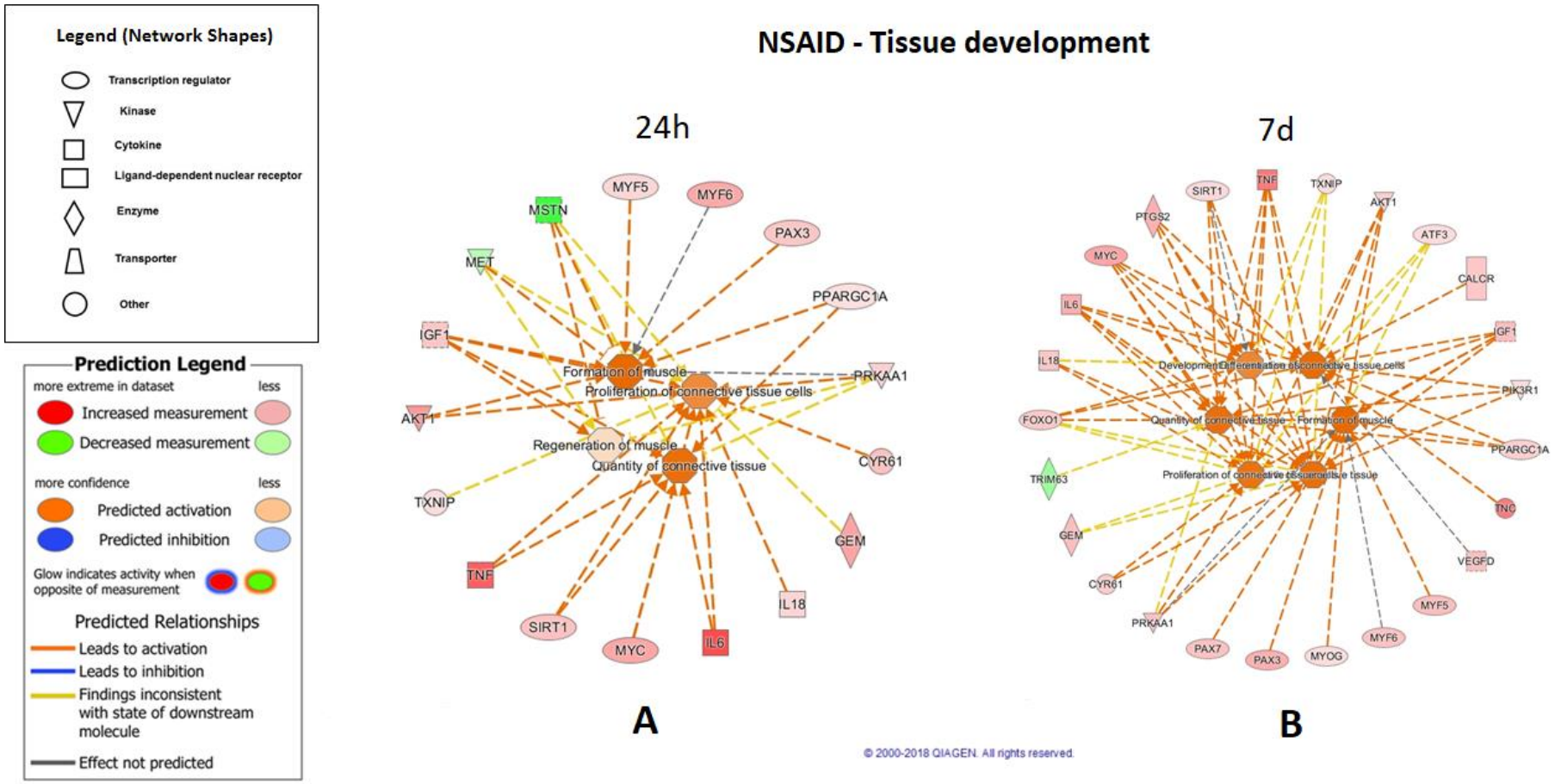

Figure 4-19: Differentially regulated muscle mRNAs (outer ring) associated with tissue development in the group consuming diclofenac (NSAID) at (A) 24 hours (24h) and (B) 7 days (7d) compared to baseline, and the cellular events predicted by Ingenuity Pathway Analysis to result from the collective changes in mRNA abundance.

Abbreviations: MYF5, myogenic factor 5; MYF6, myogenic factor 6; PAX3, paired box 3 gene; PPARGC1A, peroxisome proliferator-activated receptor gamma coactivator 1 alpha; PRKAA1, protein kinase AMP-activated catalytic subunit alpha 1; CYR61, cysteine-rich angiogenic inducer 61; GEM, GTP-binding protein; IL-18, interleukin 18; IL-6, interleukin 6; MYC, MYC proto-oncogene; SIRT1, sirtuin 1; TNF, tumour necrosis factor; TXNIP, thioredoxin interacting protein; AKT1, serine-threonine protein kinase 1; insulin-like growth factor 1; MET, MET proto-oncogene; MSTN, myostatin; ATF3, activating transcription factor 3; CALCR, calcitonin receptor; PIK3R1, phosphoinositide-3-kinase regulatory subunit 1; TNC, tenascin C; VEGFD, vascular endothelial growth factor D; MYOG, myogenin; PAX3, paired box gene 3; PAX7, paired box gene 7; TRIM63, tripartite motif containing 63; FOXO1, forkhead box protein O1; PTGS2, prostaglandin-endoperoxide synthase 2.

Schematics for predicted cellular events related to tissue development were not generated by IPA at 28d, as the number of genes that changed was insufficient to pass the threshold for reliable network predictions. Figure 4-20 relates to tissue development in the PLA group at 84d. Four mRNAs were identified as being altered in abundance relative to baseline, of which VEGFA, AKT1 and PPARGC1A were downregulated while MYOG was upregulated. Based on collective changes in abundance of these mRNAs, IPA predicted inhibition of formation of muscle with highest confidence.

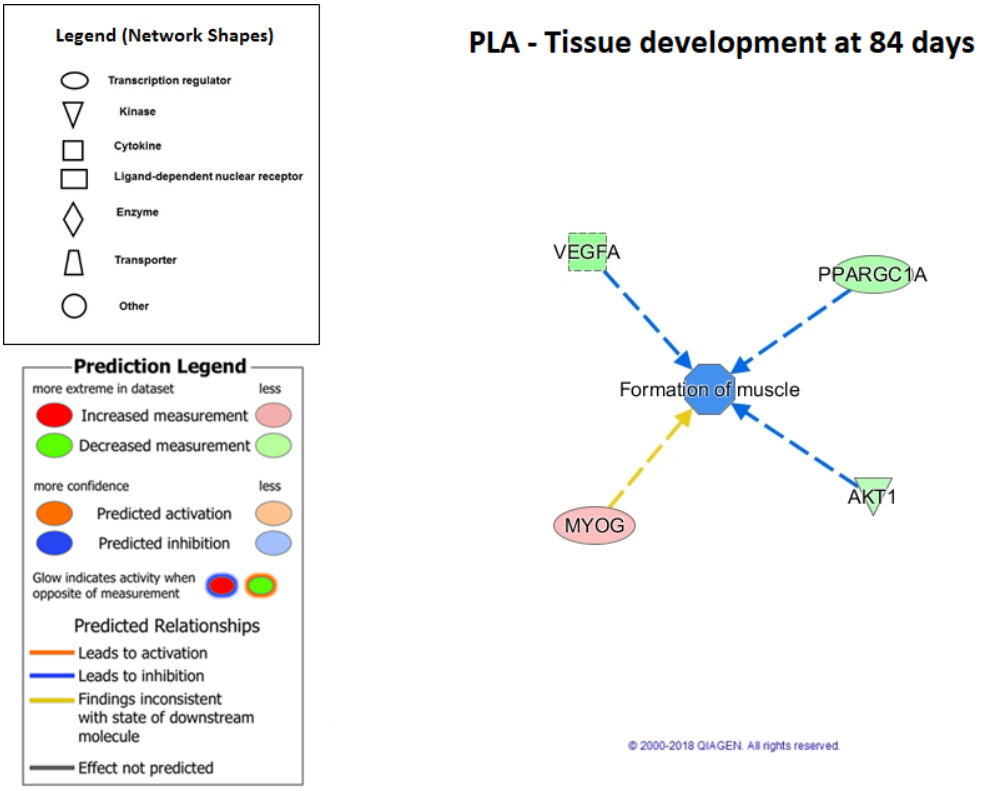

Figure 4-20: Differentially regulated muscle mRNAs (outer ring) associated with tissue development in the group consuming placebo (PLA) at 84 days (84d) compared to baseline, and the cellular events predicted by Ingenuity Pathway Analysis to result from the collective changes in mRNA abundance. Abbreviations: AKT1, serine threonine kinase 1; MYOG, myogenin; VEGFA, vascular endothelial growth factor A; PPARGC1A, peroxisome proliferator-activated receptor gamma coactivator 1 alpha.

In contrast to the PLA group, schematics for predicted cellular events related to tissue development were generated by IPA at both 28d and 84d for the NSAID group. Figure 4-21 relates to tissue development in the NSAID group at (A) 28d and (B) 84d. Twenty-two mRNAs were identified as being altered in abundance relative to baseline at 28d (Figure 4-21A). The most upregulated mRNAs from baseline were TNC and TNF, while TRIM63 was the most downregulated

transcript. Based on the collective changes in mRNA abundance from baseline, IPA predicted activation of differentiation of connective tissue cells, quantity of connective tissue and formation of muscle with highest confidence. Furthermore, IPA predicted an inhibition of differentiation of muscle comprehensively. At 84d (Figure 4-21B), 20 mRNAs were identified as being altered in abundance relative to baseline. The most upregulated mRNAs from baseline were PTGS2, TNF and GTP-binding protein (GEM). Based on the collective changes in mRNA abundance from baseline IPA predicted with high confidence the activation of differentiation of connective tissue cells, formation of muscle and proliferation of connective tissue cells.

Table 4-2 provides a summary of the gene networks predicted to be activated or inhibited with high confidence for all functions detected as altered by IPA in Figure 4-5.

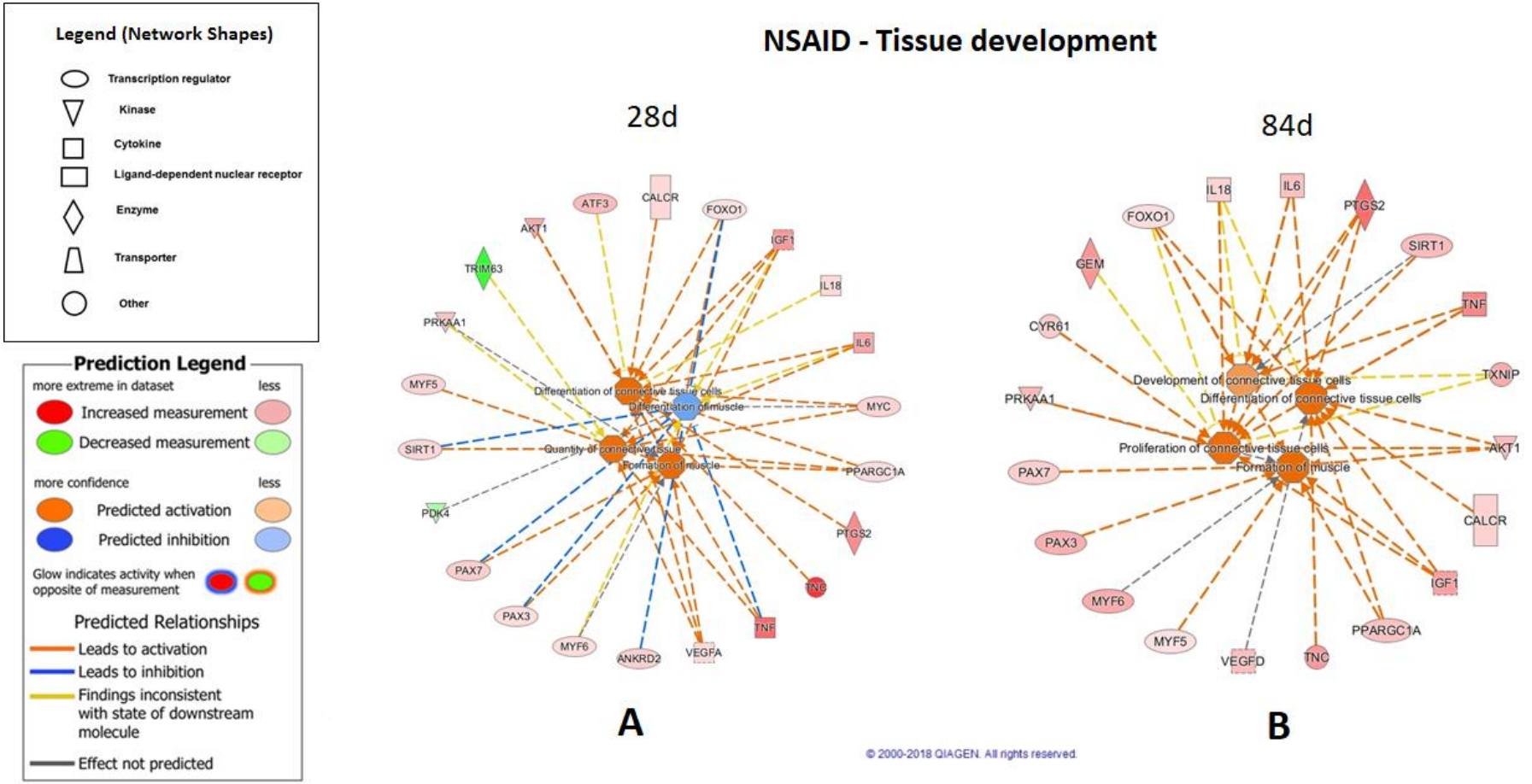

Figure 4-21: Differentially regulated muscle mRNAs (outer ring) associated with tissue development in the group consuming diclofenac (NSAID) at (A) 28 days (28d) and (B) 84 days (84d) compared to baseline, and the cellular events predicted by Ingenuity Pathway Analysis to result from the collective changes in mRNA abundance.

Table 4-2: A summary of those events associated with cellular functions predicted by IPA to be most altered based on muscle mRNA abundance change from baseline in the group consuming placebo (PLA) and the group consuming diclofenac (NSAID) at 24 hours (24h), 7 days (7d), 28 days (28d), and 84 days. Activation of an event predicted with high confidence indicated by (+). Inhibition of an event predicted with high confidence indicated by (-).

	24 h		7 d		28 d		84 d	
	PLA	NSAID	PLA	NSAID	PLA	NSAID	PLA	NSAID
Skeletal and muscular system development and function	(-) Function of muscle	(+) Formation of muscle	(-) Function of muscle (+) Differentiation of muscle	(+) Formation of muscle	No strong prediction	(+) Formation of muscle (-) Differentiation of muscle	(+) Formation of muscle	(+) Formation of muscle
Organismal injuries and abnormalities	(+) Atrophy of muscle	(-) Fibrosis of muscle (-) Atrophy of muscle (+) Wasting	(+) Atrophy of muscle (+) Tumorigenesis of tissue	No strong prediction	(+) Tumorigenesis of tissue	(-) Atrophy of muscle (+) Hypertrophy	(+) Atrophy of muscle	(+) Atrophy of muscle

	24 h		7 d		28 d		84 d	
	PLA	NSAID	PLA	NSAID	PLA	NSAID	PLA	NSAID
Lipid metabolism	(+) Concentration of lipid (+) Release of fatty acid (+) Release of lipid (+) Synthesis of fatty acid (+) Synthesis of lipid (+) Concentration of fatty acid (+) Secretion of lipid	(+) Concentration of lipid (+) Fatty acid metabolism (+) Secretion of lipid (+) Synthesis of fatty acid (+) Synthesis of lipid (+) Transport of lipid	(+) Synthesis of fatty acid (+) Concentration of lipid	(+) Lipolysis (+) Secretion of lipid (+) Synthesis of fatty acid (+) Synthesis of lipid (+) Fatty acid metabolism	(+) Concentration of fatty acid (+) Accumulation of lipid (+) Secretion of lipid	(+) Fatty acid metabolism (+) Secretion of lipid (+) Synthesis of fatty acid (+) Synthesis of lipid	(-) Fatty acid metabolism (-) Oxidation of fatty acid (-) Synthesis of lipid	(+) Fatty acid metabolism (+) Synthesis of fatty acid (+) Synthesis of lipid
Tissue development	(+) Growth of connective tissue	(+) Formation of muscle (+) Quantity of connective tissue (+) Proliferation of connective tissue cells	(+) Differentiation of muscle	(+) Formation of muscle (+) Quantity of connective tissue (+) Proliferation of connective tissue cells (+) Differentiation of connective tissue cells (+) Development of connective tissue cells	No strong prediction	(+) Differentiation of connective tissue cells (+) Quantity of connective tissue (+) Formation of muscle	(-) Formation of muscle	(+) Differentiation of connective tissue cells (+) Formation of muscle (+) Proliferation of connective tissue cells

	24 h		7 d		28 d		84 d	
	PLA	NSAID	PLA	NSAID	PLA	NSAID	PLA	NSAID
Carbohydrate metabolism	(+) Uptake of D-glucose	(+) Synthesis of D-glucose	(+) Metabolism of carbohydrate (+) Synthesis of carbohydrate	(+) Uptake of D-glucose (+) Transport of carbohydrate (+) Synthesis of D-glucose	(+) Synthesis of carbohydrate	(+) Binding of carbohydrate (+) Uptake of D-glucose (+) Synthesis of D-glucose	No strong prediction	(+) Synthesis of D-glucose (-) Oxidation of D-glucose
Skeletal and muscular disorders	(+) Atrophy of muscle	(-) Atrophy of muscle (-) Fibrosis of muscle	(+) Atrophy of muscle	No strong prediction		(-) Atrophy of muscle	(+) Atrophy of muscle	(-) Atrophy of muscle

4.3.5 Muscle protein expression

Table 4-3 shows fold change in muscle protein expression of inflammatory proteins from baseline (set at 1) in the vastus lateralis muscle in both PLA and NSAID groups at 24h, 7d, 28d and 84d. Mature TNF alpha protein expression decreased in the PLA group when compared to BL at all time-points (all $p<0.001$). Mature TNF alpha expression was also significantly lower in the NSAID group when compared to BL at 7d ($p<0.01$) and 28d ($p<0.001$). However, there was no difference between groups at any time-point in expression of inflammatory proteins. It is also noteworthy that the magnitude of fold change from baseline in each group, and for all inflammatory proteins, was relatively low, despite reaching statistical significance in the specific instances noted.

*Table 4-3: Expression levels of inflammatory proteins interleukin-6 (IL-6), tumour necrosis factor alpha precursor (TNF alpha precursor), and mature tumour necrosis factor alpha (TNF alpha mature) in vastus lateralis muscle in the group consuming placebo (PLA; n=8) and the group consuming diclofenac (NSAID; n=9) at 24 hours (24h), 7 days (7d), 28 days (28d) and 84 days (84d). Values expressed as fold change relative to mean baseline ± SEM. Baseline set at 1. **p<0.1 compared to baseline. ***p<0.001 compared to baseline.*

Protein	Treatment	24h	7d	28d	84d
IL-6	PLA	0.65 ± 0.21	0.95 ± 0.42	1.38 ± 0.47	0.91 ± 0.30
	NSAID	0.86 ± 0.24	0.62 ± 0.16	0.50 ± 0.09	0.84 ± 0.37
TNF alpha precursor	PLA	0.96 ± 0.16	0.99 ± 0.19	1.08 ± 0.30	1.19 ± 0.22
	NSAID	0.73 ± 0.15	0.90 ± 0.10	0.97 ± 0.17	0.99 ± 0.29
TNF alpha mature	PLA	0.28 ± 0.07***	0.43 ± 0.24***	0.51 ± 0.30***	0.53 ± 0.28***
	NSAID	0.75 ± 0.44	0.40 ± 0.04**	0.41 ± 0.04**	1.35 ± 0.63

4.4 Discussion

This is the first study to measure the time course of effects of chronic NSAID ingestion on resistance training-induced increase of muscle CSA, muscle volume and strength; and on the abundance of mRNAs linked to regulation of muscle mass and muscle metabolism. The major finding of this study was that 84 days of ingestion of the COX-2 inhibitor diclofenac (mechanism of action depicted in Figure 4-22) increased muscle CSA and volume gains in young, recreationally active males in response to unilateral maximal concentric training of the quadriceps above placebo. This difference in muscle CSA and muscle volume gains between groups occurred predominantly between days 28 and 84 of training. In parallel with this, gene networks associated with several cellular functions including skeletal and muscular system development and function, organismal injuries and abnormalities, tissue development and others linked to regulation of muscle mass and metabolism were found to be more altered with diclofenac ingestion than with placebo at 28 days and 84 days of training. Notably, strength gains were not increased in the NSAID group above placebo at any time-point. Furthermore, both training work output per week and total work output over the duration of the protocol were not different between groups. Therefore, the greater increase in muscle CSA and volume gains during combined diclofenac ingestion and resistance training was not accompanied by greater gains in muscle function.

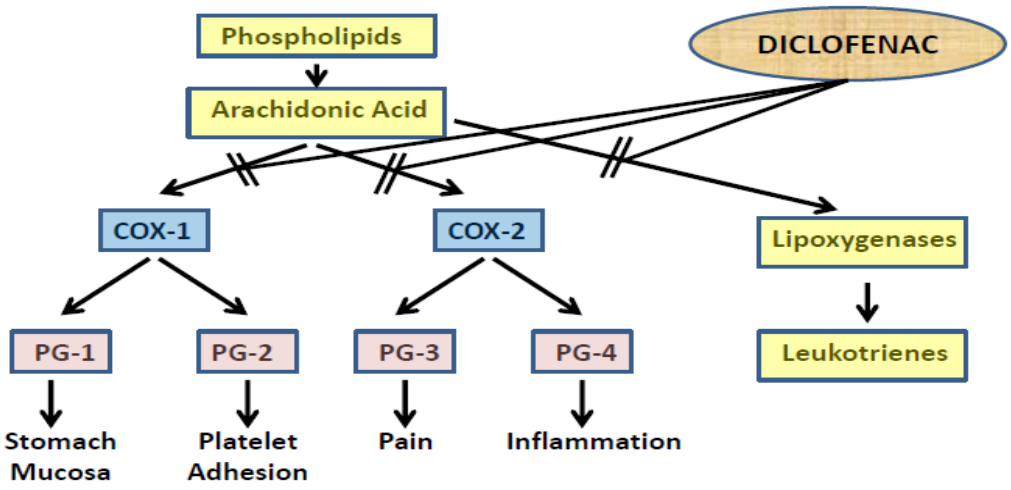

Figure 4-22: Mechanism of action of diclofenac. Diclofenac acts a competitive inhibitor of COX-1, COX-2 and lipoxygenases. However, because it preferentially inhibits COX-2, this in turn inhibits the production of those prostaglandins (PGs) that promote pain and inflammation.

Few studies have investigated the impact of chronic NSAID administration on resistance exercise-induced muscular adaptations in the young. Krentz et al. (2008) showed that in healthy, young participants performing 60 combined concentric-eccentric elbow flexor contractions 5 times per week over 6 weeks, concurrent daily ingestion of the NSAID ibuprofen had no effect on muscle hypertrophy or strength over placebo ingestion. However, the short duration of training of this trial was clearly a limitation, given the fact that the present study detected a striking difference in training-induced increase in muscle CSA and volume between NSAID and PLA groups from 28d to 84d of training (Figure 4-4). In the PLA group, neither muscle CSA nor muscle volume increased during this period (Figure 4-4A), which is not surprising, given increase in muscle mass (Ogasawara et al. 2013) and post-exercise rise in MPS (Brook et al. 2015) are known to plateau with chronic resistance training. However, while similar

muscle CSA and volume increases were noted in PLA and NSAID groups at 28d (~2.5-4.0% increase from BL), a further increase from 28d to 84d of training was noted in NSAID group alone (Figure 4-4B). During this period muscle CSA and volume increased by ~120% more in the NSAID group, which is a novel finding. NSAID augmentation of the muscular response to chronic resistance training has been reported previously, but in the elderly. Trappe et al. (2011) showed that older volunteers regularly consuming the NSAID ibuprofen demonstrated greater muscular adaptation to 12 weeks of progressive resistance training compared to placebo, with muscle volume increasing ~47% above that seen in the placebo group (Trappe et al. 2011). The magnitude of the gains in muscle mass in elderly volunteers is notably lower than the young subjects in the present study, perhaps because anabolic resistance in elderly skeletal muscle (Cuthbertson et al. 2005; Wilkes et al. 2009) limited the degree of adaptation to ibuprofen administration. This might also be proposed to be due to low-grade chronic inflammation associated with ageing (Schaap et al. 2009; Schaap et al. 2006). However, in light of the muscle inflammatory protein expression findings in the present study, this is unlikely, and will be elaborated on below.

In the current study, isometric strength of the knee extensors increased from baseline in both the PLA and NSAID group by up to ~40% ($p<0.05$) and ~50% at 84d respectively. However, no differences were noted in strength between PLA and NSAID at any time-point. This suggests that the greater muscle mass in the NSAID group is of lower quality, having not improved in function above PLA. Importantly, this is not an isolated finding among resistance training studies examining the influence of pharmacological interventions that are

purported to enhance performance, in that a linear relationship between muscle mass and strength is not always demonstrated. For instance, while it is widely perceived by athletes that anabolic steroids increase muscle mass and hence performance (Elashoff et al. 1991; Haupt and Rovere 1984; Strauss and Yesalis 1991; Wilson 1988), whether or not they increase muscle strength is inconclusive (Ariel 1973; Hervey et al. 1981; Johnson et al. 1972; Stamford and Moffatt 1974), and the limitations of these studies have been highlighted by several reviewers (Bross et al. 1998; Mooradian, Morley, and Korenman 1987; Medicine 1987). A number of potential therapies touted as showing promise with regard to functional improvements in conditions where loss of muscle mass and strength cause disability have been shown to suffer similar discrepancies in efficacy for inducing muscle hypertrophy versus increasing strength during clinical testing (Negro-Vilar 1999; Bhasin and Jasuja 2009). In this vein, one would be ill-advised to consume NSAIDs - demonstrated to elicit a number of side effects (Bhala et al. 2013; Warner et al. 2002; Warner et al. 1999; McGettigan and Henry 2013; McGettigan and Henry 2011; Bjorkman 1998) - with a view towards increasing performance in training and/or competition.

Given the greater hypertrophic response to combined NSAID ingestion and resistance training in our study, it is necessary to uncover the mechanisms by which this might have occurred. One of the aims of the present study was to determine whether the analgesic effect of diclofenac increased work output during training sessions above placebo, given their extensive use by sportspersons to prevent pain during training, perhaps towards enhancing muscle adaptation (Gorski et al. 2011; Rudgard, Hirsch, and Cox 2019). However,

neither total work output per week of training, nor work done over the entire protocol differed between PLA and NSAID (Figure 4-2), suggesting that other mechanisms might be contributory. In experiments in Chapter 3, gene networks associated with inflammation and organismal injury were found to be significantly altered from baseline in the early stages of maximal isokinetic resistance training in young volunteers (Figure 3-7). Hence, one of the aims of the present study was to determine whether modulation of this insult by diclofenac ingestion might impact on training-induced muscle hypertrophy differently from placebo ingestion. Muscle TNF alpha protein expression was found to be decreased from baseline in the PLA group at all time-points, and in the NSAID group at 7d and 28d. However, no significant difference was detected in either muscle TNF alpha or IL-6 protein expression between groups at any time-point (Table 4-3). Furthermore, despite being statistically significant, the magnitude of change in muscle TNF alpha protein expression from baseline was minor, and likely had little impact on skeletal muscle adaptation. These findings also contradict the notion that inhibition of low-grade systemic inflammation by NSAID ingestion in the elderly volunteers in the study by Trappe et al. (2011) was responsible for increased muscular adaptations above placebo. Collectively, it appears that non-overt inflammation has little impact on regulation of muscle mass, as opposed to scenarios of overt inflammation, as in the critically ill, in whom change in muscle IL-6 and TNF alpha mRNA expression has been shown to be far greater in than in controls (6.5-fold; $p<0.001$ and 2-fold; $p<0.01$, respectively) (Constantin et al. 2011b), and accompanied by dramatic muscle wasting.

Thus far, no study has identified the time course of the molecular response to combined chronic NSAID administration and resistance training. In the present study, based on collective changes in mRNAs from baseline, IPA predicted a number of cellular functions (Figure 4-5) to be altered in both groups during training. However, the magnitude of change in these functions was ~2-fold greater in the NSAID group at 28d and 84d, paralleling the greater increase in muscle CSA and volume gains in these volunteers.

Indeed, activation of cellular events linked to regulation of muscle mass was predicted by IPA in both groups at 24h and 7d. Of those related to "skeletal and muscular system development and function", differentiation of muscle in the PLA group at 7d (Figure 4-6B), and formation of muscle in the NSAID group at both 24h and 7d (Figure 4-8) were predicted to be activated with the highest confidence. In a similar vein, processes associated with "tissue development" including growth of connective tissue and regeneration of muscle were predicted to be activated in the PLA group at 24h (Figure 4-18A); and quantity of connective tissue and proliferation of connective tissue cells were predicted to be activated in the NSAID group at both 24h and 7d (Figure 4-19).

However, predictions for cellular events related to "skeletal and muscular system development and function" as a whole were not generated for the PLA group at 28d, as the magnitude of change in mRNA abundance related to this function was not sufficient at this time-point. In contrast, formation of muscle was still predicted to be activated with the highest confidence in the NSAID group at 28d and 84d (Figure 4-9), as was the activation of growth of connective tissue, quantity of connective tissue and proliferation of connective tissue cells (Figure

4-21). Furthermore, the predictions at these later time points in the NSAID group were made by IPA based on gene changes that included an upregulation of myogenic transcription factors MYF5 and MYF6, and gene transcripts linked to satellite cell activation and proliferation, PAX3 and PAX7 (Figure 4-9, Figure 4-21).

Bioinformatics analysis in the present study therefore indicate the transpiring of molecular events at 28d and 84d that corresponded temporally with the greater increase in muscle CSA and muscle volume noted in the NSAID group. Further, they indicate that this greater increase in muscle mass was not due to MRI artefacts - in particular, water content in muscle - which might have been suspected given NSAIDs are known to cause water retention, possibly through their actions on prostaglandins (Villa et al. 1997). Interestingly, network predictions by IPA also align with the key finding of an increase in muscle mass adaptations in the NSAID group above placebo without an accompanying enhancement in strength, or work output during training. At 84d, an inhibition of function of muscle was predicted in the NSAID group (Figure 4-9A), despite the aforementioned predictions related to muscle growth at 28d and 84d. In contrast, an inhibition of function of muscle was not predicted in the PLA group at 84d (Figure 4-7).

Perhaps unexpectedly, a differential response between groups was also predicted by IPA with regard to cellular events related to lipid metabolism. At 24h and 7d in both groups, IPA comprehensively predicted the activation of a number of cellular events, including increased concentration of lipid, concentration of fatty acid, synthesis of fatty acid and synthesis of lipid (PLA, Figure 4-14; NSAID,

Figure 4-16), based on collective change in abundance from baseline of many of the same gene transcripts, that included IL-6, TNF, MYC, SCD and RRAD. However, IPA also predicted an inhibition of fatty acid oxidation at 7d in the PLA group (Figure 4-14B) based on a downregulation of PPARGC1A and SIRT1 gene transcripts, while in the NSAID group, activation of fatty acid oxidation was predicted with high confidence at both 24h and 7d, based on an upregulation of the same genes (Figure 4-16B).

At 84d in the PLA group, IPA predicted an inhibition of fatty acid metabolism, fatty acid oxidation, synthesis of lipid and synthesis of fatty acid (Figure 4-15B). In contrast, in the NSAID group, in the same vein as the first week of training, activation of fatty acid oxidation was predicted with high confidence at 28d and 84d (Figure 4-16). Overall, an inhibition of fatty acid oxidation and fatty acid metabolism was predicted in both the initial and latter periods of the protocol during resistance training alone. Consumption of NSAIDs alongside resistance training, however, was predicted to activate fatty acid oxidation throughout the protocol. The reasons for this are unclear, and alterations in lipid metabolism warrant investigation in future training studies.

In summary, this study found that chronic administration of NSAIDs in young, recreationally active male volunteers over 84 days of training comprising unilateral maximal concentric knee extensions increased muscle CSA and volume gains above a group consuming placebo, and that this occurred predominantly from 28d to 84d of training. In parallel with this, greater alterations in gene networks associated with a number of cellular functions linked to regulation of muscle mass and muscle metabolism were detected in the

NSAID group relative to placebo. The augmentation of resistance training-induced muscle CSA and volume gains during combined NSAID ingestion and resistance training was not accompanied by improvements in training work output above resistance training alone as the protocol progressed, or by a greater increase in muscle strength, suggesting that these mass gains were likely of poor quality. It would therefore be incautious to recommend chronic NSAID ingestion alongside resistance training, given the muscle mass gains associated with their administration are not accompanied by a concomitant improvement in function.

CHAPTER 5. The effect of a pharmacologically mediated increase in carbohydrate oxidation on the skeletal muscle anabolic response to protein nutrition, and combined protein nutrition and exercise in elderly obese volunteers

5.1 Introduction

Thus far, experiments in Chapters 3 and 4 have focused on skeletal muscle hypertrophic adaptations in response to resistance exercise, resistance exercise plus NSAID drug intervention, and the functional and molecular events that underlie these adaptations in young, healthy participants. However, it is known that declines in muscle mass and strength occur with advancing age (Lindle et al. 1997). Furthermore, a strong relationship has been demonstrated between muscle mass as determined by D3-creatine dilution, and physical performance, mobility, and incident injurious falls in older men (Cawthon et al. 2019). Muscle mass has also been shown to predict longevity in the elderly (Srikanthan and Karlamangla 2014). Mass and strength loss is partially attributable to a failure in these individuals to increase MPS in response to protein feeding compared to the young (Cuthbertson et al. 2005). Similarly, the stimulatory effect of exercise on MPS is blunted in elderly volunteers compared to young of a comparable BMI (Kumar, Selby, et al. 2009). In both scenarios, this so called “anabolic blunting” is accompanied by the dampening of muscle anabolic signalling regulating protein translation initiation (Cuthbertson et al. 2005; Kumar, Selby, et al. 2009). The mechanistic basis of anabolic blunting is currently unresolved, but ageing *per se* (Cuthbertson et al. 2005), inactivity (Smeuninx et al. 2017) and obesity (Murton et al. 2015) have all been implicated as drivers.

Obesity is defined as abnormal or extensive fat accumulation that negatively affects health ('Obesity: preventing and managing the global epidemic. Report of a WHO consultation' 2000). It has been reported that high fat mass is associated with lower muscle quality and predicts accelerated loss of lean mass

in older adults (Koster et al. 2011), but it is not known if this is a causative association. Nevertheless, this association was confirmed even when physical activity levels were taken into account (Roubenoff 2000). In a cross-sectional study involving older volunteers, the combination of low muscle strength and obesity was found to associate more with impaired physical function than either alone (Bouchard and Janssen 2010). Indeed, it has been shown that obesity in combination with muscle impairment has a synergistic effect on the risk of developing several poor health outcomes (Dominguez and Barbagallo 2007; Aubertin-Leheudre et al. 2006).

Earlier studies in young obese volunteers demonstrated both elevated (Nair et al. 1983) and lowered (Guillet et al. 2009) post-absorptive rates of MPS compared to healthy weight controls. More recently, rates of post-absorptive MPS have been shown to be similar in young obese vs healthy-weight volunteers by Beals and colleagues (2018; 2016), which was also found to be the case in older individuals by Murton et al. (2015). However, there is growing evidence that the stimulation of MPS in response to protein feeding is blunted in obese individuals compared to normal weight controls, at least in the acute setting in both young (Beals, Sukiennik, Nallabelli, Emmons, Van Vliet, Young, Ulanov, Li, Paluska, and De Lisio 2016) and older volunteers (Murton et al. 2015; Smeuninx et al. 2017). Similarly, Beals and colleagues (2018) demonstrated that the synergistic effect of resistance exercise and protein feeding on MPS was blunted in young obese individuals when compared to normal weight counterparts. These findings are concerning particularly given the increasing prevalence of obesity across all age groups, including the elderly (Fakhouri et al. 2012), and the aforementioned

worsening of health outcomes and function by obesity in the elderly. In the case of the elderly, which is the focus of this chapter, Murton et al. (2015) found that the increase in MPS in response to acute amino acid infusion under insulin clamp conditions (to obviate any gut effects on study outcomes) was diminished in older obese volunteers compared to age and sex matched healthy-weight volunteers. This was accompanied by impaired whole-body and leg glucose disposal, collectively demonstrating poorer metabolic quality of muscle in obese older individuals. The mechanistic basis for this blunting of MPS in the obese elderly is unclear given anabolic signalling in response to feeding was not blunted compared to their healthy-weight counterparts. It has been suggested that obese adipose tissue might have an indirect effect on muscle by inducing systemic inflammation via the secretion of pro-inflammatory cytokines (adipokines) that have been shown to impact on myogenesis (O'Leary et al. 2018). However, quadriceps muscle volume was higher in the obese group suggesting the presence of low-grade systemic inflammation in the obese does not have a detrimental effect in regulating muscle mass. Furthermore, it seems unlikely that muscle inflammation was driving muscle anabolic blunting in the obese volunteers in the study by Murton et al. (2015) since muscle mRNA expression of a number of inflammatory cytokines was not elevated compared to the healthy-weight volunteers, despite the presence of low-grade systemic inflammation in the obese volunteers. Indeed, the blunting of anabolic response to protein nutrition in elderly obese individuals of Murton et al. (2015) was recently confirmed by Smeuninx et al. (2017) using protein feeding. Uncovering the underlying mechanisms is important, given there is a clearly a need for

intervention to ameliorate the poor response to muscle anabolic stimuli in the elderly obese.

Protein synthesis is the largest consumer of energy during cellular proliferation (Li et al. 2014), in that ribosomal translation is estimated to account for ~30% of the energy consumption for a differentiating mammalian cell (Buttgereit and Brand 1995). Furthermore, regulation of MPS is suggested to be highly dependent on cellular energy status, in that there exists a hierarchical arrangement in allocation of ATP to cellular ATP-consuming pathways such that MPS is very sensitive to a decrease in ATP/ADP ratio (Buttgereit and Brand 1995; Atkinson 1977; Goron et al. 2019). Therefore, the fact that carbohydrate oxidation is far more efficient than fat oxidation in terms of oxygen consumption towards ATP production (Melzer 2011) is perhaps important in the context of anabolic resistance in obesity and ageing. It is possible that the inability of muscle in the obese to switch from lipid to carbohydrate oxidation in insulin-stimulated conditions, known as metabolic inflexibility (Goodpaster and Sparks 2017; Storlien, Oakes, and Kelley 2004), transiently limits the rate of increase in muscle mitochondrial ATP resynthesis towards increasing MPS due to the greater oxygen consumption associated with fat oxidation. Metabolic inflexibility of muscle is also a feature of ageing due to the increase in fat mass (Kuk et al. 2009) and reduced mitochondrial content (Carter, Chen, and Hood 2015) that is associated with it. Hence, it is possible that poor metabolic quality and resilience of muscle drives the anabolic resistance to protein feeding in the elderly (Cuthbertson et al. 2005), which in turn might be heightened by obesity, resulting in the worsening of the anabolic response observed in the elderly obese

compared to healthy-weight controls (Murton et al. 2015; Smeuninx et al. 2017). Metabolic resilience in this context can be defined as the inability to mount an equivalent dynamic response to a physiological challenge as that seen in a young individual (Prof. P.L. Greenhaff, personal correspondence).

In the context of this background, forcibly increasing muscle carbohydrate oxidation using a pharmacological intervention might reduce the oxygen cost of ATP resynthesis in obese individuals, and possibly ameliorate anabolic resistance by increasing the energy efficiency of MPS. At least part of the regulation of fuel selection for oxidation by muscle resides at the level of the pyruvate dehydrogenase complex (PDC) (Randle 1963). This enzyme complex is comprised of three enzymes located on the inner mitochondrial membrane, and acts as a catalyst for irreversible conversion of pyruvate to acetyl-CoA (Denton, Randle, and Martin 1972) (Figure 5-1).

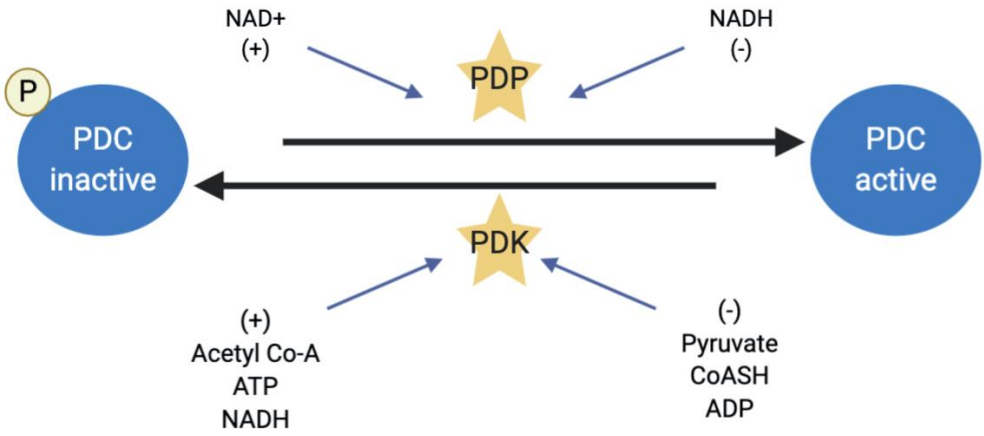

Figure 5-1. Pyruvate dehydrogenase complex (PDC) regulation. ATP, adenosine triphosphate; ADP, adenosine diphosphate; CoASH, coenzyme A; NADH, nicotinamide adenine dinucleotide (NAD) + hydrogen (H); NAD+, nicotinamide adenine dinucleotide; PDK, pyruvate dehydrogenase kinase; PDP, pyruvate dehydrogenase phosphatase.

The up-regulation of pyruvate dehydrogenase kinase-2 (PDK2) and PDK4 results in the phosphorylation and inactivation of muscle PDC (Wieland 1983), which in turn limits the rate of carbohydrate oxidation. Dichloroacetate (DCA) is a halogenated carboxylic acid that has been demonstrated to increase PDC activity (Whitehouse and Randle 1973; Whitehouse, Cooper, and Randle 1974) by the competitive inhibition of PDK2 and PDK4 (Bowker-Kinley et al. 1998). While administration of DCA does not simulate insulin secretion *per se*, it has been shown to shift cellular substrate utilisation from lipid to carbohydrate (Stacpoole 1989). DCA-mediated activation of PDC is large and rapid, resulting in full PDC activation and increased mitochondrial pyruvate flux in skeletal muscle in the resting state (Timmons et al ref, Roberts et al. ref) , which is known to increase muscle glucose oxidation (Constantin-Teodosiu et al. 1991). Indeed,

DCA has been shown to increase skeletal muscle PDC flux at rest (Howlett et al. 1999; Constantin-Teodosiu, Simpson, and Greenhaff 1999) and during ischaemic and submaximal exercise (Timmons et al. 2004; Constantin-Teodosiu et al. 2012) in several human volunteer studies.

DCA administration has also been shown to lower plasma triglycerides acutely (Hayek and Woodside 1980) and chronically (Hayek and Woodside 1981), and has also been shown to inhibit fatty acid oxidation acutely (McAllister, Allison, and Randle 1973) in animal models of obesity and diabetes respectively. It has also been demonstrated to lower plasma glucose and plasma triglycerides in insulin resistance in Type 2 diabetes in humans (Stacpoole, Moore, and Kornhauser 1978). Therefore, it might have therapeutic potential in the context of metabolic inflexibility in humans in obesity and ageing. Importantly, intravenous infusion of DCA in young healthy volunteers has been demonstrated to abolish the inhibition of carbohydrate oxidation caused by 3 days of high-fat feeding during exercise (Constantin-Teodosiu et al. 2012). In effect, DCA administration reversed metabolic inflexibility in this scenario. Studies in patients with cancer (Papandreou, Goliasova, and Denko 2011; Michelakis et al. 2010) and in models of ageing in *Caenorhabditis elegans* (Grad and Lemire 2004) have also led to the suggestion that DCA might improve metabolic resilience in the elderly by its beneficial effects on mitochondrial energetics (Stacpoole 2012). While the utility of DCA as a therapeutic tool is limited by its well-documented toxicity following chronic administration (Stacpoole 1989; Stacpoole et al. 1990), collectively, the literature indicates that it is possible that acute administration of DCA, via its ability to activate PDC, may be capable of

reversing metabolic inflexibility and improving metabolic quality and resilience in elderly obese individuals.

5.1.1 Aims and Hypothesis

The aims of the present study were to test the hypothesis that forcibly increasing muscle carbohydrate oxidation by acute DCA administration could resolve muscle anabolic resistance to protein nutrition, and exercise and protein nutrition in obese older volunteers compared to control intervention. In a randomised cross-over designed study, oral DCA (50 $mg.kg^{-1}$ body mass) was used with the aim of acutely increasing muscle carbohydrate oxidation in elderly obese volunteers over placebo control administration. Primed constant infusions of stable isotope amino acid tracers were used in both experimental paradigms to quantify the acute MPS response to protein feeding, and protein feeding plus resistance exercise.

5.2 Methods

5.2.1 Study Protocol

A randomised crossover design was used to achieve the aims of the study. Figure 5-2 shows the overall study protocol, including the experimental trial visit procedures. All participants visited the David Greenfield Human Physiology Unit on 4 separate occasions (Figure 5-2A). On the first visit participants underwent a health screening. During the second visit participants underwent (i) body composition measurements using dual energy X-ray absorptiometry (DEXA); (ii) thigh muscle volume measurements using MRI, and vastus lateralis IMCL and EMCL measurements at mid-thigh using MRS and (iii)

familiarisation to a bout of maximal intensity resistance exercise of their dominant leg on an isokinetic dynamometer so as to be able to perform the same bout on subsequent experimental trial visits correctly and without apprehension. Participants then underwent the first of two experimental trial visits (Figure 5-2B), which were conducted at least two weeks apart. The order of these trial visits was randomised based on the intervention, such that participants were administered either the placebo control drink on one visit (Control), or the intervention drink on the other visit (DCA).

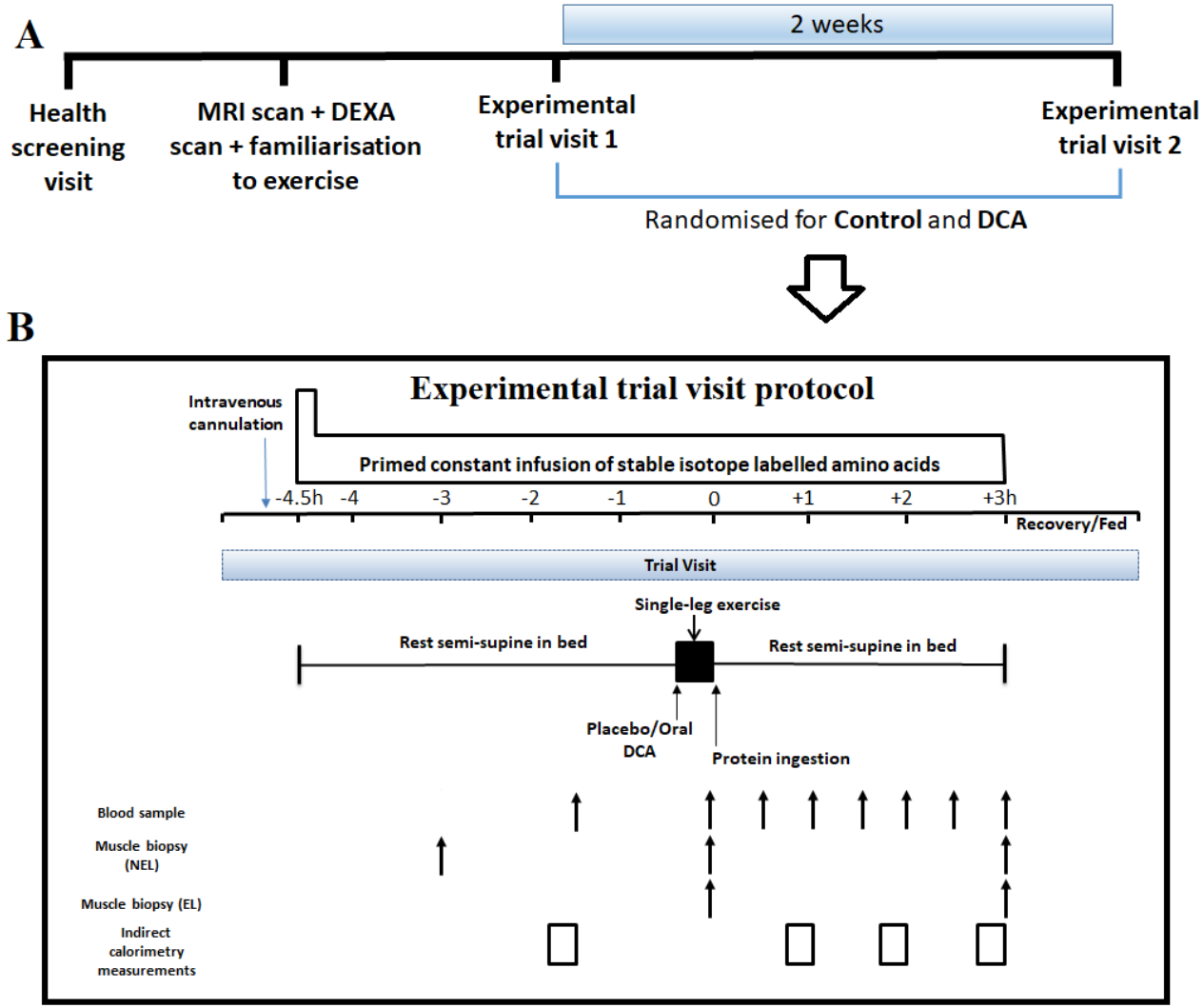

Figure 5-2. (A) Overall study schematic. Participants underwent a visit for a health screening; a visit for a dual energy X-ray absorptiometry (DEXA) scan; magnetic resonance imaging (MRI) scan and familiarisation to a bout of resistance exercise; and two experimental trial visits, randomised based on the intervention used during the visit, i.e. a placebo control drink (Control) or the intervention drink containing 50mg.kg^{-1} body mass of oral dichloroacetate (DCA). (B) Schematic of the protocol for the experimental trial visits. NEL = non-exercised leg. EL = exercised leg. DCA = dichloroacetate.

5.2.2 Participants

This study was conducted at the David Greenfield Human Physiology Unit (DGHPU), University of Nottingham. Nine obese, non-smoking, non-vegetarian males aged over 60 (age: 71.4 ± 1.6 years, BMI: 30.2 ± 1.2 $kg.m^{-2}$), who did not have a history of, or suffer from the conditions listed as exclusion criteria in General Methods (Section 2.3) were recruited to the study. The study was approved by the University of Nottingham Medical School Ethics Committee in accordance with the Declaration of Helsinki (Ethics Reference No: C18082016). Prior to taking part, all participants provided informed, written consent, underwent a routine medical screening and completed a general health questionnaire as described in General Methods (Section 2.3).

5.2.2.1 Inclusion criteria for obesity

As per the American Council for Exercise recommendations, adult males are categorised as obese when total body fat percentage is >25% (Muth 2009). However, the volunteers in the present study were >60 years old, and it is well-established that fat mass increases with age (Villareal et al. 2005). Those who were recruited had to meet a cut-off of >30% whole body fat mass, which is categorised as obese in males over 60 years of age (Gallagher et al. 2000). Body fat mass % was determined on the participants' second visit of the study protocol (Figure 5-2A) using DEXA (described in Section 5.2.4), which is a gold-standard method for body composition measurements (Kim et al. 2002; Scafoglieri et al. 2013; Clarys et al. 2010).

Hence, body fat mass % was used to determine inclusion, and not BMI. This is because the BMI range for obesity (>29 kg/m2) is a poor measure of adiposity. While it is generally assumed that BMI aligns closely with adiposity, and hence the morbidity and mortality associated with increased adiposity (Bray 1996; Cole 2002), many individuals who are overweight do not have excess fat, while others fall within the range for a normal BMI but have high body fat.

5.2.3 Isometric strength measurements

All subjects fell within 2 standard deviations of the mean isometric strength of the knee extensors for their age group (Harbo, Brincks, and Andersen 2012), so as to solely include volunteers without myopathy or overt sarcopenia. This was accomplished by measuring maximal isometric strength of the knee extensor muscles during a static voluntary contraction using an isokinetic dynamometer (as described in General Methods: Section 2.4.2) during the health screening visit.

5.2.4 Experimental trial visits

Participants underwent two experimental trial visits, conducted at least two weeks apart (Figure 5-2B). To prepare the drink for the DCA visit, a solution of oral dichloroacetate ($50mg.kg^{-1}$ body mass) was made up from sodium dichloroacetate powder for oral liquid (Sodium Dichloroacetate 50mg in 1ml S/F, C/F Powder for Oral Liquid; Veriton Pharma Limited). This solution was mixed with one-fifth of the same volume of Jucee sugar-free squash concentrate (Jucee®). For the same participant, on the Control visit a placebo control drink was prepared by mixing water and Jucee squash concentrate in the same

respective volumes as oral dichloroacetate solution and Jucee squash concentrate for the DCA visit drink. Randomisation for the interventions between visits was performed using an online randomisation tool (http://randomization.com/). Both investigator and participant were blinded to the intervention by having a research technician prepare the drink independently, and by using the same colour and flavour of squash concentrate for both drinks.

Figure 5-2B shows the experimental protocol during each trial visit. Participants arrived at the DGHPU in the morning in a fasted state, having abstained from alcohol and exercise for 48h prior to their arrival. After being weighed, participants rested in a semi-supine position on a bed. An intravenous cannula was inserted in a retrograde direction into a superficial vein on the dorsal surface of the non-dominant hand for sampling of arterialised-venous blood, as described in Section 5.2.6. The hand remained in an air-warming unit throughout the experiment to maintain arterialisation of the venous drainage of the hand (see Section 5.2.6 for rationale). A second intravenous cannula was placed in the antecubital vein of both forearms for infusion of stable isotope amino acid tracer. Patency of the lines was maintained using non-heparinised 0.9% saline infusions.

Stable isotope-labelled amino acid tracer infusions were made up aseptically on the morning of the experimental trial visit at the DGHPU by two trained members of staff. Three different tracers were used over the course of the study rather than a single amino acid tracer. This was necessary because of batch testing failure of amino acid tracers during quality control testing by our pharmacy provider (Stepping Hill Hospital, Stockport). The tracers employed in

this study were L-[ring-13C6]-phenylalanine, [D8]-L-phenylalanine and [1, 2-13C2]-Leucine. These stable isotope-labelled tracers infusates were produced at Stepping Hill Hospital, Stockport, where they had been prepared aseptically under sterile conditions. L-[ring-13C6]-phenylalanine and [1, 2-13C2]-Leucine were prepared at a concentration of 500mg in 50ml of 0.9% NaCl in sealed bottles ready for use. [D8]-L-phenylalanine was prepared at a concentration of 50mg per 10ml of 0.9% NaCl in sealed vials ready for use. All tracers had been used previously by colleagues at the University of Nottingham in human volunteer studies quantifying acute MPS, and were prepared and infused as per the guidance of Prof. Kenneth Smith, Head of the Stable Isotope Spectrometry Facility, Derby Medical School. Prof. Smith has over 30 years of experience quantifying MPS using stable isotope tracers. The amount of tracer to be infused was first calculated based on the body mass of the participant and the duration of the infusion. This volume of tracer was then dissolved in 250mL of 0.9% saline for infusion, followed by two-staged filtering through a 0.22μm filter in syringes labelled with an expiry time to remove pathogens.

Primed constant tracer infusion method was used which allowed the incorporation of the stable isotope-labelled tracers into the vastus lateralis. Initially, a priming dose (L-[ring-13C6]-phenylalanine, 0.4mg.kg^{-1} body mass; [1, 2-13C2]-Leucine, 0.7mg.kg^{-1} body mass; [D8]-L-phenylalanine, 0.4mg.kg^{-1} body mass) was administered, followed by a constant infusion (L-[ring-13C6]-phenylalanine, 0.6mg.kg^{-1}.h^{-1}; [1, 2-13C2]-Leucine, 1.0mg.kg^{-1}.h^{-1}; [D8]-L-phenylalanine, 0.6 mg.kg^{-1}.h^{-1}). Infusions lasted for 7.5h to allow for measurements of myofibrillar protein fractional synthetic rate (FSR) from

isotope incorporation in muscle in the resting fasted state, the resting fed state, and the post-exercise fed state. The commencement of the infusion served as the -4.5h time-point, the 0h time point being when participants consumed a protein drink to stimulate MPS response to feeding. The infusion, coupled with the sampling of muscle, allowed for the measurement of muscle specific protein synthesis by measuring incorporation of tracer into myofibrillar protein.

At -3h, a basal muscle biopsy sample was taken using the microbiopsy technique from the vastus lateralis muscle (described in General Methods: Section 2.6.2) in the non-dominant leg. Expired gas was then collected at -1.5h for baseline measurements of resting energy expenditure (REE) using a ventilated hood indirect calorimetry system (Quark CPET open-circuit metabolic cart; Cosmed, Quark PFT, Rome, Italy) to determine whole body substrate oxidation rates and respiratory exchange ratio (RER). For this, first the flowmeter was calibrated using a 3L calibration syringe, which was attached to an 18mm turbine flowmeter. During the flowmeter calibration, the syringe piston was pumped for 6 strokes, keeping the flow between -1 and 1 $L.s^{-1}$. Error between 1-4% was accepted. Room air calibration was conducted subsequently. Gas calibration was performed before each test using a gas calibration cylinder. With the participant lying down semi-supine, a clear perspex ventilated canopy hood with a volume of 30 litres was placed over the head and secured around the neck to collect the expired air. The hood was ventilated with room air by a vacuum pump. Flow rate was monitored and regulated by the operator carefully to ensure the fraction of CO2 in the expired air (FECO2) was between 0.5% and 0.6%. A bidirectional turbine flowmeter of 18mm diameter was used to detect the canopy ventilation

rate, which ranged from 0 – 80l/min at an accuracy of 2% (Blond et al. 2011). During testing, REE was measured for 20min. Readings were reported every 10s and the first and last 5min of data acquisition were excluded. The middle 10min were then averaged to calculate whole body fat and oxidation rates and RER. Successfully managing optimal conditions during indirect calorimetry measurements is a challenge, as errors in data acquisition can occur depending on operator expertise and volunteer compliance. Factors that can impact on VO2 and VCO2 data derived from metabolic events include restlessness, hyper- or hypoventilation, among other perturbations. Therefore, it was ensured that participants were physically comfortable during the measurements, and that repeat measurements were made in the same position (Compher et al. 2006). Thermogenic substances including nicotine in tobacco; caffeine and derivatives in coffee, tea, and chocolate; capsaicin in hot chilies, were avoided prior to the measurement (Gibney 2002). Vigorous physical activity was avoided in the hours prior to measurement to avoid higher metabolic rates (Lopes 2003). The canopy was examined regularly for leaks. A steady-state during the testing period was defined as a 5min measurement with a coefficient of variation less than 10%, and during which the average RER changed less than 5% (Compher et al. 2006).

An arterialised venous blood sample was taken at -1.5h (5ml) to measure plasma glucose and serum insulin concentration at baseline. At -0.5h, participants were seated and prepared for exercise on the isokinetic dynamometer as described in General Methods (Section 2.4). Either the Control or DCA drink was then consumed by the participant within 10-15s. Immediately after this, participants

performed a bout of single-leg resistance exercise with their dominant leg (EL) that they had been familiarised to on a prior visit (Figure 5-2A). The exercise comprised 3 sets of 10 maximal isokinetic concentric contractions of the knee extensors (as described in General Methods: Section 2.4.1) performed at an angular velocity of $90^{o}.s^{-1}$, with a 4min rest period between each set. The number of sets and contractions were chosen based on previous exercise protocols that were known to increase MPS in the elderly (Kumar, Selby, et al. 2009), and total work output per visit was calculated as the sum of the work done during the 30 maximal concentric contractions performed during each experimental trial visit. An arterialised venous blood sample was then taken for plasma glucose and serum insulin measurements. Participants then returned to a supine position, and muscle biopsy samples were obtained from both EL and the non-exercised leg (NEL). Following this, at 0h, participants consumed a drink containing 40g of whey protein (Iso:Pro Whey Protein Isolate by MYPROTEINTM) mixed with 164ml of water and 32ml of Juicee squash concentrate. This dose of whey protein has been demonstrated to increase muscle protein synthetic response in elderly males (Yang et al. 2012).

The participant then returned to a rested, semi-supine position. From this point on until +3h, arterialised-venous blood samples (5ml) were obtained at +0.5, +1, +1.5, +2, +2.5 and +3h time points to measure plasma glucose and serum insulin concentrations in the post-supplemented state. Expired gas measurements were taken using indirect calorimetry at +1, +2, and +3h to determine whole body substrate oxidation rates and RER in the post-supplemented state. At +3h, muscle biopsies were obtained from both EL and NEL. After all procedures for

the trial visit were completed at +3h, catheters were removed and volunteers were fed and monitored for 30-90min before leaving the research unit.

5.2.5 Body composition measurements using DEXA (Dual Energy X-Ray Absorptiometry)

Dual energy X-ray absorptiometry (DEXA) scans (Lunar Prodigy DEXA; GE Medical Systems, Bedford, UK) were carried out at the David Greenfield Human Physiology Unit (DGHPU) on the participants' second visit of the study (Figure 5-2A) to determine whole and regional body composition. All participants filled out a questionnaire prior to each scan to assess suitability and wore light clothing free from any metallic items. During each scan, the participant was asked to remain motionless in a supine position with arms placed by their side and hands pronated. All regional analysis was performed using specialised software (enCORE; GE Medical Systems, Chicago, IL, USA).

The DEXA scanner passes two low-dose X-ray beams through the body (40 and 70keV). X-rays pass through regions of the body at different rates as a result of the differences in elemental composition among fat, muscle tissue and bones. Regional determination of fat, lean mass and bone mineral density can then be calculated by linear decomposition of the measured X-ray beam attenuation (Pietrobelli et al. 1996). DEXA offers an accurate and convenient, non-invasive estimation of body composition. Limitations stem from the assumptions that there is a constant fractional composition of lean tissue (water, protein and mineral content), which can vary with hydration states.

Alternative techniques to assess body composition include computed tomography (CT) and MRI, however, they are expensive and the former exposes participants to higher levels of radiation. CT and MRI provide greater image resolution and accuracy, and allow for differentiation between subcutaneous and visceral adipose tissue depots (Jensen et al. 1995). Regardless, reasonable correlations (r = 0.78) between DEXA-derived visceral fat and android fat, as determined using CT, have been obtained (Hill et al. 2007).

5.2.5.1 Thigh muscle volume measurements

Participants were taken to the Sir Peter Mansfield Imaging Centre (SPMIC) on their second visit (Figure 5-2A) for measurements of muscle volume using 3T MRI. Pre-scan preparation, scanning, analysis of the scans and acquisition of data were carried out as described in General Methods (Section 2.5.3).

5.2.5.2 Mid-thigh intramyocellular lipid (IMCL) and extramyocellular lipid (EMCL) measurements

Vastus lateralis IMCL and EMCL measurements at the level of the mid-thigh were made using PRESS proton spectroscopy using 3T MR at the SPMIC on the second visit of the study (Figure 5-2A), in order to obtain an additional measure of leg fat mass. Pre-scan preparation, scanning, analysis of the scans and acquisition of data were carried out as described in General Methods (Section 2.5.4).

5.2.6 Arterialised-venous blood sampling

Blood was drawn for analyses on the experimental trial visits. For this, participants were cannulated intravenously to enable regular arterialised-venous

blood sampling. A sterile 12G cannula was inserted in a retrograde direction into a superficial vein on the dorsum of the hand. For this, ~1ml of 1% lidocaine was injected subcutaneously prior to cannula placement to minimise pain from the procedure. The cannula was subsequently kept patent by a slow running 0.9% saline infusion. To arterialise the blood, the hand was then placed in an air-warming unit set to warm the hand up to 55°C, and this was maintained for the remainder of the visit. Arterialisation by this technique has been demonstrated to produce venous concentrations of the molecules of interest that approximate arterial values, without altering core body temperature (Gallen and Macdonald 1990).

5.2.6.1 Determination of plasma glucose

Arterialised venous plasma glucose concentrations were measured using the Yellow Springs Instrument 2300 Stat Analyser (YSI; YSI Inc, Yellow Springs, Ohio, USA), which utilises steady-state measurement methodology. During the measurement, immobilised glucose oxidase in the membrane catalyses the oxidation of glucose into gluconic acid and hydrogen peroxide (Chua and Tan 1976). The glucose concentration is proportional to the difference between the plateau current generated by the sample and the initial baseline current (YSI STAT 2300 Laboratory manual 5- 4). The YSI 2300 STAT uses an aqueous–based standard, as opposed to plasma or serum-based. Once the test sample is injected into the YSI, reactions take place as follows:

$$D - glucose + oxygen \xrightarrow{glucose\ oxidase} gluconolactone + H_2O_2\ oxidase$$

Oxidation of a constant portion of the H2O2 at the platinum anode by the H2O2 sensitive electrode:

$$H_2O_2 \rightarrow 2H_+ + 2e^- + O_2$$

Completion of the circuit by a silver cathode at which oxygen is reduced to water:

$$4H + O_2 \rightarrow 2H_2O + 4e^-$$

Intra- and inter-batch precision of the YSI is reported to be strong. The coefficient of variation is 1.2% or less for glucose concentrations of 5.2 – 22.1mmol/L and 5.8% or less for glucose concentrations of 1.6-16.2mmol/L respectively. It is easy to operate, allows for quick analyses and only small samples are required (Chua and Tan 1978). Glucose measurements by YSI correlate strongly with Beckman's Haemacue device (r= 0.979) and the Glucose Analyser (r=0.997).

5.2.6.2 Determination of serum insulin

Serum insulin assays were performed by technicians at the High Performance Liquid Chromatography (HPLC) laboratory of the Metabolic Physiology Group, School of Life Sciences, University of Nottingham, from two millilitres of blood that was freshly drawn and was first allowed to clot in a Serum Separator Tube. After being centrifuged at 3.3G at 4°C for 10 minutes, the supernatant was removed, and aliquots containing the serum were stored at -80°C to retain full immuno-reactivity. Multiple (>5) freeze/thaw cycles were avoided. The level of insulin in the serum was determined by the commercially available human

radioimmunoassay (RIA) kit (Kit catalogue number: HI-14K; Merck Millipore, MA, USA) using 125I-labelled human insulin and a human insulin antiserum (YALOW and BERSON 1960). Cross-reactivity with Human Proinsulin of this RIA is less than 0.2%. The limit of sensitivity for this human insulin RIA is 2.715 µU/ml (100 µL sample size). Duplicated start-of-run and end-of-run quality controls were placed before and after the samples to check for assay drift. The average intra-assay coefficient of variation was <6%.

5.2.7 Indirect calorimetry

One of two ways (the other being direct calorimetry) and by far the most practical method of measuring energy expenditure in humans is indirect calorimetry. Indirect calorimetry quantifies resting energy expenditure (REE). The REE refers to energy expended at complete rest in a post-absorptive state. REE and whole-body substrate oxidation rates were estimated using data obtained from a Quark CPET open-circuit metabolic cart (Cosmed, Quark PFT, Rome, Italy), which operated as per the canopy dilution technique. This approach allows measurement of whole-body consumption of oxygen, and the production of carbon dioxide using a calorimeter with built-in on-stream analysers.

For the measurements, a plastic hood is placed over the subject so air drawn through a canopy by a pump can be collected and analysed. A gas analyser determines the volume and the fraction of O2 and CO2 of expired gas, which further allows it to calculate the volume of O2 inspired and CO2 expired. With the canopy hood, flow rate can be measured using a digital turbine flowmeter directly (Blond et al. 2011). The O2 sensor is paramagnetic with a measuring

range of 0 – 30%. The CO2 sensor is an infrared digital sensor with a measuring range between 0 – 10%.

During the oxidation of carbohydrates, fat, and proteins, O2 consumption and CO2 production in the tissues occur in a ratio specific to each substrate, which is known as respiratory quotient (RQ) (Frayn 1983). RQ thus indicates the relative contribution of carbohydrates, fat, and proteins to total energy expenditure. However, to measure the CO2 production and O2 consumption in the tissues requires arterial and venous blood sampling which is invasive and not feasible. Respiratory exchange ratio (RER) is a non-invasive alternative, allowing for the determination of substrate being oxidised, calculated as the ratio between the rate of CO2 released and O2 consumed through the gas exchange. An RER value of 0.707 is indicative of mixed fat oxidation, 1.0 indicates the exclusive utilisation of carbohydrates, and ~0.8 indicates protein oxidation.

Frayn's equation for calculating substrate utilisation includes calculations for urinary nitrogen excretion. However, the equations below assume accumulation and excretion of metabolic end products or intermediates or end products via ketogenesis, lipogenesis, gluconeogenesis or lactate. For this experiment, the non-protein equations of Peronnet and Massicotte (1991) were used, as urine was not collected for nitrogen excretion measurements.

$$Carbohydrate\ Oxidation: 4.585 \times VCO_2 - 3.226 \times VO_2$$

$$Fat\ Oxidation: 1.695 \times VO_2 - 1.701 \times VCO_2$$

5.2.8 Determination of myofibrillar protein fractional synthetic rate (FSR) using tracers

5.2.8.1 Overview and principles

In order to determine amino acid kinetics, a primed constant infusion of trace amounts of stable isotope amino acid, known as “tracer”, was administered into a vein in the antecubital fossa during the trial visits to minimise changes in plasma amino acid profiles. A stable isotope tracer consists of naturally occurring atoms - such as carbon, oxygen, nitrogen and sulphur - that contain extra neutrons. They behave in an identical manner to their original molecules chemically. They can be used safely in humans as they do not undergo decay, i.e. they are non-radioactive. Stable isotope tracers allow us to investigate how various stimuli, such as exercise, nutrition or hormones, alter substrate metabolism in specific tissues. Following infusion, they pass into the arterio-venous (AV) pool and arrive at the tissue of interest. They are transported by amino acid transporters, get calibrated into the intracellular pool, bind and charge their cognate tRNA, and get incorporated into the peptide. The rate of protein synthesis is estimated by measuring the incorporation of the stable isotope tracer into the peptide of a sample of tissue.

5.2.8.2 Extraction of myofibrillar protein fraction and isolation of amino acids

Myofibrillar protein extraction from muscle samples was carried out using the local standard technique (Greenhaff et al. 2008; Wilkes et al. 2009). The muscle was removed (20-25mg) from Eppendorfs that contained aliquots of frozen muscle samples. To this muscle, a homogenization buffer (HB, 10μL.mg^{-1}

tissue) was added, following which it was chopped with scissors. The homogenate was then spun in a Vibrax shaker at 1500rpm for 10min at room temperature. It was then centrifuged for 15min at 11,000g at 4°C. The supernatant (sarcoplasmic fraction) was stored for later analysis of amino acid enrichment at -20°C. The pellet (comprising collagen, myofibrils, and mitochondria) was placed on ice, washed in HB and centrifuged twice at 11,000g. After this, it was homogenised in Mito buffer, and centrifuged at 1000g. The supernatant, containing mitochondria, was stored at -20°C. The pellet, consisting of myofibrils and collagen, was then solubilized using 0.3% NaOH, and centrifuged at 13,000rpm for 10min at 4°C. To collect the collagen, it was subsequently washed using 70% ethanol, and centrifuged at 3200rpm. Further, the supernatant (comprising myofibrils) was again centrifuged at 3000rpm, washed using 70% ethanol, and collected by centrifugation. The myofibrillar extract was then hydrolysed in 0.05 M HCL Dowex 50W-X8-200.L^{-1} (Sigma Aldrich, Pool, UK) at 110°C overnight. The released amino acids were purified using ion exchange chromatography on Dowex H+ resin, and eluted in 2mol of NH4OH. Amino acids were subsequently derivatised as their N-acetyl-N-propyl ester.

5.2.8.3 Determination of muscle protein-bound and intracellular phenylalanine and leucine enrichment

Incorporation of stable isotope-labelled tracers into myofibrillar protein was determined by gas chromatography combustion isotope ratio mass spectrometry (GC–C-IRMS) analysis (Delta-plus XP, Thermo Fisher Scientific, Hemel Hampstead, UK). For separation, a 25m · 0.25mm · 1.0μ-film DB 1701 capillary

column (Agilent Technologies, West Lothian, United Kingdom) was used (Balagopal, Nair, and Stirewalt 1994; Meier-Augenstein 1999). To determine muscle intracellular stable isotope-labelled tracer enrichment, gas chromatography mass spectrometry (GC-MS - Agilent-5977a, California, USA) was used. In order to do this, the sarcoplasmic fraction was precipitated, and the aqueous supernatant underwent purification using Dowex H+ resin and derivatised as its tert-Butyldimethylsilyl (t-BDMS), after which it was transferred to the GC-MS.

5.2.8.4 Calculation of FSR

FSR was calculated by measuring the increase between two chronological biopsies of stable isotope-labelled tracer incorporation/enrichment into myofibrillar protein. For the calculations, the standard equation was used:

$$FSR\ [expressed\ as\ \%/h]\ = (\frac{\Delta E_m}{E_P} \times \frac{1}{t}) \times 100, \text{ where}$$

ΔE_m = change in labelling of muscle protein L-[ring-13C6]-phenylalanine/1, 2 13C2 Leucine/ D8 L-phenylalanine between two chronological muscle biopsy samples

E_p = mean L-[ring-13C6]-phenylalanine/ [1, 2-13C2]-Leucine/ [D8]-L-phenylalanine enrichment in the intramuscular pool

t = the time in hours between biopsies.

In the present study, the rate of tracer incorporation into myofibrillar protein from -3h to 0h in the NEL on the first trial visit was utilised to calculate baseline/fasted FSR. This was assumed to be constant across study visits.

Myofibrillar FSR in response to protein feeding was measured as the rate of tracer incorporation from 0h to +3h in the NEL during each trial visit. Myofibrillar FSR in response to both protein feeding and exercise was measured as the rate of tracer incorporation from 0h to +3h in the EL during each trial visit.

5.2.9 Statistical analysis

All values are presented as mean ± SEM unless otherwise stated. Comparisons between Control and DCA of (i) muscle FSR in response to feeding and feeding and exercise; (ii) whole body carbohydrate oxidation and fat oxidation in expired air (ii) and of plasma glucose and serum insulin curves in response to feeding were performed using a repeated measures two-way ANOVA (time and intervention as independent factors) with Tukey's or Sidak's post hoc test analysis where appropriate. Incremental total area under the curve (iAUC) for plasma glucose and serum insulin was calculated as the positive area above baseline. To identify any statistical difference in iAUC of plasma glucose and serum insulin between Control and DCA a paired sample t-test was used. Correlation between IMCL:EMCL ratio and leg fat mass, IMCL:EMCL ratio and total body fat mass, and DCA-mediated increase in CHOOx rates and DCA-mediated change in myofibrillar FSR were assessed by linear regression analysis. GraphPad Prism 8.4.1 was used for all analyses and in all cases statistical significance was set at $p<0.05$.

5.3 Results

5.3.1 Participant characteristics at baseline

Table 5-1 shows the baseline characteristics of participants, including anthropometric measurements, body and regional fat mass from DEXA measurements, the IMCL:EMCL ratio from MRS measurements, and thigh muscle volume from MRI measurements. Average body fat mass (35.6 ± 1.4%) was well above the stipulated >30% cut-off for inclusion.

Table 5-1: Participant characteristics at baseline. All values expressed as mean ± SEM. BMI = body mass index. DEXA = dual energy X-ray absorptiometry. IMCL = intramyocellular lipid, EMCL = extramyocelullar lipid. Magnetic Resonance Imaging = MRI. Magnetic Resonance Spectroscopy = MRS. Age; height; body mass; BMI; body composition measurements from DEXA including whole body fat mass and fat mass by body region; total lean mass and lean mass by body region; thigh muscle volume measured using MRI; and mid-thigh intramyocellular lipid to extramyocellular lipid (IMCL:EMCL) ratio measured using (MRS) are shown (n=9).

Age (years)	71.4 ± 1.6			
Height (cm)	180.0 ± 2.2			
Body mass (kg)	94.3 ± 4.1			
BMI (kg.m^{-2})	30.2 ± 1.2			
	Body composition measurements from DEXA			
	Body fat mass		Body lean mass	
	(kg)	(%)	(kg)	(%)
	32.1 ±2.5	35.6 ± 1.4	57.3 ± 1.5	62 ± 1.4
	Regional fat mass		Regional lean mass	
	(kg)	(%)	(kg)	(%)
Arms	3.6 ± 0.8	29.5 ± 2.5	19.1 ± 12.2	73.3 ± 0.03
Legs	9.5 ± 1.3	29.2 ± 1.8	18.8 ± 0.9	67.4 ± 0.03
Trunk	19.1 ± 1.1	40.4 ± 1.3	26.9 ± 0.6	58.7 ± 0.01
Android	3.6 ± 0.2	45.9 ± 1.5	4.1 ± 0.1	53.6 ± 0.02
Gynoid	4.8 ± 0.4	35.3 ± 1.3	8.3 ± 0.3	63.9 ± 0.01
	MRS/MRI measurements			
IMCL:EMCL Ratio	1.16 ± 0.14			
Thigh muscle volume (cm^3)	6386.2 ± 353.5			

5.3.2 Plasma glucose and serum insulin measurements

5.3.2.1 Blood glucose response to protein feeding

There were no differences in fasting concentrations of plasma glucose between Control and DCA visits (Control, 4.7 ± 0.2mmol.L^{-1}; DCA, 4.7 ± 0.2mmol.L^{-1}) (Figure 5-3A). There was a significant effect of time on the glycaemic response to protein feeding such that $F(6, 48) = 13.23$; $p<0.001$. Post-protein feeding, arterialised-venous plasma glucose rose to similar peaks on both visits, and at the same time-point, i.e. at 60min (Control, 5.0 ± 0.2mmol.L^{-1}; DCA, 5.0 ± 0.2mmol.L^{-1}). Following this, it reached a concentration of 4.5 ± 0.2mmol.L^{-1} at 180min on the DCA visit and 4.6 ± 0.2mmol.L^{-1} on the Control visit. No differences were noted at any time-point between DCA and Control. There was no difference between DCA and Control visits in incremental area under the curve (iAUC) for plasma glucose over 0-180min post-protein feeding (Figure 5-3B).

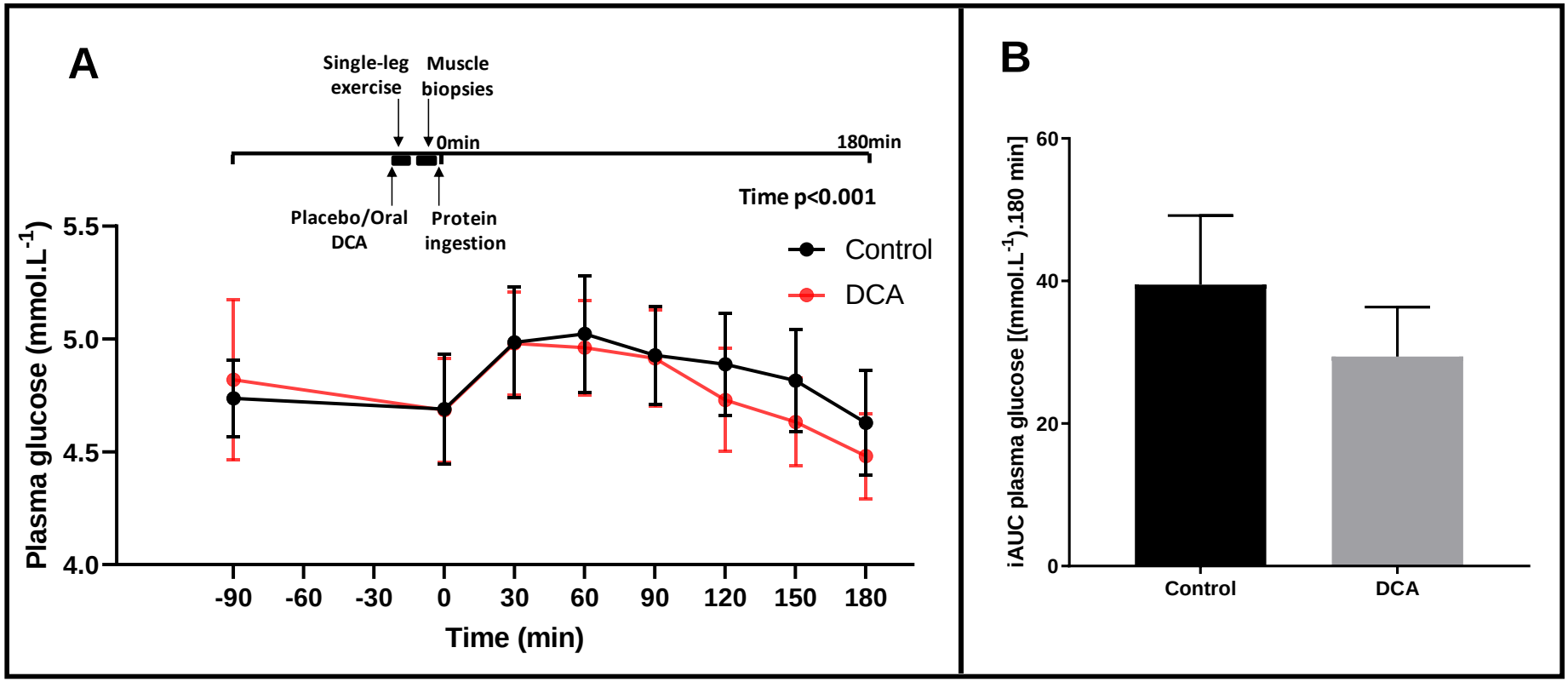

Figure 5-3*: (A) Plasma glucose (mmol.L^{-1}) in response to ingestion of 40g of protein, following the administration of an oral dichloroacetate drink (50mg.kg^{-1} body mass) on one experimental trial visit (DCA), and a placebo control drink on another experimental trial visit (Control). (B) Incremental area under the curve (iAUC) for plasma glucose [(mmol.L^{-1}).180min] over 180min post-protein ingestion on the Control and DCA visits*.

5.3.2.2 Serum insulin response to protein feeding

There were no differences in fasting concentrations of serum insulin between the Control and DCA visits (Control, 11.9 ± 1.5mIU.L^{-1}; DCA, 12.3 ± 1.5mIU.L^{-1}) (Figure 5-4A). There was a significant effect of time on the insulinaemic response to protein feeding such that $F (2.563, 41.01) = 48.82$; $p<0.001$). Following protein feeding, arterialised venous serum insulin concentration rose rapidly on both visits, reaching a peak of 54.1 ± 7.6mIU.L^{-1} at 30min on the Control visit, and 45.7 ± 6.8mIU.L^{-1} at 60min on the DCA visit. Following this, the rate of decline was the same between groups, as were serum insulin concentrations at 180min post-protein ingestion (Control, 9.9 ± 1.0mIU.L^{-1}, DCA, 13.3 ± 2.5mIU.L^{-1}). No differences were noted at any time-point between DCA and Control visits. There was no difference between DCA and Control visits in net incremental area under the curve (iAUC) for serum insulin over 0-180min post-protein feeding (Figure 5-4B).

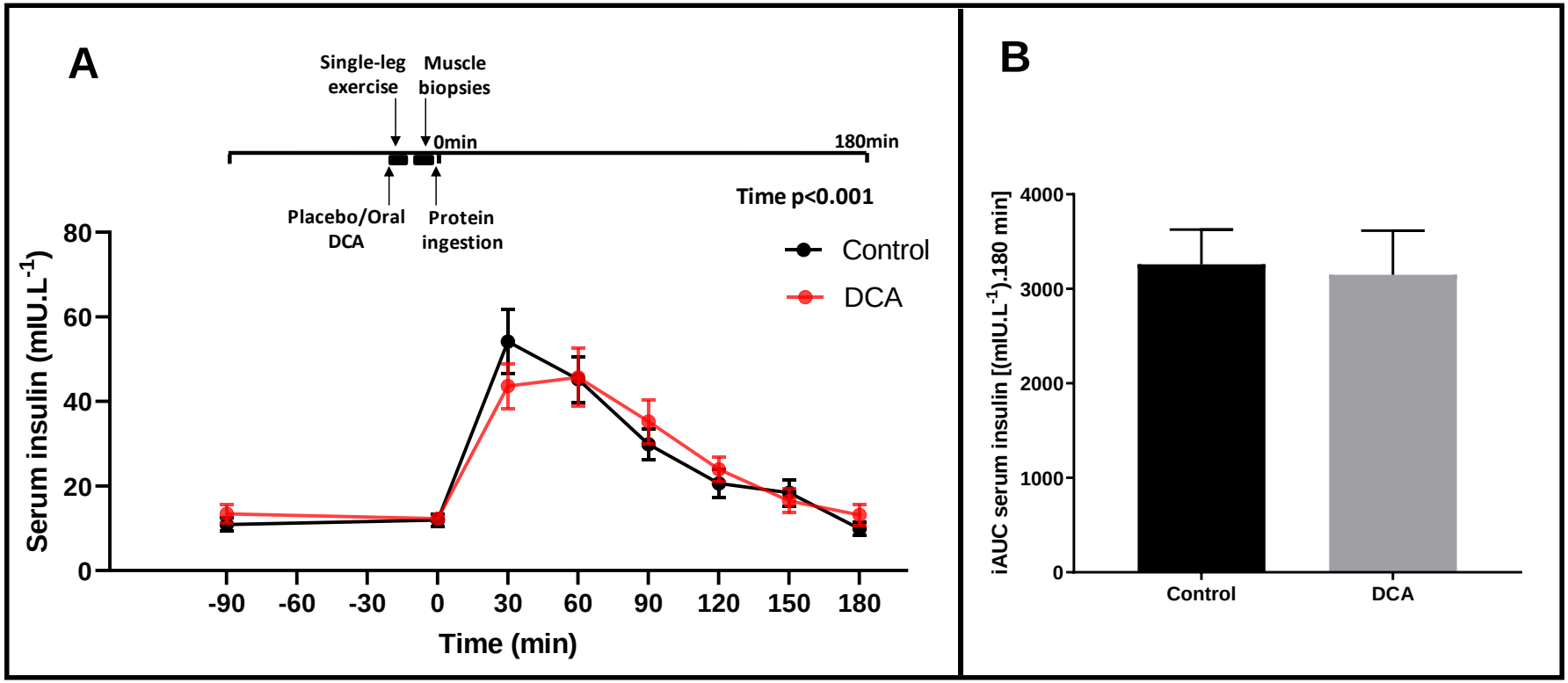

Figure 5-4*: (A) Serum insulin (mmol.L^{-1}) in response to ingestion of 40g of protein following the administration of an oral dichloroacetate drink (50mg.kg^{-1} body mass) on one experimental trial visit (DCA), and a placebo control drink on another experimental trial visit (Control). (B) Incremental area under the curve (iAUC) for serum insulin [(mmol.L^{-1}).180min] over 180min post-protein ingestion on the Control and DCA visits*.

5.3.3 Muscle function during resistance exercise

Total work output per visit was no different between the Control (2.8 ± 0.2kJ) and DCA (2.9 ± 0.1kJ).

5.3.4 Substrate oxidation

There was no difference in whole-body carbohydrate oxidation (CHO_{Ox}) rates in the baseline pre-supplemented state between Control and DCA visits (Figure 5-5). There was no change in rates of CHO_{Ox} following ingestion of Control at any time-point. However, following DCA administration, whole body carbohydrate CHO_{Ox} increased above baseline (0.04 ± 0.01g. min^{-1}) by 3-fold at 1h (0.12 ± 0.03g.min^{-1}, $p<0.05$) and 2h (0.13 ± 0.01g. min^{-1}, $p<0.01$), and ~3.5-fold at 3h (0.14 ± 0.02g. min^{-1}, $p<0.001$) (Figure 5-5). Furthermore, CHO_{Ox} at 3h post-oral DCA ingestion (0.14 ± 0.02g. min^{-1}) was more than double ($p<0.05$) that following ingestion of Control at the same time-point (0.06 ± 0.20g. min^{-1}).

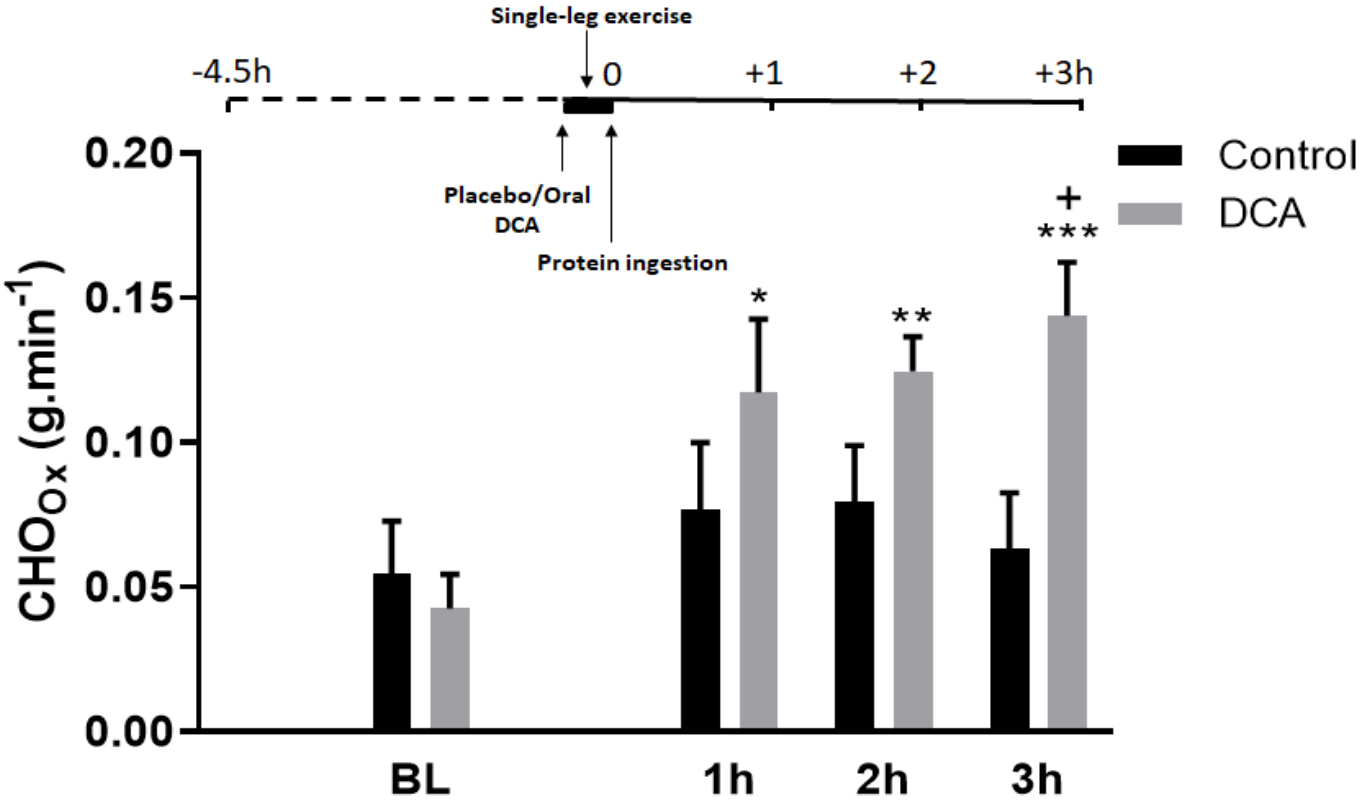

*Figure 5-5: Whole body carbohydrate oxidation rates (CHO_{Ox})($g.min^{-1}$) at baseline (BL), 1 hour (h), 2h and 3h following the administration of an oral dichloroacetate drink (50mg.kg^{-1} body mass) on one experimental trial visit (DCA), and a placebo control drink on another experimental trial visit (Control). Values are expressed as mean ± SEM. *p<0.05 compared to baseline, **p<0.01 compared to baseline, ***p<0.001 compared to baseline, +p<0.05 compared to Control at the same time-point.*

Table 5-2 shows the non-protein respiratory exchange ratio (RER), CHO_{Ox} and whole body fat oxidation rates (Fat_{Ox}) following administration of either Control or DCA. At 3h post-oral DCA administration, Fat_{Ox} was lower than ingestion of Control at the same time point. RER tended to be increased from baseline at all time-points following oral DCA administration (1h, $p<0.05$; 2h, $p<0.05$; 3h, $p<0.01$), and above Control at 3h ($p<0.05$).

*Table 5-2: Rates of carbohydrate oxidation (CHO_{Ox}), fat oxidation (Fat_{Ox}), and non-protein respiratory exchange ratio (RER) at baseline (BL), 1 hour (h), 2h and 3h following the administration of an oral dichloroacetate drink (50mg.kg^{-1} body mass) on one experimental trial visit (DCA), and a placebo control drink on another experimental trial visit (Control). Values are expressed as mean ± SEM. *p<0.05 compared to baseline, **p<0.01 compared to baseline, ***p<0.001 compared to baseline, +p<0.05 compared to Control at the same time-point.*

	BL		1h		2h		3h	
	Control	DCA	Control	DCA	Control	DCA	Control	DCA
CHO_{Ox} (mg.min^{-1})	0.06 ± 0.02	0.04 ± 0.01	0.08 ± 0.02	0.12 ± 0.03*	0.08 ± 0.02	0.13 ± 0.01**	0.06 ± 0.02	0.14 ± 0.02$^{***,+}$
Fat_{Ox} (mg.min^{-1})	0.10 ± 0.01	0.20 ± 0.01	0.12 ± 0.01	0.10 ± 0.01	0.12 ± 0.01	0.11 ± 0.01	0.13 ± 0.01	0.10 ± 0.01^{+}
RER	0.75 ± 0.02	0.74 ± 0.01	0.76 ± 0.02	0.79 ± 0.01*	0.76 ± 0.01	0.79 ± 0.01*	0.75 ± 0.01	0.81 ± 0.02$^{**,+}$

5.3.5 Muscle protein synthesis

5.3.5.1 Myofibrillar protein FSR in response to protein feeding

Myofibrillar protein FSR in NEL was 0.061 ± 0.01%.h^{-1} at baseline (Figure 5-6). Protein feeding had no effect on myofibrillar protein FSR on the Control visit when compared to baseline in NEL (0.085 ± 0.01%.h^{-1} versus 0.061 ± 0.01%.h^{-1}, p=0.47). However, protein feeding following DCA administration increased myofibrillar FSR ~2 fold in NEL (0.120 ± 0.01%.h^{-1}, p<0.05).

5.3.5.2 Myofibrillar protein FSR in response to combined protein feeding and resistance exercise

Protein feeding in combination with resistance exercise increased myofibrillar protein FSR by ~75% above baseline in EL on the Control visit (0.106 ± 0.02%.h^{-1}, p<0.05) (Figure 5-6). The response was similar following DCA administration, with myofibrillar protein FSR increased ~67% from baseline (0.102 ± 0.01%. h^{-1}, p<0.05) in the EL. There was no difference in the magnitude of myofibrillar protein FSR response from baseline when comparing control and DCA visits.

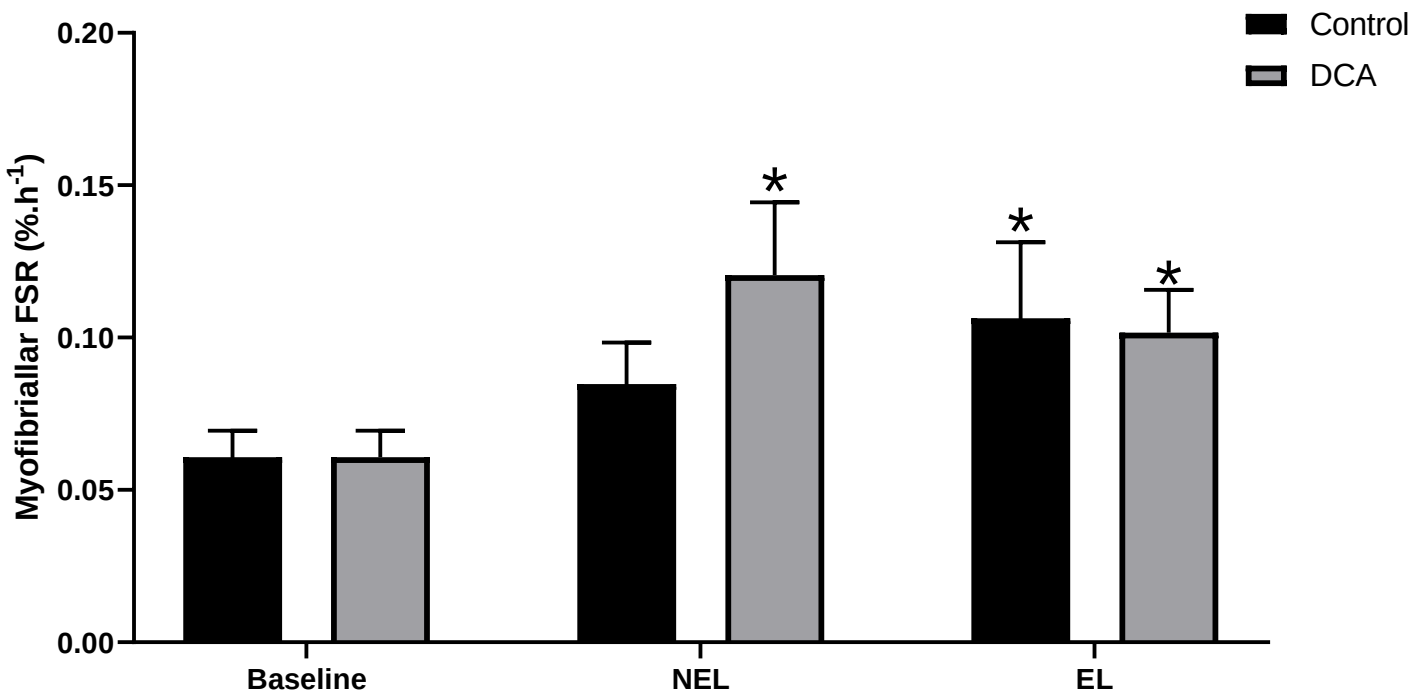

*Figure 5-6: Muscle myofibrillar protein fractional synthetic rate (FSR) (%.h^{-1}) at baseline, and three hours following ingestion of 40g of protein in the non-exercised leg (NEL) and following a bout of unilateral maximal intensity resistance exercise in the exercised leg (EL), in response to either the administration of an oral dichloroacetate drink (50mg.kg^{-1} body mass) on one experimental trial visit (DCA), or a placebo control drink on another experimental trial visit (Control).Baseline was set as the myofibrillar FSR from muscle samples obtained in the fasted state in NEL on the first of the two experimental trial visits. Values expressed as mean ± SEM. *p<0.05 compared to baseline.*

Figure 5-7 shows the correlation between the absolute change in whole body CHO_{Ox} and absolute change in myofibrillar protein FSR 3h following DCA administration in the post-fed state. Following DCA administration, a positive correlation was detected between the absolute change in CHO_{Ox} and absolute change in myofibrillar protein FSR (r^2=0.51; p<0.05).

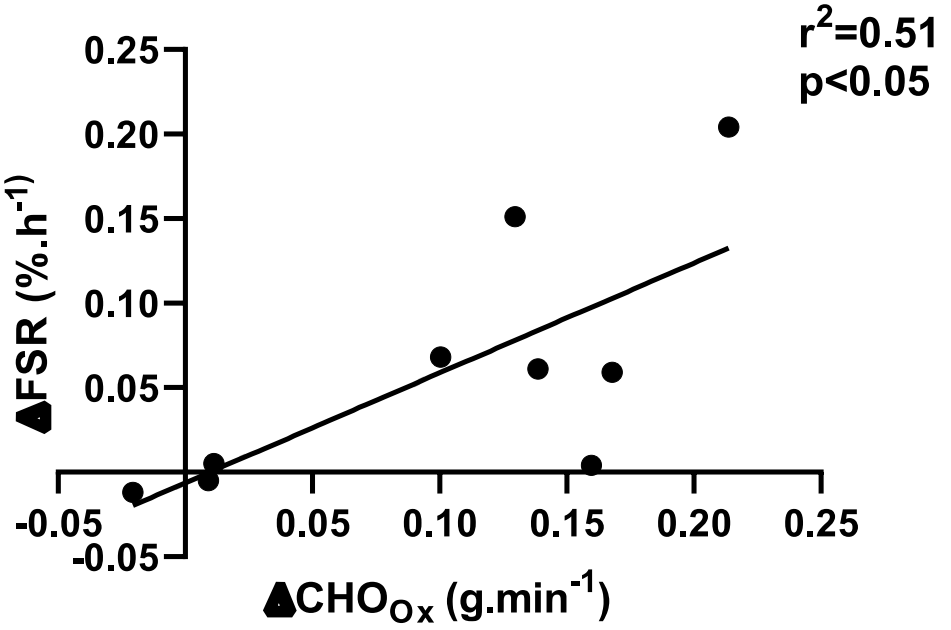

Figure 5-7: The correlation between the absolute change in whole body carbohydrate oxidation rates (ΔCHO$_{Ox}$) and the absolute change in myofibrillar protein fractional synthetic rate (ΔFSR) 3 hours following administration of oral dichloroacetate.

5.4 Discussion

The novel finding of the present study is that pharmacologically increasing carbohydrate oxidation by administration of oral dicholoroacetate in elderly obese volunteers resulted in a ~40% increase in the myofibrillar protein FSR in response to feeding compared to a placebo control intervention. However, oral

administration of dichloroacetate did not increase myofibrillar protein FSR in response to a combination of protein feeding and resistance exercise above that following the placebo control intervention.

In the present investigation, myofibrillar protein FSR in the NEL at 3h post-feeding on the Control visit was no different from the rate measured in the fasting state at baseline (Figure 5-6). This is in line with previous studies that have demonstrated a failure in elderly obese volunteers to increase MPS above baseline in response to protein nutrition (Smeuninx et al. 2017; Murton et al. 2015). The exact mechanism for this blunting of MPS in the elderly obese is unclear, given anabolic signalling is not deranged and muscle inflammation has not been shown to be greater in these volunteers relative to healthy-weight controls (Murton et al. 2015). The novel finding that oral administration of DCA and a consequent increase in carbohydrate oxidation (Figure 5-5), increased MPS in the NEL by ~40% above Control (Figure 5-6) in the elderly obese volunteers in the present study is therefore important. Thus far, this is the first study to address the question of whether the blunted anabolic response to feeding in the elderly obese might be alleviated by using a pharmacological intervention to forcibly increase muscle carbohydrate oxidation in volunteers thought to be metabolically inflexible.

5.4.1 Increase in carbohydrate oxidation by administration of DCA

In healthy weight volunteers, upon insulin stimulation muscle is able to switch from primarily oxidising fat and increasing fatty acid uptake to inhibiting lipid breakdown and increasing glucose uptake, oxidation and storage (Kelley and Mandarino 2000); so called metabolic flexibility. In the obese, however, a

condition of metabolic inflexibility exists such that the inability of muscle to switch from fat to carbohydrate oxidation in insulin-stimulated conditions is impaired (Kelley et al. 1999; Kelley and Mandarino 2000). Furthermore, ageing has been associated with metabolic inflexibility due to greater fat mass (Kuk et al. 2009), and thereby reduced metabolic quality/resilience due to reduced mitochondrial content (Carter, Chen, and Hood 2015).

DCA administration has been shown to shift substrate oxidation from lipid to carbohydrate in animals and humans (Stacpoole 1989). Importantly, it has also been shown to reverse metabolic inflexibility in the form of 3-day high fat-feeding mediated inhibition of carbohydrate oxidation in healthy, young male volunteers (Constantin-Teodosiu et al. 2012). In the present study, it was aimed to increase muscle carbohydrate oxidation maximally and thereby increase the efficiency of ATP resynthesis in the condition of lipid-induced metabolic inflexibility. Increase in both whole body glucose oxidation (Brown and Gore 1996) and skeletal muscle carbohydrate oxidation (Constantin-Teodosiu, Simpson, and Greenhaff 1999) has been demonstrated in human studies at rest in the past following administration of DCA. In the present study, rates of whole body CHO_{Ox} were unchanged following consumption of Control. However CHO_{Ox} increased markedly over time following administration of DCA, rising to nearly double the rate of Control at 3h post-supplementation (Figure 5-5).

5.4.2 Resolution of anabolic resistance to protein feeding by DCA-mediated increase in efficiency of mitochondrial ATP resynthesis

An important finding in the present study was that a positive correlation was detected (r^2=0.51, $p<0.05$) between increase in whole body CHO_{Ox} rates and the

increase in myofibrillar FSR following DCA administration (Figure 5-7). This suggests that the rise in whole body CHO_{Ox} rates observed in the post-fed state on the DCA visit was reflective of muscle level substrate oxidation. This warrants further investigation, in that is necessary to unpack the possible mechanism by which DCA-mediated increase in muscle CHO_{Ox}, and a consequent increase in efficiency of mitochondrial ATP resynthesis might have resolved anabolic resistance in these elderly obese volunteers.

It is known that the high energy demand of protein translation renders MPS a costly process. For every amino acid incorporated in ribosomal protein synthesis, 2 ATP equivalents are required to form aminoacyl tRNA, and 2 more ATP equivalents (1 GTP and 1 ATP) are required for the movement of the growing peptide chain from the A to P site of the ribosome. Hence, there is a cost of at least 4 ATP per amino acid incorporated (Bender 2012). However, because cellular generation versus cellular consumption of ATP needs to be constantly balanced to prevent energy deficits, the ATP pool of the cell is limited (Goron et al. 2019). Indeed, a clear hierarchy has been demonstrated in the responses of the various energy-consuming reactions in the mammalian cell, in that those processes and pathways linked to macromolecule biosynthesis, such as protein synthesis and RNA or DNA synthesis, are most sensitive to energy supply (Buttgereit and Brand 1995). Of further note, it has been suggested, in a commentary by Rennie (2005), that a “shutting off” of MPS occurs during exercise due to the high energy demand of protein translation. It was highlighted that average myofibrillar FSR in young healthy males (n=3) had been shown to be depressed during the course of exercise (Cuthbertson et al. 2002). Further, it

was noted that muscle contractile activity was associated with a fall in ATP/ADP ratio in experiments using perfused rat hind limb (Bylund-Fellenius et al. 1984).

Indeed, regulation of MPS is suggested to be sensitive to cellular energy status such that high cellular ATP/ADP ratios are required for it to be driven (Hardie 2003). In this regard, the role of AMP-activated protein kinase (AMPK) needs to be highlighted. AMPK has been referred to as a cellular energy sensor that responds to alterations in the cellular ATP/ADP and PCR/Cr ratios (Ponticos et al. 1998). These alterations activate AMPK by a variety of mechanisms that involve its allosteric regulation, activation by an upstream AMPK kinase, and reduction in activity of phosphatases (Hardie, Carling, and Carlson 1998). Activation of AMPK has been shown to increase fatty acid oxidation and glucose uptake in muscle (Merrill et al. 1997), and to upregulate several genes involved in muscle metabolism (Zhou et al. 2000; Bergeron et al. 2001). It acts as a regulator and sensor of intermediary metabolic processes by directing cellular events towards optimising energy availability and maintaining energy phosphate levels (Bolster et al. 2002). Hence, it has been suggested that those processes that require ATP while not being crucial to short-term survival are potential regulatory nexuses for modulation by AMPK (Hardie and Hawley 2001) (Figure 5-8).

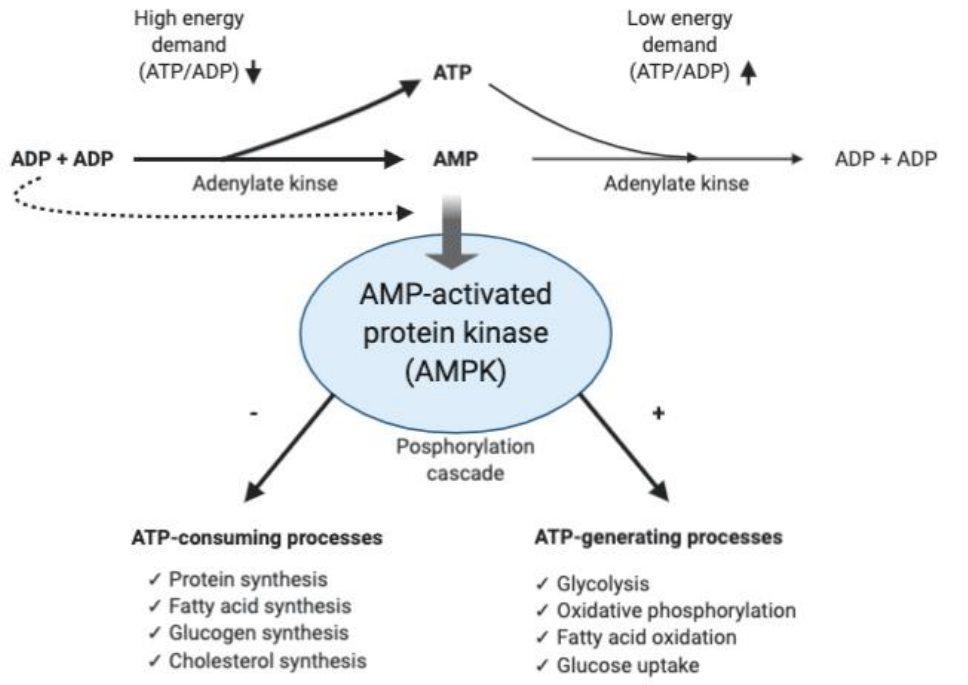

Figure 5-8: Regulation of energy metabolism by AMPK. AMP-activated protein kinase is a regulator and sensor of energy metabolism. In conditions of high ATP expenditure, the cellular ADP/ATP ratio increases, and AMPK is activated by AMP and ADP. AMPK activation stimulates processes leading to ATP production while inhibiting those, like muscle protein synthesis, that consume ATP. ATP, adenosine triphosphate; ADP, adenosine diphosphate; AMP, adenosine monophosphate. Adapted from Jose et al. (2013).

A process of prioritisation is suggested to occur in order to ensure adequate energy availability in conditions of cellular energy stress, such that the anabolic process of MPS may be inhibited by AMPK activation to support more pressing cellular functions (Bolster et al. 2002). Indeed, activation of AMPK has been shown to suppress MPS in skeletal muscle in rodents by directly influencing translation initiation via the mTOR signalling pathway (Bolster et al. 2002). It is therefore not infeasible to question whether or not an optimal increase in MPS

in response to anabolic stimuli is dependent on the type of substrate oxidised towards mitochondrial ATP resynthesis, since fatty acid oxidation consumes ~4 times more oxygen per ATP production than carbohydrate oxidation (Melzer 2011). For example, 6 molecules of oxygen are required for oxidation of a glucose molecule, while 26 molecules of oxygen are required for oxidation of a molecule of stearic acid. By forcibly increasing carbohydrate oxidation in the elderly obese volunteers in the present study, it is possible that the increased efficiency of ATP resynthesis maintained a higher cellular ATP/ADP ratio such that AMPK activation was lower relative to placebo, culminating in an attenuation of AMPK-mediated suppression of MPS in response to feeding. In support of this proposition, a recent study has shown that normalising muscle glycogen content rescued the impaired MPS response to muscle contraction in α2-AMPK-deficient mice (Knudsen et al. 2020).

5.4.3 The potential contribution of intramyocellular lipid accumulation to anabolic resistance

Earlier in this discussion it was noted that the mechanistic drivers of anabolic resistance in obesity are yet to be unravelled. It has been suggested that increased intracellular accumulation of lipids might be a contributor. In the aforementioned study by Murton et al. (2015), a negative correlation between leg fat mass and the degree of stimulation of leg muscle protein synthesis by an intravenous infusion of amino acids was observed in obese and healthy-weight older volunteers (r^2=0.183, $p<0.05$). The authors therefore proposed that resistance to the anabolic action of amino acids within the muscle tissue could perhaps be a direct result of intracellular accumulation of lipid. Novel MR

Spectroscopy findings in the present study support such a contention. A strong positive correlation was detected between mid-thigh IMCL:EMCL ratio of the vastus lateralis, and both whole body fat mass (r^2=0.69, p<0.01; Figure 5-9A) and leg fat mass (r^2=0.89, p<0.001; Figure 5-9B).

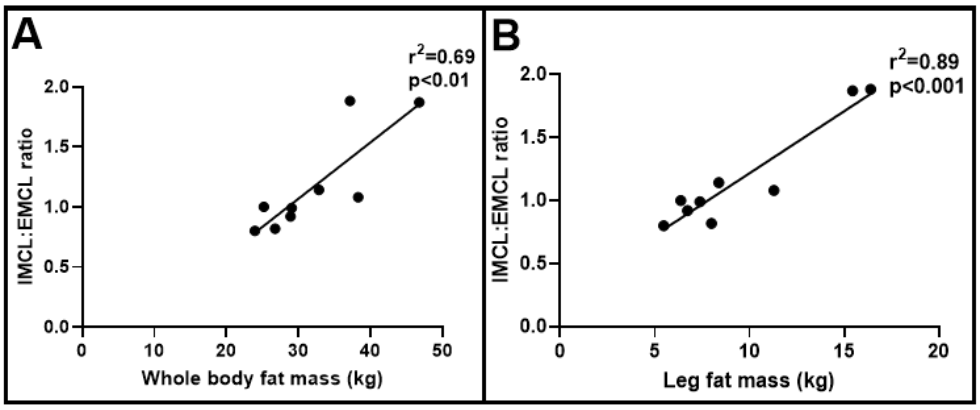

Figure 5-9: (A) The correlation between whole body fat mass from dual energy X-ray absorptiometry (DEXA) measurements and the ratio of intramyocellular to extramyocellular lipid content (IMCL:EMCL ratio) in the vastus lateralis at the level of the mid-thigh from magnetic resonance spectroscopy (MRS) measurements. (B) The correlation between leg fat mass from DEXA measurements and IMCL:EMCL ratio in the vastus lateralis at the level of the mid-thigh from MRS measurements.

When considered in the context of the major finding of the present study that a forcible increase in carbohydrate oxidation by DCA administration increased the muscle protein synthetic response to protein feeding above placebo control intervention in elderly obese individuals who are known to be anabolically resistant relative to healthy-weight controls (Murton et al. 2015; Smeuninx et al. 2017) and in whom intramyocellular content is elevated, the notion that muscle

level metabolic dysregulation in obesity might be driven by intramyocellular lipid accumulation is certainly plausible.

5.4.4 Myofibrillar FSR response to combined protein feeding and resistance exercise in DCA and Control conditions

It is known from recent work by Beals and colleagues (2018) that the synergistic effect of protein nutrition and exercise on MPS is impaired in young obese volunteers relative to lean control volunteers. In the present study in elderly obese volunteers, protein feeding + resistance exercise in response to placebo control intervention did in fact increase post-exercise muscle protein FSR in the EL (~75%) above baseline (Figure 5-6). While administration of DCA also increased post-exercise myofibrillar FSR (~67%) in the EL from baseline, this was no more than the response on the Control visit.

This is an interesting finding, given that DCA did increase the anabolic response to protein feeding alone above Control. It is important to attempt to unpack the reasons for the similar responses to combined protein feeding and exercise between the two interventions. PDC catalyses the irreversible reaction that causes carbohydrates to be committed to their oxidative fate in the mitochondria by converting pyruvate, the product of glycolysis, into mitochondrial acetyl-CoA (Denton, Randle, and Martin 1972). These acetyl groups can either be used by the tricarboxylic acid (TCA) cycle, or, when acetyl-CoA availability exceeds its rate of TCA cycle utilisation, can alternatively undergo buffering towards carnitine (Childress, Sacktor, and Traynor 1967). Such a buffering of acetyl groups in this manner is said to sustain a pool of coenzyme A (CoASH) for continuation of the PDC reaction, thus creating a readily available store of

substrate in the form of acetylcarnitine for utilisation by the TCA cycle (Lysiak et al. 1988). DCA administration has been shown to greatly increase the availability of acetyl groups to the TCA cycle at rest compared to control by stimulating PDC flux and hence acetylating the carnitine pool (Timmons et al. 1996; Timmons et al. 1997; Timmons et al. 1998). During intense exercise as well, acetylcarnitine has been demonstrated to accumulate with time in an almost linear fashion (Harris, Foster, and Hultman 1987; Howlett et al. 1999). Indeed, it has been demonstrated in canine muscle that acetyl group accumulation in mitochondria is similar during exercise in both control and DCA-supplemented states (Roberts et al. 2002). Therefore, a pool of carbohydrate oxidation-derived acetyl groups might have been available to use as a fuel towards post-exercise increase in myofibrillar FSR in both DCA and Control conditions in the present study, resulting in the similar responses observed. A potential limitation of the study therefore, was that muscle acetylcarnitine was not measured, which could have helped confirm whether the ready availability of a pool of acetyl groups was the underlying mechanism for the similarity in these myofibrillar FSR responses on the Control and DCA visits to combined protein feeding and exercise.

5.4.5 Conclusion

In summary, oral administration of dichloroacetate increased anabolic response to feeding in elderly obese volunteers compared to a placebo control intervention by forcibly increasing carbohydrate oxidation and increasing the efficiency of ATP resynthesis. The mechanism proposed is that the DCA-mediated increase in efficiency of mitochondrial ATP resynthesis allowed for the maintenance of

a higher cellular ATP/ADP ratio, such that AMPK activation was less compared to placebo, resulting in an attenuation of AMPK-mediated suppression of MPS in response to protein feeding. Administration of dichloroacetate did not enhance the anabolic response to combined protein feeding and exercise more than placebo control consumption, however. This was perhaps due to a plentiful supply of carbohydrate oxidation-derived acetyl groups being available to fuel increases in MPS in both conditions, resulting in the similar responses observed.

CHAPTER 6. General Discussion

6.1 Introduction

The overall aim of this thesis was to investigate muscle mass and metabolic regulation as a result of resistance exercise, obesity and pharmacological intervention. The experiment in Chapter 3 aimed to provide novel insight on the controversial topic of whether high-load eccentric training induces greater skeletal muscle adaptations than high-load concentric training due to greater work/inflammation during the former. Next, drawing from mRNA expression results in Chapter 3 that revealed significant alterations in gene networks associated with muscle inflammation and injury in the initial stages of resistance training, the experiment in Chapter 4 examined whether chronic NSAID administration, by influencing this early insult, might impact upon resistance training-induced muscular adaptations. Since NSAIDs are used by sportspersons to reduce pain during training, possibly to enhance muscular adaptations by performing more work, this chapter also investigated whether NSAID ingestion increased work output during training. In Chapter 5, the impaired regulation of muscle mass in response to anabolic stimuli in elderly obese individuals was examined using a pharmacological intervention aimed at diminishing anabolic blunting. This study determined whether forcibly increasing carbohydrate oxidation resolved the diminished MPS in elderly obese volunteers by increasing the efficiency of mitochondrial ATP production in a condition of lipid-induced muscle metabolic inflexibility.

6.2 Combined maximal concentric-eccentric resistance training versus maximal concentric training

Interestingly, it was found that greater work output during 12 weeks of unilateral combined maximal concentric-eccentric training did not produce greater gains in muscle CSA, muscle volume or strength than maximal concentric training alone in the contralateral leg over the course of training. Furthermore, changes in mRNA abundance from baseline associated with a number of cellular functions were altered throughout the course of training. Changes in mRNA abundance were most marked in the first week of training in both limbs, but more so during combined concentric-eccentric training, and waned thereafter.

6.2.1 Greater work output during combined concentric eccentric training does not produce greater size and strength adaptations

A similar (~4%) increase in muscle CSA and muscle volume (Figure 4-4) was detected during both combined maximal concentric-eccentric training and maximal concentric training, even though ~16% greater work was performed overall and per week during the former (Figure 4-2). These results are not without precedent. Franchi et al. (2014) detected no differences in gains in muscle volume, muscle thickness or fascicle length of the vastus lateralis over 8-10 weeks of high load (80% of 1-RM) concentric versus eccentric training when matched for workload. Cadore et al. (2014) found that ~40% greater work was performed during 6 weeks of isokinetic eccentric exercise training versus concentric training; yet, no differences were detected in ultrasound-measured muscle thickness of the vastus lateralis. Several other studies have demonstrated no differences in muscle mass adaptations between the two modalities where

high intensity resistance training protocols were used (Smith and Rutherford 1995; Farup et al. 2014; Blazevich et al. 2007).

Why greater force generation during combined maximal concentric and eccentric training did not stimulate greater muscle mass gains requires an explanation, which can be provided by considering training intensity in these experiments. Traditionally, resistance training at a load of ~70-80% of 1-RM is recommended to maximally induce muscle hypertrophy (Medicine 2009). It is not clear as to why such a high intensity of contraction is required to promote MPS. It has been argued, for instance, that given the positive correlation between force generation and electromyographic activity (Medicine 2009), greater muscle fibre recruitment at high intensities stimulates MPS more substantially. Regardless, what has been confirmed by Kumar, Selby, et al. (2009) is that a sigmoidal dose-response relationship exists between the intensity of resistance exercise performed and MPS response such that the latter plateaus at ~60-90% of 1-RM. Given both limbs performed exercise at maximal intensities in these experiments, it is likely that the post-exercise MPS response, regardless of modality, had reached this upper ceiling, culminating in the similar hypertrophic responses observed.

Another explanation concerns the metabolic cost of performing lengthening contractions. Eccentric exercise generates four times as much force as concentric exercise at the same intensity of contraction (Hoppeler 2014b). Early studies on the energy cost of lengthening actions showed EMG activity was halved and oxygen consumption was 6-fold lower during eccentric cycling versus concentric cycling at the same intensity (Bigland-Ritchie and Woods 1976).

These findings have since been replicated in both young and old subjects (LaStayo et al. 2003). Further, eccentric contractions have been shown to elicit fewer electrical signs of muscle fatigue (Molinari et al. 2006), reduced mechanical strain in the form of a reduced ATP hydrolysis rate (Ryschon et al. 1997), and lower lactate and ammonia production (Horstmann et al. 2001). This lesser metabolic cost to muscle during eccentric contractions perhaps has implications for skeletal muscle adaptations, since increases in certain metabolic stressors such as the cellular redox state, the cellular energy charge, muscle lactate and pH are known to be associated with muscle protein synthetic, and hence hypertrophic responses (Egan and Zierath 2013). These are possibly lowered during eccentric training due to the lower metabolic cost of lengthening contractions, and might explain why despite the greater work performed throughout combined maximal concentric-eccentric training in experiments in Chapter 3, greater muscle hypertrophy and strength gains were not induced.

6.2.2 The transitory nature of mRNA response to concentric-eccentric resistance training

One of the major findings in Chapter 3 was that gene networks linked to inflammation and regulation of muscle mass and muscle metabolism were detected by IPA as being significantly altered from baseline in the first week of training in both limbs, albeit to a greater extent during combined concentric-eccentric training (Figure 3-7). This is in line with literature, in that gene transcript profiling studies have demonstrated that an acute bout of eccentric exercise results in a greater inflammatory response and greater muscle damage than concentric exercise (Chen et al. 2003), accompanied by a differential

expression in genes linked to protein synthesis, muscle differentiation, stress response and early growth during a second bout (Kostek et al. 2007). However, the study design in Chapter 3 was novel in that molecular response to eccentric-exercise was examined chronically. It was found that the early response in the aforementioned gene networks had waned in both limbs by 28d of training (Figure 3-7), such that there was no difference between limbs at this point or the end of the protocol. Based on its findings it would be specious to conclude that these initial mRNA responses translate to chronic training-induced muscle hypertrophy. Hence, muscle injury and inflammation as a result of eccentric exercise, and the changes in the molecular processes regulating muscle growth were short-lived, perhaps providing one explanation for why gains in muscle CSA and volume were not greater. However, whether inflammation *per se* affects myogenesis and/or regulation of muscle mass is controversial, given the disparate adaptive responses noted in response to NSAID administration in acute exercise intervention studies in the young (Trappe et al. 2002; Mackey et al. 2016) and chronic training studies in the elderly (Trappe et al. 2011). Hence, one of the aims of Chapter 4 was to examine if NSAID ingestion influenced the early inflammatory response in the young, and muscle mass and skeletal muscle adaptations as a result.

It is unclear as to why in Chapter 3, mRNA response declined from 28 days onward during combined concentric-eccentric training despite greater work being performed throughout the protocol. A bout of eccentric-exercise-induced insult to muscle has been suggested to confer protection from similar insult during a subsequent bout of eccentric exercise for durations of up to 6 months

(McHugh 2003), though the exact mechanisms for this are unclear (Hubal et al. 2008; Hyldahl and Hubal 2014; Willoughby, McFarlin, and Bois 2003). It has been suggested that the stretch-induced muscle micro-damage that is reported as a consequence of eccentric exercise (Friden 1984; Friden, Kjorell, and Thornell 1984) is diminished with subsequent eccentric exercise sessions (Margaritelis et al. 2015). Minimal or no muscle damage has been detected after only 8 repeated sessions of eccentric exercise (Paschalis et al. 2011; Mackey et al. 2011). This phenomenon of reduction in muscle insult with repeated eccentric exercise sessions has been referred to as the "repeated bout effect" (RBE) (McHugh 2003), and is associated with a reduction in muscle soreness and quicker recovery from functional decline. Perhaps the waning in mRNA response in Chapter 3 is a reflection of this protective effect of successive bouts of eccentric exercise.

6.3 Skeletal muscle adaptations to combined chronic NSAID ingestion and resistance training

A novel finding in the experiment in Chapter 4 was that chronic NSAID ingestion augmented resistance-training induced increases in muscle CSA and volume gains above placebo, and that most of this increase occurred predominantly between days 28 and 84 of resistance training Figure 4-4. Interestingly, this paralleled greater changes in gene networks associated with a number of cellular functions linked to regulation of muscle mass and muscle metabolism in the NSAID group (Figure 4-5). Another key finding of this study was that strength gains were no different between NSAID and PLA groups

throughout the protocol. Lastly, NSAID ingestion did not improve training work output throughout the protocol, or overall.

6.3.1 Augmented muscle mass gains with combined chronic NSAID ingestion and resistance training is accompanied by greater muscle mRNA response

In experiments in Chapter 4, increase in muscle CSA and muscle volume in the NSAID group was ~120% greater than in the PLA group (Figure 4-4), which was a novel finding. It was sought to determine whether skeletal muscle mass adaptations might be impacted upon by the influence of NSAIDs on the early inflammatory response to resistance training in young volunteers in Chapter 3, and hence build on the controversial topic of the influence of inflammation on regulation of muscle mass. It was found that muscle inflammatory proteins were no different throughout training between groups, and the differences that were observed from baseline in either group were not likely to have had an impact on muscle volume and CSA adaptation (Table 4-3). Therefore, non-overt inflammation in this scenario is unlikely to have played a significant role. It was observed, however, that in the NSAID group, IPA predicted gene networks linked to skeletal and muscular development and function, organismal injuries and abnormalities and tissue development (Figure 4-5) to be more comprehensively altered at 28d and 84d of training than the PLA group at the same time-points.

6.3.2 Augmented muscle mass gains with combined chronic NSAID ingestion and resistance training is not accompanied by greater function

A major finding was that greater muscle mass gains in the NSAID group were not accompanied by greater increases in strength at any time-point (Section 4.3.2.2). This study is not the only instance in which a pharmacological intervention suggested to improve skeletal muscle adaptations has increased muscle mass while failing to improve muscle function or exercise performance. For instance, only supra-physiological doses of testosterone been shown to increase muscle strength above placebo (Bhasin et al. 1996). Moreover, anabolic steroids were abandoned as muscle size and strength promoting therapies due to myriad adverse drug reactions at the higher doses required to improve performance (Darden 1983).

Other drugs that have been geared towards improving muscle mass and function of recent have also failed at enhancing the latter. One group of such drugs are selective androgen receptor modulators (SARMs), which were promoted in the early 2000s as a new class of function-enhancing anabolic therapies, indicated in conditions associated with profound frailty, such as cancer cachexia, osteoporosis and ageing (Negro-Vilar 1999). However, despite stimulating increases in lean body mass, SARMs were found to produce only small increases in function over placebo (Bhasin and Jasuja 2009). Similarly, monoclonal antibody therapies that act as myostatin inhibitors were promoted as breakthrough drugs for muscle wasting conditions like cachexia, COPD and

sarcopenia based on animal models, yet were found to produce no functional improvements despite increases in lean mass (Becker et al. 2015; Garber 2016).

In keeping with these observations, the findings of the study in Chapter 4 indicate that the widespread use of NSAIDs by individuals engaged in sport at various levels towards improving performance is misguided, especially considering NSAIDs are associated with a number of side-effects (Bhala et al. 2013; Warner et al. 2002; Warner et al. 1999; McGettigan and Henry 2013; McGettigan and Henry 2011; Bjorkman 1998). Indeed, while the volunteers in the experiments in Chapter 4 did not present with any side-effects, diclofenac is now a prescription-only drug in the UK due to its increased risk of cardiovascular side effects (Wise 2013).

6.4 The effect of dichloroacetate on anabolic resistance in the elderly obese

The key finding in Chapter 5 was that administration of oral DCA forcibly increased whole-body carbohydrate oxidation in elderly obese volunteers which was associated with a ~40% greater increase in protein feeding induced MPS compared to placebo. Interestingly, administration of DCA did not increase MPS above placebo in response to a combination of protein feeding and resistance exercise. A novel finding of the present study was the strong correlation between mid-thigh IMCL:EMCL ratio measured using MRS, and both leg fat mass (r^2=0.89, $p<0.001$) and whole body fat mass (r^2=0.69, $p<0.05$) measured using DEXA (Figure 5-9).

6.4.1 Response to protein feeding

Ageing is associated with a decline in muscle mass and strength (Lindle et al. 1997), which in combination with obesity results in adverse physiological outcomes and increased functional disability (Stenholm et al. 2008). Determining why these individuals suffer a greater degree of anabolic resistance compared to lean controls (Murton et al. 2015; Smeuninx et al. 2017) is important, yet the mechanisms underlying this phenomenon are unclear, since it is not associated with greater derangements in anabolic signalling or more profound muscle inflammation than lean controls (Murton et al. 2015). The study in Chapter 5 attempted to examine the potential mechanisms at play using a novel approach. This experiment investigated whether forcibly increasing muscle carbohydrate oxidation to increase the efficiency of mitochondrial ATP resynthesis in elderly obese volunteers might resolve anabolic resistance in a condition of lipid-induced metabolic inflexibility.

Following DCA administration, whole body carbohydrate CHO_{Ox} increased ~3.5-fold above baseline in the post-supplemented state, while not increasing with placebo control intervention (Figure 5-5). In a similar vein, myofibrillar protein FSR in response to feeding was increased ~2-fold with administration of DCA from baseline, while not increasing with placebo control intervention (Figure 5-6). A significant correlation (r^2=0.51, $p<0.05$) was detected between increase in FSR and increase in carbohydrate oxidation following DCA administration (Figure 5-7), which warrants further investigation. A novel suggestion proposed was that via the DCA-mediated increase in efficiency of mitochondrial ATP resynthesis in metabolically inflexible obese individuals, a

higher cellular ATP/ADP ratio was maintained such that AMPK activation was less compared to placebo, resulting in an attenuation of AMPK-mediated inhibition of MPS in response to protein feeding.

6.4.2 Response to combined protein feeding and resistance exercise

Interestingly, the increase in MPS response to combined protein feeding + resistance exercise following DCA administration (~67%) was no different to the placebo control intervention (~75%) (Figure 5-6). This could be because carbohydrate flux and acetyl group accumulation in the mitochondria was similar by the end of resistance exercise in both control and DCA-stimulated states (Roberts et al. 2002), resulting in a pool of readily available carbohydrate derived acetyl groups to fuel MPS post-exercise in both conditions, therefore resulting in a similar anabolic response.

6.5 Strengths

The study in Chapter 3 was the first to examine the time course of change in skeletal muscle mass adaptations to combined concentric-eccentric training versus concentric training. Furthermore, no other study had examined the time course of molecular changes during resistance training *per se*; thus far only the pre- to post- change has been investigated, or simply the response to an acute bout of resistance exercise. This allowed for the novel finding that the increased muscle mRNA response to high intensity resistance training was transitory. Furthermore, combined maximal concentric-eccentric training, as opposed to maximal eccentric training alone was used as the exercise intervention, which

allowed for inflammation to be induced without deterring volunteers from continued participation due to soreness.

The study in Chapter 4 was the first to examine the time course of skeletal muscle adaptations to combined chronic NSAID ingestion and resistance training. Given the widespread use of NSAIDs in both training and competition, it is surprising that this has not been investigated in the young. Only one randomised controlled trial thus far has examined the effect of NSAIDs on adaptations to resistance training in the young, and over a much shorter training period (6 weeks) than the present study (Krentz et al. 2008). Indeed it was both the chronic duration of the protocol in the present study, and the examination of a temporal response to training that allowed for the finding that NSAID ingestion led to enhanced muscle mass gains above placebo predominantly after 4 weeks. Furthermore, the time course of molecular responses to combined NSAID ingestion and resistance training had never been examined. This allowed for the novel finding that increase in muscle mass with NSAID ingestion above placebo was accompanied by an increase in alteration in cellular functions associated with muscle growth.

In Chapters 3 and 4, training was performed on an isokinetic dynamometer, allowing for maximal intensity of exercise, and at a speed at which maximal fibre recruitment is known to be achieved (Tesch et al. 1990). MRI was used to determine muscle CSA and muscle volume, which is one of the most sensitive methods of measurement of these parameters (Kalia et al. 2017). IPA has been employed successfully in our research group to generate novel insight utilising an intervention study design e.g. Porter et al. (2017). For studies in Chapters 3 and 4, 93 transcripts were preselected from large scale gene array studies because

they were deemed to be central to the muscle adaptive response to resistance training (Section 3.2.6). This allowed for both an in-depth understanding and a big-picture perspective of temporal changes in cellular functions and pathways modulated by these gene targets. The results presented in Figure 3-7 and Figure 4-5 clearly show this approach generated significant novel insight on this topic, i.e. several cellular functions associated with regulation of muscle mass and metabolism were altered by both exercise modalities in Chapter 3, and in both treatment groups Chapter 4, and provided a deeper understanding of the muscle mass and functional adaptations observed.

The study in Chapter 5 was the first to test whether anabolic resistance could be increased by forcibly increasing carbohydrate oxidation so as to increase the efficiency of mitochondrial ATP resynthesis. The use of DCA as an experimental tool allowed for a novel approach to examining the mechanisms underlying the diminished metabolic response in the elderly obese. This was also the first study to measure the anabolic response to the combined stimulatory effect of protein feeding and exercise in elderly obese individuals. As part of the inclusion criteria, DEXA scans were used to determine whether a cut-off of 30% total body fat was met to confirm obese status, as opposed to BMI, which is a less robust measure of adiposity. Another strength of the study was the use of MR Spectroscopy, which allowed for non-invasive measurement of intramyocellular lipid content, and the novel finding that whole body mass and leg fat mass were strongly associated with the mid-thigh vastus lateralis IMCL:EMCL ratio (Figure 5-9). This supports the contention that

intramyocellular lipid accumulation is a driver of muscle level metabolic dysregulation (Murton et al. 2015).

6.6 Limitations

A within-subject bilateral study design was used in Chapter 3, which allowed for a minimising of inter-subject variations in response to the two modalities of training (Hubal et al. 2005; Moore, Young, and Phillips 2012), and has been used in many studies comparing the two training modalities (Moore et al. 2005; Cuthbertson et al. 2006; Kostek et al. 2007; Kumar, Selby, et al. 2009; Franchi et al. 2015). One potential limitation of such a design is the possibility of a cross-education effect. However, cross-education effects invariably apply to muscle functional adaptations (Hortobagyi, Lambert, and Hill 1997). Given primary end-point measurements of the present study were muscle hypertrophy and localised muscle mRNA responses to the two different exercise modalities (rather than functional gains), it was foreseen that cross-education would have little to no impact. Indeed, both limbs performed maximal intensity contractions, and the limb undergoing combined concentric-eccentric training performed ~16% more work throughout training when compared with the leg performing maximal concentric training. Further, muscle CSA and volume gains were no different throughout training, and muscle mRNA expression changes from baseline were similar at 28d and 84d of training when comparing limbs. Moreover, not even strength gains were different between limbs.

It might be suggested that frequency of contraction per bout might have resulted in the similar response between limbs in Chapter 3. High intensity resistance training protocols usually feature 10-12 reps per bout to induce gains in muscle

mass and strength. The concentric-eccentric training protocol might therefore be suggested to have been less well-suited to stimulate hypertrophy compared to a lower frequency higher load protocol. However, this is unlikely. While participants did perform maximal intensity exercise, and fatigued during each of the 5 bouts of 30 maximal isokinetic contractions, this resulted in a similar (~4%) increase in muscle CSA in both limbs to studies that have compared hypertrophic responses to 10-12 weeks of maximal concentric exercise training versus maximal eccentric exercise training where lower numbers of maximal, fatiguing contractions were performed (Seger, Arvidsson, and Thorstensson 1998; Farup et al. 2014).

One of the key findings in Chapter 4 was that NSAID ingestion augmented resistance-training induced muscle mass gains above placebo, but did not improve function. It might be argued that the increased muscle mass in the NSAID group was an MRI artefact due to increased water content in muscle, since ingestion of NSAIDs is known to cause water retention due to its actions on prostaglandins (Villa et al. 1997). However, an increase in gene network predictions of activation of formation of muscle, quantity of muscle and connective tissue development that occurred in parallel with the greater increase in muscle mass in the NSAID group was one of the key findings in Chapter 4, indicating that the greater muscle mass observed was underpinned by mechanisms promoting muscle growth. Nevertheless, it would have been useful to have foreseen such a possibility and perhaps adjusted T2 signal intensity during MRI measurements to pick up signs of potential water retention due to NSAID administration.

In Chapter 5, it was proposed that the similarity of the MPS response to combined protein feeding and exercise between the DCA and placebo groups was because of a similar stockpiling of acetyl groups in the mitochondria during resistance exercise, resulting in a readily available pool of carbohydrate derived acetyl groups to drive post-exercise MPS in both conditions. A possible limitation of this study therefore was that muscle acetyl groups were not measured, which could have confirmed this proposition.

6.7 Implications for future research

The novelty of the three experiments also allowed for the identification of new gaps in understanding and directions for future work. It was suggested in Chapter 3 that a threshold for muscle protein synthetic response to resistance exercise was likely reached during both concentric-eccentric and concentric training due to the intensity of exercise performed. It would therefore be useful for future studies to examine the time course of muscle adaptation to high-intensity concentric versus high-intensity eccentric training with progressively increasing workloads to examine whether increasing the relative intensity of training results in greater adaptations during the latter. It would also be useful to measure the time course of molecular responses to determine whether the greater initial muscle mRNA response observed as a result of eccentric exercise is sustained by this progressive increase in training intensity.

Chapter 3 provided a deeper understanding of the mechanisms underlying the responses to concentric versus eccentric training at high intensities. In the future, it would be worth examining the time course of change in adaptations to concentric versus eccentric endurance training. This is performed at low-

moderate intensities, at which it is known that there is scope for increase in the MPS response to training (Kumar, Selby, et al. 2009). Studies have detected a 60% increase in fibre CSA and isometric strength during eccentric training using a bicycle ergometer in frail elderly individuals, but no increase in these measures during a concentric protocol (LaStayo et al. 2003), suggesting that the greater forces generated perhaps do impart a greater stimulus for hypertrophic change at these intensities of training. Importantly, this is a population more susceptible to functional disability and poor health outcomes due to decreased muscle mass and strength (Lindle et al. 1997). Eccentric exercise at low-moderate intensities also causes less soreness and functional decline (Paulsen et al. 2012), and therefore could be extended to conditions associated with muscle wasting and/or cardiorespiratory compromise, such as chronic obstructive pulmonary disease (COPD) and coronary artery disease. Indeed, eccentric exercise using a bicycle ergometer has been found to be tolerable and complied with successfully over 5 weeks in COPD patients (Rocha Vieira et al. 2011) and 8 weeks in cardiology patients (Steiner et al. 2004). Understanding the time course of change in muscle mass and strength in response to eccentric training at these intensities might allow for an optimisation of training protocols in these vulnerable individuals.

Indeed, the findings of the study in Chapter 3 do not take away from the fact that eccentric training at low-moderate to submaximal intensities is still effective in programs of rehabilitation and prevention of orthopaedic injury. Meta-analyses are few, largely due to i) the fact that regimens are specific to the aspect of the locomotor apparatus being rehabilitated, and ii) the dearth of multicentre-trials. However, it is known that the utilisation of low-moderate intensity eccentric

training using a bicycle ergometer is superior to conventional rehabilitation regimens for regaining muscle mass secondary to immobilisation following anterior cruciate ligament (ACL) tears (Gerber et al. 2007), total knee arthroplasty (Bade and Stevens-Lapsley 2012; Marcus et al. 2011) and hip arthroplasty (Di Monaco et al. 2009). Furthermore, submaximal eccentric exercise has been shown to aid in the recovery of strength during the rehabilitation of rotator cuff tears (Bernhardsson, Klintberg, and Wendt 2011), patellar tendinopathy (Bahr et al. 2006) and tibialis posterior tendinopathy (Kulig et al. 2009).

One of the more interesting incidental findings in the study in Chapter 4 was the contrasting predictions by IPA that fat oxidation was generally inhibited during training in the PLA group, while being generally activated in the NSAID group, with a number of cellular functions linked to fat metabolism flagged as being altered with high confidence in both groups. Given such consistent alterations over a 12-week period of resistance training in lipid metabolism, it would be useful for future work to examine how chronic NSAID ingestion alongside resistance training might impact upon measures such as substrate metabolism and body composition. Furthermore, lipid is the major substrate utilised during endurance exercise (Horowitz and Klein 2000). Future research could therefore examine the time course of change in measures of lipid metabolism during combined chronic NSAID ingestion and endurance training.

Given the novel findings vis-à-vis gene network changes in Chapters 3 and 4, these experiments could be followed up by similar studies that examine the time course of change in the products of these genes using protein microarrays. This

would allow for (i) confirmation as to whether the gene expression changes observed in Chapters 3 and 4 translate to protein level changes, and (ii) an examination of the magnitude of effect of these gene changes at the protein level.

The study in Chapter 5 should be repeated with the addition of lean elderly control group and young lean and obese volunteers. Firstly, this would allow for an examination of whether anabolic resistance in the elderly obese extends to the combined effect of protein feeding and exercise. Thus far it is only known that there is a blunted response to the combined effect of these stimuli in obesity in the young (Beals, Skinner, McKenna, Poozhikunnel, Farooqi, van Vliet, Martinez, Ulanov, Li, Paluska, et al. 2018), and it would be novel to examine this in the elderly obese by having a comparator control group. Furthermore, having a lean control group would also allow for an examination of whether anabolic resistance associated with ageing alone (Cuthbertson et al. 2006; Kumar, Selby, et al. 2009) might be ameliorated by DCA. It would also be useful to replicate the present study in young obese volunteers, in whom metabolic inflexibility is also a feature (Kelley et al. 1999; Kelley and Mandarino 2000).

The results of the study in Chapter 5 indicate a possible pharmacological approach towards addressing poor anabolic response to feeding in the elderly obese. It would be worth testing these findings from this acute study chronically in these individuals. Furthermore, such a study should be accompanied by pre- and post-measurements of IMCL content in muscle using MRS, to measure whether any changes in anabolic response and body composition as a result of DCA administration are accompanied by changes in lipid content in muscle. The fact that MPS response to feeding was increased with DCA administration holds

promise for the amelioration of the poor anabolic response in other conditions in which resistance to anabolic stimuli is a feature, including muscle unloading (de Boer et al. 2008; Wall et al. 2016), infection, trauma and inflammation (Puthucheary et al. 2013). It should be noted, however, that chronic administration of DCA is associated with side-effects (Cicmanec et al. 1991; Katz et al. 1981; Stacpoole et al. 1990). In time, newer PDH kinase inhibitors might be developed for safe administration over extended periods.

6.8 Concluding remarks

This thesis examined muscle mass and metabolic regulation as a consequence of exercise, pharmacological intervention and obesity. Resistance training intervention studies were conducted that unravelled novel insights into the impact of lengthening contractions and NSAIDs on skeletal muscle adaptations. Further, these muscle mass and functional findings were examined in the context of changes in gene expression using bioinformatics analysis, advancing our knowledge while posing new questions for future research on the impact of exercise and drug use on muscle mass regulation. Pharmacological intervention was also used to examine impaired muscle mass regulation in the elderly obese linked to poor muscle metabolic quality. This resulted in the unravelling of new possibilities for reduction of morbidity and mortality in these and similarly vulnerable populations, while enhancing our understanding of the mechanisms underlying these metabolic impairments.

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APPENDICES

Appendix A

Health Questionnaire

Participant Contact Details

Today's date: ……

Please record below the participant's contact details

Title: ……

First name: ……..... **Surname**: ……..

Date of Birth:……..

Address:

House number: ……..

Street name: ……

Town/City: ……

Postcode: ……..

Email: ……..

Telephone:

Home: ……..

Work: ……..

Mobile: ……..

Preferred method of contact:

……

Please provide details of someone who can be contacted by the research team in case of an emergency

---

Title: ……

First name: ……..... **Surname** :……

Address:

House number: ……..

Street name: ……

Town/City: ……

Postcode: ……..

Email: ……..

Telephone:

Home: ……..

Work: ……..

Mobile: ……..

Preferred method of contact:

……

GP Details

Doctor/Surgery name: ……

Address: ……

……

Telephone: ……

GENERAL HEALTH QUESTIONAIRE

1.- In general would you say your health is; Please tick one response

a. …… *Much better than a year ago*

b. …… *Somewhat better than a year ago*

c. …… *About the same*

d. …… *Somewhat worse than a year ago*

e. …… *Much worse than a year age*

2. The following questions are concerned with any medical conditions you might have. Please tick one box on each line

Condition	No	Yes	Don't Know
Diabetes			
Epilepsy			
Depression			
Hayfever			
Asthma			
Eczema			
High blood pressure			
Heart problems			
Breathing problems *			
Indigestion / heartburn			
Irritable bowel			
Any other medical condition not listed above (please detail below)			

*** Other than Asthma**

Please detail any other medical condition/s:

……
……
……

If you answered '*yes*' to any of the above, when did you first start having the problem and what medication (if any) do you use?

……
……
……
……
……

3. Do you take any regular medication? Yes…….. No……..

If yes please provide further information: (drug, dose, frequency, duration)

……

……

4. Do you have a tendency to faint in certain situations?

Yes……. No……. Don't Know…….

eg. on standing, in a warm room or after fasting

5. On average, how many cigarettes do you smoke a day? ……..

Did you ever smoke in the past? ……

If so, when did you quit? ……

6. Have you had any previous injuries* which affected any of your lower limbs?

* An injury which limited your walking ability for more than 2 days in the last year.

Yes……. No…….

If yes please provide further information:

……

……

……

……

……

……

7. Do you drink alcohol:

Yes……. No…….

If yes please provide further information: (units per average week)

……

……

DIET AND HEALTH

The following questions relate to your diet and your health

Please tick next to your answer.

1. Are you on a medically prescribed diet? eg diabetic, gluten-free etc

   Yes…….. No…….. Don't Know……..

   If '*yes*', what diet are you following?

   ……

   ……

2. Do you suffer from any food allergies? Eg nuts, wheat, milk

   Yes…….. No…….. Don't Know……..

   If '*yes*', which foods are you allergic to?

   ……

   ……

3. Do you regularly (at least once a week) take **vitamin** and / or **mineral** supplements

   Yes…….. No…….. Don't Know……..

   If '*yes*', which supplements do you take?

   ……

   ……

4. Do you regularly (at least once a week) take any other supplements for your health? Eg. Creatine, Carnitine, Echinacea, St John's Wort, etc

   Yes…….. No…….. Don't Know……..

   If '*yes*', which supplements do you take?

   ……

   ……

Work and leisure activities

This section asks about the type of work you do if you work and followed by the duration and intensity of any work and leisure activities

Physical Activity Record (Please think of a **usual day** during the last 3-months)

Please tell us the type and amount of physical activity involved in your work.

		Please mark one box only
A.	I am not in employment (e.g. retired, retired for health reasons, unemployed, full-time carer etc.)	
B.	I spend most of my time at work sitting (such as in an office)	
C.	I spend most of my time at work standing or walking. However, my work does not require much intense physical effort (e.g. shop assistant, hairdresser, security guard, childminder, etc.)	
D.	My work involves definite physical effort including handling of heavy objects and use of tools (e.g. plumber, electrician, carpenter, cleaner, hospital nurse, gardener, postal delivery workers etc.)	
E.	My work involves vigorous physical activity including handling of very heavy objects (e.g. scaffolder, construction worker, refuse collector, etc.)	

Clinical Physical Activity Questionnaire (CPAQ) (Please think of a **usual week** during the last 3-months)

If you do not work or are not at college, please go straight to Part B (leave Part A blank).

When completing the questionnaire, please consider:

- **MODERATE** activity as any activity that gets you mildly sweaty and out of breath, (e.g. brisk walking, carrying loads, digging, climbing ladders)
- **VIGOROUS** activity as any activity that involves hard physical effort and makes you breathe much harder than normal (e.g. heavy lifting, aerobics, or fast bicycling)

Please tick here if you DO NOT complete ANY physical activity as outlined below in the normal week ☐

Part A: AT WORK/COLLEGE		MODERATE	VIGOROUS
1. How much time do you spend doing physical activity **at work/college** every week? The activity much be done for at least **10** minutes at a time. Please include time spent travelling to/from work or college (if **moderate or vigorous** in nature).	Monday Tuesday Wednesday Thursday Friday Saturday Sunday	Minutes ……. Minutes ……. Minutes ……. Minutes ……. Minutes ……. Minutes ……. Minutes …….	Minutes ……. Minutes ……. Minutes ……. Minutes ……. Minutes ……. Minutes ……. Minutes …….
Part B: AT HOME/LEISURE TIME			
2. How much time do you spend doing **sport/exercise when not at work or college?** The activity much be done for at least 10 minutes at a time. Please include time spent travelling to/from events (if **moderate or vigorous** in nature).	Monday Tuesday Wednesday Thursday Friday Saturday Sunday	Minutes ……. Minutes ……. Minutes ……. Minutes ……. Minutes ……. Minutes ……. Minutes …….	Minutes ……. Minutes ……. Minutes ……. Minutes ……. Minutes ……. Minutes ……. Minutes …….

Have you have been a subject in any other research study in the last three months which involved: taking a drug; being paid a disturbance allowance; having an invasive procedure (eg blood sample >50ml) or being exposed to ionising radiation?

If yes please provide further information:

……
……
……
……
……
……
……
……
……
……

MUSCLE HEALTH QUESTIONNAIRE

Only answer these questions if you are **65 and older**

The following questions relate to muscle health. Please answer each question as accurately as possible. If you would like any question explained or if you would like help, please speak with the researcher.

1. Do you suffer from muscle discomfort such as aches / pains or tiredness?

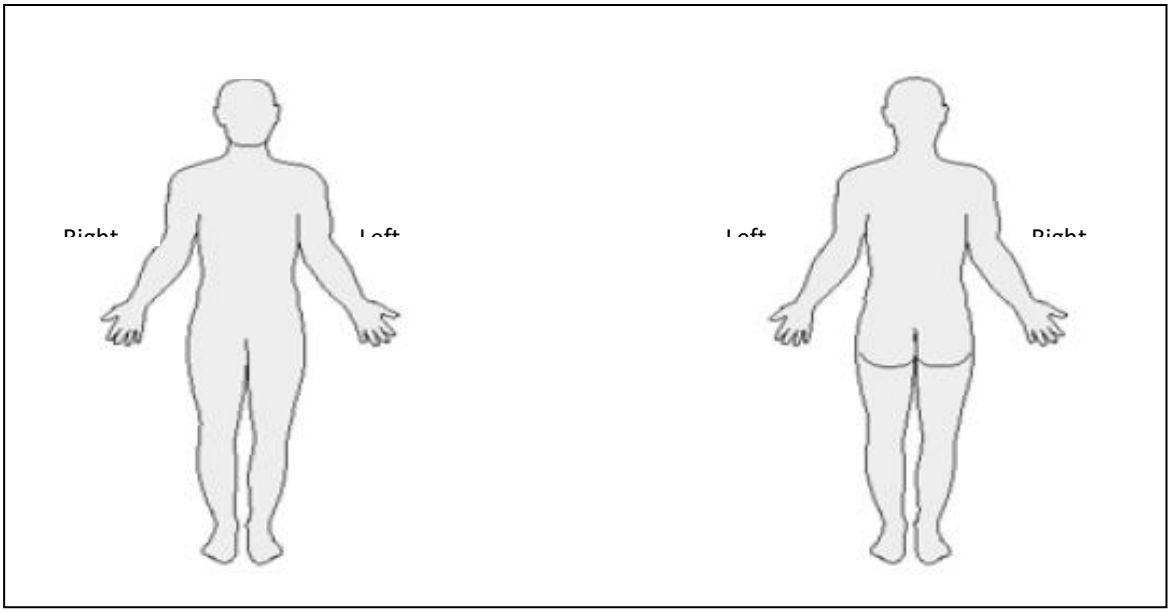
Yes…….. No……..

If no, please go to Page 12.

2. Is the discomfort:

All over the body ……. **OR** In a certain area of the body ……..

If the discomfort is in a certain area of the body, please indicate on the diagram below where it is.



3. Please tick which box best describes your discomfort.

	On most days	At it's worst	At it's best
No discomfort			
Mild			
Distressing			
Horrible			
Excruciating			

4. Look at the words below which can be used to describe muscle discomfort. If any of these describe your muscle discomfort, please indicate its intensity by placing a tick in the appropriate column.

	None	Mild	Moderate	Severe
Weakness				
Loss of strength				
Throbbing				
Shooting				
Stabbing				
Cramping				
Gnawing				
Stiffness				
Tender				
Numb				
Aching				
Tiring/exhausting				
Unbearable				

5. On a scale of 1 to 10, please circle where your discomfort is **usually**.

No discomfort or **1** **2** **3** **4** **5** **6** **7** **8** **9** **10** **Worst possible**

6. How often do you feel this discomfort?

☐ ☐ ☐ ☐ ☐

Rarely **Occasionally** **Often** **Frequently** **All the time**

7. Approximately, how long have you had this discomfort?

☐ ☐ ☐ ☐ ☐

1-2 weeks **1-2 months** **Over 6 months** **1 year** **Over a year**

8. Is your muscle discomfort due to a previous injury/accident? ☐ **Yes** ☐ **No**

……

……

……

9. What kinds of things make the discomfort better (e.g. heat, pain killers, rest)?

……

……

……

……

10. What kinds of things make the discomfort worse (e.g. walking, standing, lifting)?

……

……

……

……

……

11. If you experience muscle discomfort, has it got worse since taking the statin?

☐ **Yes** ☐ **No**

Thank you for completing the questionnaire.

HEIGHT AND WEIGHT

Height		cm
Weight		kg
BMI (researcher can calculate if required)		**kg**/m^2
BP Standing		mm/hg Heart Rate: …….. BPM
BP Sitting		mm/hg Heart Rate: …….. BPM
BP Supine		mm/hg Heart Rate: …….. BPM
Cybex Strength	1)	nm
	2)	nm
	3)	nm
Dominant side:		

Research Associate completing this form:

Signature: . . . . . Designation . . . . . .

Date: | | | . | | | . | 2 | 0 | 1 | |

Appendix B

List of genes used on low-density RT-PCR array microfluidic cards (Applied Biosystems Inc., Foster City, CA, USA).

Symbol	Gene Name
ABLIM2	Actin Binding LIM Protein Family Member 2
ACTC1	Actin, alpha, cardiac muscle 1
ACTN3	Alpha-actinin-3
ACVRC1	Activin A receptor 1
ADORA1	Adenosine A1 receptor
AKT1	Serine-threonine protein kinase 1
ANKRD1	Ankyrin repeat domain 1
ANKRD2	Ankyrin repeat domain 2
ASB5	Ankyrin repeat and SOCS box containing 5
ATF3	Activating transcription factor 3
CALCR	Calcitonin receptor
CD34	Hematopoietic progenitor cell antigen CD34
CDH15	Cadherin 15
CEBPB	CCAAT/enhancer binding protein (C/EBP), beta

Symbol	Gene Name
CLEC3B	C-type lectin domain family 3, member B
CTSL1	Cathepsin L
CYR61	Cysteine-rich angiogenic inducer 61
DNAJB4	DnaJ (Hsp40) homolog, subfamily B, member 4
EFEMP1	EGF containing fibulin-like extracellular matrix protein 1
EIF2B	Eukaryotic translation initiation factor 2B, subunit 2 beta
FABP3	Fatty acid binding protein 3, muscle and heart
FBXO32	F-box protein 32
FIGF	Cellular oncogene c-fos
FOXO1	Forkhead box protein O 1
FOXO3	Forkhead box protein O 3
GADD45a	Growth arrest and DNA damage-inducible protein alpha
GADD45b	Growth arrest and DNA damage-inducible protein
GEM	GTP-binding protein overexpressed in muscle
GSK3b	Glycogen synthase kinase 3 beta
HDAC9	Histone deacetylase

Symbol	Gene Name
HEMk1	HemK methyltransferase family member 1
HGF	Hepatocyte growth factor
HIF1a	Hypoxia inducible factor 1, alpha subunit
HSPA1a	Heat shock protein (70 kDa) 1A
HSPA1b	Heat shock protein (70 kDa) 1B
IFRD1	Interferon-related developmental regulator 1
IGF1	Insulin-like growth factor 1
IGJ	JCHAIN
IL-18	Interleukin 18
IL-6	Interleukin-6
ITGA10	integrin subunit alpha 10
JKAMP	Mitogen-activated protein kinase 8
Jun	c-jun
MAFF	MAF bZIP transcription factor F
MAP3K12	Mitogen-activated protein kinase kinase kinase 12
MDF1	MAD (yeast Mitosis arrest deficient) related
MET	MET proto-oncogene

Symbol	Gene Name
MSTN	Myostatin
MYC	MYC proto-oncogene
MYF5	Myogenic factor 5
MYF6	Myogenic factor 6
MYH1	Myosin heavy chain 1
MYH2	Myosin heavy chain 2
MYH3	Myosin heavy chain 3
MYH4	Myosin heavy chain 4
MYH7	Myosin heavy chain 7
MYOG	Myogenin
NEDD4	Neural precursor cell expressed developmentally down-regulated protein 4
NFKb1	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1
NFKb2	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 2
PAX3	Paired box 3
PAX7	Paired box 7
PDK2	Pyruvate dehydrogenase kinase, isoenzyme 2

Symbol	Gene Name
PDK4	Pyruvate dehydrogenase kinase 4
PDP1	Pyruvate dehyrogenase phosphatase catalytic subunit 1
PFK1	Phosphofructokinase 1
PIK3R1	Phosphoinositide-3-kinase regulatory subunit 1
PPARGC1A	Peroxisome proliferator-activated receptor gamma coactivator 1 alpha
PRKAA1	Protein kinase AMP-activated catalytic subunit alpha 1
PSMA1	Proteasome subunit alpha 1
PTGS1	Cyclooxygenase 1
PTGS2	Prostaglandin-endoperoxide synthase 2
PTK2	Protein tyrosine kinase 2
Rabb15	Member RAS oncogene family
RPS6	Ribosomal protein S6
RPS6KA	Ribosomal protein S6 kinase
RRPTOR	Regulatory associated protein of MTOR, complex 1
RRAD	Ras Related Glycolysis Inhibitor And Calcium Channel Regulator

Symbol	Gene Name
SCD	Stearoyl-Coenzyme A desaturase 1
SIRT1	Sirtuin 1
SREBF2	Sterol Regulatory Element Binding Protein
SRF	Serum Response Factor
TGF1b	Transforming growth factor, beta 1
TNC	Tenascin C
TNF	Tumor necrosis factor alpha
TRIM63	Tripartite motif containing 63
TSC2	Tuberous Sclerosis Complex Subunit 2
Tubb2A	Tubulin beta 2A
Tubb	Tubulin beta
TXNIP	Thioredoxin-interacting protein
VEGFA	Vascular endothelial growth factor A
YWZHA	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase
ZFP36	Zinc finger protein 36, C3H type

Appendix C

Differential expression of targeted gene transcripts in concentric (CON) and combined concentric + eccentric (ECC + CON) trained legs at 24 hours (24h), 7 days (7d), 28 days (28d) and 84 days (84d). Values expressed as fold change relative to mean baseline.

Gene Name	CON								ECC + CON							
	24h	7d	28d	84d	24h	7d	28d	84d	24h	7d	28d	84d	24h	7d	28d	84d
	Fold change from mean baseline				p-value				Fold change from mean baseline				p-value			
ABLIM2	1.93	1.47	1.08	0.78	0.40	0.50	0.27	0.53	4.22	2.77	1.39	1.87	0.18	0.99	0.97	0.73
ACTC1	34.85	4.46	5.63	9.10	0.02	0.03	0.96	0.28	52.85	22.25	4.29	18.01	0.00	0.03	0.08	0.03
ACTN3	2.08	0.93	1.63	1.25	0.28	0.25	0.41	0.63	0.87	1.33	0.08	1.04	0.29	0.24	0.89	0.15
ACVRC1	3.24	3.72	1.91	1.41	0.08	0.71	0.40	0.36	26.70	29.73	1.39	14.17	0.03	0.04	0.35	0.45
ADORA1	2.45	3.20	2.12	2.26	0.07	0.02	0.40	0.44	7.72	7.85	1.87	3.87	0.00	0.03	0.12	0.04
AKT1	1.89	1.55	1.95	0.88	0.27	0.41	0.25	0.63	2.78	3.17	1.93	2.49	0.40	1.00	0.39	0.57
ANKRD1	11.28	12.30	4.75	1.40	0.18	0.50	0.55	0.13	30.78	8.19	1.46	8.09	0.00	0.43	0.24	0.04
ANKRD2	6.31	1.80	1.28	1.41	0.13	0.46	0.59	0.48	8.50	2.10	0.98	1.15	0.01	0.60	0.93	0.64
ASB5	6.66	2.46	1.42	1.71	0.20	0.09	0.76	0.43	4.00	3.00	1.92	1.88	0.13	0.87	0.33	0.88
ATF3	2.86	2.13	1.51	2.04	0.28	0.25	0.73	0.14	8.67	3.41	2.20	2.94	0.10	0.86	0.27	0.52
CALCR	1.22	1.71	1.79	1.56	0.99	0.19	0.38	0.11	1.80	2.42	1.92	6.54	0.91	0.89	0.15	0.28
CD34	1.38	2.01	1.81	1.72	0.53	0.10	0.30	0.06	1.56	2.60	1.78	2.41	0.88	0.92	0.15	0.32
CDH15	1.54	2.53	1.34	1.70	0.97	0.17	0.30	0.09	2.41	2.90	1.45	3.53	0.51	0.53	0.34	0.15
CEBPB	2.10	1.68	1.42	1.75	0.40	0.29	0.95	0.56	2.24	1.99	1.50	1.98	0.75	0.63	0.42	0.73
CLEC3B	1.07	3.58	3.87	1.63	0.96	0.01	0.27	0.15	1.31	5.97	1.78	3.55	0.53	0.45	0.20	0.36
CTSL1	6.02	4.06	1.57	2.04	0.06	0.01	0.94	0.29	3.14	2.99	3.18	2.04	0.72	0.85	0.27	0.96
CYR61	5.56	5.99	3.02	6.17	0.03	0.00	0.06	0.00	7.52	5.31	2.53	5.61	0.07	0.45	0.15	0.11
DNAJB4	2.25	2.85	2.04	1.25	0.44	0.14	0.80	0.62	3.74	3.01	1.71	3.40	0.20	0.86	0.78	0.33
EFEMP1	3.40	8.88	1.46	2.02	0.03	0.00	0.63	0.21	4.83	24.06	1.94	4.33	0.62	0.07	0.36	0.29

Gene Name	CON								ECC + CON							
	24h	7d	28d	84d	24h	7d	28d	84d	24h	7d	28d	84d	24h	7d	28d	84d
	Fold change from mean baseline				p-value				Fold change from mean baseline				p-value			
EIF2B	1.28	1.31	1.17	1.12	0.51	0.93	0.60	0.41	1.80	1.74	1.01	1.39	0.81	0.18	0.65	0.70
FABP3	1.93	1.23	1.68	1.13	0.91	0.51	0.66	0.80	3.48	3.40	1.66	1.97	0.13	0.61	0.77	0.79
FBXO32	2.07	1.95	1.79	2.70	0.26	0.13	0.68	0.26	1.74	3.00	2.61	2.71	0.63	0.96	0.23	0.67
FIGF	1.07	2.39	1.00	1.43	0.37	0.38	0.48	0.26	3.38	5.18	1.66	3.17	0.49	0.28	0.74	0.62
FOXO1	1.57	1.46	1.18	1.60	0.32	0.16	0.40	0.04	1.94	2.72	1.16	2.37	0.89	0.82	0.95	0.87
FOXO3	1.53	1.46	1.33	1.39	0.93	0.87	0.60	0.20	1.75	2.57	1.10	2.19	0.58	0.72	0.18	0.69
GADD45a	2.52	4.59	1.26	1.53	0.13	0.04	0.73	0.24	9.56	8.00	2.35	5.64	0.01	0.40	0.14	0.04
GADD45b	4.36	3.12	2.21	1.62	0.02	0.03	0.36	0.06	8.11	3.98	1.56	3.16	0.39	0.41	0.38	0.62
GEM	1.86	5.68	2.14	5.26	0.26	0.65	0.43	0.07	3.59	6.82	1.09	8.43	0.41	0.73	0.88	0.43
GSK3b	1.25	1.07	1.15	1.44	0.72	0.75	0.96	0.19	1.76	1.27	0.87	1.36	0.67	0.18	0.91	0.96
HDAC9	1.96	1.59	1.58	1.46	0.47	0.14	0.78	0.28	2.65	2.01	1.90	2.27	0.38	0.62	0.25	0.57
HEMk1	1.44	1.72	1.44	1.23	0.34	0.08	0.99	0.45	1.77	2.47	1.56	2.60	0.97	0.95	0.29	0.79
HGF	2.53	19.38	2.92	2.75	0.04	0.03	0.32	0.04	11.87	26.80	3.00	8.61	0.01	0.00	0.29	0.02
HIF1a	1.76	5.68	2.08	1.61	0.05	0.16	0.50	0.06	4.20	7.28	2.13	3.59	0.17	0.08	0.41	0.16
HSPA1a	3.12	2.09	1.76	2.23	0.18	0.10	0.76	0.36	3.82	2.84	1.85	1.36	0.44	0.93	0.45	0.58
HSPA1b	2.16	1.94	1.69	1.33	0.50	0.78	0.80	0.71	4.89	3.38	1.71	1.63	0.09	0.51	0.86	0.75
IFRD1	1.31	1.62	1.12	1.03	0.65	0.19	0.74	0.27	1.41	1.12	0.80	1.02	0.68	0.10	0.93	0.50
IGF1	5.78	2.47	2.18	2.35	0.46	0.49	0.47	0.02	3.00	9.05	2.11	4.51	0.14	0.02	0.38	0.01
IGJ	3.22	16.18	2.24	2.17	0.11	0.03	0.50	0.12	4.54	13.16	3.38	3.82	0.55	0.23	0.17	0.41
IL18	1.36	4.48	1.67	2.38	0.86	0.02	0.98	0.05	7.99	3.75	1.43	6.25	0.01	0.49	0.84	0.51
IL6	11.64	8.06	1.86	2.19	0.04	0.00	0.46	0.03	33.98	25.06	3.39	3.24	0.01	0.06	0.23	0.67
ITGA10	2.11	11.12	1.53	2.26	0.10	0.02	0.68	0.11	2.08	3.68	1.30	8.15	0.99	0.95	0.45	0.42
JKAMP	2.11	1.94	2.04	1.51	0.50	0.93	0.90	0.35	2.80	3.10	1.87	4.82	0.44	0.73	0.80	0.53
Jun	2.21	1.76	2.36	1.84	0.41	0.23	0.54	0.20	1.97	2.18	1.66	2.97	0.84	0.76	0.70	0.91
MAFF	8.53	4.00	2.05	1.83	0.10	0.09	0.83	0.52	3.26	3.92	2.75	3.17	0.36	0.92	0.56	0.90
MAP3K12	5.28	4.77	2.33	2.28	0.06	0.01	0.09	0.01	3.27	7.50	1.83	7.48	0.36	0.09	0.12	0.14

Gene Name	CON								ECC + CON							
	24h	7d	28d	84d	24h	7d	28d	84d	24h	7d	28d	84d	24h	7d	28d	84d
	Fold change from mean baseline				p-value				Fold change from mean baseline				p-value			
MDF1	6.27	5.30	6.65	1.70	0.50	0.75	0.00	0.30	9.56	16.03	3.79	28.33	0.52	0.13	0.23	0.60
MET	1.49	1.83	1.31	1.76	0.45	0.24	0.78	0.24	1.58	2.13	1.62	1.93	0.66	0.74	0.44	0.84
MSTN	0.62	1.04	1.52	2.22	0.17	0.75	0.78	0.34	1.66	2.13	2.01	3.05	0.55	0.69	0.61	0.79
MYC	20.34	5.76	2.32	1.66	0.04	0.01	0.86	0.29	8.09	5.78	1.94	2.95	0.12	0.75	0.33	0.81
MYF5	1.84	3.57	1.48	1.35	0.05	0.04	0.75	0.21	1.99	3.06	1.68	4.10	0.72	0.75	0.20	0.40
MYF6	1.39	2.01	1.80	2.51	0.95	0.52	0.89	0.01	4.12	3.70	2.30	3.61	0.11	0.40	0.32	0.07
MYH1	0.64	1.81	0.35	1.81	0.14	0.24	0.01	0.19	4.37	3.76	0.54	1.72	0.71	0.59	0.13	0.26
MYH2	0.94	1.19	1.51	1.35	0.23	0.52	0.91	0.57	2.09	2.42	1.96	3.02	0.85	0.65	0.58	0.69
MYH3	1.39	1.69	4.56	10.13	0.54	0.04	0.46	0.12	2.37	8.04	2.37	15.28	0.82	0.70	0.17	0.05
MYH4	1.59	1.72	1.81	2.29	0.87	0.43	0.86	0.56	5.48	3.05	3.51	5.73	0.42	0.90	0.17	0.29
MYH7	1.62	1.77	1.76	1.33	0.86	0.49	0.51	0.51	1.62	1.59	1.87	1.15	0.73	0.42	0.25	0.72
MYOG	2.00	3.04	1.42	4.57	0.79	0.13	0.73	0.06	4.46	1.50	2.18	1.49	0.26	0.43	0.38	0.86
NEDD4	0.80	1.05	1.60	1.87	0.30	0.36	0.96	0.29	2.26	1.63	1.27	1.82	0.98	0.38	0.71	0.95
NFKb1	2.84	2.03	1.11	1.57	0.09	0.07	0.46	0.16	1.85	3.12	1.81	2.10	0.92	0.70	0.34	0.99
NFKb2	4.79	3.51	1.98	2.39	0.01	0.17	0.69	0.05	6.53	5.66	1.60	4.65	0.05	0.10	0.84	0.09
PAX3	0.90	1.48	1.38	1.88	0.06	0.25	0.26	0.39	3.28	3.47	3.01	5.96	0.43	0.56	0.62	0.38
PAX7	1.51	2.23	1.38	1.47	0.92	0.14	0.54	0.08	2.23	2.44	1.43	3.99	0.43	0.83	0.34	0.18
PDK2	0.91	1.01	0.98	0.79	0.14	0.69	0.59	0.95	1.11	1.81	0.86	1.39	0.18	0.27	0.67	0.77
PDK4	4.15	2.03	1.61	2.07	0.21	0.36	0.69	0.45	4.19	3.58	2.81	1.96	0.73	0.92	0.33	0.87
PDP1	0.86	1.26	1.44	1.53	0.74	0.59	0.79	0.19	0.49	1.06	1.85	1.95	0.06	0.19	0.30	0.98
PFK1	11.44	3.90	1.78	3.93	0.06	0.01	0.39	0.11	4.12	7.49	2.21	5.59	0.03	0.03	0.30	0.03
PIK3R1	3.56	1.74	1.27	2.14	0.20	0.16	0.91	0.20	2.49	2.47	1.36	2.24	0.84	0.79	0.55	0.94
PPARGC1A	0.95	1.16	1.42	1.06	0.32	0.56	0.48	0.87	1.72	3.28	1.49	2.27	0.43	0.73	0.86	0.91
PRKAA1	2.50	2.27	2.02	2.03	0.16	0.53	0.80	0.11	2.86	4.25	2.04	2.75	0.33	0.29	0.98	0.54
PSMA1	1.70	1.62	1.19	1.53	0.31	0.24	0.63	0.31	1.83	1.85	1.31	1.45	0.96	0.54	0.60	0.94
PTGS1	3.35	3.63	1.79	1.10	0.05	0.00	0.55	0.18	3.54	8.09	1.52	7.12	0.06	0.15	0.34	0.14

Appendices

Gene Name	CON								ECC + CON							
	24h	7d	28d	84d	24h	7d	28d	84d	24h	7d	28d	84d	24h	7d	28d	84d
	Fold change from mean baseline				p-value				Fold change from mean baseline				p-value			
PTGS2	2.10	8.77	1.39	1.57	0.06	0.13	0.55	0.76	9.48	10.76	1.60	1.73	0.01	0.31	0.79	0.76
PTK2	4.80	2.98	1.54	1.75	0.12	0.09	0.98	0.30	2.37	2.25	1.83	1.54	0.81	0.87	0.38	0.82
Rabb15	16.95	5.45	1.99	2.67	0.04	0.00	0.80	0.12	18.88	5.71	2.18	7.34	0.00	0.14	0.23	0.42
RPS6	2.16	1.85	1.84	1.30	0.08	0.34	0.79	0.17	3.13	3.23	1.72	2.34	0.05	0.52	0.43	0.10
RPS6KA	2.29	1.93	1.20	1.01	0.16	0.08	0.94	0.28	1.88	2.16	1.06	2.08	0.96	0.51	0.73	0.82
RRPTOR	2.12	1.67	1.32	1.29	0.37	0.87	0.45	0.21	2.24	2.63	1.19	2.09	0.65	0.77	0.70	0.65
RRAD	4.40	2.91	1.53	2.00	0.25	0.07	0.89	0.69	11.78	4.58	1.72	2.06	0.01	0.08	0.34	0.99
SCD	6.41	6.54	5.49	3.18	0.15	0.35	0.24	0.07	54.13	116.56	1.67	7.00	0.36	0.20	0.57	0.50
SIRT1	1.58	1.41	1.61	1.16	0.76	0.70	0.65	0.42	2.35	2.95	1.71	2.32	0.98	0.85	0.78	0.98
SREBF2	2.11	1.80	1.54	1.21	0.21	0.72	0.84	0.30	3.20	3.40	1.76	3.61	0.12	0.50	0.65	0.22
SRF	2.67	1.53	1.16	1.16	0.07	0.19	0.43	0.27	1.99	2.29	1.40	2.07	0.81	0.84	0.32	0.50
TGF1b	3.48	8.43	1.97	1.75	0.04	0.00	0.36	0.04	2.34	7.92	1.85	5.02	0.58	0.09	0.17	0.09
TNC	51.38	58.53	14.32	2.51	0.00	0.01	0.13	0.15	69.88	150.08	5.72	13.50	0.00	0.02	0.05	0.02
TNF	2.44	4.48	2.84	2.00	0.28	0.10	0.19	0.37	6.12	8.17	3.04	3.99	0.11	0.15	0.30	0.28
TRIM63	2.25	1.81	1.39	2.44	0.70	0.65	0.99	0.17	3.42	1.42	1.62	1.62	0.85	0.38	0.45	0.75
TSC2	2.35	2.47	1.36	1.62	0.23	0.10	0.70	0.34	2.06	2.94	2.02	2.34	0.94	0.97	0.37	0.83
Tubb2A	3.66	3.26	3.42	1.38	0.07	0.04	0.32	0.32	4.65	2.94	1.19	3.17	0.30	0.97	0.82	0.74
Tubb	4.57	4.11	1.91	1.22	0.04	0.03	0.09	0.29	3.96	6.63	1.23	2.86	0.13	0.41	0.97	0.22
TXNIP	1.04	1.40	1.66	1.17	0.90	0.80	0.79	0.60	3.30	4.30	2.55	2.59	0.46	0.62	0.36	0.46
VEGFA	1.22	1.14	1.25	0.74	0.67	0.68	0.70	0.89	1.47	1.91	1.25	1.21	0.34	0.53	0.46	0.54
YWZHA	2.45	2.42	1.70	1.38	0.07	0.12	0.84	0.46	4.40	5.33	1.45	2.74	0.05	0.20	0.62	0.30
ZFP36	7.11	2.83	2.14	1.66	0.12	0.44	0.74	0.27	10.97	4.69	1.61	3.58	0.30	0.31	0.68	0.47

Appendix D

Differential expression of targeted gene transcripts in the group consuming placebo (PLA; n=8) and the group consuming diclofenac (NSAID; n=9) from baseline at 28 days (28d) and 84 days (84d) of training. Values expressed as fold change relative to mean baseline.

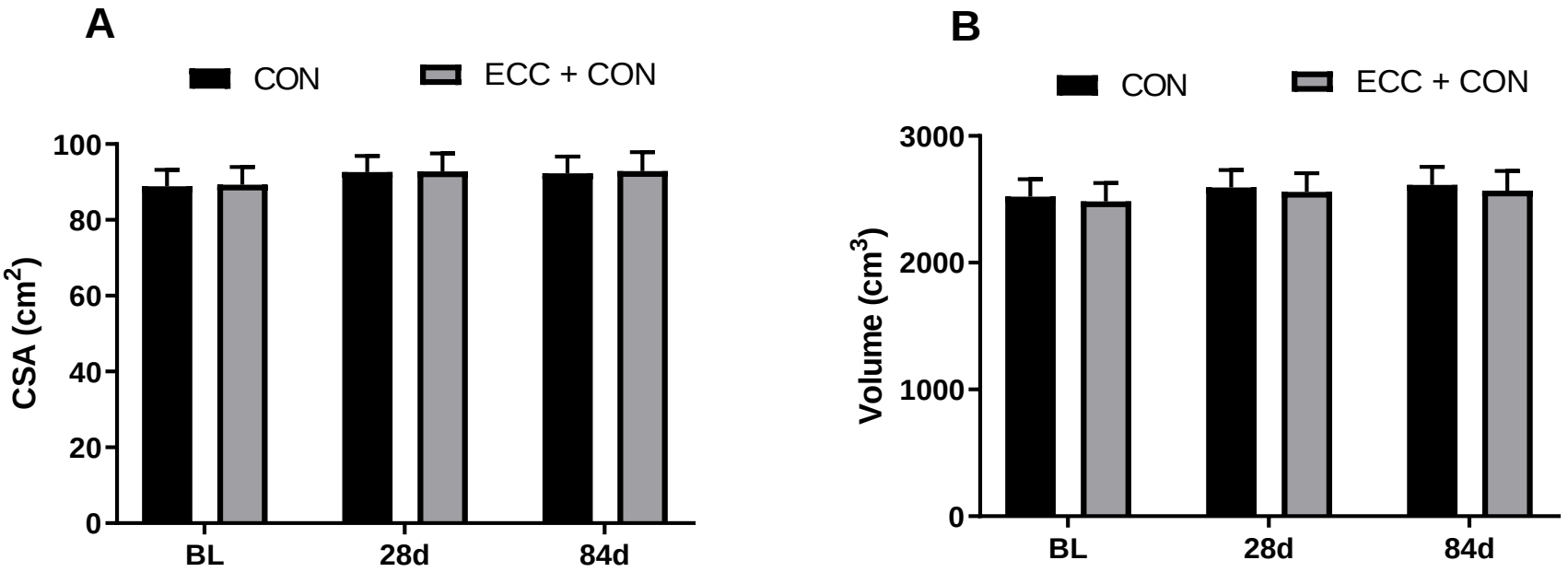
Gene Name	PLA								NSAID							
	24h	7d	28d	84d	24h	7d	28d	84d	24h	7d	28d	84d	24h	7d	28d	84d
	Fold change from mean baseline				p-value				Fold change from mean baseline				p-value			
ABLIM2	1.93	1.47	1.08	0.78	0.40	0.50	0.27	0.53	5.27	2.21	1.62	1.64	0.11	0.33	0.60	0.00
ACTC1	34.85	4.46	5.63	9.10	0.02	0.03	0.96	0.28	39.85	32.06	15.87	8.23	0.12	0.03	0.03	0.84
ACTN3	2.08	0.93	1.63	1.25	0.28	0.25	0.41	0.63	7.28	4.55	1.72	5.28	0.90	0.83	0.49	0.81
ACVRC1	3.24	3.72	1.91	1.41	0.08	0.71	0.40	0.36	9.01	5.67	5.86	5.68	0.03	0.05	0.16	0.18
ADORA1	2.45	3.20	2.12	2.26	0.07	0.02	0.40	0.44	5.38	6.64	3.81	2.95	0.14	0.03	0.19	0.36
AKT1	1.89	1.55	1.95	0.88	0.27	0.41	0.25	0.63	4.41	2.40	3.02	2.45	0.07	0.78	0.01	0.70
ANKRD1	11.28	12.30	4.75	1.40	0.18	0.50	0.55	0.13	11.86	17.29	8.48	10.89	0.05	0.40	0.13	0.36
ANKRD2	6.31	1.80	1.28	1.41	0.13	0.46	0.59	0.48	4.24	2.22	1.77	1.42	0.51	0.56	0.74	0.04
ASB5	6.66	2.46	1.42	1.71	0.20	0.09	0.76	0.43	5.10	1.59	1.66	0.78	0.63	0.66	0.98	0.02
ATF3	2.86	2.13	1.51	2.04	0.28	0.25	0.73	0.14	2.09	2.09	9.31	1.69	0.31	0.67	0.35	0.22
CALCR	1.22	1.71	1.79	1.56	0.99	0.19	0.38	0.11	0.98	2.16	1.77	1.93	0.03	0.94	0.62	0.30
CD34	1.38	2.01	1.81	1.72	0.53	0.10	0.30	0.06	0.86	1.55	1.90	1.42	0.02	0.60	0.68	0.12
CDH15	1.54	2.53	1.34	1.70	0.97	0.17	0.30	0.09	2.32	2.65	1.15	1.95	0.28	0.88	0.20	0.05
CEBPB	2.10	1.68	1.42	1.75	0.40	0.29	0.95	0.56	1.37	1.21	1.10	1.60	0.12	0.37	0.52	0.16
CLEC3B	1.07	3.58	3.87	1.63	0.96	0.01	0.27	0.15	0.73	2.26	5.96	1.86	0.01	0.98	0.20	0.16
CTSL1	6.02	4.06	1.57	2.04	0.06	0.01	0.94	0.29	1.87	1.60	1.36	0.89	0.20	0.71	0.86	0.04
CYR61	5.56	5.99	3.02	6.17	0.03	0.00	0.06	0.00	2.69	2.85	3.31	4.49	0.55	1.00	0.39	0.64
DNAJB4	2.25	2.85	2.04	1.25	0.44	0.14	0.80	0.62	4.34	3.70	2.71	2.91	0.29	0.36	0.02	0.58
EFEMP1	3.40	8.88	1.46	2.02	0.03	0.00	0.63	0.21	2.26	5.92	11.59	2.66	0.43	0.28	0.07	0.52

Gene Name	PLA								NSAID							
	24h	7d	28d	84d	24h	7d	28d	84d	24h	7d	28d	84d	24h	7d	28d	84d
	Fold change from mean baseline				p-value				Fold change from mean baseline				p-value			
EIF2B	1.28	1.31	1.17	1.12	0.51	0.93	0.60	0.41	2.48	2.08	1.58	2.30	0.88	0.21	0.58	0.58
FABP3	1.93	1.23	1.68	1.13	0.91	0.51	0.66	0.80	3.03	2.11	1.72	2.22	0.67	0.18	0.90	0.39
FBXO32	2.07	1.95	1.79	2.70	0.26	0.13	0.68	0.26	1.27	1.13	1.52	0.77	0.10	0.38	0.51	0.04
FIGF	1.07	2.39	1.00	1.43	0.37	0.38	0.48	0.26	1.49	3.35	1.42	2.59	0.05	0.57	0.77	0.70
FOXO1	1.57	1.46	1.18	1.60	0.32	0.16	0.40	0.04	1.39	2.14	1.43	1.68	0.01	0.93	0.94	0.05
FOXO3	1.53	1.46	1.33	1.39	0.93	0.87	0.60	0.20	2.42	2.28	1.58	2.36	0.45	0.28	0.37	0.58
GADD45a	2.52	4.59	1.26	1.53	0.13	0.04	0.73	0.24	25.93	11.73	2.93	12.35	0.68	0.58	0.42	0.95
GADD45b	4.36	3.12	2.21	1.62	0.02	0.03	0.36	0.06	2.81	1.81	2.11	1.51	0.96	0.84	0.63	0.23
GEM	1.86	5.68	2.14	5.26	0.26	0.65	0.43	0.07	3.25	3.17	4.30	4.28	0.79	0.62	0.10	0.23
GSK3b	1.25	1.07	1.15	1.44	0.72	0.75	0.96	0.19	1.04	1.17	0.94	1.04	0.07	0.27	0.44	0.03
HDAC9	1.96	1.59	1.58	1.46	0.47	0.14	0.78	0.28	1.82	1.46	1.33	1.10	0.19	0.44	0.86	0.03
HEMk1	1.44	1.72	1.44	1.23	0.34	0.08	0.99	0.45	1.26	2.08	1.61	1.95	0.06	0.96	0.78	0.37
HGF	2.53	19.38	2.92	2.75	0.04	0.03	0.32	0.04	4.58	5.17	7.26	3.31	0.38	0.02	0.01	0.74
HIF1a	1.76	5.68	2.08	1.61	0.05	0.16	0.50	0.06	3.72	4.08	3.94	2.91	0.57	0.05	0.00	0.55
HSPA1a	3.12	2.09	1.76	2.23	0.18	0.10	0.76	0.36	1.56	1.57	1.44	0.93	0.17	0.69	0.94	0.04
HSPA1b	2.16	1.94	1.69	1.33	0.50	0.78	0.80	0.71	3.51	2.61	1.93	2.53	0.48	0.66	0.71	0.83
IFRD1	1.31	1.62	1.12	1.03	0.65	0.19	0.74	0.27	1.56	1.29	1.89	1.24	0.16	0.11	0.92	0.04
IGF1	5.78	2.47	2.18	2.35	0.46	0.49	0.47	0.02	2.51	3.14	4.41	3.54	0.31	0.37	0.00	0.30
IGJ	3.22	16.18	2.24	2.17	0.11	0.03	0.50	0.12	2.15	3.85	6.68	4.20	0.23	0.41	0.21	0.85
IL18	1.36	4.48	1.67	2.38	0.86	0.02	0.98	0.05	1.76	4.26	1.90	1.83	0.39	0.66	0.89	0.35
IL6	11.64	8.06	1.86	2.19	0.04	0.00	0.46	0.03	15.52	4.65	3.43	3.42	0.03	0.42	0.58	0.74
ITGA10	2.11	11.12	1.53	2.26	0.10	0.02	0.68	0.11	2.24	3.06	3.12	1.95	0.46	0.79	0.34	0.43
JKAMP	2.11	1.94	2.04	1.51	0.50	0.93	0.90	0.35	3.36	2.52	2.03	2.54	0.80	0.67	0.31	0.83
Jun	2.21	1.76	2.36	1.84	0.41	0.23	0.54	0.20	0.82	1.85	0.90	1.68	0.01	0.72	0.40	0.16
MAFF	8.53	4.00	2.05	1.83	0.10	0.09	0.83	0.52	4.96	1.48	2.35	0.99	0.82	0.43	0.95	0.08

Gene Name	PLA								NSAID							
	24h	7d	28d	84d	24h	7d	28d	84d	24h	7d	28d	84d	24h	7d	28d	84d
	Fold change from mean baseline				p-value				Fold change from mean baseline				p-value			
MAP3K12	5.28	4.77	2.33	2.28	0.06	0.01	0.09	0.01	2.23	3.28	4.72	2.76	0.41	0.36	0.04	0.86
MDF1	6.27	5.30	6.65	1.70	0.50	0.75	0.00	0.30	15.73	9.37	15.29	9.08	0.30	0.16	0.00	0.21
MET	1.49	1.83	1.31	1.76	0.45	0.24	0.78	0.24	0.83	1.28	1.25	1.04	0.03	0.44	0.76	0.05
MSTN	0.62	1.04	1.52	2.22	0.17	0.75	0.78	0.34	0.48	1.33	0.74	1.22	0.00	0.18	0.17	0.02
MYC	20.34	5.76	2.32	1.66	0.04	0.01	0.86	0.29	10.92	5.27	2.55	2.50	0.82	0.54	0.65	0.25
MYF5	1.84	3.57	1.48	1.35	0.05	0.04	0.75	0.21	2.36	2.69	1.83	1.63	0.32	0.71	0.71	0.17
MYF6	1.39	2.01	1.80	2.51	0.95	0.52	0.89	0.01	4.98	3.70	1.58	3.26	0.63	0.62	0.75	0.51
MYH1	0.64	1.81	0.35	1.81	0.14	0.24	0.01	0.19	4.43	5.10	4.35	5.75	0.94	0.75	0.97	0.84
MYH2	0.94	1.19	1.51	1.35	0.23	0.52	0.91	0.57	2.11	2.14	1.41	2.13	0.33	0.16	0.54	0.27
MYH3	1.39	1.69	4.56	10.13	0.54	0.04	0.46	0.12	3.13	3.32	5.62	3.83	0.88	0.03	0.05	0.99
MYH4	1.59	1.72	1.81	2.29	0.87	0.43	0.86	0.56	2.56	2.85	2.68	4.06	0.73	0.90	0.44	0.82
MYH7	1.62	1.77	1.76	1.33	0.86	0.49	0.51	0.51	0.75	0.99	1.02	0.89	0.01	0.17	0.26	0.02
MYOG	2.00	3.04	1.42	4.57	0.79	0.13	0.73	0.06	1.94	2.44	1.24	1.69	0.44	0.74	0.50	0.13
NEDD4	0.80	1.05	1.60	1.87	0.30	0.36	0.96	0.29	1.92	1.77	0.93	1.64	0.65	0.25	0.18	0.29
NFKb1	2.84	2.03	1.11	1.57	0.09	0.07	0.46	0.16	1.85	1.95	1.61	1.23	0.22	0.85	0.96	0.08
NFKb2	4.79	3.51	1.98	2.39	0.01	0.17	0.69	0.05	5.43	4.42	2.89	2.33	0.14	0.19	0.10	0.44
PAX3	0.90	1.48	1.38	1.88	0.06	0.25	0.26	0.39	2.71	4.24	1.54	2.68	0.73	0.26	0.86	0.32
PAX7	1.51	2.23	1.38	1.47	0.92	0.14	0.54	0.08	1.38	2.67	1.87	2.19	0.01	0.66	0.41	0.31
PDK2	0.91	1.01	0.98	0.79	0.14	0.69	0.59	0.95	1.43	1.47	1.21	1.49	0.10	0.08	0.26	0.08
PDK4	4.15	2.03	1.61	2.07	0.21	0.36	0.69	0.45	6.13	1.34	2.30	0.91	0.49	0.23	0.72	0.08
PDP1	0.86	1.26	1.44	1.53	0.74	0.59	0.79	0.19	0.49	1.02	2.17	0.82	0.01	0.17	0.96	0.05
PFK1	11.44	3.90	1.78	3.93	0.06	0.01	0.39	0.11	3.28	3.79	4.09	2.94	0.70	0.13	0.06	0.21
PIK3R1	3.56	1.74	1.27	2.14	0.20	0.16	0.91	0.20	0.90	1.54	1.51	0.97	0.04	0.73	0.98	0.08
PPARGC1A	0.95	1.16	1.42	1.06	0.32	0.56	0.48	0.87	1.73	2.65	1.67	2.18	0.05	0.81	0.69	0.47
PRKAA1	2.50	2.27	2.02	2.03	0.16	0.53	0.80	0.11	2.27	3.16	2.31	2.70	0.19	0.58	0.17	0.96

Gene Name	PLA								NSAID							
	24h	7d	28d	84d	24h	7d	28d	84d	24h	7d	28d	84d	24h	7d	28d	84d
	Fold change from mean baseline				p-value				Fold change from mean baseline				p-value			
PSMA1	1.70	1.62	1.19	1.53	0.31	0.24	0.63	0.31	1.39	1.43	1.40	1.10	0.06	0.53	0.88	0.02
PTGS1	3.35	3.63	1.79	1.10	0.05	0.00	0.55	0.18	1.94	3.23	3.92	2.86	0.13	0.19	0.05	0.64
PTGS2	2.10	8.77	1.39	1.57	0.06	0.13	0.55	0.76	8.39	5.42	6.52	8.01	0.32	0.13	0.10	0.07
PTK2	4.80	2.98	1.54	1.75	0.12	0.09	0.98	0.30	1.44	1.44	2.02	1.12	0.16	0.57	0.81	0.08
Rabb15	16.95	5.45	1.99	2.67	0.04	0.00	0.80	0.12	20.05	4.07	5.76	2.79	0.14	0.35	0.19	0.78
RPS6	2.16	1.85	1.84	1.30	0.08	0.34	0.79	0.17	3.61	2.05	1.87	1.90	0.92	0.41	0.40	0.13
RPS6KA	2.29	1.93	1.20	1.01	0.16	0.08	0.94	0.28	2.00	1.72	1.53	1.31	0.49	0.51	0.88	0.01
RRPTOR	2.12	1.67	1.32	1.29	0.37	0.87	0.45	0.21	2.09	2.40	1.62	2.11	0.09	0.46	0.58	0.25
RRAD	4.40	2.91	1.53	2.00	0.25	0.07	0.89	0.69	5.86	4.55	1.79	2.06	0.17	0.78	0.97	0.89
SCD	6.41	6.54	5.49	3.18	0.15	0.35	0.24	0.07	9.46	5.03	12.12	13.12	0.73	0.16	0.12	0.02
SIRT1	1.58	1.41	1.61	1.16	0.76	0.70	0.65	0.42	3.01	2.18	1.69	2.35	0.61	0.12	0.74	0.61
SREBF2	2.11	1.80	1.54	1.21	0.21	0.72	0.84	0.30	2.93	2.56	2.29	2.35	0.75	0.87	0.12	0.84
SRF	2.67	1.53	1.16	1.16	0.07	0.19	0.43	0.27	1.51	1.42	1.13	1.15	0.05	0.27	0.56	0.01
TGF1b	3.48	8.43	1.97	1.75	0.04	0.00	0.36	0.04	1.74	3.86	3.57	2.24	0.17	0.30	0.15	0.56
TNC	51.38	58.53	14.32	2.51	0.00	0.01	0.13	0.15	23.82	6.81	36.50	4.42	0.01	0.01	0.00	0.39
TNF	2.44	4.48	2.84	2.00	0.28	0.10	0.19	0.37	10.17	8.45	8.01	4.33	0.01	0.04	0.00	0.09
TRIM63	2.25	1.81	1.39	2.44	0.70	0.65	0.99	0.17	2.20	0.69	0.59	0.51	0.39	0.10	0.19	0.01
TSC2	2.35	2.47	1.36	1.62	0.23	0.10	0.70	0.34	0.96	1.43	1.71	1.14	0.05	0.53	0.88	0.08
Tubb2A	3.66	3.26	3.42	1.38	0.07	0.04	0.32	0.32	2.53	2.40	1.89	2.68	0.37	0.92	0.50	0.80
Tubb	4.57	4.11	1.91	1.22	0.04	0.03	0.09	0.29	4.79	4.43	6.39	3.63	0.04	0.11	0.01	0.38
TXNIP	1.04	1.40	1.66	1.17	0.90	0.80	0.79	0.60	2.50	2.60	1.34	2.93	0.38	0.52	0.45	0.69
VEGFA	1.22	1.14	1.25	0.74	0.67	0.68	0.70	0.89	1.15	1.24	1.46	1.26	0.03	0.19	0.94	0.06
YWZHA	2.45	2.42	1.70	1.38	0.07	0.12	0.84	0.46	7.28	4.25	3.37	3.38	0.03	0.09	0.00	0.33
ZFP36	7.11	2.83	2.14	1.66	0.12	0.44	0.74	0.27	6.03	2.86	1.99	2.37	0.48	0.85	0.72	0.47

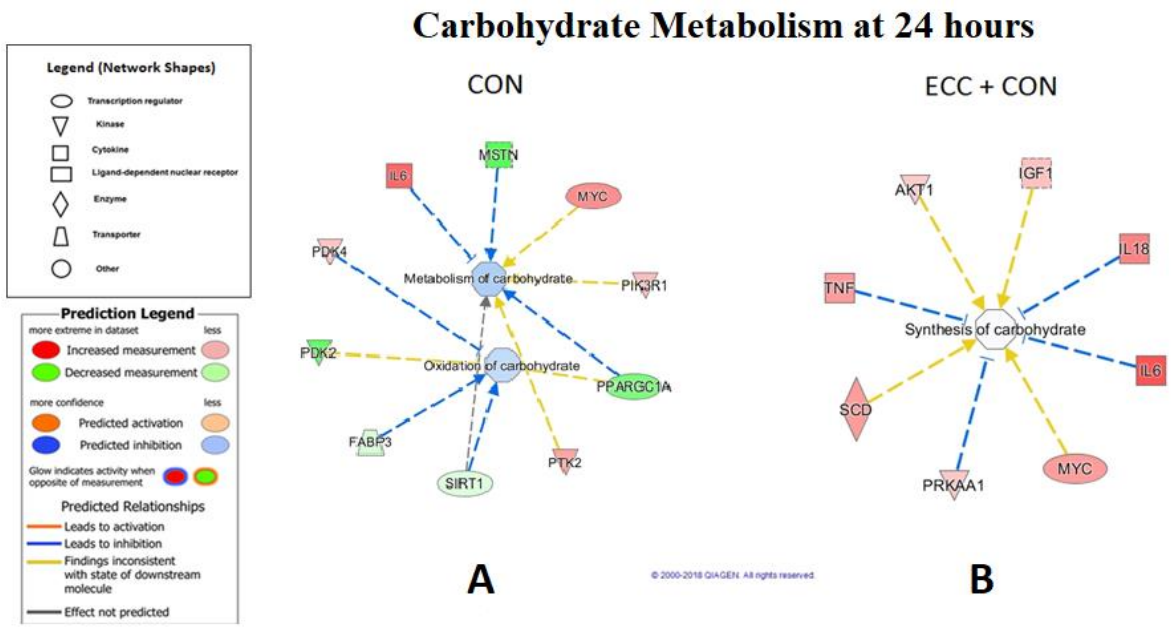
Appendix E



(A) Mid-thigh quadriceps muscle cross-sectional area (cm^2), and (B) quadriceps muscle volume (cm^3) in concentric (CON) and combined concentric + eccentric (ECC + CON) trained legs at baseline (BL), 28 days (28d) and 84 days (84d) of training. Values expressed as mean ± SEM.

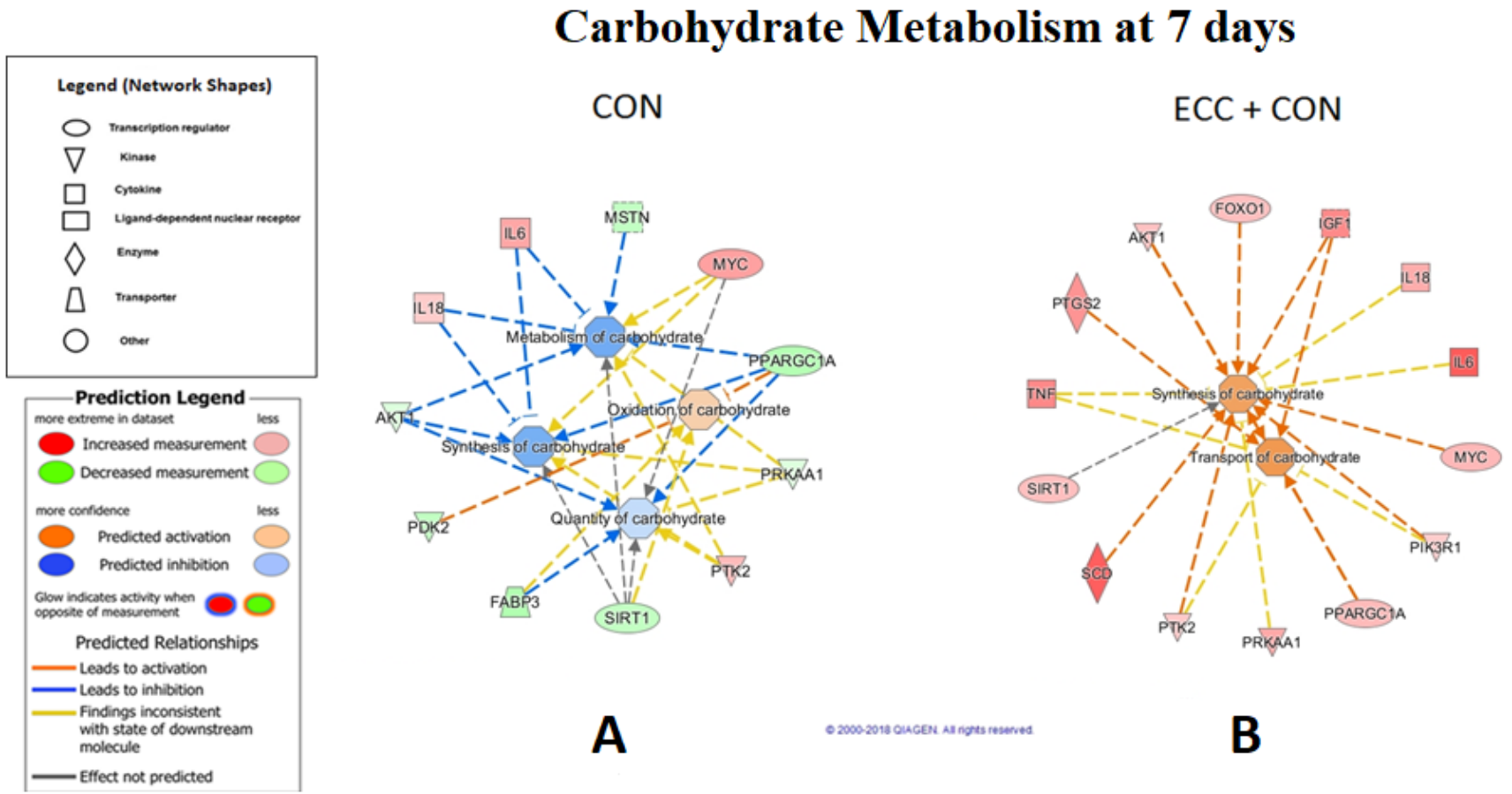
Appendix F

Ingenuity Pathway Analysis predictions for altered cellular functions in experiments in Chapter 3 not included in main text.



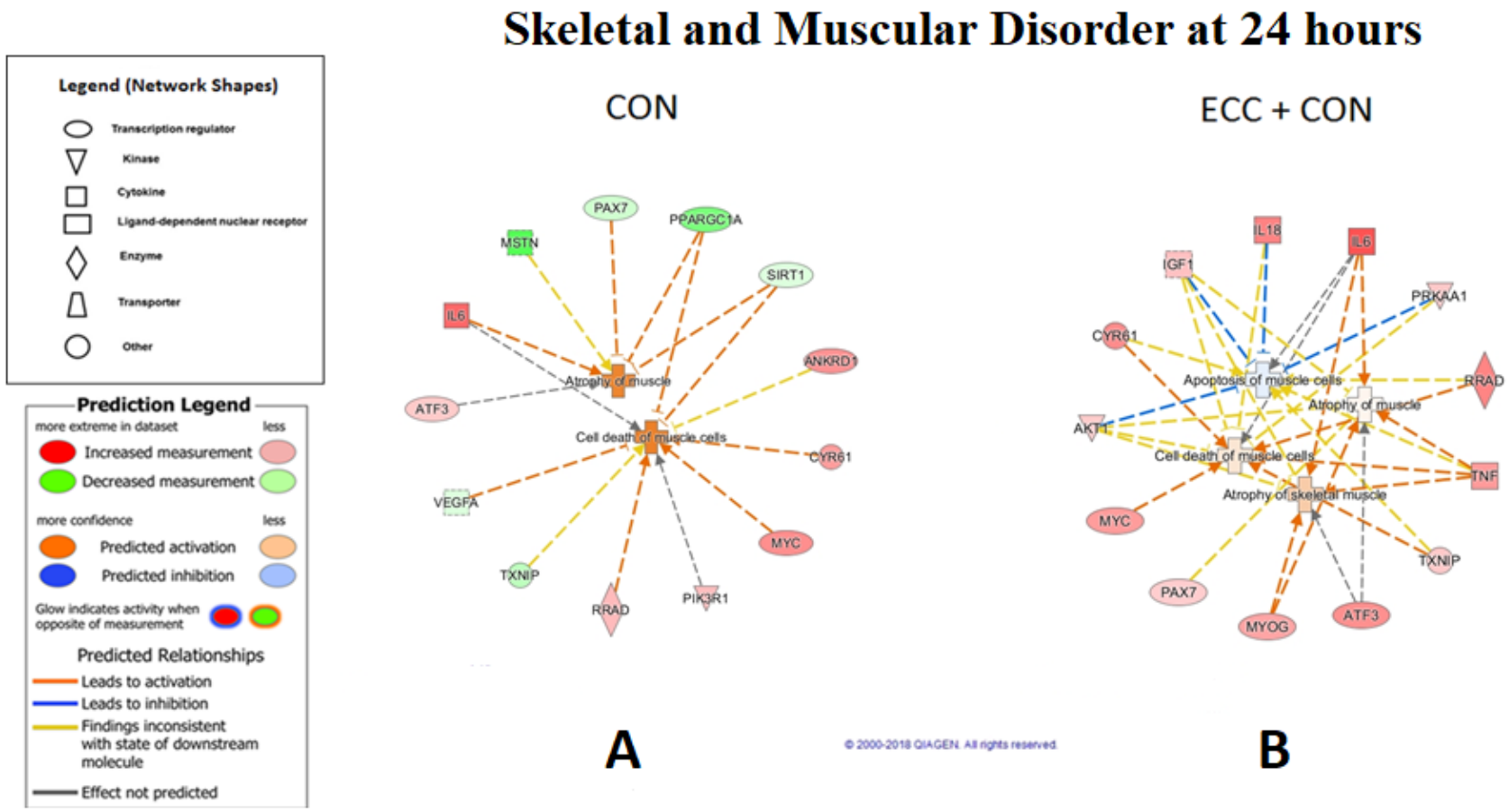
Differentially regulated muscle mRNAs (outer ring) associated with carbohydrate metabolism in (A) concentric (CON) and (B) combined eccentric + concentric (ECC + CON) trained legs at 24 hours of training compared to baseline, and the cellular events predicted by Ingenuity Pathway Analysis to results from the collective changes in mRNA abundance.

Abbreviations. MSTN, Myostatin; PIK3R1, phosphoinositide-3-kinase regulatory subunit 1; PPARGC1A, peroxisome proliferator-activated receptor gamma coactivator 1 alpha; PTK2, protein tyrosine kinase 2; SIRT1, sirtuin 1; FABP3, fatty acid binding protein 3; PDK2, pyruvate dehydrogenase kinase 2; PDK4, pyruvate dehydrogenase kinase 4; IL-6, interleukin 6; IGF1, insulin-like growth factor 1; IL-18, interleukin 18; MYC, MYC proto-oncogene; PRKAA1, protein kinase AMP-activated catalytic subunit alpha 1; SCD, stearoyl-CoA desaturase; TNF, tumour necrosis factor; AKT1, serine-threonine protein kinase 1.



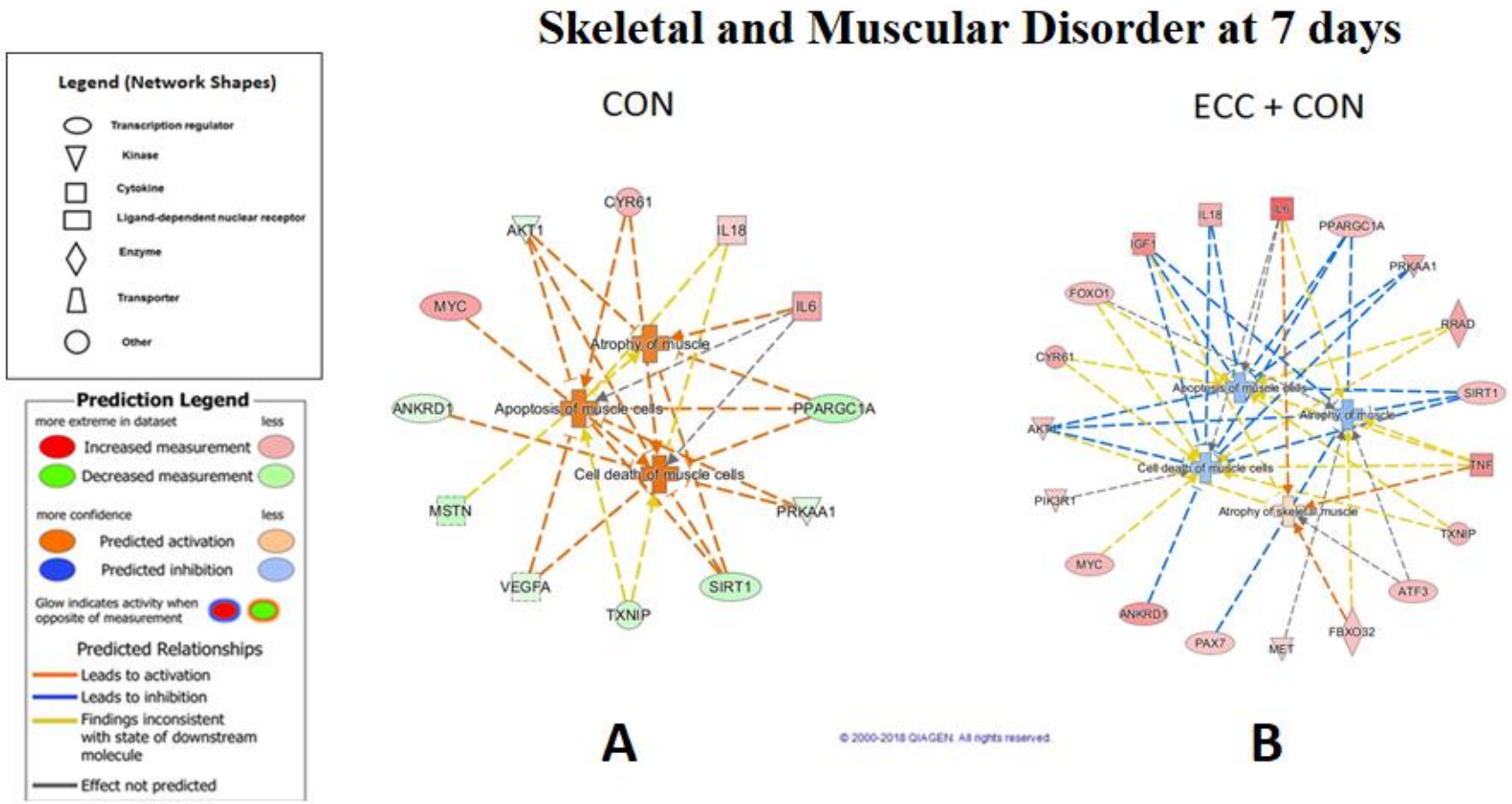
Differentially regulated muscle mRNAs (outer ring) associated with carbohydrate metabolism in (A) concentric (CON) and (B) combined eccentric + concentric (ECC + CON) trained legs at 7 days of training compared to baseline, and the cellular events predicted by Ingenuity Pathway Analysis to results from the collective changes in mRNA abundance.

Abbreviations: MSTN, myostatin; MYC, MYC proto-oncogene; PPARGC1A, peroxisome proliferator-activated receptor gamma coactivator 1 alpha; PRKAA1, protein kinase AMP-activated catalytic subunit alpha 1; PTK2, protein tyrosine kinase 2; SIRT1, sirtuin 1; FABP3, fatty acid binding protein; PDK2, pyruvate dehydrogenase kinase 2; AKT1, serine-threonine protein kinase 1; IL-18, interleukin 18; IL-6, interleukin 6; FOXO1, forkhead box protein O 1; IGF1, insulin-like growth factor 1; PIK3R1, phosphoinositide-3-kinase regulatory subunit 1; SCD, stearoyl-CoA desaturase; TNF, tumour necrosis factor; PTGS2, prostaglandin-endoperoxide synthase 2.



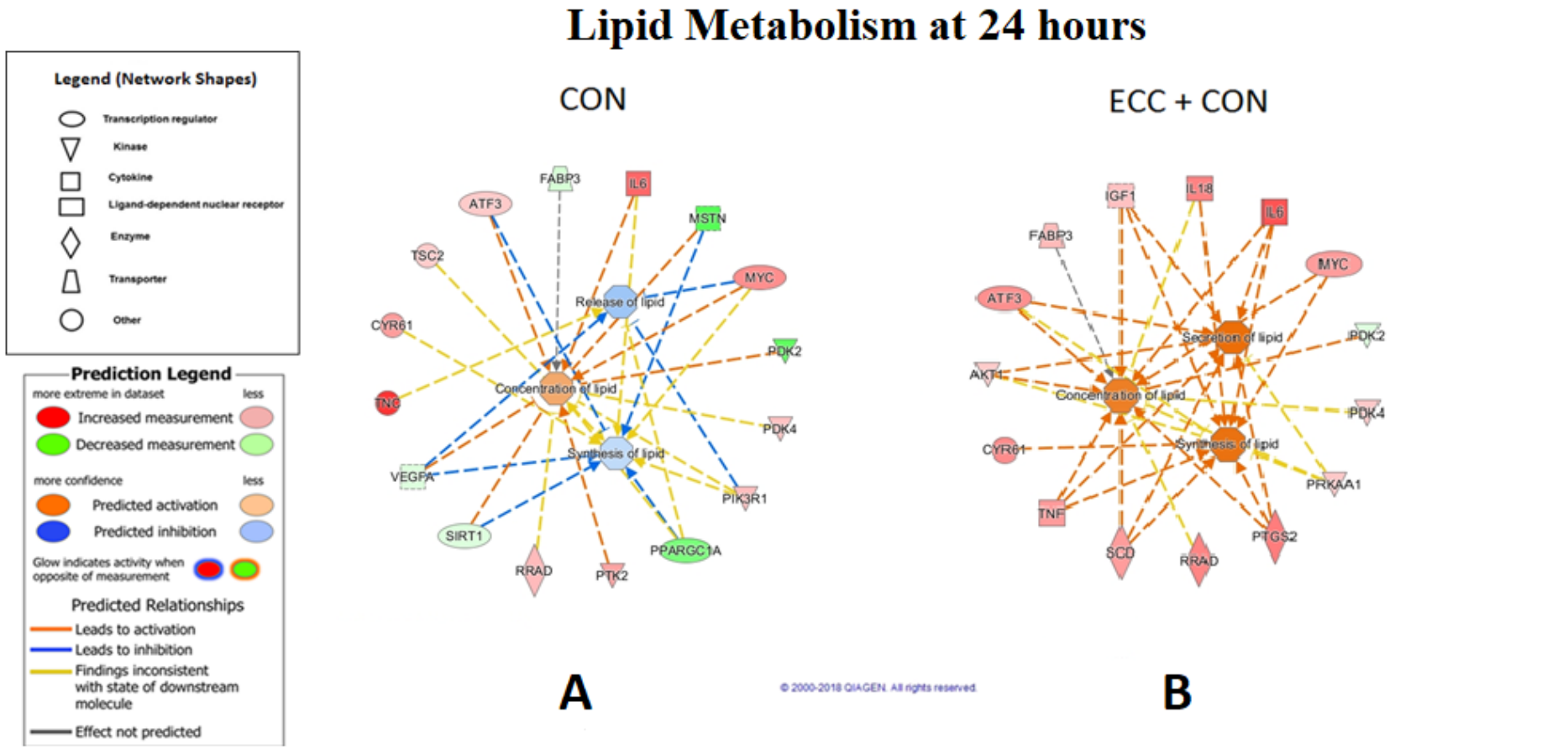
Differentially regulated muscle mRNAs (outer ring) associated with skeletal and muscular disorder in (A) concentric (CON) and (B) combined eccentric + concentric (ECC + CON) trained legs at 24 hours of training compared to baseline, and the cellular events predicted by Ingenuity Pathway Analysis to results from the collective changes in mRNA abundance.

Abbreviations: PAX7, paired box gene 7; PPARGC1A, peroxisome proliferator-activated receptor gamma coactivator 1 alpha; SIRT1, sirtuin 1; ANKRD1, ankyrin repeat domain 1; cysteine-rich angiogenic inducer 61; MYC, MYC proto-oncogene; PIK3R1, phosphoinositide-3-kinase regulatory subunit 1; RRAD, ras related glycolysis inhibitor and calcium channel regulator; VEGFA, vascular endothelial growth factor A; TXNIP, thioredoxin interacting protein; ATF3, activating transcription factor 3; MSTN, myostatin; IL-18, interleukin 18; IL-6, interleukin 6; ; PRKAA1, protein kinase AMP-activated catalytic subunit alpha 1; MYOG, myogenin; AKT1, serine-threonine protein kinase 1; CYR61, cysteine-rich angiogenic inducer 61; IGF1, insulin-like growth factor 1.



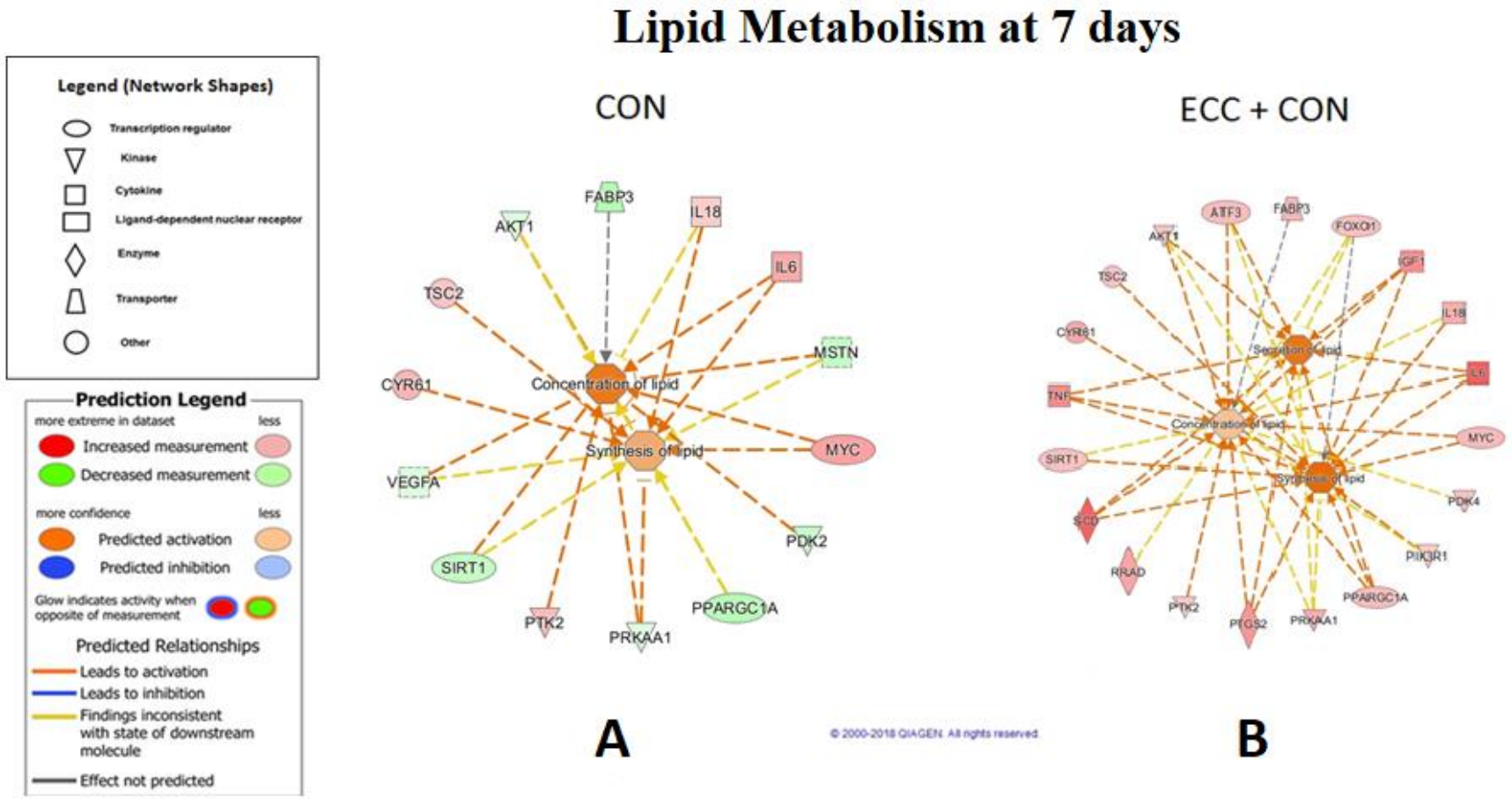
Differentially regulated muscle mRNAs (outer ring) associated with skeletal and muscular disorder in (A) concentric (CON) and (B) combined eccentric + concentric (ECC + CON) trained legs at 7 days of training compared to baseline, and the cellular events predicted by Ingenuity Pathway Analysis to results from the collective changes in mRNA abundance.

Abbreviations: CYR61, cysteine-rich angiogenic inducer 61; ; IL-18, interleukin 18; PPARGC1A, peroxisome proliferator-activated receptor gamma coactivator 1 alpha; PRKAA1, protein kinase AMP-activated catalytic subunit alpha 1; SIRT1, sirtuin 1; TXNIP, thioredoxin interacting protein; ; VEGFA, vascular endothelial growth factor A; MSTN, myostatin; ANKRD1, ankyrin repeat domain 1; MYC, MYC proto-oncogene; AKT1, serine-threonine protein kinase 1; RRAD, ras related glycolysis inhibitor and calcium channel regulator; TNF, tumour necrosis factor; ATF3, activating transcription factor 3; FBXO32, F-box protein32; MET, MET proto-oncogene; PAX7, paired box gene 7; PIK3R1, phosphoinositide-3-kinase regulatory subunit 1; FOXO1, forkhead box protein O 1; IGF1, insulin-like growth factor 1.



Differentially regulated muscle mRNAs (outer ring) associated with lipid metabolism in (A) concentric (CON) and (B) combined eccentric + concentric (ECC + CON) trained legs at 24 hours of training compared to baseline, and the cellular events predicted by Ingenuity Pathway Analysis to results from the collective changes in mRNA abundance.

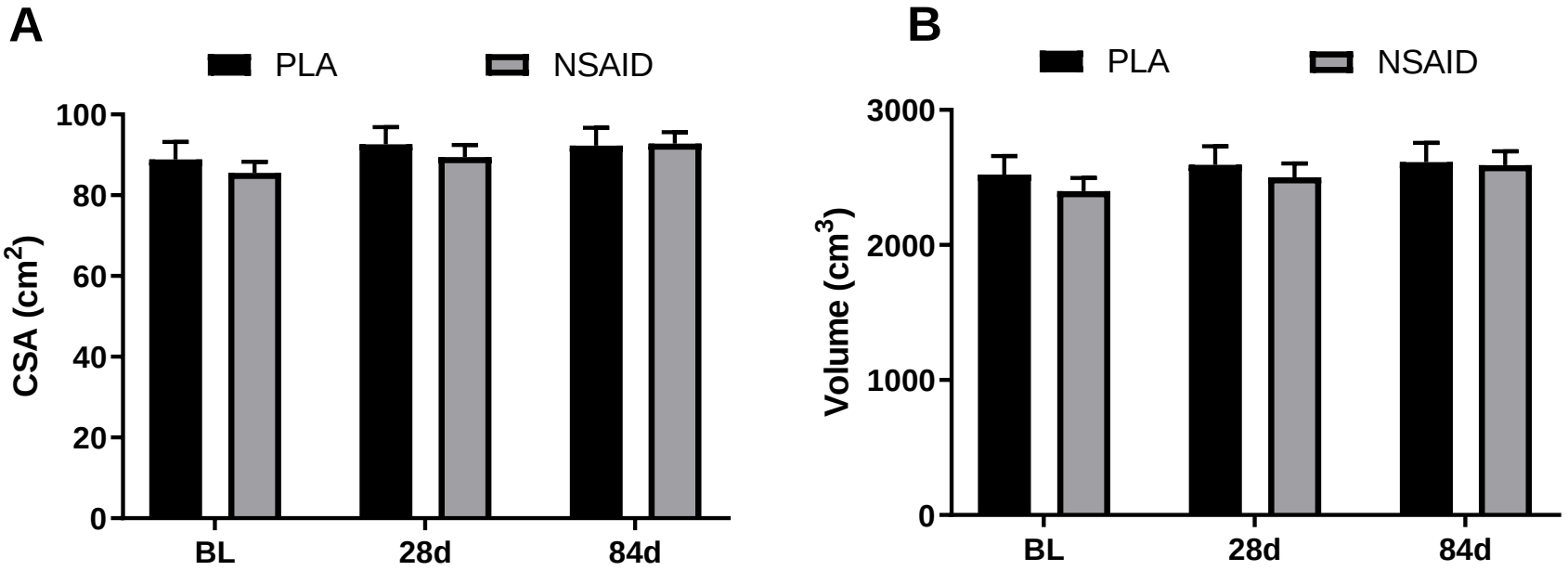
Abbreviations: MSTN, myostatin; MYC, MYC proto-oncogene; PDK2, pyruvate dehydrogenase kinase 2; PDK4, pyruvate dehydrogenase kinase 4; PIK3R1, phosphoinositide-3-kinase regulatory subunit 1; PPARGC1A, peroxisome proliferator-activated receptor gamma coactivator 1 alpha; PTK2, protein tyrosine kinase 2; RRAD, Ras related glycolysis inhibitor and calcium channel regulator; SIRT1, sirtuin 1; vascular endothelial growth factor A, VEGFA; TNC, tenascin C; CYR61, cysteine-rich angiogenic inducer 61; TSC2, tuberous sclerosis complex 2; ATF3, activating transcription factor 3; FABP3, fatty acid binding protein 3; IL-6, interleukin 6; PRKAA1, protein kinase AMP-activated catalytic subunit alpha 1; PTGS2, prostaglandin-endoperoxide synthase 2; SCD, stearoyl-CoA desaturase; TNF, tumour necrosis factor; AKT1, serine-threonine protein kinase 1; IGF1, insulin-like growth factor 1.



Differentially regulated muscle mRNAs (outer ring) associated with lipid metabolism in (A) concentric (CON) and (B) combined eccentric + concentric (ECC + CON) trained legs at 7 days of training compared to baseline, and the cellular events predicted by Ingenuity Pathway Analysis to results from the collective changes in mRNA abundance.

Abbreviations: IL-6, interleukin 6; IL-18, interleukin 18; FABP3, fatty acid binding protein 3; MSTN, myostatin; MYC, MYC proto-oncogene; PPARGC1A, peroxisome proliferator-activated receptor gamma coactivator 1 alpha; PRKAA1, protein kinase AMP-activated catalytic subunit alpha 1; PTK2, protein tyrosine kinase 2; SIRT1, sirtuin 1; VEGFA, vascular endothelial growth factor A; CYR61, cysteine-rich angiogenic inducer 61; TSC2, tuberous sclerosis complex 2; AKT1, serine-threonine protein kinase 1; FOXO1, forkhead box protein O1; IGF1, insulin-like growth factor 1; PDK4, pyruvate dehydrogenase kinase 4; PIK3R1, phosphoinositide-3-kinase regulatory subunit 1; PTK2, protein tyrosine kinase 2; RRAD, Ras related glycolysis inhibitor and calcium channel regulator; SCD, stearoyl-CoA desaturase; TNF, tumour necrosis factor; ATF3, activating transcription factor 3.

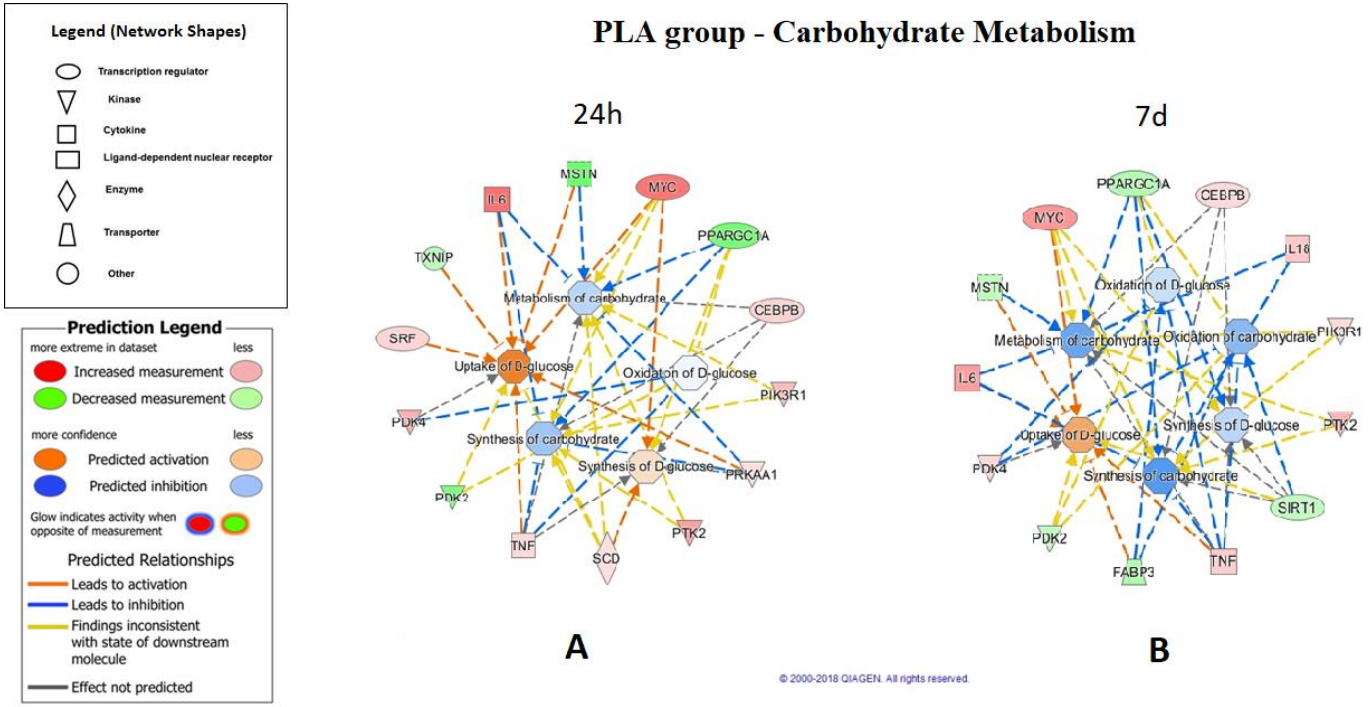
Appendix G



(A) Mid-thigh quadriceps muscle cross-sectional area (cm^2), and (B) quadriceps muscle volume (cm^3) in the group consuming placebo (PLA; n=8) and the group consuming diclofenac (NSAID; n=9), at baseline (BL), 28 days (28d) and 84 days (84d) of training. Values expressed as mean ± SEM.

Appendix H

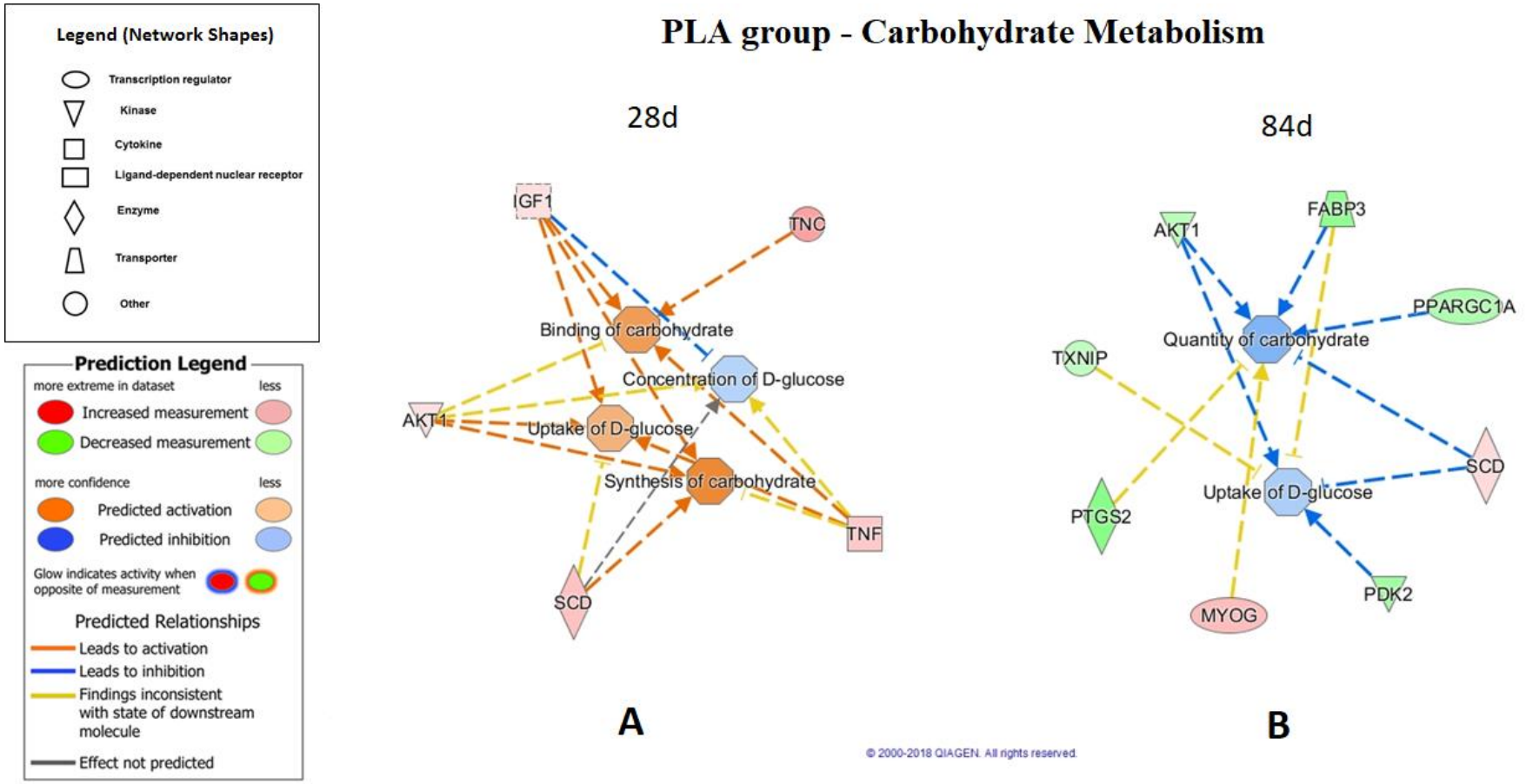
Ingenuity Pathway Analysis predictions for altered cellular functions in experiments Chapter 4 not included in main text.



Differentially regulated muscle mRNAs (outer ring) associated with carbohydrate metabolism in group consuming placebo (PLA) at (A) 24 hours (24h) and (B) 7 days (7d) compared to baseline, and the cellular events predicted by Ingenuity Pathway Analysis to result from the collective changes in mRNA abundance.

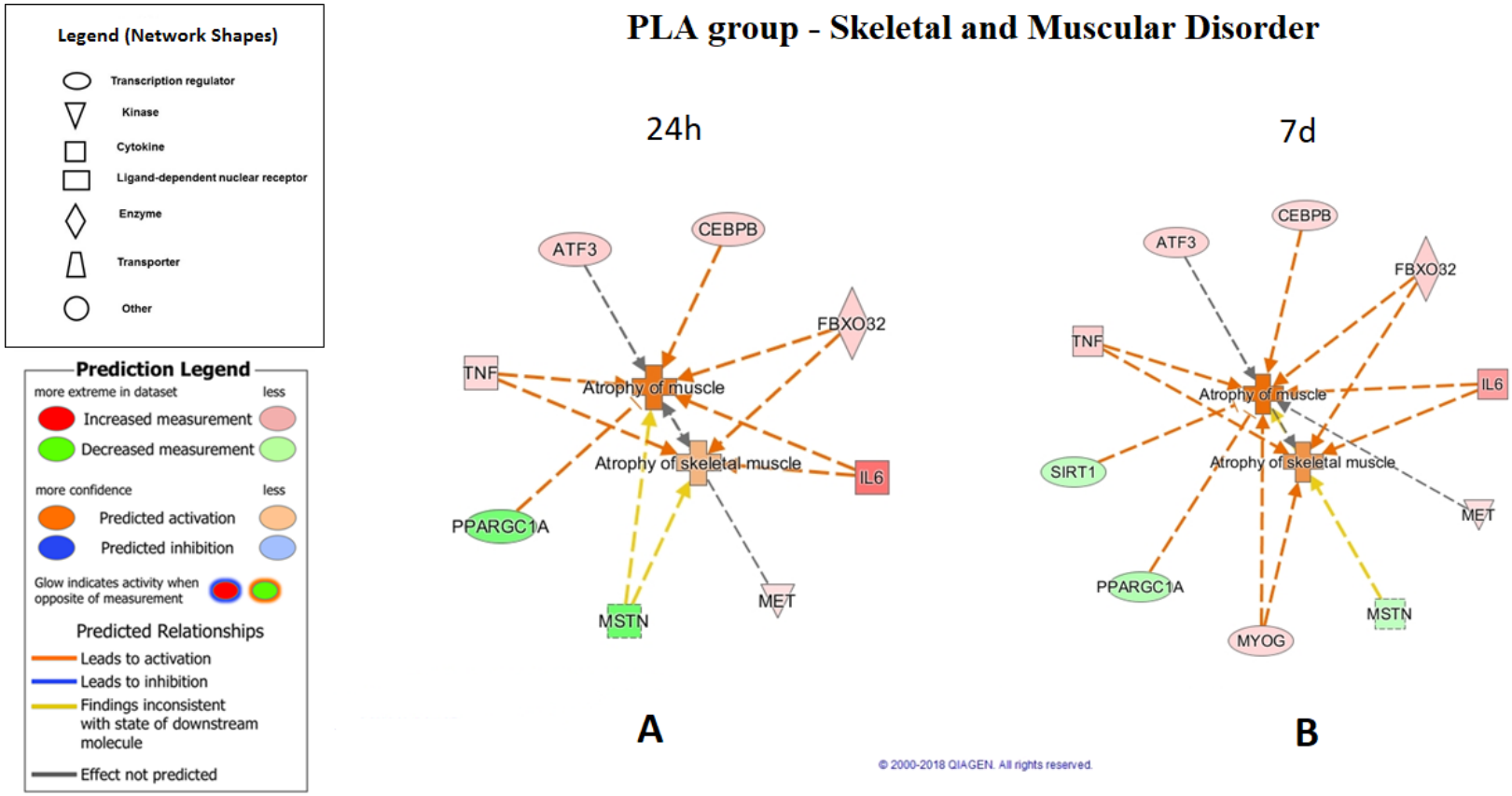
Abbreviations: MSTN, myostatin; MYC, MYC proto-oncogene; PPARGC1A, peroxisome proliferator-activated receptor gamma coactivator 1 alpha; CEBPB, CCAAT enhancer binding protein beta; PIK3R1, phosphoinositide-3-kinase regulatory subunit 1; PRKAA1, protein kinase AMP-activated catalytic subunit alpha 1; PTK2, protein tyrosine kinase 2; SCD, stearoyl-CoA desaturase; TNF, tumour necrosis factor; PDK2, pyruvate dehydrogenase kinase 2; PDK4, pyruvate dehydrogenase kinase 4; TXNIP, thioredoxin interacting protein; IL-6, interleukin 6; IL-18, interleukin 18; PIK3R1, phosphoinositide-3-kinase regulatory subunit 1; FABP3, fatty acid binding protein 3.

Appendices



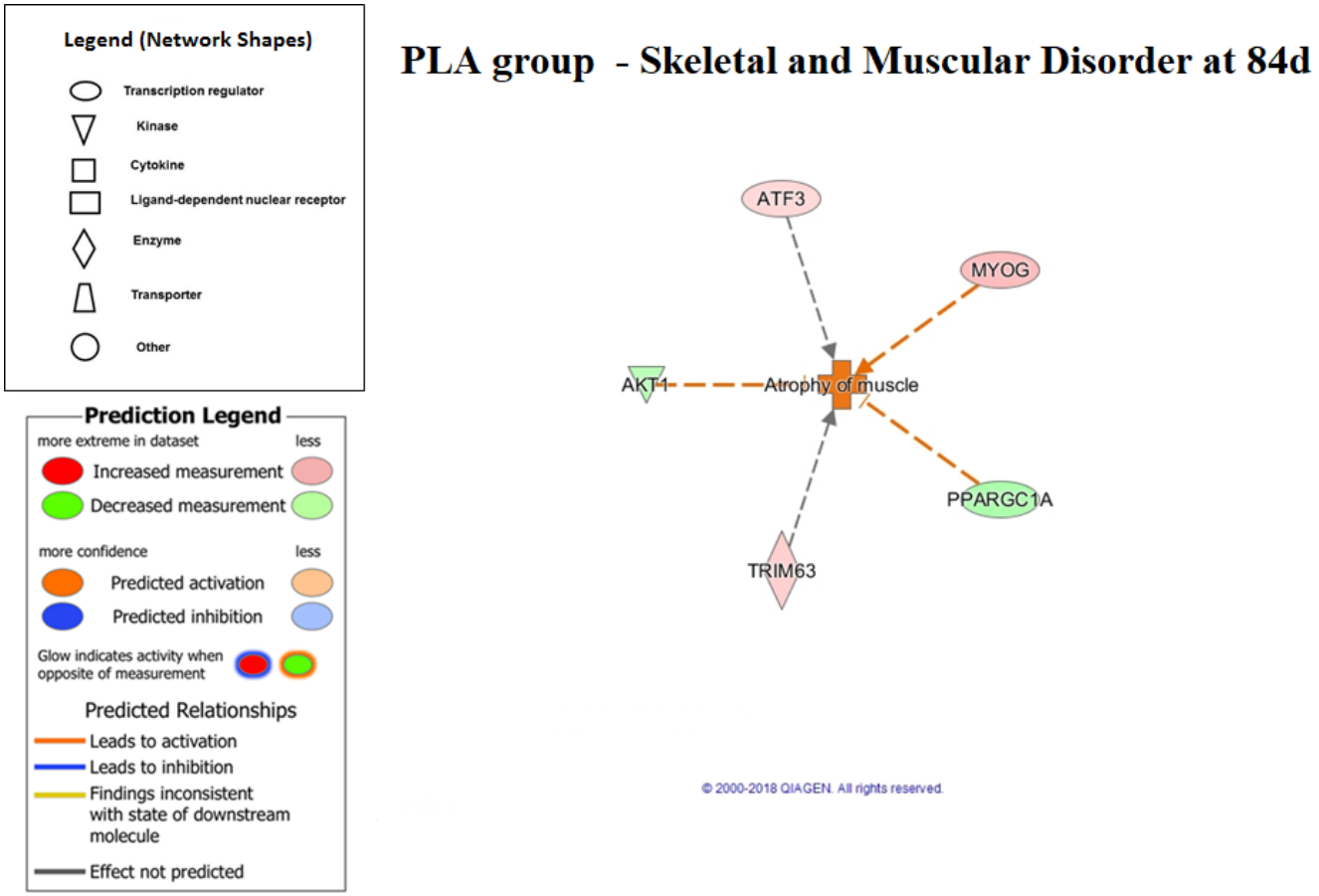
Differentially regulated muscle mRNAs (outer ring) associated with carbohydrate metabolism in group consuming placebo (PLA) at (A) 28 days (28d) and (B) 84 days (84d) compared to baseline, and the cellular events predicted by Ingenuity Pathway Analysis to result from the collective changes in mRNA abundance.

Abbreviations: TNC, tenascin C; TNF, tumour necrosis factor; SCD, stearoyl-CoA desaturase; AKT1, serine-threonine protein kinase 1; IGF1, insulin-like growth factor 1; FABP3, fatty acid binding protein 3; PPARGC1A, peroxisome proliferator-activated receptor gamma coactivator 1 alpha; PDK2, pyruvate dehydrogenase kinase 2; MYOG, myogenin; PTGS2, prostaglandin-endoperoxide synthase 2; TXNIP, thioredoxin interacting protein.



Differentially regulated muscle mRNAs (outer ring) associated with skeletal and muscular disorder in group consuming placebo (PLA) at (A) 24 hours (24h) and (B) 7 days (7d) compared to baseline, and the cellular events predicted by Ingenuity Pathway Analysis to result from the collective changes in mRNA abundance.

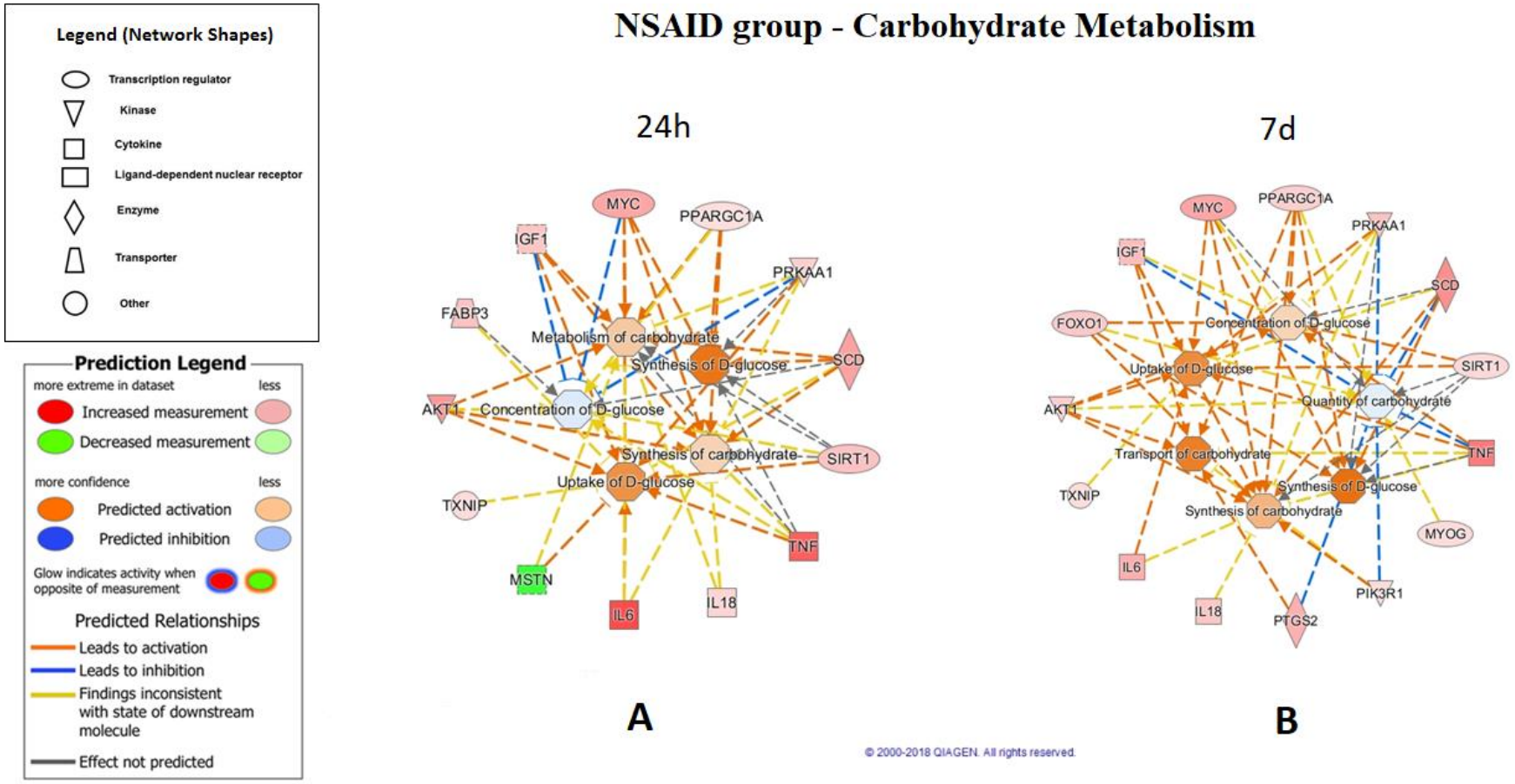
Abbreviations: CEBPB, CCAAT enhancer binding protein beta; FBXO32, F-box protein32; IL-6, interleukin 6; MET, MET proto-oncogene; MSTN, myostatin; PPARGC1A, peroxisome proliferator-activated receptor gamma coactivator 1 alpha; TNF, tumour necrosis factor; ATF3, activating transcription factor 3; MYOG, myogenin; SIRT1, sirtuin 1.



Differentially regulated muscle mRNAs (outer ring) associated with skeletal and muscular disorder in group consuming placebo (PLA) at 84 days (84d) compared to baseline, and the cellular events predicted by Ingenuity Pathway Analysis to result from the collective changes in mRNA abundance.

Abbreviations: MYOG, myogenin; PPARGC1A, peroxisome proliferator-activated receptor gamma coactivator 1 alpha; TRIM63, tripartite motif containing 63; AKT1, serine-threonine protein kinase 1; ATF3, activating transcription factor 3.

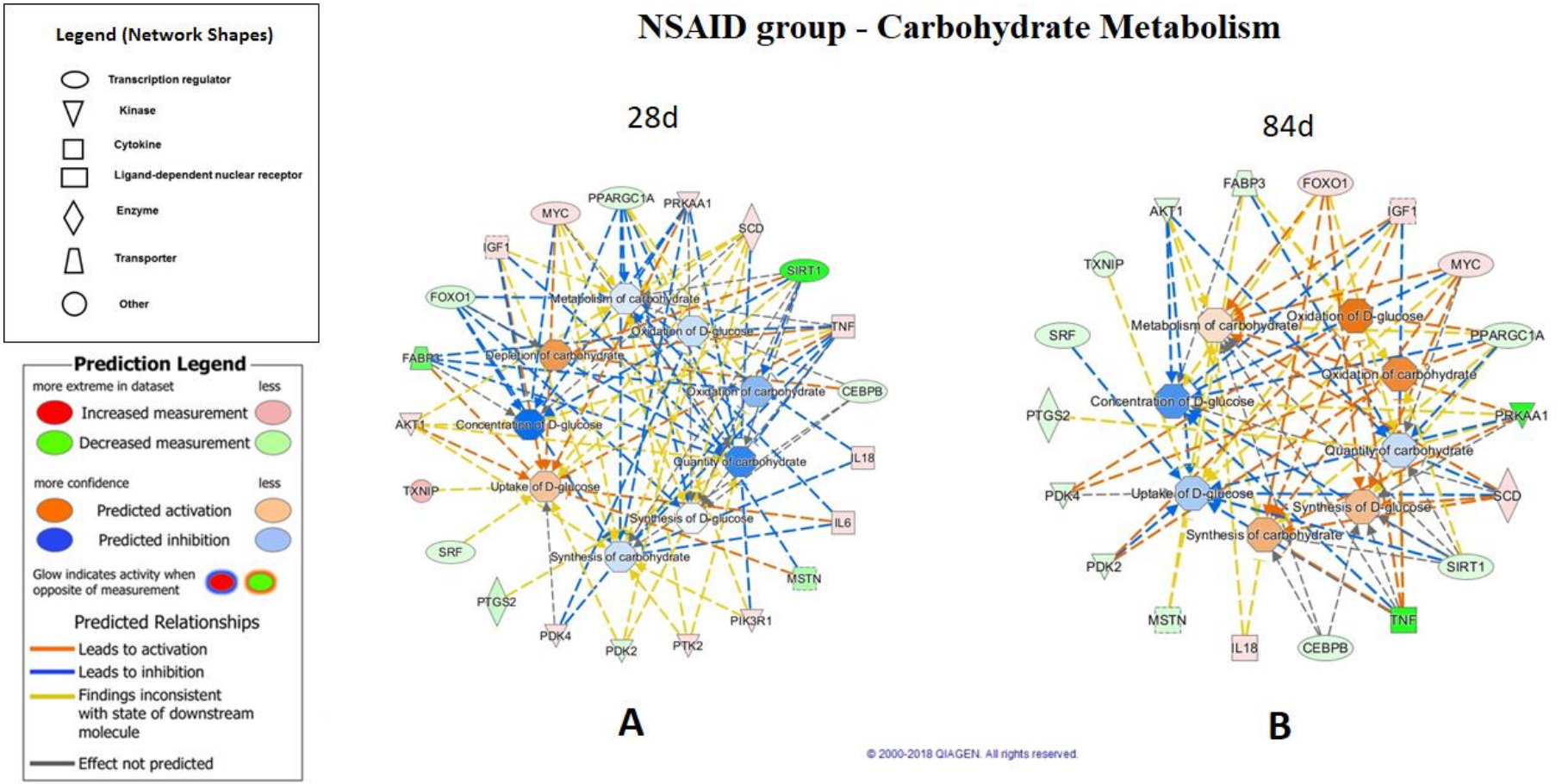
Appendices



Differentially regulated muscle mRNAs (outer ring) associated with carbohydrate metabolism in group consuming diclofenac (NSAID) at (A) 24 hours (24h) and (B) 7 days (7d) compared to baseline, and the cellular events predicted by Ingenuity Pathway Analysis to result from the collective changes in mRNA abundance.

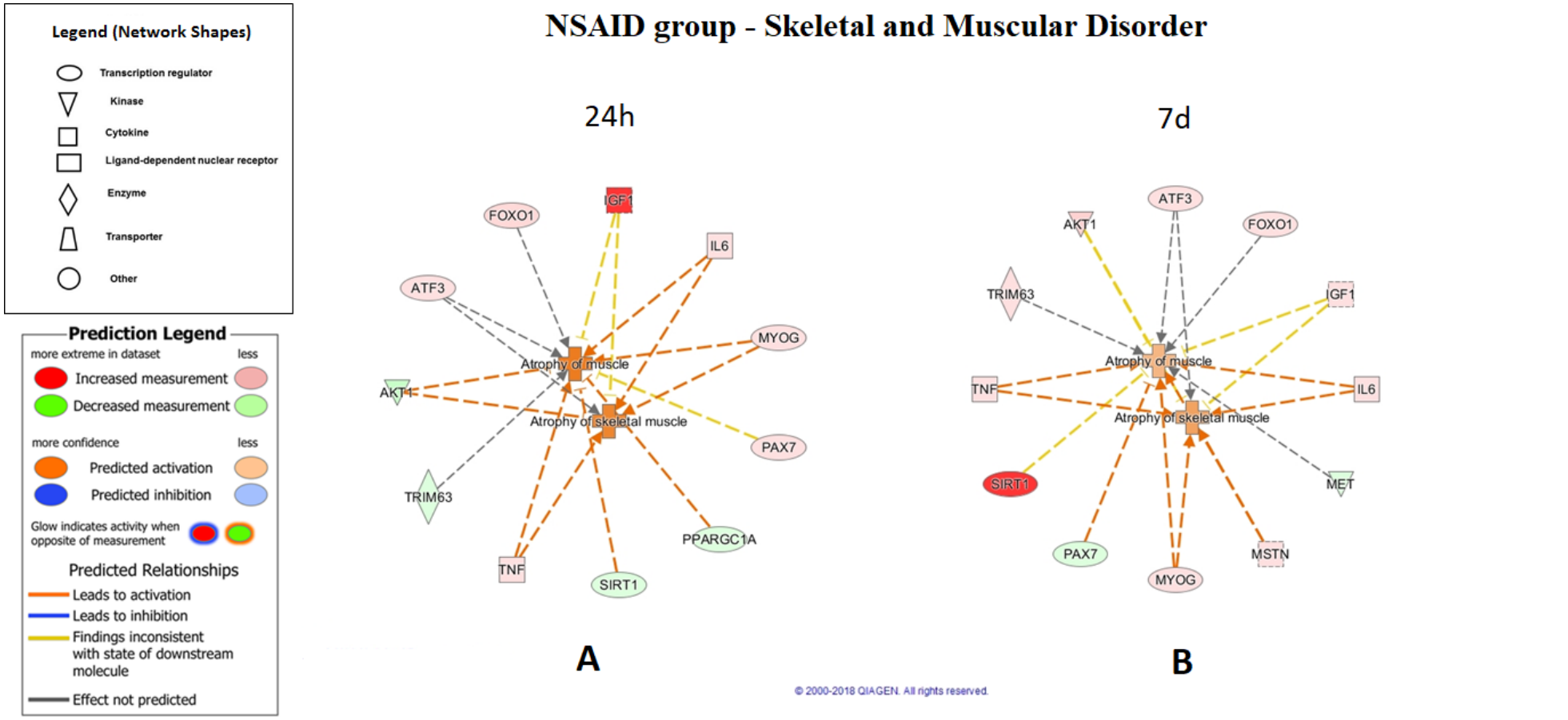
Abbreviations: PPARGC1A, peroxisome proliferator-activated receptor gamma coactivator 1 alpha; PRKAA1, protein kinase AMP-activated catalytic subunit alpha 1; SCD, stearoyl-CoA desaturase; SIRT1, sirtuin 1; TNF, tumour necrosis factor; IL-18, interleukin 18; IL-6, interleukin 6; MSTN, myostatin; TXNIP, thioredoxin interacting protein; AKT1, serine-threonine protein kinase 1; FABP3, fatty acid binding protein 3; IGF1, insulin-like growth factor 1; MYC, MYC proto-oncogene, MYOG, myogenin; PIK3R1, phosphoinositide-3-kinase regulatory subunit 1; PTGS2, prostaglandin-endoperoxide synthase 2; FOXO1, forkhead box protein O 1.

Appendices



Differentially regulated muscle mRNAs (outer ring) associated with carbohydrate metabolism in group consuming diclofenac (PLA) at (A) 28 days (28d) and (B) 84 days (84d) compared to baseline, and the cellular events predicted by Ingenuity Pathway Analysis to result from the collective changes in mRNA abundance.

Abbreviations: PRKAA1, protein kinase AMP-activated catalytic subunit alpha 1; PPARGC1A, peroxisome proliferator-activated receptor gamma coactivator 1 alpha; SCD, stearoyl-CoA desaturase; SIRT1, sirtuin 1; TNF, tumour necrosis factor; CEBPB, CCAAT enhancer binding protein beta; IL-18, interleukin 18; IL-6, interleukin 6; MSTN, myostatin; PIK3R1, phosphoinositide-3-kinase regulatory subunit 1; PTK2, protein tyrosine kinase 2; PDK2, pyruvate dehydrogenase kinase 2; PDK4, pyruvate dehydrogenase kinase 4; PTGS2, prostaglandin-endoperoxide synthase 2; SRF, serum response factor; TXNIP, thioredoxin interacting protein; AKT1, serine-threonine protein kinase 1; FABP3, fatty acid binding protein 3; FOXO1, forkhead box protein O1; IGF1, insulin-like growth factor 1; MYC, MYC proto-oncogene.



Differentially regulated muscle mRNAs (outer ring) associated with skeletal and muscular disorder in group consuming diclofenac (NSAID) at (A) 24 hours (24h) and (B) 7 days (7d) compared to baseline, and the cellular events predicted by Ingenuity Pathway Analysis to result from the collective changes in mRNA abundance.

Abbreviations: ; IGF1, insulin-like growth factor 1; IL-6, interleukin 6; MYOG, myogenin; ; PAX7, paired box gene 7; PPARGC1A, peroxisome proliferator-activated receptor gamma coactivator 1 alpha; SIRT1, sirtuin 1; TNF, tumour necrosis factor; TRIM63, tripartite motif containing 63; AKT1, serine-threonine protein kinase 1; ATF3, activating transcription factor 3; FOXO1, forkhead box protein O 1; MET, MET proto-oncogene; MSTN, myostatin; MYOG, myogenin; PAX7, paired box gene 7.

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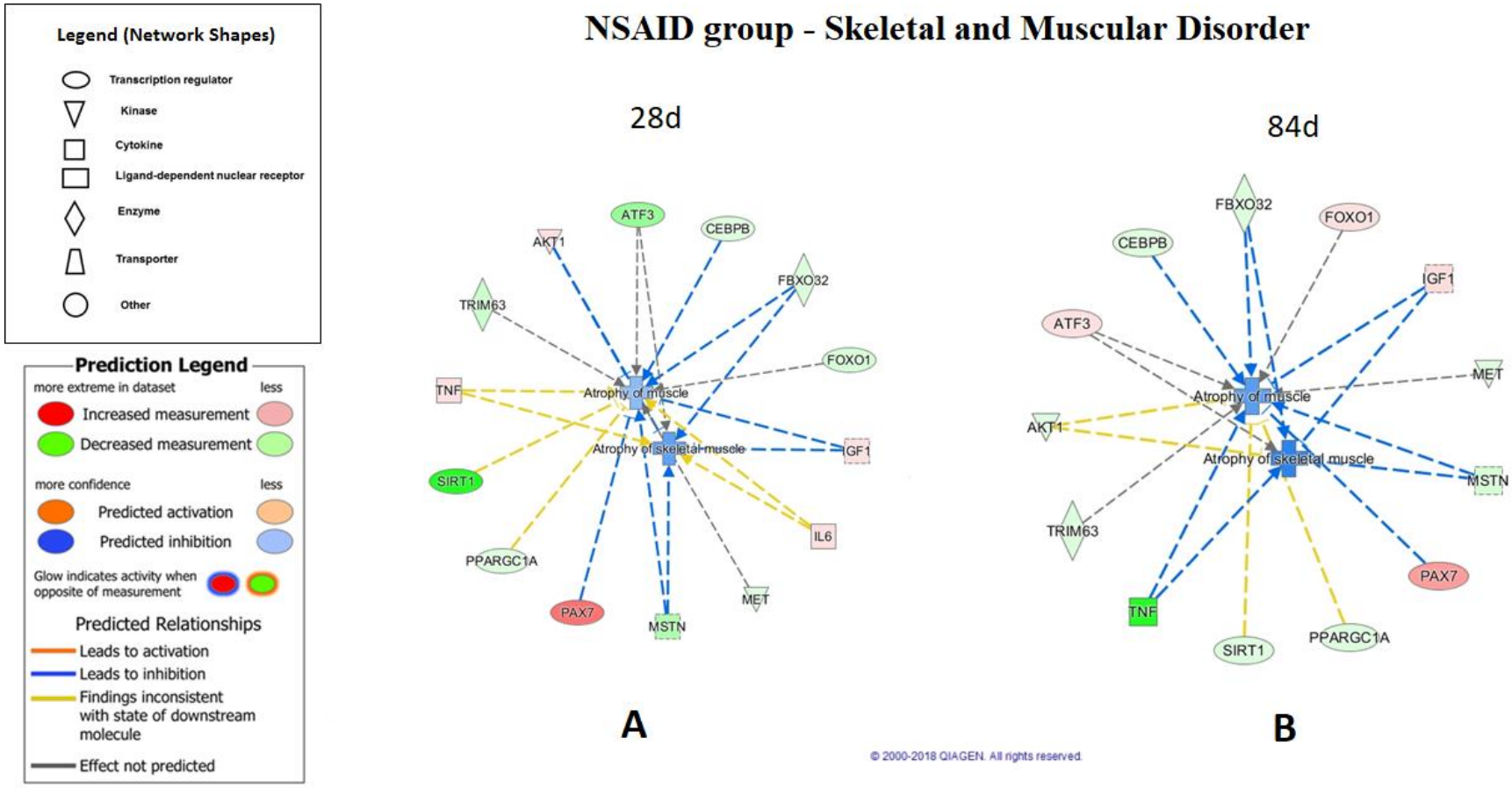

Differentially regulated muscle mRNAs (outer ring) associated with skeletal and muscular disorder in group consuming diclofenac (PLA) at (A) 28 days (28d) and (B) 84 days (84d) compared to baseline, and the cellular events predicted by Ingenuity Pathway Analysis to result from the collective changes in mRNA abundance.

Abbreviations: ATF3, activating transcription factor 3; CEBPB, CCAAT enhancer binding protein beta; FBXO32, F-box protein 32; FOXO1, forkhead box protein O 1; IGF1, insulin-like growth factor 1; IL-6, interleukin 6; MET, MET proto-oncogene; PAX7, paired box gene 7; PAX7, paired box gene 7; PTGS2, prostaglandin-endoperoxide synthase 2; PPARGC1A, peroxisome proliferator-activated receptor gamma coactivator 1 alpha; SIRT1, sirtuin 1; TNF, tumour necrosis factor; TRIM63, tripartite motif containing 63; AKT1, serine-threonine protein kinase 1; MSTN, myostatin 1.