THE ROLE OF ADIPOSE AND SKELETAL MUSCLE DERIVED CYTOKINES IN PRIMARY HUMAN MYOGENESIS: IMPLICATIONS FOR AGEING SKELETAL MUSCLE.

by

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Abstract

Sarcopenia is the age-related loss of skeletal muscle mass and function; inflammation is thought to be one aetiological factor in its development. Adipose tissue accumulates with advancing age and adipose-derived cytokines (adipokines) contribute to inflammaging. Skeletal muscle myogenesis is one adaptaive mechanism by which skeletal muscle mass is sustained throughout the human lifespan. The effect of the adipose inflammatory milieu on such myogenesis is unknown, as is the relative importance of its constituent adipokines to myogenesis. This work demonstrates that conditioned medium generated from obese subcutaneuous adipose tissue has a detrimental effect on *in vitro* primary human myogenesis. Resistin is shown to be – in part – responsible for this phenomenon and is demonstrated to inhibit myogenesis by activating the classical NFκB pathway. Resistin is further shown to be a metabolic stressor of primary human myotubes, promoting increased oxygen consumption, fatty acid oxidation and lipid accumulation. It is important to identify more avenues for the development of pharmacological interventions in sarcopenia. To that end, this thesis also demonstrates for the first time that the myokine IL-15: 1) is pro-myogenic in primary human cultures; 2) can mitigate the detrimental effects of an inflammatory environment on myogenesis; and 3) supports myogenesis at autocrine concentrations.

200-word limit

Dedication

To my extraordinary wife Emma,

(who objects vociferously to this overt sentimentality)

mo chuisle, mo chroí.

On striving to not make Epics from our local academic rows:

Epic (1960) – Patrick Kavanagh

Acknowledgements

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To my parents, thank you for laying the foundations. Mum, you continue to do a hundred things before half past nine. Any ability that I have to marry competence with efficiency undoubtedly comes from you. Dad, you are still missed every day, in ways that are both large and small, but often in the face of adversity, I smile and think to myself '*Illegitimi non carborundum'*. Thank you to my siblings, Margaret, Anne, Patrick and Catherine as well as their spouses and children. The family WhatsApp group (while overwhelmingly active at times) has kept us connected despite the distance. My wife's wonderful family have been a part of my life for almost 14 years and I couldn't ask for a better second family.

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Emma, you are a much better wife than physics lab partner. Your formal objections to the sentiments herein have been received, logged and ignored. Thank you for your continued unconditional support and patience – I love you. I can't wait to find out what happens next.

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List of Abbreviations

All abbrevations are defined where they are first mentioned in the text. Standard units (e.g. °C) and commonly used chemical formulae (e.g. CO_2) are not defined.

ACC	Acetyl-CoA carboxylase
ADP	Adenosine diphosphate
Akt	Protein kinase B
AMPK	5' AMP-activated protein kinase
ANT	Adenine nucleoide translocase
ATP	Adenosine triphosphate
AVS	Adipokine varibility score
BAT	Brown adipose tissue
bFGF	Basic fibroblast growth factor
BMI	Body mass index
CAP1	Adenylyl cyclase-associated protein 1
CAP2	Adenylyl cyclase-associated protein 2
CCL11	C-C motif chemokine 11
CCL2	C-C motif chemokine 2
CCL20	C-C motif chemokine 20
CCL3	C-C motif chemokine 3
CCL4	C-C motif chemokine 4
COX	Cyclooxygenase

COXIV	Cytochrome c oxidase subunit IV
CPT-1	Carnitine palmitoyl transferase 1
CRP	c-reactive protein
CSA	Cross-sectional area
Ct	Cycle threshold
CXCL10	C-X-C motif chemokine 10
DAG	Diacylglycerol
DAMPs	Damage-associated molecular patterns
DAPI	4',6-diamidino-2-phenylindole
DEXA	Dual-energy X-ray absorptiometry
DGAT1	Diglyceride acyltransferase
Dkk1	Dickkopf-related protein 1
DMEM	Dulbecco's Modified Eagle Medium
DMEM/F12	Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12
DMSO	Dimethyl sulfoxide
ECAR	Extracellular acidification rate
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent Assay
EMSA	Electrophoretic mobility gel shift assays
ERK 1/2	Extracellular signal–regulated kinases 1 and 2
ERS	Endoplasmic reticulum stress

EWGSOP	European Working Group on Sarcopenia in Older People
FABP4	Fatty acid binding protein 4
FACS	Fluorescence activated cell sorting
FAP	Fibroadipogenic progenitor cells
FAS	Fatty acid synthase
FATP	Fatty acid transport protein
FBS	Fetal bovine serum
FCCP	Carbonyl cyanide-p-trifluoromethoxyphenylhydrazone
FDA	Federal drug administration
FFA	Free fatty acid
FFM	Fat free mass
FM	Fat mass
FOXO1	Forkhead box protein O1
G-CSF	Granulocyte colony stimulating factor
GDP	Gross domestic product
GH	Growth hormone
gp120	Envelope glycoprotein GP120
HEK 293	Human embryonic kidney 293
HGF	Hepatocyte growth factor
HIV	Human immunodeficiency virus
IF	Immunofluorescence
IGF-1	Insulin-like growth factor 1

IKKβ	IκB-kinase-β
IL-10	Interleukin 10
IL-15	Interleukin 15
IL-15	Interleukin 15
IL-15rα	Interleukin 15 receptor alpha subunit
IL-1β	Interleukin 1β
IL-2	Interleukin 2
IL-2Rβ	Interleukin 2 receptor beta subunit
IL-4	Interleukin 4
IL-6	Interleukin 6
IL-7	Interleukin 7
IL-9	Interleukin 9
IMAT	Intramuscular adipose tissue
IMCL	Intramyocellular lipid
IR	Insulin resistance
IV	Intravenous
IκBα	Inhibitor of kappa B
JAK1	Janus kinase 1
LBM	Lean body mass
LIF	Leukemia inhibitory factor
LMN	Lower motor neuron/s
LRP-1	Low density lipoprotein-like receptor-1

LSP-*IL15*	Long signal peptide IL-15
MACS	Magnetic activated cell sorting
MAFbx	Muscle atrophy F-box protein
MHC	Myosin heavy chain
MMP-1	Matrix metalloproteinase 1
MMP-10	Matrix metalloproteinase 10
MMP-12	Matrix metalloproteinase 12
MMP-13	Matrix metalloproteinase 13
MMP-2	Matrix metalloproteinase 2
MMP-3	Matrix metalloproteinase 3
MMP-7	Matrix metalloproteinase 7
MMP-8	Matrix metalloproteinase 8
MND	Myonuclear domain
MPS	Muscle protein synthesis
MRF4	Myogenic regulatory factor 4
MRFs	Myogenic regulatory transcription factors
mTORC1	Mammalian target of rapamycin complex 1
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl) -2-(4-sulfophenyl)-2H-tetrazolium inner salt
MTT	Myotube thickness
MURF-1	Muscle RING finger 1 protein
MYF5	Myogenic factor 5

MyoD	Myogenic differentiation 1 protein
NFI	Nuclear fusion index
NFκB	nuclear factor-κB
NMJ	Neuromuscular junction
NSAID	Non-steroidal antiinflammatory drug
NW	Normal weight
OB	Obese
OCR	Oxygen consumption rate
OW	Overweight
p38 MAPK	p38 mitogen-acivated protein kinases
PAX3	Paired box protein 3
PAX7	Paired box protein 7
PBS	Phosphate-buffered saline
PCG-1α	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
PI3k	Phosphoinositide 3-kinase
PITX2	Paired-like homeodomain transcription factor 2
PPARγ	Peroxisome proliferator-activated receptor gamma
PPARδ	Peroxisome proliferator-activated receptor delta
PVDF	Polyvinylidene difluoride
RDI	Recommended daily intake
rIL-15	Recombinant interleukin 15

RIPA buffer	Radioimmunoprecipitation assay buffer
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT	Resistance training
rTNFα	Recombinant tumour necrosis factor alpha
SARMS	Selective androgen receptor modulators
SAT	Subcutaneous adipose tissue
SC	Satellite cell
SCD	Stearoyl-CoA desaturase
SCFA	Subcutaneous fat area
SDS	Sodium dodecyl sulfate
SDS-PAGE	SDS polyacrylamide gel electrophoresis
SKM-IR	Skeletal muscle insulin resistance
SNPs	Single nucleotide polymorphisms
SR	Sarcoplasmic reticulum
SREBP1	Sterol regulatory element-binding protein 1
SREBP2	Sterol regulatory element-binding protein 2
SSP-*IL15*	Short signal peptide IL-15
STAT3	Signal transducer and activator of transcription 3
SUnSET	Non-Radioactive Surface Sensing of Translation

SVF	Stromal vascular fraction
TAG	Triacylglycerols
TBS-T	Tris buffered saline with Tween 20
TBX1	T-box transcription factor 1
TLR4	Toll-like receptor 4
TMB	Tetramethylbenzidine
TNFα	Tumour necrosis factor alpha
TNFα	Recombinant TNFα
tPA	Tissue plasminogen activator
TPCA-1	5-(p-Fluorophenyl)-2-ureido]thiophene-3-carboxamide
Tris-HCl	Tris hydrochloride
UCP1	Uncoupling protein 1
UCP2	Uncoupling protein 2
UCP3	Uncoupling protein 3
uPA	Urokinase
VAT	Visceral adipose tissue
VFA	Visceral fat area
VL	Vastus lateralis
YY1	Ying-Yang 1
βME	Beta-mercaptoethanol
γc	Gamma cytokine receptor

Style Guide

This thesis adheres to International Organization for Standardization (ISO) guidelines for the presentation of units of measurement, including currencies (i.e. using approved abbreviations, with a space between the measurement and unit). Two exceptions are made. First, the ISO abbreviation of year (a) is not commonly used in the biological literature. Thus, for ease of reading, ‘yr’ is used to reference both singular and multiple years. Second, there is no ISO standard abbreviation for week; ‘wk’ is used here. All other abbreviations (except common chemical formulae e.g. CO_2) are defined where they first appear in the text. The Vancouver referencing style is employed. Integers from one to nine are spelled out, unless the number is immediately followed by a scientific unit, a unit of time or reference to fold-change.

For ease of reading, figures and tables appear immediately following the paragraph in which they are first referenced in the text.

Chapter 1

Introduction and Background

1. Introduction and Background

1.1 General Introduction

Skeletal muscle mass begins to decline continuously in middle age, at a rate of 3-8 % per decade (1). This phenomenon is known as sarcopenia; a term derived from the Greek *sarx,* meaning flesh and *penia,* meaning loss (2–5). In addition to this loss of muscle mass, definitions of sarcopenia also describe a decline in muscle strength and power as well as impaired physical performance (6). Indeed, the European Working Group on Sarcopenia in Older People (EWGSOP) guidelines require the presence of low muscle mass and either low muscle strength or poor physical performance for the diagnosis of sarcopenia (6). Globally, 5-10 % of those over 65 yr meet such criteria and the prevalence of sarcopenia is increased in advanced age. (7). In many individuals, sarcopenia is accompanied by an increase in adipose tissue mass, a condition that is termed sarcopenic obesity (8). Adipose tissue mass typically accumulates throughout middle age at a rate of 0.21 kg/yr in men and 0.14 kg/yr in women, peaking in old age (4). Sarcopenia and sarcopenic obesity are associated with an increased risk of cardiovascular, metabolic and musculoskeletal diseases as well as poorer quality of life scores and an increased incidence of falls and fractures (9–12). These clinical conditions have profound personal and societal costs; sarcopenia increases all-cause mortality 4-fold (13). Indeed, the UK is expected to spend an additional 2.3 % of gross domestic product (GDP) on age-related healthcare services by 2062 (14). In 2004, it was estimated that sarcopenic individuals in the United States required excess healthcare spending of ~ USD 900 per annum compared to their non-sarcopenic counterparts (15). There are few effective therapeutic interventions available for the prevention or reversal of sarcopenia. Avoidance of

sedentary behaviour (16) and resistance exercise training, supported by adequate nutrition are partially effective in reversing sarcopenia (17–19). However, even lifelong resistance training does not facilitate the retention of skeletal muscle mass and strength at the levels that are observed in the young (20). No pharmacological interventions are currently approved for the prevention or reversal of sarcopenia, although some selective androgen receptor modulators (SARMs) and the myostatin antagonist bimagrumab have produced promising results (21). To develop effective interventions for sarcopenia, it is essential that we continue to characterise the mechanisms underlying its development.

The aetiology of sarcopenia is complex and incompletely understood. A decrease in skeletal muscle fibre size and fibre number, as well as impaired myofibre contractile performance, are well characterised in ageing and underlie the deficiencies in skeletal muscle strength and physical performance seen in the elderly. However, the cellular and molecular changes that are known to promote such deficiencies in myofibre performance are manifold, and many questions remain to be answered as to their origins and relative importance in sarcopenia. The factors that are most often considered include altered satellite cell (SC) function, neuromuscular junction (NMJ) signalling deficits, a decline in neural and myofibre mitochondrial health, insulin resistance, inadequate nutrition, and a decline in physical activity. Often the interdependence of these events is understated, and it is not fully known which of these factors represent a primary pathophysiological insult in ageing and which occur secondarily as sarcopenia advances. The issue of SC function in ageing is of particular importance to the *in vitro* primary human myogenesis (the formation of skeletal myofibres) model that we employ in the experiments detailed in this thesis.

Adult SC physiology is considered further in Section 1.6.3.1, while our experimental model is appraised in Chapter 7.

Inflammation is increasingly considered to be part of the molecular aetiology of sarcopenia. Indeed, a profound inflammatory burden is well established as a major contributor to the cachexia of chronic illness (22). Ageing is often accompanied by chronic low-grade inflammation, referred to as “inflammaging” (23). Plasma c-reactive protein (CRP) levels, a biomarker of generalised systemic inflammation, increase with age (24). Cross-sectional and prospective studies of elderly populations have positively associated high systemic levels of pro-inflammatory cytokines (e.g. Tumour necrosis factor alpha [TNFα] and interleukin 6 [IL-6]) with reduced skeletal muscle mass (25–27). Furthermore, total adipose tissue mass and visceral adipose tissue (VAT) mass are negatively correlated with skeletal muscle mass (28,29).

Importantly, given the frequent co-existence of sarcopenia with adipose tissue accumulation, obesity is independently associated with inflammation (30–33). Adipose tissue is an endocrine organ that secretes pro-inflammatory cytokines, known as adipokines (34). Adipokines have distal effects on many tissues, including skeletal muscle (35) and are implicated in the pathology of diabetes, cardiovascular disease and cognitive decline (36–38). Intra-abdominal visceral adipose tissue (VAT) is in particular associated with an increased risk of the adverse cardiovascular and metabolic outcomes that are often attributed to adipokines (39,40). Indeed VAT is a prolific secretor of pro-inflammatory cytokines (41), and ageing is associated with increased VAT mass (2,5,42). Subcutaneous adipose tissue (SAT) mass does not appear to increase with age in men for a given body

mass index (BMI) (43), nor did it increase in a 2 yr prospective study of elderly African American women despite significant VAT gain (2). However, when younger and older cohorts are not BMI matched, an absolute gain of SAT in conjunction with increased body weight and BMI is evident with advancing age (44). Despite the prominent attention that VAT receives as a secretor of adipokines, SAT also secretes pro-inflammatory adipokines to a lesser extent (45–47). Furthermore, VAT represents a small proportion of total adipose tissue mass (12.7 % in endurance runners aged 27 to 69 yr (48)). Thus, SAT may be underappreciated as a contributor to the systemic inflammatory burden. Intramuscular adipose tissue (IMAT) mass also increases with age (42). However, little is known about adipokine secretion by IMAT due to the technical difficulties associated with its biopsy in adequate quantities for study.

Recent work demonstrated an atrophic effect of adipokines secreted by adipocytes on human fetal myotubes (47). Myotube atrophy was most pronounced in adipocytes derived from VAT. However, the stromal vascular fraction (SVF) of adipose tissue, which includes preadipocytes and macrophages, is a more prolific secretor of pro-inflammatory cytokines than mature adipocytes (46). Consequently, adipokine secretion by human adipose tissue – not just that by adipocytes – must be characterised and its effect on aged human myofibre size and function determined. It must also be ascertained whether obesity alters the adipose tissue inflammatory milieu and the effects of these milieux on skeletal muscle detailed. Furthermore, there exists a need to identify which adipokine constituents of such milieux are most important with regard to skeletal myofibre size and function in ageing. TNFα and IL-6 are well studied in this respect (Section 1.7.2, 1.7.3). However, the effects

of other adipokines (e.g. resistin, leptin and visfatin) on myofibre size, development and function are not well characterised.

Additionally, it is important to identify more avenues for the development of pharmacological therapeutic interventions in sarcopenia. To that end, interleukin 15 (IL-15) is a myokine that is known to promote the formation of C2C12 myotubes in culture and can prevent the loss of skeletal muscle mass in mice exposed to systemic inflammation (49,50). It has been proposed that IL-15 is expressed by skeletal muscle in response to such pro-atrophic environments, to mitigate the loss of skeletal muscle mass (51). However, the effect of IL-15 on human myofibre development, maintenance and hypertrophy remains unexplored. Furthermore, given the high concentrations of recombinant IL-15 (rIL-15) used in such studies, it is unclear whether autocrine skeletal muscle IL-15 secretion is necessary for adult myofibre maintenance and hypertrophy (50,52). If the pro-myogenic and hypertrophic actions of IL-15 are preserved in the skeletal muscle of elderly human participants, then IL-15 signalling may represent a promising therapeutic target in sarcopenia and cachexia.

1.2 Skeletal Muscle Development, Structure and Function

Skeletal muscle is a contractile tissue that facilitates voluntary movement and protects the viscera. Such movement encompasses gross and fine motor actions as well as the voluntary control of the urinary and gastrointestinal systems. Skeletal muscle has other important functions, e.g. as the body's principal site for insulin-stimulated glucose

disposal and as an endocrine tissue (53). While myofibres – which are discussed in detail below – make up the preponderance of cells within skeletal muscle, other cells exist within the tissue. These include perineural cells and neurons as well as cells that make up the skeletal muscle vasculature, including pericytes, adventitial cells, endothelial cells and smooth muscle cells (54). Under normal physiological conditions few resident immune cells are present and are limited to a sparse population of macrophages (55). Importantly, fibroblasts also exist between myofibres. Fibroblasts are the principal producers of skeletal muscle extracellular matrix which provides skeletal muscle with stability and facilitates force transmission (56).

The principal cellular units of skeletal muscle are the multinucleate muscle fibres termed myofibres. Embryonically, skeletal muscle originates from the mesoderm, with most muscles being derived from somite budding of the paraxial trunk mesoderm (57). The embryonic myogenic program is similar to that of adult myogenesis (Section 1.6.3.1). The molecular markers that define embryonic muscle precursor cells are broadly the same as those in adult SC, namely paired box protein 3 (PAX3) and paired box protein 7 (PAX7), although the differentiation of some non-somite derived head and neck muscles is initially defined by alternative transcription factors, e.g. Paired-like homeodomain transcription factor 2 (PITX2) and T-box transcription factor 1 (TBX1) (57). The myogenic regulatory transcription factors (MRFs) – Myogenic factor 5 (MYF5), MyoD, myogenin and myogenic regulatory factor 4 (MRF4) – are the master regulators of both adult and embryonic myogenesis, regardless of skeletal muscle mesodermic origin. MYF5 expression generally precedes that of MyoD and MRF4 as embryonic or

adult precursor cells commit to their myogenic lineage as myoblasts. However, the sequence of their expression varies with anatomical location (57). Myogenin expression occurs last, coinciding with the fusion of myoblasts to form terminally differentiated multinucleated myofibres. There exists a certain amount of functional redundancy in the embryonic expression of the MRFs, with severe defects in muscle formation occurring only in myogenin deficient mice (58) or those with combined MYF5, MyoD and MRF4 gene knockouts (59,60).

Mature adult myofibres are long, cylindrical, multinucleate structures of a diameter ~ 10 – 100 μm and they extend from millimetres to centimetres in length (61). Individual muscle fibres, groups of fibres and each muscle are ensheathed by connective tissue layers termed the endomysium, perimysium and epimysium respectively (Fig. 1.1). The myofibre membrane is referred to as the sarcolemma, and the cytoplasm is termed the sarcoplasm. Myonuclei exist in the sarcoplasm, between the sarcolemma and the contractile apparatus of the myofibre – the myofibrils. Myofibrils comprise repeated arrangements of contractile units called sarcomeres that are visible by electron microscopy. This arrangement is responsible for the striated appearance of skeletal muscle (Fig. 1.1). The A and I bands correspond to the contractile thick (myosin) and thin filaments respectively (Fig.1.1). An electro-dense line, the Z disc is evident in the I band, representing the area of the sarcomere where α-actinin anchors actin filaments and desmin envelops the myofibrils, binding them together. Titin forms intermediate filaments that span the sarcomere and connects the myosin thick filaments to the Z disc (61). Such filaments are untethered in the I band region of the sarcomere, conferring a

passive resistance to stretching and elastic recoil properties on the muscle (62).

The thick myosin filaments comprise multiple (~ 200) myosin molecules. Each myosin molecule is formed by two heavy polypeptide chains and four light chains. The heavy chains are wrapped around one another, and their heads at one end are folded to form the myosin heads. Two light chains are associated with each myosin head to form the complete myosin molecule. Myosin molecules are bundled together to form myosin filaments, with myosin heads and a portion of the molecule called the arm protruding from the side of the filament to facilitate myosin interaction with the thin filaments (Fig. 1.1). Thin filaments comprise a double-stranded F-actin molecule. The strands are composed of polymerised G-actin molecules, each of which binds one molecule of ADP, forming the so-called active sites for muscle contraction. Tropomyosin molecules are wrapped around each F-actin helix and troponin molecules (complexes of troponin I, T and C subunits) are attached to the side of thin filament (62).

Human muscle fibres are commonly subdivided into ‘slow’ type I fibres and ‘fast’ type IIa and IIb fibres. Different isoforms of myosin exist and such fibres may be molecularly defined by their predominant isoform of myosin and the speed of that isoform’s ATPase activity. Type I fibres display slower contraction and relaxation speeds as well as power outputs than type II fibres but display greater endurance and reliance on oxidative rather than glycolytic metabolism. Type IIa fibres are considered to be an intermediate between type I and type IIb fibres with respect to these measures (63).

1.3 The Neuromuscular Junction and Myofibre Depolarisation

The NMJ consists of the terminal end of a lower motor neuron (LMN), a synaptic cleft and the skeletal muscle fibre that it innervates. The motor neurons contain the neurotransmitter acetylcholine within synaptic vesicles (Fig. 1.2). Depolarisation of the neuron induces the release of synaptic vesicles into the synaptic cleft where they bind to sarcolemmal acetylcholine-gated ion channels (receptors). The acetylcholine channels allow the flux of positive ions, principally the influx of sodium (Na+) ions, thus depolarising the sarcolemma. Sarcolemmal invaginations – the transverse tubules – extend throughout the muscle fibre to facilitate the spread of an action potential throughout the large cell. A specialised endoplasmic reticulum, termed the sarcoplasmic reticulum (SR) extends in a network between the myofibrils, and comes into contact with the transverse tubules to form triads of tubules sandwiched between SR cisternae (Fig. 1.2). The SR contains calcium-ATPase pumps that transport calcium into their cisternae, where they are sequestered by the protein calsequestrin. Transverse tubule depolarization causes SR calcium channels to open, releasing calcium into the sarcoplasm where it facilitates skeletal muscle contraction (62).

1.4 Skeletal Muscle Contraction

The sliding filament mechanism describes the interaction between myosin heads and the active sites of thin filaments to form myosin-actin cross bridges that initiate sarcomeric contraction. Myosin heads cleave adenosine triphosphate (ATP), and retain

the adenosine diphosphate (ADP) and phosphate ion products while storing the energy produced by extension of its head. Calcium ions bind to the troponin-tropomyosin complex, uncovering the active sites, thus facilitating the binding of the myosin head. The head tilts on binding, initiating a power stroke facilitated by the earlier cleavage of ATP. ADP and phosphate then detach from the head, allowing its detachment from the active site (62).

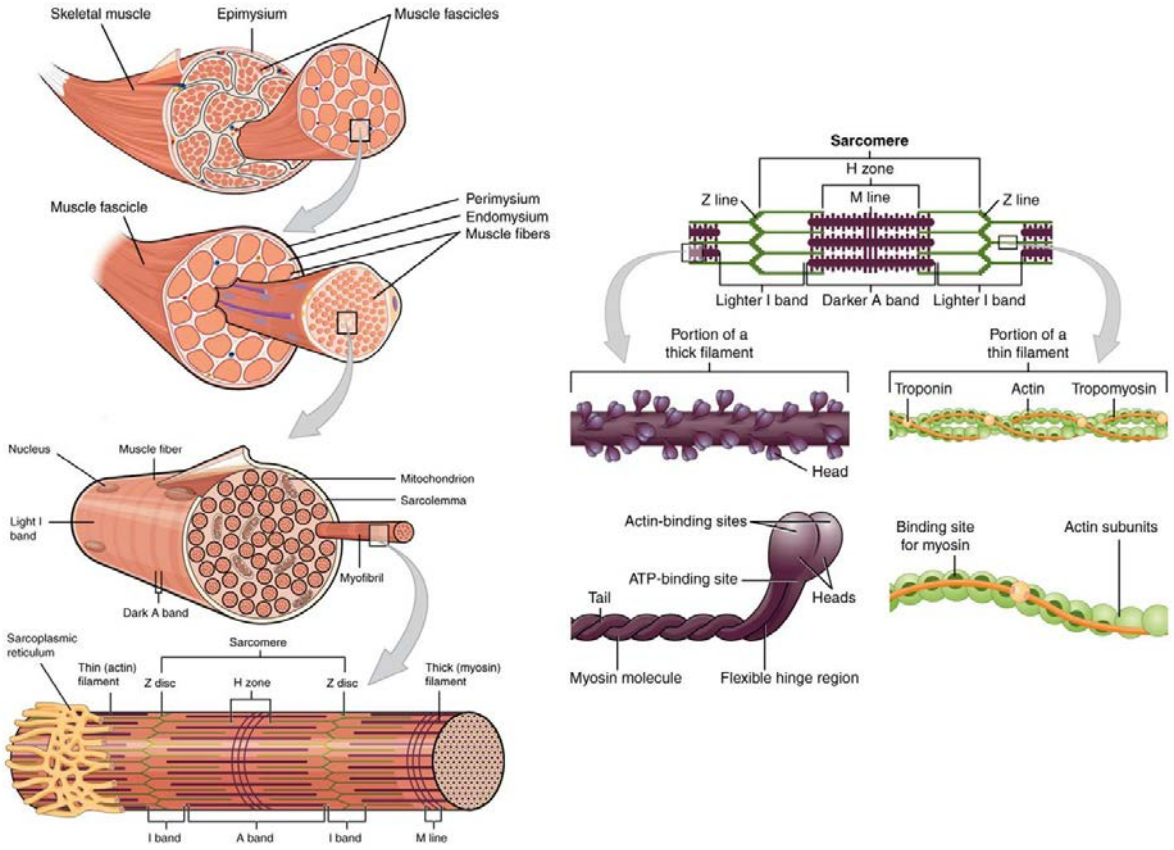

Figure 1.1. Skeletal Muscle Gross and Cellular Structure. Adapted from: OpenStax, Anatomy & Physiology. OpenStax CNX. Feb 26, 2016, under a Creative Commons Attribution License 4.0.

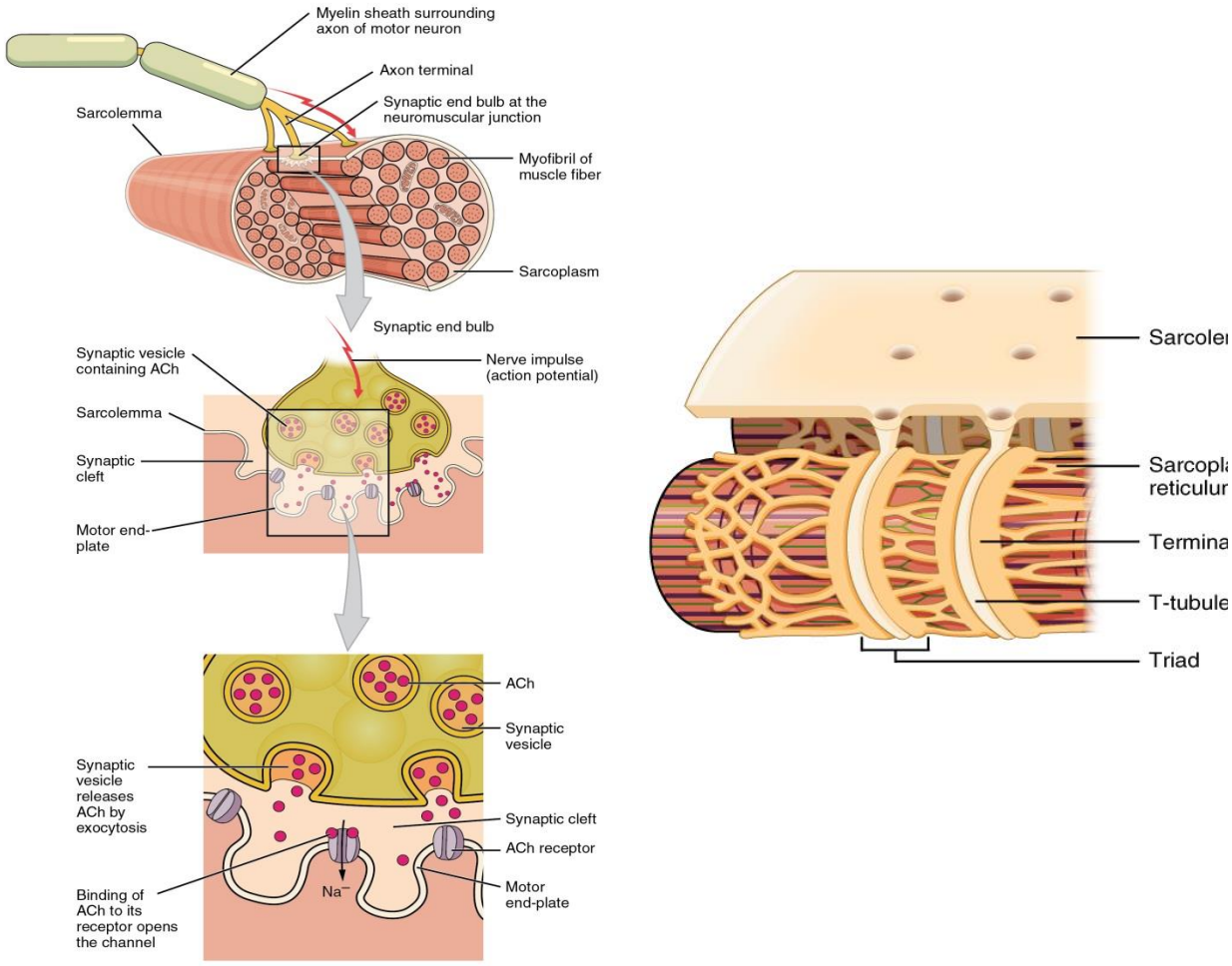

Figure 1.2. Lower Motor Neuron and Skeletal Muscle Fibre Structure and Function. Adapted from: OpenStax, Anatomy & Physiology. OpenStax CNX. Feb 26, 2016, under a Creative Commons Attribution License 4.0.

1.5 Primary Myogenic Cultures

The isolation of SC, their culture as myoblasts and differentiation of such myoblasts to form surrogate *in vitro* myofibres – termed myotubes – has been extensively used in the exploration of skeletal muscle biology (64–70). The immortalised murine myoblast C2C12 cell line was developed by Blau et al. (71), building on the work of Yaffe and

Saxel (72) and it is widely used (64–68). Its suitability for the study of skeletal myofibre physiology is often justified by observations that C2C12 form multinucleated contractile cells that contain sarcomeres and express myosin and actin (64,71,73). The C2C12 cell line is convenient as it is immortal. However, its relevance to human myofibre physiology remains underexplored. Indeed, the expression of genes involved in myogenesis varies in both timing and magnitude between primary human and primary mouse myogenic cultures (74). C2C12 cells could suffer from the additional problem of accumulating chromosomal alterations and epigenetic changes with extended passaging, thus altering their phenotype further from that of both murine and human primary myoblasts (75).

Some evidence exists to suggest that primary human myogenic cultures retain at least some of the characteristics of their donors. Such myotubes retain the insulin resistance of their donors (76), display increased palmitate oxidation rates (77) and fail to upregulate adiponectin receptor gene expression in response to globular adiponectin or leptin (78). Myotubes from obese participants accumulate lipid (79) and do not increase their expression of cytochrome c Oxidase Subunit IV (COXIV) in response to leptin stimulation, unlike myotubes from their lean counterparts (78). However, primary human myotubes are not a perfect surrogate for *in vivo* myofibres. They express immature, fetal forms of myosin heavy chain (MHC) (80), generate smaller contractile forces than intact skeletal muscles (81–83) and handle intracellular calcium differently from mature skeletal muscle (84).

Methods of SC isolation and myogenic culture conditions vary. No evidence exists to

suggest which protocol produces the most physiologically relevant myotubes. One priority in SC isolation is obtaining cultures that are free from the most likely contaminating cell type – fibroblasts (69,70). Fluorescence-activated cell sorting (FACS), or magnetic activated cell sorting (MACS) can be used to select cells based on a SC marker, e.g. α7 integrin (70) or CD56 (69). Alternatively, skeletal muscle digests can be pre-plated in an uncoated culture vessel to which fibroblasts preferentially adhere, before moving the cell suspension to a coated (e.g. 0.2 % gelatin) flask (73,85). This approach, combined with differential trypsinisation at passages one and two has consistently produced cultures in our lab that are negative for the fibroblast marker TE7 (86) (see Section 2.4.1).

Myoblast growth and differentiation conditions vary in terms of the media used. Firstly, the media can be divided into high and low glucose base media. Base media that promote the rapid expansion and differentiation of cultures are often high in glucose, e.g. Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12, 3.1 g/L glucose) (87) or high glucose DMEM (4.5 g/L glucose) (83). These glucose concentrations far exceed the fasting blood glucose of a non-diabetic individual (88). Indeed, in rodent L6 myotubes, 4.5 g/L glucose in combination with insulin (an additive often included in primary human myogenic culture media (69,87)), induces myotube insulin resistance (89). Some media that are used for the culture of human myogenic cells are supplemented with additional growth factors e.g. epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) to facilitate their rapid expansion (87). The concentrations of EGF and bFGF used (10 ng/mL and 1 ng/mL respectively in a

commercially available formulation from PromoCell, Heidelberg, Germany (69)) exceed normal serum concentrations approximately 10-fold (90,91). We are unaware of any comparison of primary human myoblast culture and differentiation media to determine which conditions produce the most physiologically relevant myotubes. In the absence of such evidence, we employ a low glucose (1.1 g/L) base medium (Ham's F10) supplemented with 20 % fetal bovine serum (FBS) for myoblast growth or 6 % horse serum to induce myoblast differentiation.

1.6 Sarcopenia

The decline in skeletal muscle mass and quality in sarcopenia has been attributed to several important changes in skeletal muscle fibre size, type and distribution. Here, the performance of sarcopenic skeletal muscle is first described. The myofibre changes that underlie such alterations in skeletal muscle performance are then considered in detail. Finally, the cellular and molecular events underpinning such changes in fibre performance are elaborated upon. The major known aetiological factors underpinning sarcopenia are summarized in Fig. 1.3.

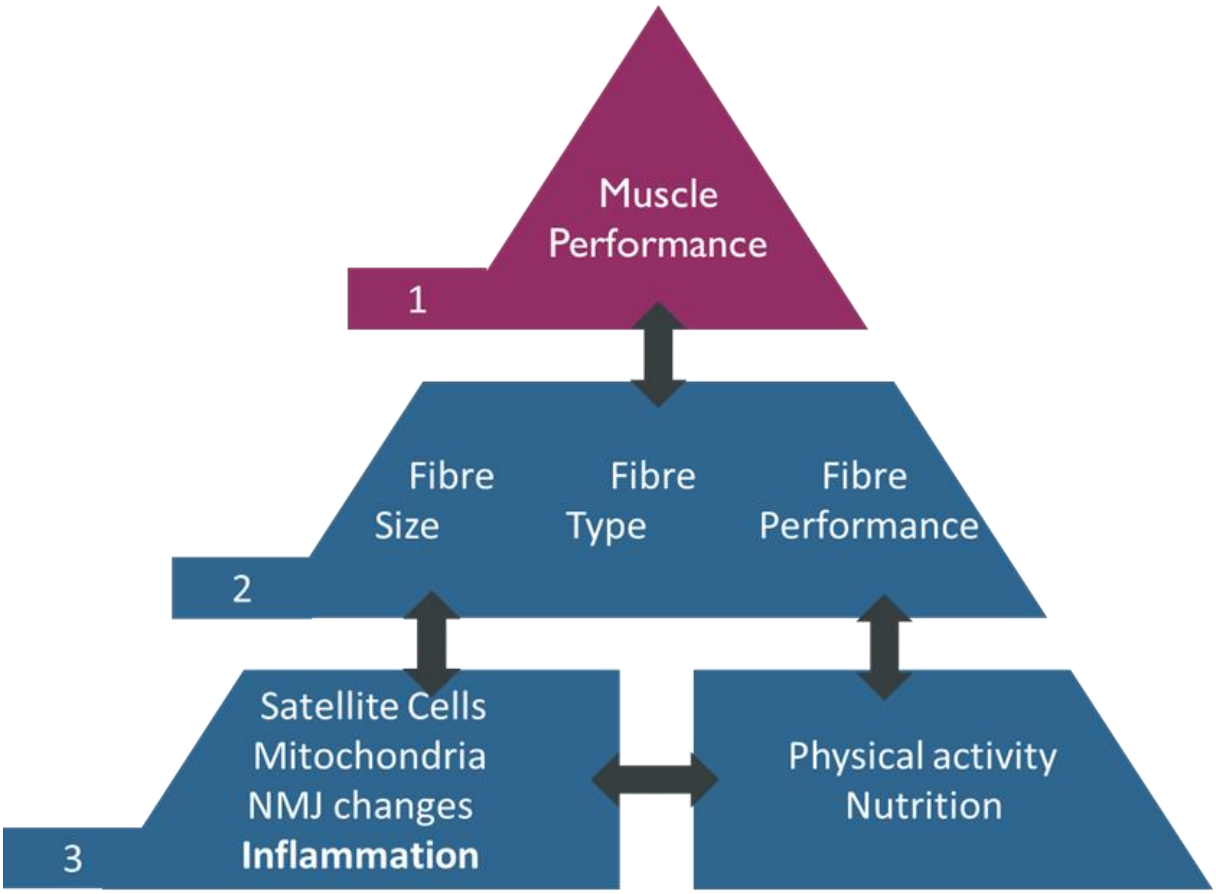

Figure 1.3. Sarcopenia is a multifactorial phenomenon with many aetiological factors underpinning the decline in skeletal muscle performance. Skeletal muscle fibre alterations (level 2) and cellular and molecular changes (level 3) underpin the loss of skeletal muscle mass and quality observed in sarcopenia. Many of these changes are interdependent. NMJ = neuromuscular junction.

1.6.1 Level 1: Skeletal Muscle Performance in Sarcopenia

The presence of low muscle mass in addition to reduced muscle strength or impaired physical performance is required for the diagnosis of sarcopenia (6). The methods commonly used to measure such outcomes are summarised in Table 1.1. The EWGSOP guidelines summarise the diagnostic cut off points that have been used for these

outcomes in large studies of sarcopenia. However, no firm cut-off points were proposed by the working group, and the guidelines cite the need to establish firm reference values across ethnic groups (6). Poor skeletal muscle performance has important effects on activity in ageing. Low muscle mass (92,93) and poor muscle strength (92,94) predict a loss of physical independence. Prospective studies of sarcopenic individuals have reported a significant increase in the incidence of falls (12,95) and fractures (95,96), although co-existent low bone mineral density might be necessary to increase fracture risk (96).

Sarcopenia is also independently associated with pathology at sites distinct from skeletal muscle itself, e.g. diabetes (97), cardiovascular disease (10), osteoarthritis (98) and cognitive impairment (99). A detailed discussion of the pathophysiological link between sarcopenia and these conditions is outside the scope of this thesis. The burden of such medical complications likely explains the link between sarcopenia and increased cancer (100,101) and postoperative (102,103) mortality.

Table 1.1. Assessment of Muscle Mass, Muscle Strength and Physical Performance for the Diagnosis of Sarcopenia.

Variable	Measurement
Muscle mass	CT MRI DXA BIA Total/partial body potassium per fat-free soft tissue Anthropometry
Muscle strength	Handgrip strength Knee extension/flexion Peak expiratory flow
Physical performance	SPPB Gait speed Timed get-up-and-go test Stair climb power test

CT = Computed tomography, MRI = Magnetic resonance imaging, DXA = Dual-energy X-ray absorptiometry, BIA = bioimpedance analysis, SPPB = short physical performance battery. Adapted from (6).

1.6.2 Level 2: Skeletal Muscle Fibres in Sarcopenia

1.6.2.1 Skeletal Muscle Fibre Size in Sarcopenia

It is now well established that ageing is typified by a reduction in the size of individual skeletal muscle fibres, with the type II, so-called fast twitch fibres being most severely affected (104). Indeed, Lexell and Taylor showed that the mean vastus lateralis (VL) cross-sectional area of type I fibres did differ significantly in men aged 69-85 compared to young controls aged 19-35; type II fibres were ~ 35 % smaller in an elderly cohort (105). Many studies have reported similar findings (Table 1.2). The VL is by far the most studied muscle in this regard, perhaps due to its functional importance in ageing as a major knee extensor

and as a byproduct of its accessibility for muscle biopsy. Very few studies have explored the effect of ageing on skeletal muscle fibre size in other muscles, yet there is some indication that the reduction in type I fibre size may be more variable outside the VL, with no decline observed in the tibialis anterior by Jakobsson et al. (106) and a profound decline (21%) described in the biceps brachii by Monemi et al. (107).

Table 1.2. Percentage Changes in Skeletal Muscle Fibre Size in Elderly Cohorts Compared to Young Controls. Adapted from Lexell J Gerontol A Biol Sci Med Sci. 1995 with the addition of selected contemporary literature.

Reference	Sex	Elderly Age Range	Muscle	Type I Fibres (%)	Type II Fibres (%)
Tomonaga 1977	M/F	60-90	VL	-7	-52
Larsson, 1978	M	*	VL	-1	-25
Scelsi,1980	M/F	65-89	VL	-7	-24
Essén-Gustavsson, 1986	M	70	VL	-15	-19
	F	70	VL	-25	-45
Lexell, 1991	M	69-84	VL	-6	-35
Jakobsson, 1990	M/F	66-77	TA	0	-30
Monemi 1998 (diameter)	M	58-83	Mas.	-13	-26
			BB	-21^	-32^
Verdijk 2007	M	76±1	VL	n.s.	-27
Nilwik 2013	M	71±1	VL	n.s.	-30
Kramer 2017	F	79±2	VL	n.s.	-22

Fibre size measured as cross-sectional area unless otherwise stated. ^Denotes fibre diameter measurement. VL = vastus lateralis, TA = tibialis anterior, Mas. = masseter, BB = Biceps brachii, n.s. = non-significant difference vs controls. Where no age range is detailed, mean ± SEM age are quoted.

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1.6.2.2 Skeletal Muscle Fibre Number in Sarcopenia

Studies of muscle fibre changes with age demonstrate reductions in total muscle cross-

sectional area (CSA) which exceed those which could be accounted for by reductions in muscle fibre area alone; in one study, 80-yr-olds displayed a ~ 33 % reduction in soleus CSA compared to young controls, but a reduction of only ~ 20 % in mean muscle fibre CSA (108). One possible explanation for such a discrepancy is a reduction in absolute fibre number. Indeed it was in the vocal muscles (109) and the pectoralis minor (110) that a reduction in fibre number was first demonstrated, with the numbers of type I fibres being retained until a more advanced age than type II fibres which declined in number only beyond 60 yr in the pectoralis minor and 80 yr in the vocal fold. Subsequent studies by Lexell on whole cadaveric VL showed that ageing is associated with a loss of total fibre number and that fibre number correlates more strongly with muscle area than does mean fibre area, again highlighting the importance of fibre loss in ageing (111,112). Furthermore, preferential loss of type II fibres, distinct from alterations in their size has emerged as an age-related phenomenon since it was first described (109,110). LMN are lost with advancing age, with those innervating type II fibres being disproportionally affected (113). This phenomenon is discussed further in Section 1.6.3.3.

1.6.2.3 Skeletal Muscle Fibre Contraction in Sarcopenia

The decline in individual fibre performance represents an additional potential mechanism underlying age-related declines in skeletal muscle performance. Isolated fibres from old individuals appear to have reduced shortening velocity (114,115) and force production (115,116) in both type I and type IIa fibres compared to those of young controls, although no age-related difference in CSA-normalised fibre performance has been shown in

sedentary populations (117). An age-associated decline in MHC concentration within individual fibres (regardless of their fibre type) is thought to underlie reductions in their force production (115). It has been suggested (118) that age-related changes in fibre shortening velocity are due to myosin molecule defects that produce a slower myosin-actin sliding speed (119). Isolated skeletal muscle fibre performance is modifiable in old age. One year of resistance exercise training in elderly women increased the specific force of type I and type IIa fibres, as well as the shortening velocity of type I fibres, even in the absence of an increase in fibre CSA (118). Immobilisation adversely affects the shortening velocity of elderly muscle fibres (115).

It is clear from the preceding discussion that sarcopenia is characterised by changes in skeletal muscle fibre number, size, distribution of type and individual fibre performance. However, skeletal muscle exists in proximity to other tissues that alter its mechanical function. Adipose tissue mass has generally been found to increase in parallel with sarcopenia, a phenomenon termed sarcopenic obesity (3,4), and ageing is in particular associated with increased IMAT e.g. frail elderly have increased quantities of IMAT, and decreased lean body mass compared to their age-matched non-frail counterparts (120). In addition to its proposed inflammatory and atrophic effects (Section 1.7.1), IMAT is increasingly thought to mechanically influence skeletal muscle function. Greater accumulation of IMAT is associated with poorer physical performance (121,122), and a recent finite element analysis model of human skeletal muscle concluded that accumulation of IMAT reduces skeletal muscle mechanical performance by altering muscle stiffness (123). Pennation angle is the angle at which skeletal muscle fibres insert

into a tendon relative to the longitudinal axis of a muscle (124), and this is an additional feature of muscle architecture that may influence skeletal muscle performance in the elderly. Pennate muscle arrangements allow a greater number of fibres to exist within a given muscle volume, at the expense of force production along the longitudinal axis of a muscle (124). The elderly have reduced pennation angles within the VL compared to their young counterparts (125), perhaps in part due to the age-associated reductions in fibre number and area previously discussed. 12 wk of ski training in elderly participants significantly increased VL pennation angle and induced a 7.1 % increase in VL thickness as measured by ultrasound (126). In one study, obese elderly women had a significantly increased pennation angle in the major knee extensor muscles, yet they displayed significantly lower knee extension torque normalised to muscle CSA (~ 25 % of normal weight controls) (127). Indeed, the accumulation of IMAT described in the obese participants may represent an underlying cause of the increased muscle pennation angles (123). This, coupled with an insufficient compensatory increase in muscle fibre CSA, may compromise longitudinal muscle force production.

1.6.3 Level 3: Cellular and Molecular Determinants of Skeletal Myofibre Performance in Ageing

Having discussed how myofibre changes underpin an age-related decline in skeletal muscle performance, an overview of the important determinants of such changes is necessary. The inflammatory drivers of sarcopenia are the principal focus of this thesis and are considered in detail in Section 1.7. However, our current understanding is of

sarcopenia as a multifactorial pathophysiological process, in which the aetiological factors that are often cited as underlying a decline in myofibre and skeletal muscle performance span a continuum from the molecular to the integrated physiological. Indeed, as our understanding of the causes of sarcopenia is incomplete, it is often difficult to appreciate which factors represent the primary pathophysiological insult and which are merely aggravators of sarcopenia. In addition to inflammation, the factors that are most often considered include altered SC function, neuromuscular pathophysiology, a decline in mitochondrial health, inadequate nutrition, and a decline in physical activity. They are considered here, with some attempt being made to infer their importance and interdependence as events in the development of sarcopenia. Their relationship to inflammaging is also considered. The accumulation of intramyocellular lipid, insulin resistance (IR) and their relationship to sarcopenia are considered briefly in a later section.

1.6.3.1 Skeletal Muscle Satellite Cells and Adult Myogenesis in Sarcopenia

SC are muscle precursor cells and exist in a niche between the basal lamina and the sarcolemma of myofibres (128). They are self-renewing and can give rise to differentiated myofibres. Thus SC meet the fundamental definition of a stem cell (129).

Quiescent SC express high levels of the transcription factor Pax7 (130,131) and in some muscles Pax3 (132). Pax7 is essential for normal postnatal skeletal muscle development (130). SC proliferate upon activation and daughter cells either commit to a myogenic lineage as myoblasts or retain high levels of Pax7 expression and renew the SC pool. Those SC committed to myogenesis – the proliferating myoblasts – express MyoD and

MYF5. Expression of the differentiation factors myogenin and MRF4 is necessary for myoblasts to fuse to each other and to existing myofibres, thus forming mature, multi-nucleated myotubes (129).

SC are required for optimal hypertrophic responses of skeletal muscle to stimuli and are essential for muscle repair. SC are conditionally depleted by > 90% in tamoxifen-treated Pax7-DTA mice (133). When synergist muscles are removed, no difference in the degree of initial plantaris hypertrophy is observed compared to vehicle-treated mice (134). However, the hypertrophic response to synergist muscle removal plateaus beyond two weeks, with vehicle-treated mice displaying a 1.5 times greater increase in muscle size than the SC-depleted counterparts after eight weeks (133). Furthermore, it has previously been thought that SC remain quiescent in their niche until skeletal muscle is exposed to a challenge e.g. exercise or trauma. However recent murine evidence challenges this paradigm with 20 % of hindlimb myofibres containing new tdTomato-labelled SC after a 2 wk lineage-labelling period (135).

The myonuclear domain (MND) – the volume of cytoplasm regulated by each nucleus in an individual myofibre (136) – increased by 32 % in tamoxifen-treated Pax7-DTA mice over their controls up to 2 wk (134). Unfortunately, the MND was not measured in the follow-up study, but an inability to expand the domain further may in part explain the importance of SC abundance to long-term skeletal muscle hypertrophy (133). Plantaris hypertrophy in response to synergist removal was similar in young (five months) and aged (25 months) Wistar rats. Yet, the aged rats had 26 % more myonuclei per unit of fibre length compared to their younger counterparts. Myonuclear accretion appears to be

particularly important in sustaining skeletal muscle hypertrophy in ageing. The importance of SC to skeletal muscle repair is further underlined by murine studies of SC depletion, in which muscle regeneration in response to cardiotoxin-induced skeletal muscle injury is severely impaired (137–139).

Having established that SC are important in murine skeletal muscle regeneration, maintenance and hypertrophy, it is plausible that any impairment of their function in human ageing may contribute to sarcopenia. The number of SC within human skeletal muscle decreases with age, specifically within the atrophy-prone type II muscle fibres (140–142). Animal studies have suggested that SC proliferative capacity is impaired with age (143–145). Under standard culture conditions, myoblasts derived from young and old participants display no differences in terms of growth and differentiation capacity (146). The culture of myoblasts from young participants in medium supplemented with elderly serum does not affect their desmin expression or differentiation to myotubes (147). However, culture of myoblasts from elderly participants in autologous serum adversely affects their differentiation to myotubes (148). This literature should be interpreted with caution as primary human myotubes were cultured in a variety of media and it is unclear which conditions produce the most physiologically representative myotubes (see Section 1.5). Pertinently, given the alterations in MND observed in SC-depleted mice and in aged rats (133,137–139,149), changes in MND have also been seen in the skeletal muscle fibres of elderly human participants. In the atrophy-prone type IIa fibres of the VL in healthy, sedentary elderly men and women, both nuclear numbers and MND are significantly decreased (Nuclear number, 27%; MND, 37%) (136). Only nuclear number

appears to be significantly diminished in type I fibres (136).

The mechanisms underlying the loss of SC in aged skeletal muscle are unclear and their discovery is hampered by the requirement to remove SC from their niche in order for a comprehensive study to take place. Such removal forces SC to differentiate into myoblasts, which are not SC, but an intermediate cell in the development of myofibres (150). As such, assertions that increased reactive oxygen species (ROS) production (151,152) and reduced antioxidant activity in myoblasts derived from elderly participants (152,153) are representative of SC physiology are inaccurate. However, the idea that mitochondrial and metabolic dysfunction might underlie the loss of SC activity in ageing has merit, particularly given the evidence of mitochondrial-driven skeletal muscle loss in mature myofibres (Section 1.6.3.2).

The reduction in skeletal muscle SC content appears to be only partially reversible with increased physical activity. In young participants, a single bout of resistance training increases VL SC in type I and type II fibres at 48 h post-exercise (154). In the elderly, resistance training increases SC content in type I fibres at 48 h (155,156) but type II fibre SC content is not increased at this time point (155,156), increasing only at 72 h and remaining significantly (14 %) lower than the SC numbers observed in the young (155). 12 wk resistance training studies in the elderly have shown that SC content increases only in type II fibres over the longer term (17,142) and remains ~ 25 % lower than values seen in healthy, untrained young adults (142).

Endurance exercise for increasing skeletal muscle SC content in the elderly is less well studied. A single bout of endurance training did not increase type I or type II fibre SC

numbers in sedentary older men (156). However, 10 d of increased training load in endurance-trained young individuals may increase type II myofibre SC content (35 ± 28%) as well as myonuclear density (16 ± 6 %), within type II myofibres (157). Contrastingly, 10 wk of cycle training in untrained young men increased type II fibre SC content (158). In a study of elderly lifelong endurance runners, type II fibres were smaller and had fewer SC than the fibres of their young active controls. However, lifelong endurance running preserved type II fibre SC number relative to fibre area, a phenomenon not seen in sedentary elderly (159).

It is clear from the above discussion that skeletal myofibres are not static entities with a fixed number of myonuclei that simply increase and decrease their myofibrillar content over time. They are dynamic cells, in which SC activation and myoblast fusion play an important role over the course of an adult lifespan. Primary human myogenic cultures (see Section 1.5) offer a useful, if imperfect *in vitro* model for examining such physiology in the context of adipose-derived inflammation.

1.6.3.2 Myofibre Mitochondrial Dysfunction and Sarcopenia

Outside of the SC niche, within mature skeletal muscle fibres, mitochondrial deficits may contribute to declining muscle function and mass with age. Such deficits have been suggested to include respiratory chain deficiencies, enhanced ROS production and mitochondria-driven skeletal muscle apoptosis (160,161). Mitochondrial mass is decreased in sedentary older adults, but habitual physical activity preserves it (162,163), along with the expression of peroxisome proliferator-activated receptor gamma

coactivator 1-alpha (PCG-1α) (163,164), the so-called ‘master regulator of mitochondrial biogenesis’ (161).

Skeletal muscle mitochondrial DNA content declines with age, with substantial deletions being most commonly described. It is thought that these deletions are inherited or acquired in early life, with their burden increasing throughout the lifespan by clonal expansion. This results in a mixture of respiratory deficient affected (~ 5-14 %) and unaffected skeletal muscle fibres (161,165). However, only 5 % of these abnormal fibres are atrophied in healthy older adults (166). It is unclear whether respiratory deficient fibres are functionally affected in terms of fatigability or force production, although respiratory complex IV deficiency in mice does produce a progressive functional decline (167).

Older studies of rodents, in which mitochondria were mechanically isolated, suggested that ageing is associated with an increase in skeletal muscle ROS production (168–170). However, recent evidence from saponin-permeabilised fibres suggests that this method of mitochondrial isolation exaggerates the difference in skeletal muscle mitochondrial function between young and old animals (171). Increased ROS production is not consistently demonstrated in permeabilised fibre bundles from aged rodents (171,172), and skeletal muscle ROS production does not appear to be predictive of the degree of atrophy within individual rodent muscles (172). Furthermore, a recent human study found no difference in such ROS production in the skeletal muscle of very old (> 75 yr) participants compared to younger elderly (60-75 yr) participants, despite the very old having smaller VL myofibres (173).

Myofibres isolated from the VL of physically active elderly men have enhanced

mitochondrial-derived markers of apoptosis, e.g. 3-fold increase in endonuclease G compared to their young counterparts (174) and aged human mitochondria are more sensitivity to permeability transition, which facilitates the release of mitochondrial pro-apoptotic proteins (173,174). It is therefore plausible that sarcopenia may in part be mediated by apoptosis. Indeed, mitochondria-driven skeletal muscle apoptosis is active in aged rats and correlates with the progression of sarcopenia (175), although direct evidence of nuclear apoptosis leading to skeletal muscle fibre atrophy or loss in human muscle is lacking (160). It would appear that this feature of sarcopenia is not amenable to reversal by physical activity (173,174).

It is difficult to say with any certainty whether the mitochondrial deficits in skeletal myofibres that are described here are primary events in the development of sarcopenia. Given that physical activity supported by appropriate nutrition (Section 1.6.4.1) is the only intervention known to ameliorate sarcopenia, the susceptibility of physically active elderly to LMN loss and mitochondria-derived apoptosis must be considered a significant indicator of their importance in sarcopenia. It is also challenging to place this mitochondrial pathophysiology in the context of inflammaging. If such deficits represent primary events in sarcopenia, it is possible that they might, in fact, contribute to the burden of inflammation. For example, mitochondrial damage-associated molecular patterns (DAMPs), released from damaged mitochondria stimulate the innate immune system (176). However, adipokines might also be detrimental to mitochondrial function. This is discussed further in Section 1.7.

1.6.3.3 Neuromuscular Signalling in Sarcopenia

Neuromuscular signalling is broadly impaired with age. Loss of LMN was first described in human post-mortem tissues 40 yr ago (113), with the number of lumbosacral limb motor neurons declining by ~ 20 % by the eighth decade. LMN innervating type II fibres are disproportionally affected, with denervated fibres either lost or reinnervated by adjacent LMN. This leads to the phenomenon known as 'fibre type grouping' in which motor unit size increases and fibres within these larger motor units are restricted to one myosin heavy chain isoform (177,178). Such fibre type grouping is not ameliorated by physical activity; lifelong runners are similarly affected (179). The mechanisms underlying the loss of LMN are incompletely understood. Diminished mitochondrial complex I activity has been observed in the spinal neurons of the elderly, compared to foetal controls. Such neurons were also smaller and had a reduced mitochondrial DNA content (180). This implicates mitochondrial pathology in the loss of LMN with age. Other work in aged rats demonstrated morphologically abnormal mitochondria and the apoptosis markers caspase 3 and cytochrome c in axon terminals, near the NMJ (181). Caspase 3 colocalised with dynein in this study, raising the possibility of retrograde caspase 3 transport to the cell soma, where it may affect neuronal viability (181).

In addition to the 'dying back' phenomenon, in which axonal terminal mitochondrial pathology might affect neuronal viability, such pathology may also affect neurotransmitter release and synaptic function. Garçia et al. described axonal terminal mitochondria in aged rats that were two to three times larger than elsewhere in the neuron. They were electron-deficient, with ruptured mitochondrial matrices and lacked cristae. Furthermore,

the mitochondria displayed an increased tendency towards fusion and thus the formation of megamitochondria (181). Crucially, synaptic mitochondria play a role in regulating synaptic function, notably by buffering intracellular calcium to regulate exocytosis and endocytosis of synaptic vesicles and by providing ATP to facilitate synaptic vesicle recruitment from the reserve pool to active zones (182). It is therefore plausible that dysfunctional aged mitochondria might affect axonal synaptic signalling and thus skeletal muscle function, although this requires further study in humans. In aged rodents, fragmentation of the normal, continuous 'pretzel-like' post-synaptic acetylcholine receptor pattern occurs (183), and NMJ denervation precedes the development of sarcopenia, lending credence to the hypothesis that such NMJ changes drive the muscular changes of sarcopenia (184). However, whether the primary event is neurological or muscular remains to be seen (183). The relationship between these neural deficits and inflammaging remains underexplored (185).

1.6.4 Sarcopenia: Consequences and Management

Given its complex aetiology, many potential therapeutic strategies and targets exist to counteract sarcopenia, but no panacea is likely to be discovered. Therefore, to develop an evidence-based, multifaceted approach to the management of sarcopenia. we must continue to explore its aetiology. The inflammatory crosstalk between skeletal muscle and adipose tissue in ageing – the principal focus of this thesis – is one such avenue of exploration. Physical activity, diet and existing pharmaceutical interventions for the amelioration of sarcopenia are considered below.

1.6.4.1 Physical Activity and Diet

It is evident from the preceding discussion of the cellular and molecular pathophysiology driving sarcopenia that physical activity can partially reverse such changes. Indeed, decreased physical activity is common in ageing (186,187), and this phenomenon represents both a cause of and potential therapeutic intervention for sarcopenia. Cross-sectional studies have shown that skeletal muscle mass (measured by dual-energy X-ray absorptiometry, DEXA) has a strong positive association with self-reported physical activity levels in those over age 60 (16,188). Habitual endurance training can increase knee extensor peak isometric force, knee extensor one-repetition maximum and peak grip strength, albeit without an increase in total lean body mass (189). Some older studies found that masters endurance athletes did not have improved skeletal muscle strength, power or mass compared to their sedentary counterparts (190–192). It is suggested (189) that this may be reflective of the decrease in skeletal muscle fibre size that may be seen with marathon training in the young (193).

Although power and strength decrease at a similar rate in masters weightlifters compared to untrained controls, in one study, they generated on average 32 % more absolute peak power and 32% greater isometric knee extensor force than their sedentary counterparts. Furthermore, they reached their absolute peak power 13 % faster (20). Thus, resistance training may represent a useful intervention in the prevention of sarcopenia. Indeed, prospective RT interventions (12-24 wk duration) in which daily protein intake exceeds 0.9 g protein/kg/d have been shown to increase skeletal muscle mass, strength (17,18) and

whole body protein mass in the elderly (194). However, the current recommended daily intake (RDI) of 0.8 g protein/kg/d appears insufficient to prevent loss of lean body mass (LBM) or to facilitate increases in skeletal muscle mass following resistance training in an older population. Campbell et al. demonstrated that consuming a euenergetic diet containing the RDI of protein for 14 wk did not facilitate increases in whole body muscle mass in resistance trained elderly and promoted the accrual of body fat in both resistance training and sedentary groups (19). While mid-thigh muscle area was increased by resistance training, the sedentary group lost muscle area (19). Such observations in longer-term studies are in keeping with acute observations that older individuals require a greater quantity of protein than their younger counterparts to maximally stimulate muscle protein synthesis (MPS) at rest (195,196) and post-exercise (197). The importance of adequate protein intake to support MPS in the elderly is evident. However, the financial constraints of living on a fixed income, an inability to prepare meals due to frailty, difficulties in chewing and swallowing as well as nutrient malabsorption often conspire to prevent sufficient nutritional intake in this population (198). Dietary supplementation with vitamin D, ursolic acid and omega-3 fatty acids have also been proposed as strategies that might ameliorate sarcopenia (199); detailed discussion of their merits is outside the remit of this thesis.

Maintenance of physical activity, supported by adequate nutrition into old age is therefore partially effective at preventing and reversing sarcopenia. Additionally, physically active older adults (55 to 64 yr) have reduced fat mass (4.9 kg in men, 2.5 kg in women) compared to their sedentary counterparts (200). This is pertinent to the central hypothesis

of this thesis – that adipose-derived inflammation is one aetiological factor in sarcopenia.

1.6.4.2 Pharmacological Interventions in Sarcopenia

No effective pharmacological interventions exist for the treatment of sarcopenia. Myostatin is a negative regulator of muscle growth (201); its inhibition is therefore of considerable interest as a target in anti-sarcopenia drug development. A myriad of potential myostatin inhibitors have been developed and trialled to different extents, including anti-myostatin antibodies, myostatin propeptide, soluble myostatin receptors as well as follistatin and similar proteins (follistatin is an endogenous inhibitor of myostatin) (202). In a phase 2 clinical trial, the myostatin neutralising antibody LY2495655 significantly increased LBM and improved physical performance in elderly who had fallen in the previous year (203). Such treatment requires monthly subcutaneous injection of the antibody and is therefore unlikely to be a viable preventative intervention for the wider elderly population.

Growth hormone (GH) and its analogs are often considered as potential interventions for sarcopenia. Systemic GH concentrations decline with age (204). GH administration to elderly increases LBM and decreases adipose tissue mass (205,206). GH has been approved by the federal drug administration (FDA) for the treatment of the muscle wasting associated with chronic human immunodeficiency virus (HIV) infection (207). However, the efficacy and safety of GH for the long-term (> 1 yr) treatment and prevention of sarcopenia has not been established, despite the fact that it is often used off-label in the nebulous discipline of ‘anti-ageing medicine’ (208).

Systemic testosterone concentrations decline with age (209,210), and testosterone administration improves skeletal muscle mass and performance (211–213). However,

concerns remain regarding the safety of testosterone, particularly with regard to cardiovascular events (214), prostate cancer (215–217) and polycythaemia (218) and indeed, that an increased risk of these events exists with testosterone administration remains equivocal. SARMs are androgen receptor ligands. Some have shown an ability to increase lean body mass in phase I and II clinical trials (219–221). However, none has met the criteria for approval.

It is therefore evident that continued exploration of the mechanisms underlying sarcopenia is necessary, to diversify the pharmacological targets at our disposal.

1.7 Sarcopenic Obesity and Inflammaging

The pro-inflammatory, endocrine function of adipose tissue is particularly pertinent in ageing as adipose tissue mass has generally been found to increase with age (3,4). Ageing is consistently associated with increases in VAT and IMAT, regardless of sex or race (2,4,5,42). Ageing is also associated with chronic low-grade inflammation, referred to as “inflammaging” (23). Plasma CRP levels, a biomarker of generalised systemic inflammation, increase with age (24); a study of almost 9,000 adults in the USA found the median plasma CRP to be 1.4 m/L in those aged 20-29 and 2.7 mg/L in those aged 80 or older (24). Importantly, given the association of ageing with adipose tissue accumulation, obesity is also independently associated with inflammation (30–33).

Roubenoff has proposed that loss of LBM and the resulting reduction in energy expenditure, promote the gain of adipose tissue that is observed in ageing (222). Such a

hypothesis has merit; resting energy expenditure decreases with age (5,223), and this decrease can largely be explained by a reduction in LBM (224). Furthermore, low resting energy expenditure is associated with an increased risk of obesity (225–227). The complexity of the aetiology of sarcopenia has been established in earlier sections of this chapter. Thus, the possibility that adipose tissue accumulation might be both a cause and a consequence of LBM loss is not surprising. Reduced overall activity levels are also likely to play a large part in age-related adiposity (228).

1.7.1 Adipose Tissue Structure and Function

Adipose tissue is located in two principal anatomical depots throughout the body: subcutaneously, between skeletal muscle and the dermis (SAT), and intra-abdominally, surrounding the viscera (VAT). Adipose tissue serves to protect underlying tissues and provides thermal insulation (229).

Adipocytes may be subdivided into white and brown adipocytes (229). White adipocytes function as a lipid storage depot, storing fuel as triglycerides when it is in surplus, and releasing free fatty acids to facilitate ATP generation in times of fuel scarcity (229). Their cytoplasm is dominated by a large unilocular lipid droplet. The storage of triglycerides is facilitated by expansion of existing adipocytes (hypertrophy) and formation of new adipocytes (hyperplasia) (230).

Brown adipocytes exist in discrete anatomical areas in both SAT and VAT, and their number decreases with age (231). Brown adipocytes are thermogenic cells, under the

control of the sympathetic nervous system. They produce heat to maintain temperature homeostasis via uncoupled oxidative phosphorylation that is facilitated by uncoupling protein 1 (UCP1) (229). Their high rate of fatty acid oxidation is facilitated by triglycerides that are stored within many small lipid droplets (229).

In addition to its energy storage and mechanical protective functions, adipose tissue is an endocrine organ that is a prolific source of pro-inflammatory cytokines (adipokines). Proteomics approaches have identified over 200 potential adipokines that are expressed by human subcutaneous adipocytes (34). Adipokines are now known to have distal effects on many tissues, not just skeletal muscle (35) and are implicated in the development of insulin resistance, cardiovascular disease and cognitive decline (36–38).

Hypertrophic adipocytes are associated with a greater secretion of pro-inflammatory adipokines (45), but adipokine secretion in obesity is not restricted to adipocytes themselves. Other cell types reside within adipose tissue, (e.g. mesenchymal stem cells, preadipocytes, endothelial cells, pericytes, T cells, and M2 macrophages) and are collectively termed the SVF. *In vitro*, the SVF is a more prolific secretor of pro-inflammatory cytokines (e.g. IFNγ, IL-1β, TNFα) than mature adipocytes (46). Notably, co-culture of the SVF with mature adipocytes produces a unique highly inflammatory cytokine profile (46). Thus, the cytokine secretion by adipose tissue as a whole, not just that by its individual cellular components would appear to be of the most physiological relevance. Increased numbers of resident macrophages have been found in the obese adipose tissue of both mice (232,233) and humans (234); furthermore, obesity may induce a switch from the M2 anti-inflammatory macrophage state to the M1 proinflammatory state (235,236). A study

of mice has suggested that absolute numbers of adipose tissue macrophages do not increase in lean elderly, but there is a relative increase in M1 macrophages (235).

Gross anatomical depots display different adipokine secretory profiles. VAT accumulation is associated with an increased risk of adverse cardiovascular and metabolic outcomes (39,40), a phenomenon that is often attributed to its prolific secretion of pro-inflammatory cytokines (41). In obese participants, portal vein (which drains VAT) IL-6 concentrations are 1.5 times those of the systemic circulation, although leptin concentrations are significantly lower (237). Adipocyte hypertrophy (238,239) and macrophage accumulation (234,240) are two important determinants of adipose tissue inflammation and are more prominent in VAT than SAT. Unfortunately, little is known about adipokine secretion by IMAT due to the technical difficulties associated with its biopsy in adequate quantities for study. This is an area which requires further study, given the proximity of IMAT to skeletal muscle fibres.

Lipids may also accumulate within skeletal muscle myofibres (intramyocellular lipid; IMCL). Fatty acids are transported into skeletal muscle fibres and are channelled towards 1) storage as triacylglycerols (TAG); 2) mitochondrial oxidation; or 3) conversion to signalling molecules (including eicosanoids, phospholipids and ceramides). Dysfunctional intramyocellular fatty acid handling and IMCL accumulation have a well-established if incompletely understood association with skeletal muscle insulin resistance (SKM-IR). Particularly, the accumulation of specific lipid intermediates, namely diacylglyerol (DAG) and ceramides has been suggested to mediate SKM-IR. Dysfunctional fatty acid uptake by skeletal muscle and IMCL lipid accumulation is – at least in part – an age-related

phenomenon, having been observed to occur in lean and overweight non-diabetic men (241). In addition to their effects on skeletal muscle insulin sensitivity, IMCL may contribute to sarcopenia. Diet-induced obesity and IMCL accumulation inhibit rodent skeletal MPS (242–244); furthermore, lipid infusion in healthy young men inhibits MPS (245).

Both insulin and amino acids promote MPS via mammalian target of rapamycin complex 1 (mTORC1) signalling, raising the possibility that insulin resistance in ageing might reduce MPS (246–248). However, considerable evidence exists to suggest that it is the vasodilatory effects of insulin, which most affect MPS. In healthy young adults, nitric oxide synthase inhibition prevents insulin-induced increases in MPS (249). In one study, femoral amino acid-glucose infusion increased MPS in the young, but not in the elderly; following the infusion, plasma arterial insulin concentrations were significantly lower in the elderly (250). It must be remembered that amino acid ingestion alone is capable of enhancing MPS in the elderly (251,252) (Section 1.6.4.1). Despite this, under conditions of combined carbohydrate and protein feeding – an experimental condition that better reflects real-world nutrient consumption and allows for an endogenous insulin response – MPS rates are diminished in the elderly compared to their young counterparts (250,253). Pertinently, leg blood flow does not increase in response to hyperinsulinaemia in older people (254). Additionally, insulin-induced inhibition of whole body (255) and leg proteolysis (256) is diminished in the elderly.

1.7.2 Adipokines and Skeletal Muscle Mass

Having described the phenomena of sarcopenic obesity and inflammaging and having

highlighted the prolific production of pro-inflammatory cytokines by adipose tissue, it is important to outline broadly what is known about the effect of such adipokines on skeletal muscle mass.

In sarcopenia, the multifaceted nature of LBM loss leads to a substantial and therefore easily detectible change in LBM. If adipokines indeed represent one facet of the sarcopenic process, then one might expect to see a decrease in LBM in younger obese individuals, although the magnitude of the LBM loss in the young is likely to be considerably smaller given the multifactorial nature of sarcopenia. Additionally, skeletal muscle loss may not be as great in young obese individuals, as obesity represents an overload stimulus acting on anti-gravity muscles, promoting increased absolute muscle mass and strength (257). However, skeletal muscle strength relative to total body mass is reduced in the obese, indicating that the increase in total strength is functionally inadequate (257).

Despite the confounding factor of obesity being an overload stimulus on skeletal muscle, limited cross-sectional studies exist to suggest that visceral fat area (VFA) correlates negatively with skeletal muscle mass in the young. In one study of healthy young men of normal BMI, a significant negative correlation existed between VFA and total skeletal muscle mass ($R=-0.57$, $P < 0.001$); a negative correlation with skeletal muscle mass also existed for subcutaneous fat area (SCFA) ($R=-0.55$, $P < 0.001$) (258). In a slightly older, largely middle-aged population, those with coexisting visceral obesity and low muscle mass had a larger VFA than those with visceral obesity or low muscle mass alone, although this did not reach significance (259). Genetic factors may play a role in such a

phenomenon, with Asian-American young women being particularly prone to coexistent obesity and low BMI (260).

In the elderly, the association between systemic inflammation, adipose tissue mass and sarcopenia is more pronounced. The cross-sectional Health, Aging, and Body Composition (Health ABC) Study of 3075 men and women aged 70–79 years demonstrated that those with high systemic concentrations of TNFα and IL-6 had a smaller mid-thigh muscle cross-sectional area and decreased grip strength (27). Furthermore, longitudinal studies of large elderly cohorts have positively associated high systemic concentrations of IL-6, CRP and TNFα and with loss of skeletal muscle mass (25,26). Indeed, the risk of losing 40 % of grip strength over 3 yr was increased 2 to 3-fold in those with a combination of increased serum IL-6 (> 5 pg/mL) and CRP (> 6.1 μg/mL) (25). Cross-sectional studies of the body composition of individuals across the lifespan found that total adipose tissue mass (28) and VAT mass (29) negatively correlated with skeletal muscle mass. A 2 yr prospective study of 379 Korean men and women also demonstrated that VAT area, as measured by DEXA, predicted appendicular lean soft tissue area at 2 yr ($r2 = -0.203$, $p < 0.001$) (259). Caution must be exercised in inferring causation from such correlative data, but the consistent finding of a negative association between adipose tissue mass and skeletal muscle mass/LBM adds considerable weight to the validity of exploring adipokine-mediated sarcopenia.

Indeed, the anti-myogenic and pro-atrophic actions of some adipokines are now well established. High systemic concentrations of widely produced cytokines such as IL-6 and TNFα, which are also adipokines, contribute to the substantial loss of skeletal muscle

mass in the cachexia of chronic disease (261,262). While inflammation-driven sarcopenia and cachexia undoubtedly have mechanisms in common, the degree of inflammation in cachexia is far greater than that seen in ageing and obesity (263,264), and thus cachexia is not considered in detail here, for fear of conflating the two phenomena.

A large study of adults aged 19-80 has shown that plasma IL-6 levels increase with age, independently of BMI (264). Interestingly, correction for VAT mass eliminates such age-associated differences in plasma IL-6 concentration (265). Although IL-6 appears necessary for normal skeletal muscle hypertrophy. (266–269), higher concentrations of IL-6 have been reported to induce skeletal muscle atrophy (270,271), and IL-6 has been shown to inhibit myogenic differentiation of C2C12 cultures (272). 14 d of local IL-6 infusion into rodent tibialis anterior muscle can induce a significant decrease in total and myofibrillar TA content (273). The dose of IL-6 infusion in this study was 0.7 $pg \cdot muscle^{-1} \cdot h^{-}$, This is equivalent to plasma concentrations observed in the elderly (264).

TNFα is another cytokine which is well established as an inducer of myofibre atrophy. In chapter 5 of this thesis, recombinant TNFα (rTNFα) stimulation of primary human myogenic cultures is employed a pro-inflammatory stimulus that impairs myotube formation *in vitro*. Thus, its effects on skeletal muscle mass are considered in some detail below. This section also serves as a case-study, highlighting the breadth of evidence supporting a role for a better-studied adipokine in the aetiology of sarcopenia. While adipokines like IL-6 and TNFα are well established as promoting loss of skeletal muscle mass, there exist many adipokines within the adipose inflammatory milieu that are less well characterised. The function of several such cytokines is explored in this thesis and

consideration is given in the following sections to the limited existing evidence of their effect on skeletal muscle mass.

1.7.3 TNFα and Skeletal Muscle Mass

In a study of ~550 adults aged 19-80, plasma TNFα levels increased significantly with age, independent of BMI (264). Plasma TNFα is also significantly increased in obese participants, independent of age (32). Adipose tissue is an important source of TNFα; its adipose tissue expression increased 2.5-fold in aged mice (33). A similar increase in subcutaneous adipose tissue TNFα (*TNFA*) gene expression has been described in obese mice and humans (274,275). A study comparing the systemic (radial arterial blood) and visceral (portal venous blood) concentrations of adipokines in the obese found that their TNFα concentrations did not differ significantly (276), although VAT *TNFA* expression increased with non-alcoholic fatty liver disease severity in a study of those with class III obesity (BMI > 40 kg/m^2) (277).

In considering the possible effects of adipose-derived TNFα on skeletal muscle size, quality and function, it is useful to consider studies that have explored the effect of exogenous TNFα on skeletal muscle, both *in vitro* and *in vivo*.

Strong evidence exists to suggest that TNFα activates muscle catabolic pathways, including the 28S ubiquitin proteasome. *In vitro*, stimulation of C2C12 myotubes with rTNFα induced myotube atrophy and increase the expression of the ubiquitin E3 ligases MAFbx and MURF-1 (65,278). Furthermore, such rTNFα stimulation (100 ng/mL) can also increase the protein expression of unphosphorylated forkhead box protein O1 (FOXO1) and forkhead box protein O3 (FOXO3); when myotubes are transfected with FOXO1

siRNA, the rTNFα-induced increase in MAFbx and MURF-1 protein expression is attenuated (65). *In vivo,* Intravenous (IV) rTNFα administration (100 μg/kg) to rats increases skeletal muscle ubiquitin gene expression, as well as increasing the ubiquitination of skeletal muscle proteins (279,280). The same dose of rTNFα, delivered intraperitonally to mice can induce increases in both the mRNA and protein expression of MAFbx and MURF-1 at 24 hours post-injection (281). Furthermore, *ex-vivo* soleus muscle force production was reduced by 25 % in such mice, and ubiquitinylation of troponin T increased significantly. Identical TNFα administration to MURF-1 knockout (MURF-1$^{-/-}$) mice does not affect *ex-vivo* soleus force production or the proportion of ubiquitinylated troponin T (281). In certain circumstances, TNFα appears to also have anti-myogenic actions. While physiological concentrations of rTNFα (5 pg/mL) have been shown to enhance the differentiation of C2C12 cultures and TNFα expression is increased in regenerating murine skeletal muscle (282), concentrations of rTNFα above 50 pg/mL appear to inhibit myogenic differentiation *in vitro* (282,283).

The absolute concentrations of TNFα that myotubes and skeletal muscle are exposed to in the studies detailed here generally far exceed those concentrations seen in the plasma of elderly individuals (interquartile range 13.73 to 20.54 pg/mL in (264)). Local resting skeletal muscle interstitial concentrations of TNFα, as measured by microdialysis appear to be similar to plasma concentrations (284–286). However, we are unaware of measurements having been made in the elderly or the obese. Interstitial concentrations of TNFα in areas of skeletal muscle lying adjacent IMAT may be higher, given that SCAT and VAT are known to secrete TNFα. Furthermore, the magnitude and rapidity of the

effects seen with rTNFα on skeletal muscle *in vivo* and *in vitro* (e.g. 25% reduction in soleus force production at 24 h after a single TNFα IP injection (281)) may suggest that lower concentrations of TNFα might have important effects on skeletal muscle mass and function over time.

1.7.4 Resistin and Skeletal Muscle Mass

Resistin is a pro-inflammatory adipokine that is produced predominantly by monocytes and macrophages in humans, with a smaller proportion produced by adipocytes (287). However, given the importance of M1 macrophage accumulation in ageing and obesity (Section 1.7.1), adipose tissue secretion of resistin may be of some consequence in sarcopenic obesity. It has been suggested that resistin may initiate a pro-inflammatory 'feed-forward loop' by inducing the expression of TNFα and IL-6, a phenomenon that has been observed in leukocytes (288). In turn, interleukin 1β (IL-1β), TNF-α and IL-6 can induce resistin expression by leukocytes (288). However, it is unclear whether this phenomenon occurs in adipose tissue or skeletal muscle. Plasma and serum resistin concentrations are positively correlated with BMI (289,290) although no independent effect of age has been reported (289). Surgical removal of half of the total VAT in rats leads to systemic reductions (26 %) in resistin concentrations (28).

The effect of resistin on sarcopenic muscle is understudied, but limited evidence suggests that it might have an important effect on skeletal muscle mass and quality. Plasma resistin concentrations have been reported to have an inverse relationship with quadriceps torque in old (69-81 yr), but not in young (18-30 yr), participants (291). Transfection of a human

resistin eukaryotic expression vector into C2C12 cells was reported to increase myoblast proliferation, but reduced the expression of desmin and resulted in thinner myotubes (292). However, this study used a murine cell line and did not investigate the effect of exogenous resistin on myotube formation. Notably, human resistin displays only 59 % amino acid sequence homology to murine resistin. Thus, mouse models may be inadequate for the investigation of its human physiology (293).

1.7.5 Leptin and Skeletal Muscle Mass

Leptin is an adipocyte-derived cytokine. Its plasma concentrations are largely determined by adipose tissue mass and food intake. Leptin secretion increases in response to energy intake and decreases during periods of fasting. Leptin crosses the blood-brain barrier, suppressing food intake via its actions in the arcuate nucleus (294).

Unsurprisingly, plasma leptin concentrations are greater in obese participants (10.4 ± 1.3 ng/mL) compared to non-obese controls (7.6 ± 0.5 ng/mL) (32). Interestingly, murine models suggest that leptin may exert hypertrophic effects on skeletal muscle and thus have the potential to reverse sarcopenia. 10 d of subcutaneous leptin injection (10 μg/d) in old mice significantly increased quadriceps mass and extensor digitorum longus fibre area (c. 20%) (295). In another study leptin deficient (ob/ob) mice with a body weight 2.5 times that of lean controls displayed a 40 % reduction in quadriceps mass (296).

However, studies in human participants suggest that such dramatic hypertrophic effects are not seen with plasma leptin fluctuations within the physiological range. In adults under

50 yr, serum leptin concentrations are positively correlated with fat free mass (FFM) in men, but not in women (297). However, men in the highest quintile for FFM also displayed the highest BMI and fat mass (FM); the resulting anti-gravity overload stimulus represents an important confounder. Indeed, when multiple linear regression analyses were carried out, FFM explained only 6 % of the variation in serum leptin, independent of FM (297). In late middle-aged and elderly participants, sarcopenia with coexistent visceral obesity has been associated with significantly greater plasma leptin concentrations than in those with sarcopenia alone (2.6 ng/mL ± 2.3 vs 5.7 ng/mL ± 2.3) (298). None the less, skeletal muscle atrophy, as measured by thigh muscle CSA corrected for body weight does not differ between the groups (298). However in another study, when appendicular skeletal muscle mass was measured by DEXA in a healthy elderly population, plasma leptin was negatively associated with skeletal muscle mass, even after correction for FM (299). Such a relationship may not hold true in very frail elderly who have low absolute plasma leptin levels and muscle mass compared to healthy controls (300). Work to date has drawn correlations between plasma leptin concentrations and measurements of skeletal muscle mass. We are unaware of any work demonstrating a clear causative effect of leptin on human skeletal muscle growth and development (e.g. using an *in vitro* myotube model).

1.7.6 Visfatin and Skeletal Muscle Mass

Visfatin (also known as Nicotinamide phosphoribosyltransferase – Nampt) is a relatively recently discovered pro-inflammatory adipokine/biosynthetic enzyme. As with resistin, visfatin upregulates production of IL-1β, TNF-α and IL-6 by monocytes (301). Visfatin

plasma concentrations correlate positively with BMI (302–304), but the effect of age (independent of BMI) is unclear. Endurance exercise training increases skeletal muscle visfatin activity in mice (305) and humans (306). In chicken myoblasts, recombinant human visfatin decreased their expression of the MRFs MyoD and MRF4, while increasing MYF5 expression, but myogenesis was not examined (307).

1.7.7 Adipokines and Skeletal Muscle Metabolism

Sarcopenia describes a loss of skeletal muscle mass and quality. It is clear from the previous discussion that alterations in myofibre metabolism, may in part underpin the loss of skeletal muscle mass and performance and that adipokines such as IL-6 (Section 1.7.2) and TNFα (Section 1.7.3) are capable of altering skeletal muscle protein turnover.

Little evidence exists to suggest what effect other, less well-studied adipokines e.g. leptin and resistin might have on skeletal muscle protein turnover. Limited evidence exists to suggest that such adipokines may alter energy substrate uptake and metabolism. For example, resistin – which features prominently in this thesis – inhibited glucose uptake and impaired glycogen synthesis during a 24 h stimulation of rat L6 myotubes (308,309). Another study found that resistin inhibited palmitate uptake by L6 myotubes (310). However, whether primary human myotubes or human skeletal muscle behave in a similar fashion to an immortalised rat cell line is unknown. Indeed, certain adipokines are better studied with regard to energy substrate uptake and metabolism, but their study has highlighted that caution is advisable when extrapolating studies in rodent cell lines to human physiology. For example, TNFα infusion in healthy humans induces skeletal

muscle insulin resistance (311), yet TNFα has been found by different investigators to increase (312), decrease (313) and not influence (314,315) glucose uptake by L6 myotubes. IL-6 infusion in human participants increases whole-body and skeletal muscle lipolysis, without affecting glucose turnover (316–318), yet IL-6 suppresses glucose uptake by L6 myotubes (319).

1.8 IL-15

Given the dearth of pharmacological therapeutic interventions in sarcopenia (Section 1.6.4), it is important to explore additional avenues for the development of such interventions. To that end, IL-15 is a myokine that is known to promote the formation of C2C12 myotubes in culture and can prevent the loss of skeletal muscle mass in mice exposed to systemic inflammation (49,50). Its biological role in human skeletal muscle is not fully understood.

1.8.1 IL-15 Background

IL-15 was first identified as a 14 kDa four-helix bundle cytokine with T-cell stimulatory activity (320,321). It is similar in structure to IL-2 and IL-6 (322). The human and murine forms of IL-15 have a homology of 73 % at both the nucleotide and amino acid levels; it has been suggested that such homology is sufficient for IL-15 function to be largely conserved between the species (323,324).

Two mRNA isoforms of *IL15* are generated. Long signal peptide IL-15 (LSP-*IL15*) is

encoded by exons 3-5 of the gene (325). The short signal peptide IL-15 (SSP-*IL15*) transcript encodes an identical mature IL-15 peptide. However, the signal peptide portion of SSP-IL-15 lacks the first exon of the LSP-*IL15* transcript, with additional alternative exons discovered in both the human and murine forms of SSP-*IL15* (326–329). SSP-*IL15* is highly transcribed in the heart, with prominent expression also in thymus and testes (325). LSP-*IL15* expression is high in skeletal muscle, placenta, heart, lung, liver, thymus, and kidney (320,325). The presence of upstream AUGs in the 5' UTR of a transcript impairs translation (330,331). The 5' UTR of *IL15* contains multiple such AUGs (320). Eliminating many of these AUGs improves *IL15* translation (332). Indeed, SSP-*IL15* is more efficiently translated and this is thought to be due to translation initiation occurring at its alternative exon, thus avoiding the AUG-containing 5' UTR (333). However, it is the protein generated by the inefficiently translated LSP-*IL15* which is better secreted (325,334,335). Such secretion of IL-15 is facilitated by its specific alpha receptor (IL-15rα) (336).

1.8.2 IL-15 Receptor Complexes and Subunits

The IL-15 receptor is a heterotrimeric complex which consists of two signalling subunits - the gamma cytokine receptor (γc) and the interleukin 2 receptor beta subunit (IL-2Rβ) and one non-signalling subunit (interleukin 15 receptor alpha subunit, IL-15rα) which confers IL-15 specificity on the receptor complex (336). The γc subunit forms part of receptors for multiple cytokines, including IL-2, IL-4, IL-7 and IL-9 (337). IL-15rα contains an exon 2-encoded sushi domain that facilitates the high-affinity binding of IL-15 to IL-15rα via

extensive ionic interactions (338,339). Multiple isoforms (generated through alternative splicing) of IL-15rα have been described (323,339); the first three to be characterised are equally capable of binding IL-15 (323). These isoforms of IL-15rα are expressed across multiple human cell types (339) and have a broad intracellular distribution. Notably, IL-15rα is prominently expressed at the nuclear membrane, and its co-localisation with p300 suggests that a portion of IL-15rα is located inside the membrane (339). The presence of an abundant intracellular receptor for IL-15 may suggest an intracellular function for the poorly secreted SSP-IL-15 (339).

IL-15rα facilitates the secretion of IL-15. Transfection of human embryonic kidney 293 (HEK 293) cells with IL-15 DNA in conjunction with an IL-15rα expressing plasmid enhances IL-15 secretion (335). This mechanism is conserved in murine myotubes – primary myotube cultures from IL-15rα knockouts do not secrete IL-15 (52). IL-15rα also exists in soluble isoforms (sIL-15rα), which bind IL-15 and may act as either agonists or antagonists at the cell membrane IL-15rα/β/γ receptor complex (340). Intracellular IL-15 signal transduction has been characterised in immune cells, but nothing is known about such intracellular signalling in skeletal muscle. In lymphocytes janus kinase 1/ signal transducer and activator of transcription 3 (JAK1/STAT3) activation takes place via IL-15rβ and JAK3/STAT5 activation occurs via the γc subunit (341). The phosphoinositide 3-kinase/protein kinase B (PI3k/Akt) and MAPK pathways are also activated (341). The functional importance of IL-15 activation of these pathways remains to be established in skeletal muscle.

1.8.3 IL-15 in Skeletal Muscle

A role for IL-15 in skeletal muscle myogenesis and hypertrophy was first suggested 20 years ago when it was discovered that IL-15 stimulation of murine C2C12 myoblast cultures is as effective as insulin-like growth factor 1 (IGF-1) stimulation in increasing MHC accumulation at the myoblast and myotube stages (49). Additionally, IL-15 stimulation promoted the development of larger myotubes (49). In another study, stimulation of rat extensor digitorum longus muscle with human rIL-15 (100 ng/mL) decreased its proteolytic rate, but did not affect protein synthesis (342). Furthermore, stimulation of murine C2C12 myotubes with IL-15 can ameliorate the increase in cathepsin L activity seen with TNFα and dexamethasone stimulations (50). In mice with experimentally-induced sepsis, intraperitoneal pre-treatment with IL-15 (10 μg/kg) reduced mRNA expression of the E3 ligases MAFbx and MURF-1 at 24 h (50). *IL15* expression has been shown to be increased in the unloaded, atrophic limbs of old rats and quail (51). Such an increase in *IL15* mRNA expression might be a physiological attempt to counteract atrophic stimuli (51). Aged mice (28 months) have been observed to display a non-significant decrease in LBM and lower muscle and serum IL-15 levels than their younger controls (343). Our limited knowledge of IL-15 biology suggests that an age-related reduction in skeletal muscle IL-15 might contribute to this murine ‘sarcopenia’. However, definitive mechanistic evidence is lacking.

In addition to its apparent role in the maintenance of skeletal muscle mass, IL-15 may play a metabolic role within skeletal muscle. The observation that an increase in IL-15 expression (soleus and gastrocnemius) accompanied improved glucose tolerance in

endurance-trained Zucker Diabetic Fatty Rats led to speculation that IL-15 may promote enhanced insulin sensitivity (344). Co-incubation of rIL-15 (10 and 100 ng/mL) with rat muscle strips and endoplasmic reticulum stress inducers (dithiothreitol or tunicamycin) reduced the expression of ERS markers (345). ERS is known to impair insulin signalling (345). Definitive evidence of anti-diabetic actions of IL-15 remain to be presented. Additionally, IL-15 may promote enhanced oxidative metabolism in skeletal muscle and thus improved endurance capacity. Stimulation of murine C2C12 myoblasts with rIL-15 (50 μg/mL) reduced the activity of the non-oxidative metabolism enzymes creatine kinase and lactate dehydrogenase and attenuated the hydrogen peroxide (H_2O_2) induced increase in cellular ROS (346). Furthermore, mice that overexpress IL-15 can run for twice as long as their wild-type counterparts; expression of mediators of oxidative metabolism, (peroxisome proliferator activated receptor delta [PPAR δ], sirtuin 1, peroxisome proliferator activated receptor gamma coactivaror 1 alpha [PGC1α]), is increased in the skeletal muscle of such mice (347).

Studies characterising the biological actions of IL-15 in human skeletal muscle are lacking. We are unaware of any studies demonstrating the secretion of IL-15 by primary human myotubes cultured from healthy muscle. Endurance training has been demonstrated to increase basal IL-15 protein expression, without a change in IL-15 mRNA or plasma IL-15 (348). In another study, an acute bout of resistance exercise increased IL-15 protein expression, yet its basal expression was unaffected by resistance training (349). Furthermore, following an acute bout of resistance exercise, the myofibrillar protein synthesis rate has been observed to display a significant and substantial positive

relationship with skeletal muscle IL-15rα expression (350). Interestingly, single nucleotide polymorphisms (SNPs) in exons 7 and 4 of the IL-15rα gene are associated with an enhanced hypertrophic training response (351). The physiological basis for these changes is unknown. It is clear from animal studies that IL-15 may have important functions in skeletal muscle. Such functions implicate IL-15 as a potential therapeutic target in sarcopenia and skeletal muscle metabolic dysfunction. However, the biological actions of IL-15 in human skeletal muscle remain to be characterised.

1.9 Hypotheses and Aims

The two central hypotheses of this thesis are:

1. That there exist understudied adipokine constituents of the adipose inflammatory milieu that are differentially secreted by lean and non-lean adipose tissue and that the enhanced secretion of these adipokines is detrimental to myogenesis and is in part responsible for sarcopenia.
2. That the pro-myogenic and hypertrophic actions of IL-15 previously described in rodent studies would be retained in human myogenic cultures, but that cultures derived from the skeletal muscle of elderly individuals would be more resistant to the hypertrophic effects of IL-15 than those of young individuals.

Therefore, the objectives of this work were as follows:

1. To determine the effect of the adipose inflammatory milieux secreted by lean and non-lean adipose tissue on primary human myogenesis and to identify understudied adipokine constituents that may be consequential in mediating such effects (Chapter 3).
2. To identify in the adipose inflammatory milieux a candidate adipokine of apparent myogenic consequence and detail its myogenic actions and the mechanism of such action (Chapter 4).
3. To consider whether the candidate adipokine has metabolic effects on primary human myocytes (Chapter 5).
4. To detail the myogenic actions of IL-15 in primary human myogenic cultures derived from young and old participants and to establish whether its purported pro-myogenic and hypertrophic actions can be sustained in a pro-inflammatory environment.

The specific objectives that were identified to address these aims are outlined in the introductory sections of each data chapter.

Chapter 2

Materials and Methods

2. Materials and Methods

All procedures were carried out at room temperature unless otherwise stated.

2.1 Ethical Approval and Subject Recruitment

Samples obtained from healthy participants were obtained and used under approval granted by the University of Nottingham Medical School Ethics Committee. The participants were volunteers, recruited through poster advertising that was approved by the ethics committee. Young participants were aged 18-30 yr; elderly participants were > 65 yr. Potential participants with no history of cardiovascular disease or diabetes were invited to a medical screening at the University of Nottingham Human Physiology Unit, comprising a 12-lead electrocardiogram, blood pressure monitoring and blood sampling for routine haematology and biochemistry analyses (full blood count, urea and electrolytes, clotting factors). Informed consent was obtained from participants during this visit and the participants were advised that they were free to withdraw at any time. The results of medical screening were reviewed by a clinician before participants were invited to attend for the main study visit.

Adipose tissue samples were obtained from participants with knee and hip osteoarthritis were obtained intra-operatively from middle-aged and elderly patients undergoing total joint replacement surgery under the approval of the University of Birmingham Research Ethics Committee. The demographics of this population is presented in Chapter 3. All patients gave informed consent before surgery. Those with a known secondary cause of osteoarthritis were excluded, as were patients who had ever received IV or oral immunosuppressive medications or those who had received any intra-articular steroid injections in the preceding six months.

2.2 Subject Data and Sample Collection

Healthy participants attended the University of Nottingham Human Physiology Unit for the main study visit. 12 young (age 22.2 ± 0.6 yr, BMI 21.9 ± 0.9) and 6 older (age 70.3 ± 1.9 yr, BMI 23.5 ± 1.9) participants were recruited. Three biological replicates were used for each cell culture experiment and participant details for each experiment are outlined in Appendix Table 1. Participants were asked to abstain from alcohol consumption and exercise training for 48 h prior to the visit and to arrive having fasted for a minimum of 6 h. Each subject underwent a DEXA scan to determine their body composition, for which they gave separate informed consent. Subject height and weight were recorded, and BMI was calculated as weight (kg) divided by height (m) squared. A single blood sample (30 mL) was collected by venipuncture in the antecubital fossa, using a butterfly needle and syringe. The blood was divided between serum separating blood tubes and tubes containing EDTA. The tubes were placed on ice until further processing could take place (within 3 h). A VL biopsy was obtained under local anaesthetic by the Unit clinician, using the suction-modified Bergström technique (352). The biopsy was placed in sterile phosphate-buffered saline (PBS) and was kept on ice; subsequent processing took place within 3 h.

Skeletal muscle and SAT samples obtained from participants with knee and hip osteoarthritis were obtained intra-operatively by the lead surgeon, placed in sterile DMEM/F12 on ice and transported to the laboratory on the same day for further processing.

2.3 Blood Sample Processing

Serum separating blood tubes were centrifuged at 3000 rpm for 10 min, and the serum was aliquoted into 1 mL cryovials, snap frozen in liquid nitrogen and stored at -80 °C. EDTA blood tubes were snap-frozen in liquid nitrogen and stored at - 80 °C.

2.4 Tissue Culture

All reagents used were from Sigma-Aldrich, St. Louis, MO, USA, unless otherwise stated. Tissue culture was carried out under sterile conditions in a class II safety cabinet.

2.4.1 Primary Human Myoblast Isolation and Culture

Primary human skeletal myoblasts were isolated from skeletal muscle using a protocol provided by Dr Andrew Bennett (FRAME laboratory, University of Nottingham). Muscle samples were minced for 10 min with a scalpel, digested with 5 mL 1x trypsin-EDTA and placed on an orbital rotator for 15 min at 37 °C. An equal volume of growth medium (Ham's F10 with 2 mM L-glutamine, 100 µg/mL penicillin/streptomycin, 20 % heat-inactivated FBS) was added and muscle digests centrifuged at 460 g for 5 min. The resulting pellets were resuspended in growth medium and then incubated for 20 min at 37 °C, 5 % CO_2 in an uncoated 75 cm^2, vented tissue culture flask. The cell suspensions were then removed to vented flasks or plates coated with 0.2 % gelatin and cultured in an appropriate volume of medium (Table 2.1). Our isolation technique has consistently produced cultures in our lab that produce desmin positive multinucleated myotubes that are negative for the fibroblast marker TE7(86) (Appendix Fig. 2.1).

Commercially available primary human myoblasts (Thermo Fisher cat. No. A12555),

isolated from a female aged 21 yr were used for some of the work described in this thesis. They were cultured in the same media and conditions as the cultures that we isolated in-house.

Table 2.1 Tissue Culture Vessels and Media Volumes.

Culture Vessel Type	Medium Volume (mL)
Flask - 75 cm^2	12 mL
Flask - 25 cm^2	4 mL
Plate – 6 well	2 mL
Plate – 12 well	1.5 mL
Plate – 24 well	1 mL

Cultures were fed with growth medium at 48 h and every 48-72 h thereafter. If necessary, myoblasts were subcultured (1:3) at 70 % confluence by trypsin-induced dissociation from their vessel. Briefly, the growth medium was removed from the vessel and replaced with an equal volume of PBS. The PBS was aspirated and replaced with 0.05 % Trypsin-EDTA. The culture vessel was placed in an incubator at 37 °C for 5 -10 min until > 80 % of cells were observed to be detached by light microscopy. An equal volume of growth medium was added to neutralise the trypsin-EDTA. The cell suspension was centrifuged at 460 g for 5 min and the resulting pellet resuspended in growth medium and transferred to new 0.2 % gelatin-coated flasks or plates.

For cryopreservation, myoblasts were detached from their vessel as described above and

resuspended in a medium containing 65 % growth medium, 25 % FBS and 10 % Dimethyl sulfoxide (DMSO) (Adapted from (73)). Following transfer to a freezing container, (Mr Frosty, Thermo Scientific, MA, USA) samples were stored at - 80 °C overnight before being moved to long-term liquid nitrogen storage.

Myoblasts and Myotubes were used experimentally between passages two and five.

2.4.2 Differentiation of Primary Human Myoblasts to Myotubes

Myoblasts were grown to 90 % confluence before switching to culture medium that promoted their differentiation to myotubes (Ham's F10 with 2mM L-glutamine, 100 µg/mL penicillin/streptomycin, 6 % heat-inactivated horse serum). The cells were maintained in this differentiation medium for 8 d, after which time multinucleated myotubes were predominant in the culture.

2.4.3 Recombinant Cytokine Stimulation of Primary Human Myoblasts and Myotubes

Much of the work presented in this thesis involves the stimulation of myoblasts and myotubes with recombinant cytokines. The details of cytokine concentrations, as well as the timing and duration of such stimulations, are described in the relevant data chapters. Broadly, stimulations were achieved by the addition of the relevant cytokine to an appropriate medium. The details of the cytokines used in this work are outlined in Table 2.2.

Table 2.2. Recombinant Cytokines Used in this Work.

Recombinant Protein	Source	Sequence	Manufacturer	Catalogue No.
Resistin	E. Coli	Full length (aa 15 – 110)	Cambridge Bioscience	GFH107
Leptin	E. Coli	Full length (aa 1 – 146)	Cambridge Bioscience	GFH37
Visfatin	E. Coli	Full length (AA 1-491 and N-term His tag	Cambridge Bioscience	4907-50
IL-15	E. Coli	114 AA	Cambridge Bioscience	GFH86
TNFα	E. Coli	115 AA N-terminal methionine	Cambridge Bioscience	GFH111

2.4.4 Generation of Adipose Conditioned Medium

SAT was obtained intraoperatively from patients of varying BMI undergoing total hip and total knee replacement surgery. SAT was incubated in myotube differentiation medium at a ratio of 1 g tissue to 10 mL medium for 24 h. Larger samples were divided into segments of ~ 1 g to ensure that the surface area of adipose tissue exposed to medium remained approximately constant. At 24 h the adipose conditioned medium (ACM) was removed,

aliquoted into 5 mL sample containers and stored at - 80 °C. For experimental use, the ACM was diluted 1:2 with differentiation medium, to ensure a sufficient nutrient composition to sustain myogenic differentiation.

2.5 Isolation of Total Protein from Primary Human Myoblasts and Myotubes

For protein extraction, myotubes were lysed in ice-cold radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris HCl, 150 mM NaCl, 1.0 % (v/v) NP-40, 0.5 % (w/v) Sodium Deoxycholate, 1.0 mM EDTA, 0.1 % (w/v) sodium dodecyl sulfate [SDS], 0.01 % (w/v) sodium azide, protease inhibitor cocktail, phosphatase inhibitor cocktail, pH of 7.4). 15 µL/cm^2 growth surface of RIPA was used. A cell scraper was used to aid dissociation of cells from the culture plate. Cell lysates were then removed to a microcentrifuge tube and sonicated on ice for 1 min before being centrifuged at 14,000 g for 10 min at 4 °C to pellet cell debris. The total protein concentration of each cell lysate supernatant was quantified by a bicinchoninic acid assay (Thermo Scientific), according to the manufacturer's instructions. Lysates were stored at - 80 °C

2.6 SDS Polyacrylamide Gel Electrophoresis and Immunoblotting

Total protein lysates of known concentration (see Section 2.5 above) were mixed 3:1 with 4x Laemmli sample loading buffer (0.5M Tris-HCl pH 6.8, 20 % (v/v) Glycerol, 10 % (w/v) SDS, 0.1 % (w/v) bromophenol blue, 10 % (v/v) beta-mercaptoethanol [βME]) and water to generate polyacrylamide gel loading stocks of known concentration. Samples were boiled for 5 min.

SDS polyacrylamide gel electrophoresis (SDS-PAGE) gels were cast in empty gel loading

cassettes (Thermo Fisher) using 37.5:1 Acrylamide:Bisacrylamide ProtoGel 30 % (National Diagnostics). The volume of ProtoGel used was calculated as Vp = (X x Vt)/30 where, Vp = Volume of 30 % ProtoGel, X = % Monomer desired in gel, Vt = Total volume of gel casting solution. For resolving gels, Vt comprised the volume of ProtoGel required, 4x ProtoGel resolving buffer (1.5M Tris-HCl pH 8.8, 10 % SDS) diluted to 1x, and deionised water. Stacking gels comprised the volume of ProtoGel required for a 4 % gel, 4x ProtoGel stacking buffer (0.5M Tris-HCl pH 6.6, 0.4% SDS) diluted to 1x, and deionized water. An appropriate gel percentage was chosen according to the size of the protein of interest. In general, proteins < 20 kDa were run on gels of 14 % (w/v) acrylamide, proteins > 60 kDa were run on gels of 10 % (w/v) acrylamide. All other gels were 12 % (w/v) acrylamide.

Equal amounts of total protein were loaded onto SDS-PAGE gels; when samples were run across multiple gels, an internal control sample was run. Proteins were separated by electrophoresis (50 mA) in 1x Tris-Glycine SDS-PAGE Buffer (0.025M Tris base, 0.192M glycine, and 0.1 % (w/v) SDS). Proteins were then transferred onto polyvinylidene difluoride (PVDF) membrane (Amersham Hybond-P, GE Healthcare, Buckinghamshire, UK) in an electroblotting buffer (0.025M Tris base, 0.192M glycine, 20 % (v/v) methanol at pH 8.4) using the 'Standard SD' program of the Trans-Blot Turbo semi-dry transfer system (Bio-Rad, CA, USA). Membranes were blocked for 1 h and agitated overnight with primary antibody diluted in TBS-T at 4 °C (Table 2.3). The membranes were washed for 4 x 5 min in 1xTBS-T (50 mM Tris hydrochloride [Tris-HCl], 150 mM sodium chloride [NaCl], 0.1% (v/v) Tween 20 at pH 7.5) and agitated for 1 h at room temperature with horseradish peroxidase-conjugated secondary antibody (Table 2.3). TBS-T washes were repeated and

the membranes incubated with ECL Prime Western blotting detection reagents (Amersham Biosciences, Amersham, UK) according to the manufacturer's instructions. Bands were visualised on the ChemiDoc MP imaging system (Bio-Rad). An open-source, public domain software package (Image J v1.47) was used for band quantification (353).

Table 2.3. Immunoblotting Antibody Details.

Protein	Clonality	Species	Manufacturer	Cat. Number	Block (w/v TBS-T)	Dilution
GAPDH	MC	Rabbit	CST	5174	5% milk	1:2000
Histone H3	MC	Mouse	CST	3638	5% milk	1:2000
IL-15	MC	Rabbit	Generon	EPR1542Y	5% milk	1:1000
NF-κB p65	MC	Mouse	CST	6956	5% BSA	1:2000
Phospho – NF-κB p65	MC	Rabbit	CST	3033	5% BSA	1:2000
β-actin	MC	Mouse	Sigma	A4700	5% milk	1:5000
ERK	PC	Rabbit	CST	9102	5% BSA	1:1000
Phospho-ERK (Thr202/Tyr 204)	PC	Rabbit	CST	9102	5% BSA	1:1000
Akt	MC	Rabbit	CST	4691	5% BSA	1:1000
Phospho-Akt (Ser 473)	PC	Rabbit	CST	9271	5% BSA	1:1000
Myogenin	MC	Rabbit	Abcam	ab124800	5% BSA	1:1000
MyoD1	MC	Rabbit	Abcam	ab126726	5% milk	1:1000
MAFbx	MC	Rabbit	Abcam	ab168372	5% BSA	1:1000
MURF-1	MC	Rabbit	ECM Biosciences	MP3401	5% BSA	1:1000
Total myosin heavy chain	MC	Rabbit	R&D Systems	MAB4470	5% BSA	1:1000
Puromycin	MC	Mouse	Millipore	MABE343	5% BSA	1:1000

Secondary antibodies diluted 1:15000 in TBS-T. Sigma Aldrich, sheep anti-mouse IgG HRP-linked whole antibody (GFH37-1000). Sigma-Aldrich, donkey anti-rabbit IgG HRP linked whole antibody (GE NA934V).

2.7 Isolation of Total RNA

All work involving ribonucleic acids (RNA) was carried out on surfaces treated with a nuclease control reagent (RNaseZap®, Thermo Fisher). Dedicated RNA pipettes, which were cleaned regularly with the nuclease control solution were used in conjunction with sterile DNase and RNase free filter pipette tips. DNase and RNase free microcentrifuge tubes were also used. All reagents were used exclusively for RNA extractions.

For RNA extraction, 1 mL TRIzol® Reagent (Life Technologies, CA, USA) was added to myoblasts and myotubes grown in 12-well plates or to 100 mg skeletal muscle. The TRIzol® lysates were homogenized by pipetting up and down several times, transferred to microcentrifuge tubes and 200 µL chloroform was added. Following incubation for 2-3 min, the samples were centrifuged for 15 min at 12,000 g and 4 °C. The upper aqueous phase was removed to a new tube and 500 µL isopropanol added. Following a 10 min incubation the samples were centrifuged for 30 min at 12,000 g and 4 °C. The supernatants were removed, the pellets washed with 1 mL 75 % ethanol and the samples were centrifuged for 5 min at 7500 g and 4 °C. The wash was removed and the RNA pellets were vacuum dried for 30 min. The pellets were resuspended in 30 µL RNase-free water and the samples placed in a heat block set at 60 °C for 5 min to ensure complete solubilisation. The RNA yield and quality was determined using a microvolume spectrophotometer (NanoDrop 2000, Thermo Fisher). A 260/280 nm ratio of > 1.7 was considered acceptable for sample use in RT-qPCR. RNA samples were stored at - 80 °C.

2.8 Real-Time Quantitative PCR

A one-step master mix pre-mixed with SYBR green (Primerdesign, Southampton, UK),

was used and 5 µL reaction volumes were prepared in triplicate, in 384 well plates (Table 2.4). One-step RT-qPCR was carried out on a Light-Cycler 480 (Roche, Basel, Switzerland) using the following cycler conditions: 1) Reverse transcription - 10 min, 55 °C; 2) Polymerase activation – 8 min, 95 °C; 3) Denaturation, annealing and extension – 10 s, 95 °C plus 60 s, 60 °C, repeated for 45 cycles; 4) Melting curve – stepwise temperature increase to 95 °C. Non-template controls were run for each primer used and a reference gene was quantified on each plate (See Table 2.5 for primer details). For analysis, the threshold fluorescence was set using a logarithmic view of the curve and was placed in the linear phase of the reaction, above the background signal. Cycle threshold (Ct) values for each sample were defined by the cycle number at which the curve crossed the threshold (see Appendix Table 2). Relative quantities of RNA were calculated as:

$$Relative\ quantity\ RNA = \frac{2^{Ct\ control\ target\ gene - Ct\ sample\ target\ gene}}{2^{Ct\ control\ reference\ gene - Ct\ sample\ reference\ gene}}$$

Table 2.4. SYBR green RT-qPCR Reaction Volumes.

Reagent	Volume per Reaction (µL)
One-step SYBR green master mix	2.5
Primer mix	0.25
RNA	Equivalent to 10 ng
H_20	To 5 µL

Table 2.5. RT-qPCR Primer Details.

Gene	Manufacturer	Primer Sequences
IL15	Primerdesign	F: 5'-GGAAACCCCTTGCCATAGC-3'
		R: 5'-GATGGAAATACTTCTCAAATGTGGT-3'
IL15RA	Life Technologies	F: 5'-CGCTGGGCTCAGCATCTC-3'
	(in-house design)	R: 5'-AGCTGCTCTGCACACATGGA-3'
IL15RB	Life Technologies	F: 5'-GGCTACCTCTTGGGCATCTG-3'
	(in-house design)	R: 5'-TCGAGTTGTAGAAGCATGTGAACTG-3'
MYF5	Primerdesign	F: 5'-CACCTCCAACTGCTCTGATG-3'
		R: 5'-TAAGGAGTTTTTATCTGTGGCATATAC-3'
MYOD1	Primerdesign	F: 5'-CGCCTGAGCAAAGTAAATGAG-3'
		R: 5'-GCCCTCGATATAGCGGATG-3'
MYOG	Primerdesign	F: 5'-GCCCTGATGCTAGGAAGCC-3'
		R: 5'-CTGAATGAGGGCGTCCAGTC-3'
FBX032	Primerdesign	F: 5'-AACTCAAATACAAAATAGGACGCTTT-3'
(MAFbx)		R: 5'-CCTTCGCCTTCTCAAAACAAAC-3'
TRIM63	Primerdesign	F: 5'-GACGCCCTGAGCCATT-3'
(MURF-1)		R: 5'-CCTCTTCCTGATCTTCTTCTTCAAT-3'
PPARG	Primerdesign	F: 5'-GAATAAAGATGGGGTTCTCATATCC-3'
		R: 5'-AACTTCACAGCAAACTCAAACTT-3'
PPARD	Primerdesign	F: 5'-TGATGCCCAGTACCTCTTCC-3'

		R: 5’-CTCGGTTTCGGTCTTCTTGAT-3’
ACACB	Primerdesign	F: 5’-ATGGCCTCCACCGTTGTC-3’
		R: 5’-CTTCTGTCCACTCCACTGTCA-3’
PDK1	Primerdesign	F: 5’-CCAAAGCATCAGAGCCATCATT-3’
		R: 5’-AGGATTACTTGAGCCCAGAAGAT-3’
UCP2	Primerdesign	F: 5’-CTCTCCCAATGTTGCTCGTA-3’
		R: 5’-ATGAGGTTGGCTTTCAGGAG-3’
UCP3	Primerdesign	F: 5’-CCTACAGAACCATCGCCAGG-3’
		R: 5’-GCTTCTCCTTGAGGATGTCGTA-3’
SLC2A4	Primerdesign	F: 5’-ATGGCTGTGGCTGGTTTCT-3’
(GLUT4)		R: 5’-AGCAGGAGGACCGCAAATA-3’
PPARGC1A	Primerdesign	F: 5’-TTGCTAAACGACTCCGAGAAC-3’
		R: 5’-GACCCAAACATCATACCCCAAT-3’
ACTB	Primerdesign	Proprietary
GAPDH	Primerdesign	Proprietary
TMEM8C	Primerdesign	F: 5’-AACTCAAATACAAAATAGGACGCTTT-3
(Myomaker)		R: 5’-CCTTCGCCTTCTCAAAACAAAC-3’

F = Forward primer, R = reverse primer

2.9 Enzyme-Linked Immunosorbent Assay

Enzyme-linked immunosorbent assays (ELISAs) were used, according to manufacturer’s instructions for the quantification of human IL-15 (Human IL-15 Quantikine ELISA Kit; R&D Systems, Minneapolis, MN, USA; sensitivity 2 pg/mL) in myotube culture supernatants

and human resistin (Human Resistin Quantikine ELISA Kit; R&D Systems; sensitivity 55 pg/mL) in ACM. Standards were prepared by a series of 2-fold dilutions of the top standard in accordance with the manufacturer's instructions. No blocking step is required with Quantikine ELISA kits. The prescribed amount of sample or standard was added – in duplicate – to microplates pre-coated with a monoclonal antibody against the target protein. Eight standards were included, including a blank (0 pg/mL) standard. Plates were incubated at room temperature for 2 h, the samples aspirated from the plate and the wells washed four times with the wash buffer provided. 200 µL of the provided monoclonal antibody, conjugated to horseradish peroxidase was added to each well for the prescribed time. The wells were washed as before and 200 µL of tetramethylbenzidine (TMB) substrate solution added for 30 min. Finally, 50 µL 2 N sulfuric acid was added to each well and plates were read with an absorbance microplate reader (ELx808, Biotek, Winooski, VT, USA) at the wavelengths prescribed by the kit manufacturer. The mean of duplicate standard and sample values were calculated and corrected for the mean blank value. A standard curve was generated using the standard values (Microsoft Excel, 2016). A coefficient of determination (R^2) value of > 0.99 was considered acceptable. The slope equation of the curve was used to calculate the sample concentrations. Any sample for which the calculated concentration was below the published sensitivity of the ELISA was considered to be negative (0 pg/mL).

2.10 Multiplex Immunoassay

Cytokine and chemokine concentrations were quantified in ACM by multiplex magnetic bead-based immunoassay (Luminex® Screening Assay, R&D Systems) according to the

manufacturer's instructions. 50 µL of a 1x antibody magnetic bead stock (Adiponectin, Serpin E1, Aggrecan, Amphiregulin, CCL11, CCL2, CCL3, CCL20, Chemerin, CXCL10, Dkk1, Galectin-1, gp120, IL-1β, IL-10, IL-15, IL-7, visfatin, TNFα, Galectin-3BP, Lipocalin-2, CCL4, FABP4, LIF, Leptin, IL-6, Resistin, MMP-1, MMP-2, MMP-3, MMP-7, MMP-8, MMP-9, MMP-10, MMP-12, MMP-13) was added to each well of a flat bottom black plate. 50 µL of undiluted sample or standard were added in duplicate to the plate. The plate was sealed with foil and incubated for 2 h at room temperature on an orbital rotator at 800 rpm. The plate was washed three times with a magnetic plate washer (Bio-Plex Pro™ Wash Station, Bio-Rad) using the wash buffer provided. 50 µL of a biotinylated antibody cocktail was added to each well; the plate was resealed and incubated for 1 h on the orbital rotator. The wash steps were repeated as before and 50 µL of the provided streptavidin-PE was added to the wells. The plate was incubated on the orbital rotator for 30 min and the wash steps repeated for a final time. Finally, the beads were resuspended in 200 µL wash buffer and the analytes were quantified by the Luminex® 200 multiplex analyser (Luminex® Corporation, Austin, Texas, USA).

2.11 Free Fatty Acid Assay

A colourimetric free fatty acid (FFA) quantification assay (Abcam, Cambridge, UK) was used to measure ACM FFA concentrations. The assay converts FFA to their CoA derivatives which are then oxidised, with colour formation occurring in proportion to their concentration. Briefly, a standard curve was prepared as described in the kit protocol and 50 µL acyl-CoA synthase reagent was added to an equal volume of sample or standard in a 96 well plate and incubated for 20 min at 37 °C. The oxidation reaction mix was then

added for 30 min at 37 °C in the dark. The absorbance of each well was read at 570 nm using an absorbance microplate reader (ELx808, Biotek).

2.12 HDL and LDL/VLDL Assay

A colourimetric HDL and LDL/VLDL cholesterol assay kit *(*Abcam, Cambridge, UK) was used to measure cholesterol concentrations in ACM. Cholesterol oxidase oxidises free cholesterol to produce a product that reacts with a probe to provide a colour change in proportion to free cholesterol concentration. Cholesterol esters can be hydrolysed to free cholesterol separately, allowing the separate quantification of free cholesterol and total cholesterol. In brief, a standard curve and the reaction mix were prepared according to the manufacturer's instructions. 50 µL reaction mix was added to an equal volume of sample or standard in a 96 well plate and incubated for 60 min at 37 °C in the dark. The absorbance of each well was read at 570 nm using an absorbance microplate reader (ELx808, Biotek).

2.13 Cell Proliferation Assay

A colourimetric tetrazolium salt-based cell proliferation assay (CellTiter 96® Aqueous One Solution; Promega, Madison, WI, USA) was used. This assay uses 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt (MTS), which is reduced to a coloured formazan product by metabolically active cells. Thus, the assay indicates the number of metabolically active cells in culture at any given timepoint. Myoblast growth medium was replaced with 100 µL serum-free Ham's

F10 base medium to prevent FBS interference with absorbance readings (354). 20 µL MTS reagent was added to each well the plate was incubated at 37 °C, 5 % CO_2 for 3 h. Each condition was assayed in quadruplicate. The absorbance of each well was read at 450 nm using an absorbance microplate reader (ELx808, Biotek).

2.14 Immunofluorescence Staining of Primary Human Myotubes

Myotubes were differentiated for 8 d in 24-well culture plates (See Section 2.4). The culture medium was removed and the cells fixed with 2 % (v/v) formaldehyde in PBS for 30 min. Following permeabilisation in 100 % methanol for 10 min, wells were washed three times with 500 µL PBS and then blocked with 5 % (v/v) goat serum in PBS for 30 min. An anti-desmin primary antibody (Thermo Fisher, MA5-13259) was diluted in 1 % (v/v) BSA/PBS and 150 µL was added per well for 1 h. Wells were washed as before and incubated with 150 µL/well secondary antibody (Goat anti-Mouse IgG Secondary Antibody, Alexa Fluor 488, Thermo Fisher, R37120) for 1 h in the dark. Each well was washed once with PBS and 150 µL/well DAPI (4',6-diamidino-2-phenylindole)/PBS (1:5000, Cell Signalling Technology) was added for 5 min in the dark. Wells were washed with PBS, a drop of mountant added to each well (ProLong Diamond Antifade, Thermo Fisher) and a coverslip applied.

2.15 Quantification of Myotube Thickness, Myotube Number and Nuclear Fusion Index

24-well plates of immunofluorescence (IF) stained (desmin/DAPI) myotubes were imaged on an epifluorescence/brightfield microscope (Leica DMI6000). Triplicate wells were

stimulated for each biological replicate. Multiple images were taken in each well for the quantification of myotube thickness (MTT), myotube number and nuclear fusion index (NFI). For the quantification of MTT, 15 images of each well were obtained using the 63x objective, the first image being obtained at a fixed starting point and subsequent images selected by moving to the next field of view in a predefined pattern. For assessment of myotube number and NFI, five images of each well were obtained in the same fashion, using the 20x objective. Image analysis was carried out by a blinded researcher, using Image J software (353) (Figure 2.1). A myotube was defined as a desmin positive object, containing 3 or more nuclei. The MTT was calculated by taking the average of five measurements obtained along its length, and the centremost myotube in each field of view was chosen for measurement (Fig. 2.1, A). The NFI was defined as the number of nuclei incorporated into myotubes, expressed as a proportion of the total visible nuclei in each field of view (Fig. 2.1, B). In order to calculate a NFI that is closer to the true NFI of the cultures, the measurement was made inside a fixed grid superimposed on each image. This allowed nuclei outside of the grid to be considered in defining a desmin positive structure as a myotube, without considering them for nuclear counting purposes. The true NFI of our cultures is still likely to be higher than the values quoted in this thesis, as some desmin positive structures containing fewer than 3 nuclei likely had additional nuclei outside of the field of view and/or the focal plane of the microscope. MTT data in this thesis comprises 450 total measurements from 90 myotubes per treatment condition for each subject (n), n=3 participants. NFI comprises values from 45 images per treatment condition for each subject, n = 3 participants. This strategy is comparable to previously published work (146,147,355–357) although a higher magnification was chosen for our

MTT measurements to allow greater discrimination between myotubes.

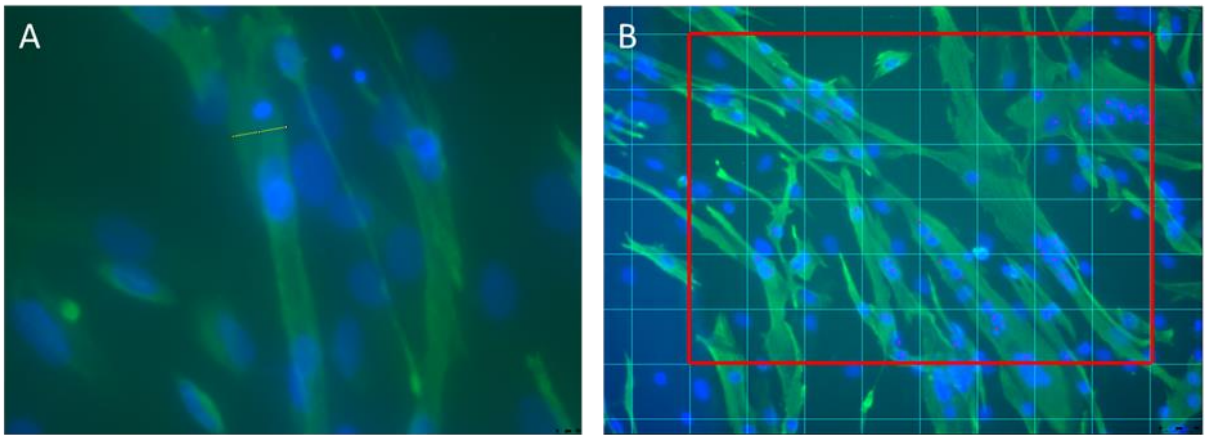

Figure 2.1. Quantification of myotube thickness and nuclear fusion index. Myotubes were fixed, immunofluorescence stained for desmin (green) and with DAPI (blue) and imaged on an epifluorescence microscope. A) For measurement of myotube thickness, images were taken using the 63x objective, the first image being obtained at a fixed starting point and subsequent images selected by moving to the next field of view in a predefined pattern. The thickness of each myotube was calculated by taking the average of 5 measurements (one shown in yellow) obtained along its length and the centremost myotube in each field of view was chosen for measurement. B) For measurement of nuclear fusion index, (NFI) images were taken using the 20x objective in a similar fashion. The NFI (the number of nuclei clearly incorporated into myotubes, expressed as a proportion of the total visible nuclei) was measured within the confines of the red box.

2.16 Immunoprecipitation of Resistin from Adipose Conditioned Media

All procedures were carried out in a class II safety cabinet, and all reagents were sterile. 70 µL Protein A Sepharose® beads (Abcam, ab193256) were incubated with 1 µg anti-resistin primary antibody (polyclonal rabbit IgG, Thermo Fisher PA1-1049) or 1 µg rabbit IgG isotype control (Sigma, 12-370). The antibody-bead mixture was incubated for 4 h at 4 °C on a shaker. The beads were centrifuged at 3,000 g for 2 min at 4 °C and the supernatant was discarded. 1 mL PBS was added. This wash step was repeated twice. 5

mL ACM was added to each bead-antibody conjugate and the ACM-bead-antibody mixture was incubated for 24 h at 4 °C under rotary agitation. The mixture was centrifuged at 3,000 g for 2 min at 4 °C and the supernatant (ACM) was retained and stored at - 80 °C. The antibody-bead conjugates were washed in PBS as before. The antigen-antibody complexes were eluted from the sepharose beads by the addition of 50 µL 2x Laemmli sample loading buffer (see Section 2.6). The elutes were incubated at 50 °C for 10 min. The mixture was centrifuged at 3,000 g for 2 min at 4 °C and the supernatant (elute 1) was retained and stored at – 80 °C. 50 µL 2x Laemmli sample loading buffer was again added to the beads and the procedure repeated (elute 2). Both elutes were stored at – 80 °C in advance of their use in immunoblotting for the detection of resistin (see Section 2.6).

2.17 Oil Red O Staining

Oil Red O staining was carried out on 24-well plates of myotubes that had been previously IF stained using a protocol adapted from Koopman et al. (358). 500 µL PBS was added to each well and the plate incubated at 37 °C for 30 min. The coverslips were carefully removed with forceps, and the wells washed three times in PBS. Microscopic examination of the wells and detached coverslips confirmed that no cellular detachment from the plate occurred. An Oil Red O stock solution (0.5 % (w/v) Oil red O in 60 % triethyl-phosphate in ddH_2O) was diluted 3:2 in ddH_2O. This working solution was filtered through a 0.22 µM syringe filter to remove Oil Red O precipitate. 150 µL Oil Red O working solution was added to each well for 1 h. The wells were washed as before in PBS, a drop of mountant (ProLong Diamond Antifade, Thermo Fisher) added to each well and a coverslip applied.

Brightfield imaging was carried out on a microscope (Leica DMI6000, Fig. 2.2, A). The Oil Red O positive area was quantified by Image J analysis. Briefly, each image was converted to a grayscale 8-bit format (Fig. 2.2, B) and the threshold was adjusted to highlight pixels of intensity between 90 and 110 (Fig. 2.2, C). The total Oil Red O area of this binary image (Fig. 2.2, D) was calculated by the program.

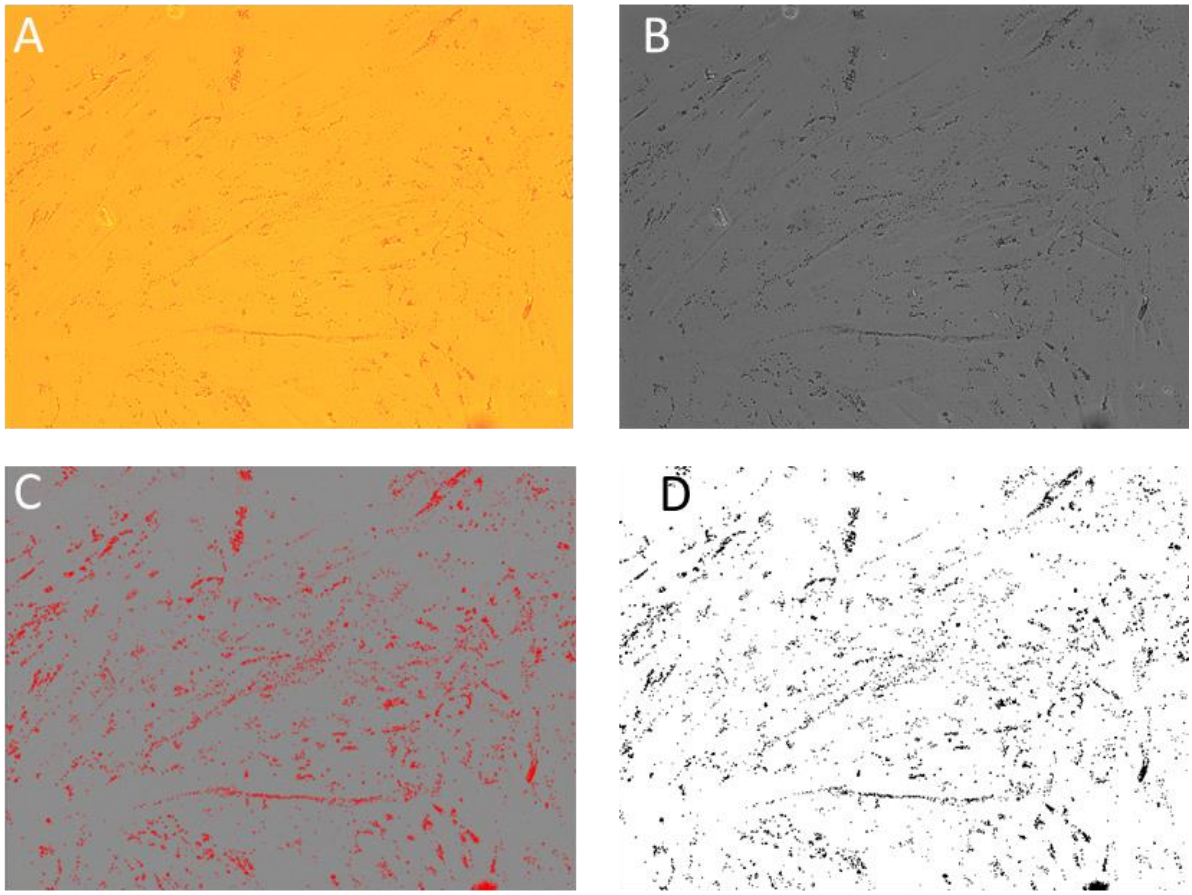

Figure 2.2. Conversion of brightfield microscope images of Oil Red O stained myotubes to binary images suitable for quantification. A,B) Each image was converted to a grayscale 8-bit format. **C)** The threshold was adjusted to highlight pixels of an intensity between 90 and 110. **D)** The total Oil Red O area of this binary image was calculated by the program.

2.18 Mitotracker® Stain

Primary human myotubes were stained with Mitotracker® Green FM, which stains mitochondria independent of their membrane potential and Mitotracker® Orange CM-H2TMRos which accumulates in mitochondria in a membrane-dependent fashion (Thermo Fisher). Both reagents were reconstituted at 1 mM and diluted together in myotube differentiation medium (Green 1:10,000, Orange 1:5000). Culture medium was removed from the myotubes and the wells washed once in PBS. 500 µL Mitotracker® stain medium was added to each well, the plate wrapped in foil and incubated for 45 min at 37 °C. The wells were washed three times with 500 µL PBS; the final wash was not removed. The plates were imaged immediately on an epifluorescence microscope (Leica DMI6000). Five images were taken in each well using the 20x objective, with triplicate wells imaged for each treatment condition in each independent experiment. Image acquisition settings were fixed throughout. The mean fluorescence intensity of the green and red channels of each image was quantified using Image J software. Mitotracker® Orange intensity was corrected for the Mitotracker® Green FM intensity – a surrogate for total mitochondrial mass.

2.19 Seahorse XFe96 Analysis of Myotube Metabolic Function

The Seahorse XFe96 analyser (Agilent Technologies, Santa Clara, California, United States) measures oxygen consumption rate (OCR) and the extracellular acidification rate (ECAR) of cells in 96 well plates to assess their metabolic function. A mitochondrial stress test (Seahorse XF Mito Stress Test) was used; oligomycin, Carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP) and a mixture of rotenone and antimycin A are

serially injected by the analyser to assess aspects of mitochondrial function. Oligomycin inhibits ATP synthase (mitochondrial complex V), reducing OCR. The remaining OCR represents proton leak and non-mitochondrial respiration. Basal ATP production may be calculated by subtraction of this OCR from basal OCR. FCCP uncouples respiration, producing unfettered maximal oxygen consumption by complex IV. Rotenone (complex I inhibitor) and antimycin A (complex III inhibitor) completely inhibit mitochondrial respiration, enabling calculation of proton leak by subtraction from oligomycin OCR values. Baseline ECAR values, indicative of lactate production and thus non-oxidative metabolism, are also obtained during a Mito Stress Test. The test was carried out in accordance with the manufacturer's recommendations. 25,000 myoblasts per well were seeded to 0.2 % gelatin-coated Seahorse XFe96 Cell Culture Microplates. At 24 h the cells were differentiated. Treatment conditions were assayed in quadruplicate – further details can be found in the relevant data chapter. The assay was run at differentiation day 8. An XFe96 sensor cartridge was hydrated in Seahorse XF Calibrant Solution (Agilent Technologies) overnight at 37 °C in a non-CO_2 incubator. On the day of the assay, XF Cell Mito Stress Test assay medium was prepared (XF base medium, 5.5 mM glucose, 1 mM sodium pyruvate, 1 mM glutamine, pH 7.4, filter sterilised). Myotube differentiation medium was replaced with 175 µL/well assay medium and the plate was incubated for 1 h at 37 °C in a non-CO_2 incubator. Oligomycin, FCCP and Rotenone/antimycin A were reconstituted according to the Mito Stress Test Kit instructions and 25 µL of each added to the appropriate wells of the Seahorse utility plate. The utility plate is loaded into the analyser with the cell microplates to facilitate drug delivery during the stress test. The analyser was calibrated and the plate loaded. The assay configuration for each of the four

test phases (basal, oligomycin injection, FCCP injection, rotenone/antimycin A injection) was as follows: mix 3 min, wait 2 min, measure OCR/ECAR 3 min, repeated for three cycles.

The XF Cell Mito Stress Test may also be modified to allow the measurement of endogenous and exogenous fatty acid oxidation and this procedure was carried out according to the manufacturer's instructions. Briefly, the protocols for the seeding of myoblasts and for carrying out the Mito Stress Test were repeated as described above. However, 24 h before the assay, the growth medium was replaced with a substrate limited medium (DMEM: 0.5 mM glucose, 1 mM glutamine, 0.5 mM carnitine, 1 % FBS) to deplete endogenous substrates within the cell. 45 min prior to the assay the medium was replaced with assay medium (111 mM NaCl, 4.7 mM KCl, 1.25 mM $CaCl_2$, 2 mM $MgSO_4$, 1.2 mM $NaHPO_4$, 2.5 mM glucose, 0.5 mM carnitine, 5 mM HEPES, pH 7.4 at 37 °C). For each treatment condition, four assay conditions were utilised to allow endogenous and exogenous fatty acid oxidation to be calculated: BSA controls (added to wells just prior to assay commencement as per the manufacturer) with and without the CPT-1 inhibitor etomoxir (400 μM in FAO assay medium added 15 min prior to the assay) and palmitate-BSA (added to wells just prior to assay commencement as per the manufacturer) with and without the CPT-1 inhibitor etomoxir (400 μM in FAO assay medium added 15 min prior to the assay). Endogenous and exogenous fatty acid oxidation were calculated from the Mito Stress Test data as per the manufacturer's guidance (Fig. 2.3).

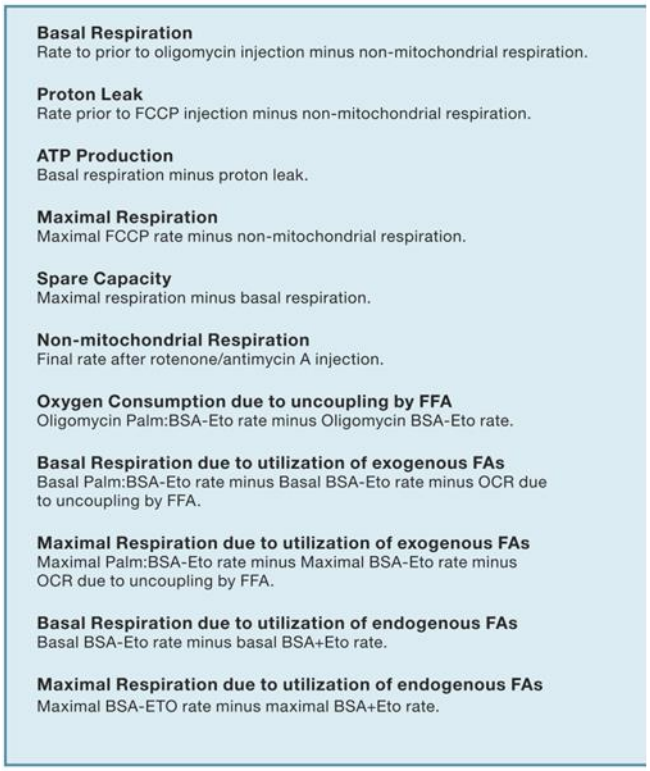

Figure 2.3. XF FAO Assay Parameters. From the Seahorse Bioscience Technical Brief 'Simultaneously Measuring Oxidation of Exogenous and Endogenous Fatty Acids using the XF Palmitate-BSA FAO Substrate with the XF Cell Mito Stress Test'. Available from: https://www.agilent.com/cs/library/applications/5991-7149EN.pdf

2.20 Non-Radioactive Surface Sensing of Translation (SUnSET) Protein Synthesis Assay

A SUnSET protein synthesis assay was employed in myogenic cultures that had been stimulated with recombinant cytokines. On the day of the assay, the culture medium was removed and replaced with differentiation medium containing 1 μM puromycin (Sigma Aldrich) for 30 min. Cultures were then prepared for immunoblotting (Section 2.6), and the resulting blots were probed with an anti-puromycin primary antibody (Table 2.3). Protein synthesis was estimated by Image J densitometric analysis of each lane (Section 2.6),

corrected for ponceau stain densitometry values. Immunoblots were stained with ponceau S (0.5 % (w/v) ponceau S in 1 % acetic acid) for 5 min, washed for 1 min in 1x TBS-T. The blots were then imaged on the ChemiDoc MP imaging system (Bio-Rad). Image J v1.47 was used for the densitometric quantification of each lane.

2.21 Data Handling and Statistical Analysis

A brief description of the data analysis strategy used is intended here. Details of the every tests used for each data set may be found in the appropriate figure legend. All data analysis was carried out using SPSS Statistics 21 (IBM, NY, USA) and GraphPad Prism v5.03 (GraphPad Software, CA, USA). Shapiro-Wilk tests were used to evaluate the normality of data sets and the Levene test was employed to assess the equality of variances within data groups. If possible, data were transformed so that a normal distribution and equal variances were achieved. For parametric data involving two treatment conditions unpaired t-tests were used. Such non-parametric data were analysed by Mann-Whitney U tests. For parametric data involving multiple treatment conditions, one-way analysis of variance (ANOVA) with post-hoc Bonferroni correction was preferred, to limit type I error. Where data violated the assumptions of ANOVA, Mann-Whitney U tests were used, with post-hoc Holm's sequential Bonferroni adjustment to limit the type I error associated with multiple comparisons.

A p value of < 0.05 was considered statistically significant although this should not be confused with the biological significance of a finding, which is considered in the discussion of each data set. In general, data are presented as mean ± standard error of the mean

(SEM) of biological replicates or independent experiments.

Chapter 3

The Subcutaneous Adipose Inflammatory Milleu and Myogenesis

3.1 Background

Adipose tissue mass typically accumulates from middle age at a rate of 0.21 kg/yr in men and 0.14 kg/yr in women, peaking in old age (4). The coexistence of sarcopenia with this increase in adipose tissue mass is termed sarcopenic obesity (8).

Cross-sectional studies of body composition across the lifespan have found that total adipose tissue mass (28) and VAT mass (29) are negatively correlated with skeletal muscle mass. Furthermore, a prospective study of 379 Korean men and women also demonstrated that VAT area, (measured by DEXA) predicted 20 % of the variance in appendicular lean soft tissue area at 2 years (r^2 = -0.203, $p < 0.001$) (259).

Ageing is also accompanied by an increased systemic inflammatory burden (264). This inflammation has been associated with the loss of LBM with age. Indeed, longitudinal studies of elderly cohorts have positively correlated high systemic concentrations of IL-6, CRP and TNFα and with loss of skeletal muscle mass (25,26). Adipose tissue is now known to be a prolific secretor of pro-inflammatory cytokines. Proteomic approaches have identified hundreds of potential adipokines that are expressed and likely secreted by human subcutaneous adipocytes (34). The association of adipose tissue accumulation and systemic inflammation with sarcopenia, coupled with the status of adipose tissue as an endocrine organ, suggest that there is substance to the hypothesis that sarcopenia is – in part – an adipokine-driven phenomenon. This chapter uses a broad definition of the term adipokine, to encompass all molecules that are secreted by adipose tissue and which might have distal effects on other tissues, rather than restricting the definition to traditional cytokines.

A lone study has examined the effect of an inflammatory milieu secreted by human SAT and VAT adipocytes on primary human myotubes, demonstrating that VAT adipocytes from OB individuals induce myotube atrophy (47). However, the SVF of adipose tissue, which includes preadipocytes and macrophages, is a more prolific secretor of pro-inflammatory cytokines than mature adipocytes (46). Consequently, adipokine secretion by human adipose tissue – not just that by adipocytes – must be characterised and its effect on human myofibre size and function determined. It must also be ascertained whether obesity alters this adipose tissue inflammatory milieu as well as its myogenic, atrophic and metabolic effects on skeletal muscle.

The prolific secretion of pro-inflammatory cytokines by VAT is addressed in Section 1.7.1. We have noted that SAT also secretes pro-inflammatory adipokines (45–47) and that VAT represents a small proportion of total adipose tissue mass (12.7 % in endurance runners aged 27 to 69 years (48)). Thus, SAT may be underestimated as a contributor to the systemic inflammatory burden and is the focus of this work.

The anti-myogenic and pro-atrophic actions of some adipokines are now well established. TNFα and IL-6 are well studied in this respect (270,271,273,279,281,359). However, such effects of many other adipokines are not well characterized, and it is not known which components of the subcutaneous adipose inflammatory milieu are most consequential in the pathogenesis of sarcopenic obesity.

Therefore, the objectives of this chapter were as follows:

1. To quantify the secretion of prominent known adipokines from the SAT of middle-aged and elderly participants and to identify whether the inflammatory milieux differ

between lean (normal weight; NW) and non-lean (overweight; OW and obese; OB) elderly individuals.

2. To identify the adipokine constituents of these milieux which may be most consequential in the pathophysiology of sarcopenic obesity.
3. To determine whether these inflammatory milieux alter the development of myotubes in primary myogenic cultures derived from young and old participants.
4. To ascertain whether these inflammatory milieux alter myotube oxidative metabolism.

3.2 Methods

Subcutaneous adipose tissue samples from subjects (aged 45 – 89 yr) of varying BMI (Table 3.1) were maintained in myoblast differentiation medium for 24 h (1 g adipose tissue per 10 mL medium). The media were aliquoted and stored at -80 °C. The concentrations of known adipokines were determined in lean (BMI < 25, n = 13), and non-lean (BMI > 25; overweight, n = 17, obese, n = 15) ACM by multiplex magnetic bead-based human cytokine assays. The concentrations of fatty acids, total cholesterol and free cholesterol were determined in ACM by using enzyme-based colorimetric assay kits (Abcam, UK). Next, the effect of the NW and OB adipose inflammatory milieux on young and old myotube formation was examined. Subconfluent myoblasts from young and old lean, healthy participants (n = 3 per group) were switched to unconditioned differentiation medium, NW ACM or OB ACM. Each young biological replicate was paired with one from

the old experimental group, with both replicates being stimulated with the same NW and OB ACM sample. NW and OB ACM samples were chosen from the small pool of such samples with sufficient media available to facilitate an experiment in which media were renewed every 2 d. A combination of inadequate OW ACM samples of sufficient volume and limitations on the number of available myogenic culture wells led us to eliminate OW ACM from these experiments. At 8 d, myotubes were fixed, IF stained for desmin and with DAPI and imaged on an epifluorescence microscope. These images were used to quantify MTT and NFI. To examine the effect of ACM on myotube mitochondrial metabolism, commercially available primary human myoblasts from a 21 yr old female were used in conjunction with a Seahorse® XFe96 analyser XF Mito Stress Test. We demonstrate in Chapter 4 that such myogenic cultures behave similarly to the ones that we routinely isolate. Four independent experiments were conducted, using genetically identical myotubes that had been derived from four different manufacturer vials and were cultured separately. Myotubes were stimulated with NW (n = 5), OW (n = 7) and OB (n = 7) ACM for 24 h prior to the assay.

Table 3.1. Population Demographics. Subcutaneous adipose tissue samples were obtained from participants undergoing total hip and knee replacement surgery.

	Normal weight	Overweight	Obese
Gender (n, M/F)	4/7	7/10	9/6
Hip/Knee (n)	7/4	11/6	11/4
Age (yr)	68.1 ± 3.3 [a]	72.3 ± 2.2 [a]	68.1 ± 2.0[a]
BMI	23.8 ± 0.3[a]	27.3 ± 0.4[b]	33.1 ± 0.7[c]
WHR	0.88 ± 0.02 [a]	0.92 ± 0.01[a]	0.95 ± 0.02 [b]

Different symbols indicate that values are significantly different ($p < 0.05$) one-way ANOVA with post-hoc Bonferroni adjustment. Values are mean ± SEM. Normal weight age range = 49 to 81 yr, overweight age range = 56 – 85 yr, obese age range = 55 – 81 yr.

3.3 Results

3.3.1 Characterising the Inflammatory Milieu Secreted by Subcutaneous Adipose

The concentrations (mean ± SEM) of ACM adipokines in lean (NW) and non-lean (OW and OB) ACM are represented in Fig. 3.1 and Table 3.2. The data are presented in both table and dot plot formats to allow the absolute values and the true spread of the datasets to be appreciated. The concentrations of resistin (lean 1207 ± 225 pg/mL, non-lean 1778 ± 109 ng/mL; $p = 0.01$ by unpaired t test) and serpin E1 (lean 4033 ± 967 pg/mL, non-lean 13328 ± 3110 pg/mL; $p = 0.02$ by Mann Whitney U test) were significantly elevated in non-lean ACM. There was a trend for the concentrations of fatty acid binding protein 4 (FABP4) to be lower in non-lean ACM (lean 56 x 10^4 ± 12x10^4 ng/mL, non-lean 33 x10^4 ± 37x10^4 pg/mL; $p = 0.06$ by Mann Whitney U test).

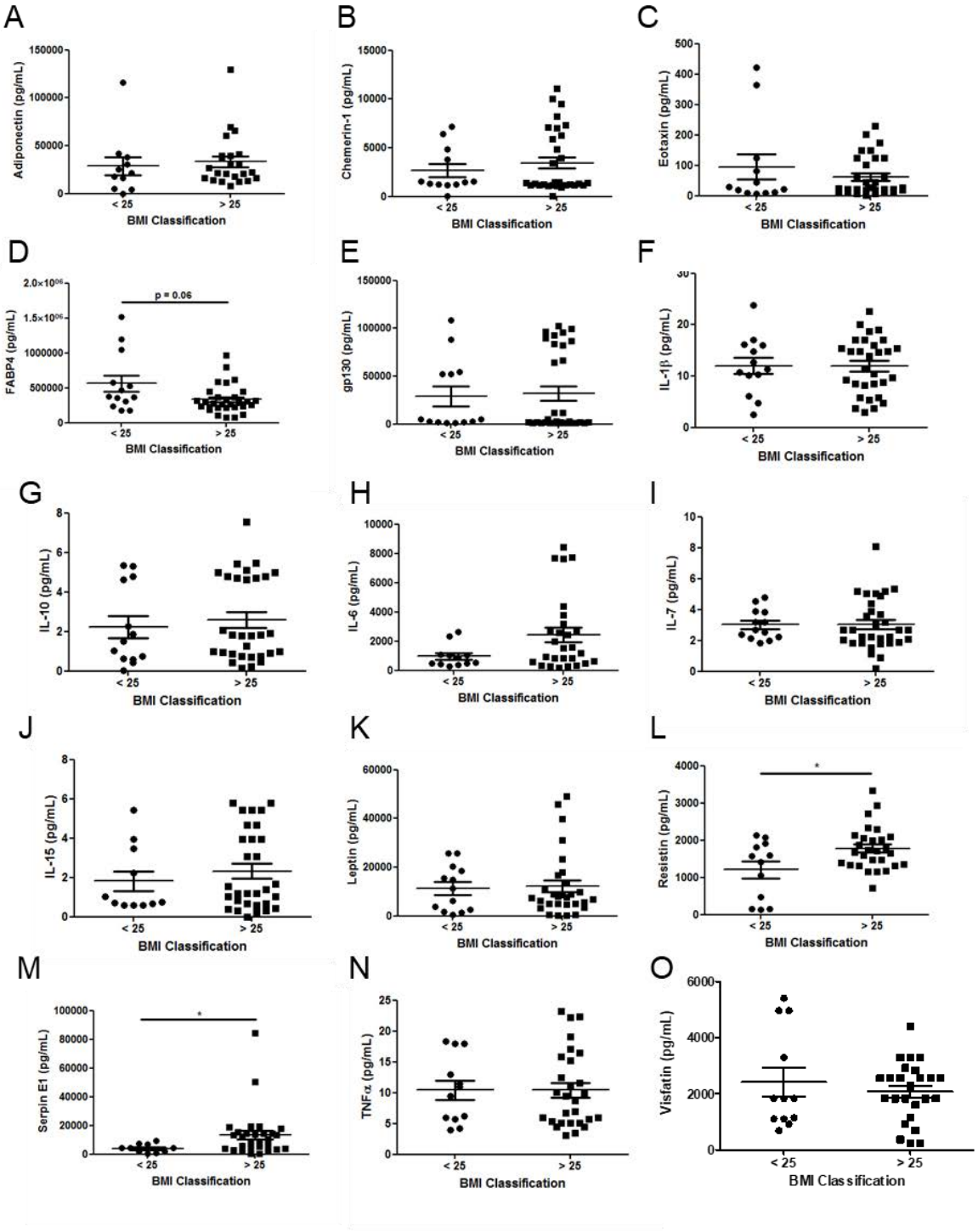

Figure 3.1. The concentration of prominent known adipokines in adipose conditioned medium with respect to BMI. Missing data did not fall within the assay range or were eliminated due to being greater two standard deviations from the mean. * $p < 0.05$, ** $p < 0.01$ by unpaired t test or Mann Whitney U test.

Table 3.2. The Inflammatory Secretory Profile of Lean and Non-Lean Adipose Conditioned Medium

	Lean (BMI < 25) (Mean ± SEM, pg/mL)	Non-Lean (BMI > 25) (Mean ± SEM, pg/mL)	P-value (Lean vs. non-lean)
Adiponectin	28716 ± 4524	28841 3779	0.18
Aggrecan	579 ± 15	513 ± 23	0.07¶
Amphiregulin	532 ± 137	703 ± 67	0.22
Chemerin-1	2655 ± 673	3450 ± 583	0.44
Eotaxin	95 ± 41	61 ± 12	0.81
FABP4	56 $x10^4$ ± 12 $x10^4$	33 $x10^4$ ± 4 $x10^4$	0.06¶
Galectin-1	5.3 $x10^4$ ± 0.6 $x10^4$	5.2 $x10^4$ ± 0.3 $x10^4$	0.85
IL-10	2.25 ± 0.56	2.59 ± 0.39	0.62
IL-15	1.81 ± 0.52	2.32 ± 0.37	0.45
IL-1β	12.02 ± 1.59	11.94 ± 1	0.96
IL-6	968 ± 220	2438 ± 495	0.12¶
IL-7	3.02 ± 0.27	3.04 ± 0.29	0.96
Leptin	11335 ± 2592	12210 ± 2467	0.83
MCP-1	2372 ± 924	1540 ± 406	0.34
MIP1a	363 ± 54	303 ± 32	0.33
MIP1b	101 ± 37	125 ± 24	0.58
MIP3a	85 ± 25	164 ± 51	0.97
Resistin	1207 ± 225	1778 ± 109	0.01

Serpin E1	4033 ± 967	13328 ± 3110	0.02¶
TNFα	10.43 ± 1.57	10.43 ± 1.17	0.99
Visfatin	2420 ± 507	2077 ± 212	0.91¶

Data analysed as lean vs non-lean by unpaired t-test except values marked ¶, which were analysed by Mann Whitney U test.

The variable nature of the concentrations of each cytokine between individuals is somewhat evident in Fig. 3.1. However, such a graphical representation of the data does not adequately communicate the degree to which each ACM differs from that of the average NW individual. In Fig. 3.2, the data are expressed as a log10 transformation of their fold change from the NW mean for each cytokine so that the distance of a given point from 0 on the y-axis scale represents an equivalent change in adipokine concentration when compared to a mirrored point on the other side of the 0 line. It is evident that OW ACM (Fig. 3.2, A) and OB ACM (Fig. 3.2, B) adipokine concentrations are highly variable with respect to the NW mean value. It is also evident that lesser variability exists in the adipokine concentrations found in the ACM of NW individuals, with respect to the NW ACM population mean (Fig. 3.2).

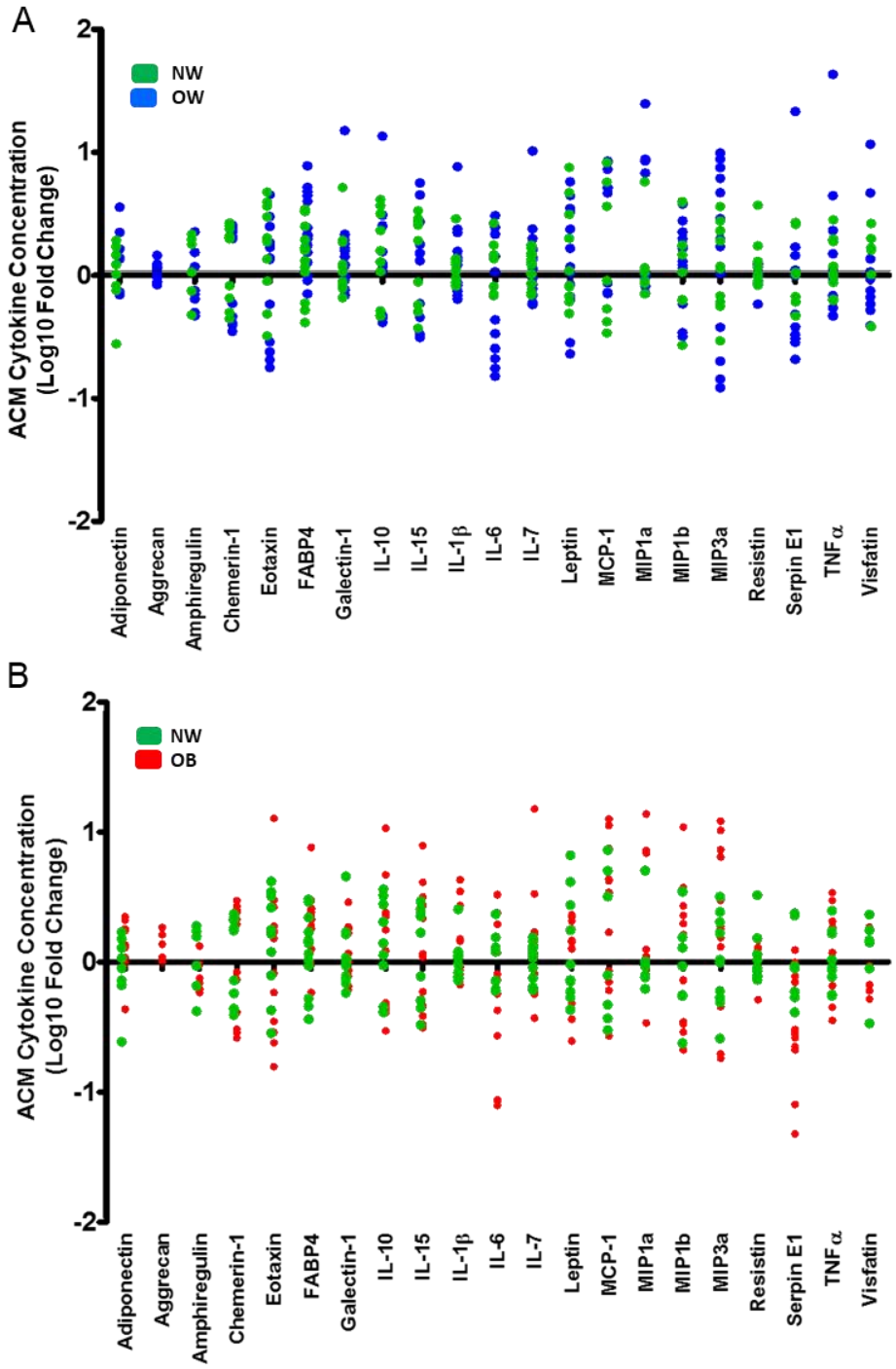

Figure 3.2. The concentration of selected known adipokines in adipose conditioned medium is highly variable. Missing data did not fall within the assay range or were eliminated due to being greater two standard deviations from the mean. Data are expressed as Log10 of their fold change from the NW mean for each cytokine. Log10 transformation allows the linear graphical representation of positive and negative fold change values. **A)** OB and NW ACM adipokine concentrations **B)** OW and NW ACM adipokine concentrations.

To quantify the degree to which the ACM of each subject in this study differs from that of the standard NW individual, an Adipokine Variability Score (AVS) was designed (see Appendix Fig. 3.1 and the associated text). It is evident from these data that it should not be assumed that the ACM concentration of a given adipokine is predictive of an adipose secretory pattern for all the adipokines that were measured.

Linear regression analyses were performed, comparing BMI with the concentrations of adipokines that were significantly different or approached such significance in comparisons of lean and non-lean ACM (Fig. 3.1, Table 3.2). These analyses indicate that BMI is responsible for – at most – a small proportion of the variance in ACM adipokine concentrations (e.g. resistin $R^2 = 0.06$, $p = 0.14$) (Fig. 3.3).

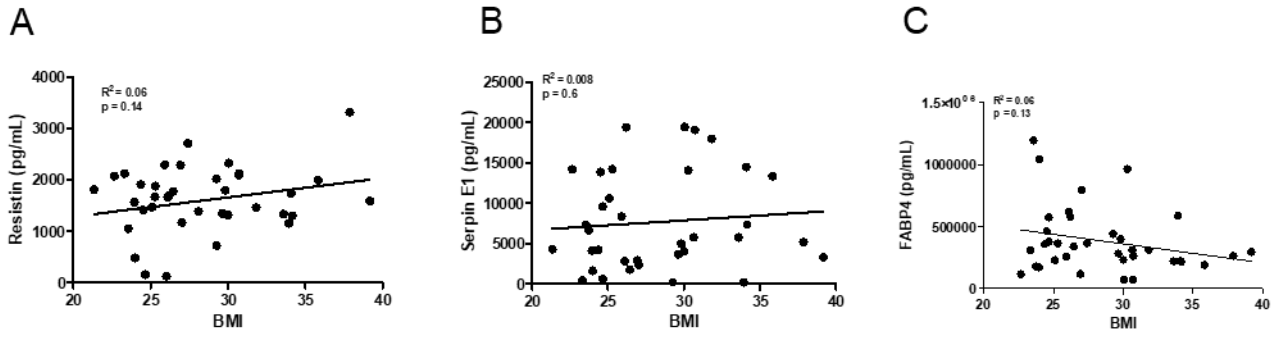

Figure 3.3. BMI is a minor determinant of the concentration of individual adipokines in adipose conditioned medium. A-C) Linear regression analyses of resistin, serpin E1 and fatty acid binding protein 4 (FABP4) ACM concentrations vs. BMI. Missing data did not fall within the assay range or were eliminated due to being greater two standard deviations from the mean.

The multiplex magnetic bead-based immunoassay was supplemented by the use of colourimetric assays, designed to quantify free fatty acids, total cholesterol and free cholesterol. No significant differences in their concentrations were observed between the

lean and non-lean ACM samples (Fig. 3.4).

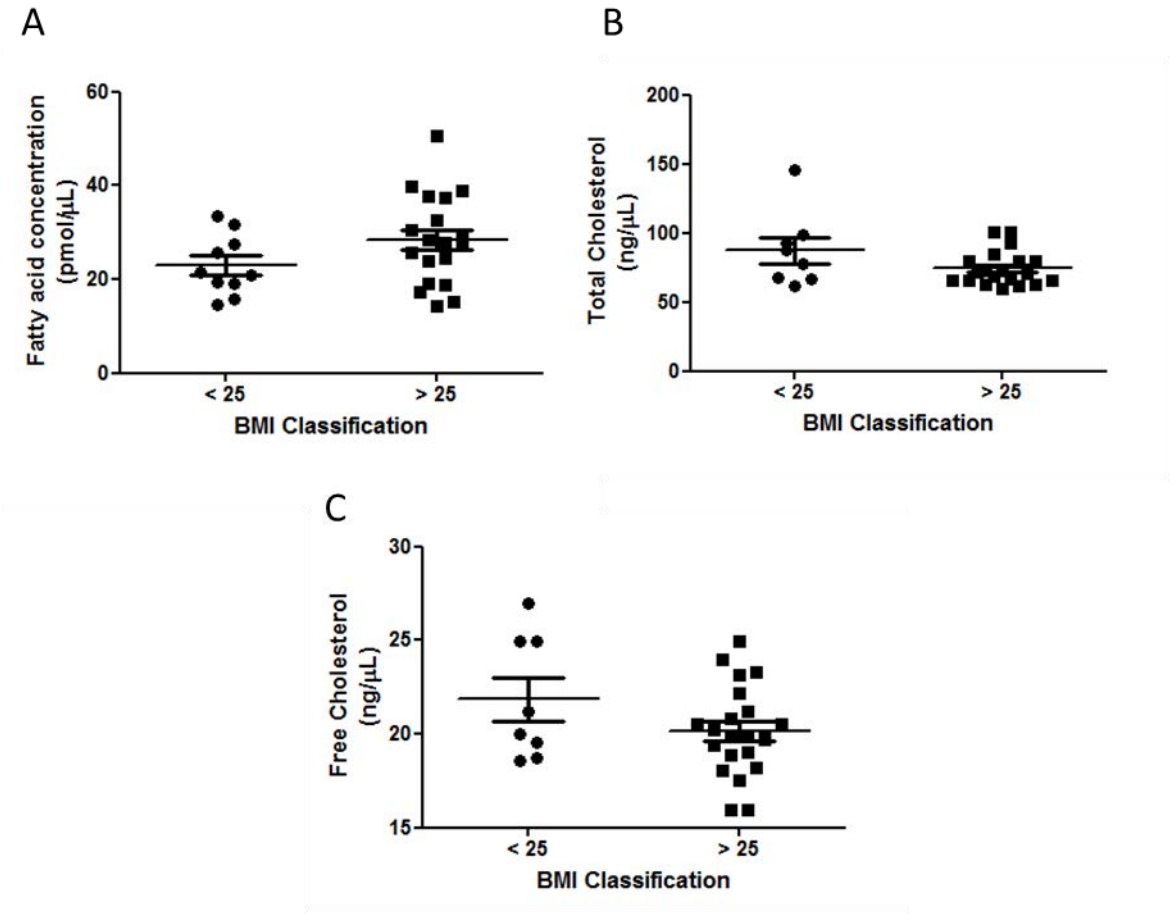

Figure 3.4. The concentration of fatty acids and cholesterol in adipose conditioned medium with respect to BMI. Missing data were eliminated due to being greater two standard deviations from the mean.

3.3.2 The Effect of Adipose Conditioned Medium on the Differentiation of Primary Human Myotubes from Young and Old Participants

Myotubes from elderly participants that were stimulated with OB ACM (See Table 3.3) were significantly thinner (30% ± 5%, p = 0.009) than their NW ACM counterparts (Fig 3.5, B). The NFI of elderly myogenic cultures was also diminished (42 ± 6%, p = 0.0003) by

OB ACM compared to NW ACM (Fig. 3.5, C). Young myotubes were not significantly affected by stimulation with the same ACM samples (Fig. 3.5 B, C).

Table 3.3. Adipokine Concentrations (pg/mL) for the Adipose Conditioned Media that were Selected for Use in the Differentiation of Primary Human Myotubes.

	NW-1 ADI105	**OB-1 ADI119**	**NW-2 RHH120**	**OB-2 ADI117**	**NW-3 ADI110**	**OB-3 RHH158**
Adiponectin	21150	26398	15944	15939	18021	7995
FABP4	$151x10^4$	$31 x10^4$	$46 x10^4$	$22 x10^4$	$11 x10^4$	$10 x10^4$
IL-10	0.01	4.7	5.3	5.4	4.6	0.73
IL-1β	23.9	17.1	16.2	14.8	2.4	5.4
IL-6	475	1564	527	7621	488	309
Leptin	1553	8942	416	23426	1270	17923
Resistin	1417	1465	483	1742	1586	2020
TNFα	No read	No read	3.9	No read	5.9	9.5
Visfatin	1827	2558	1096	3289	1827	No read

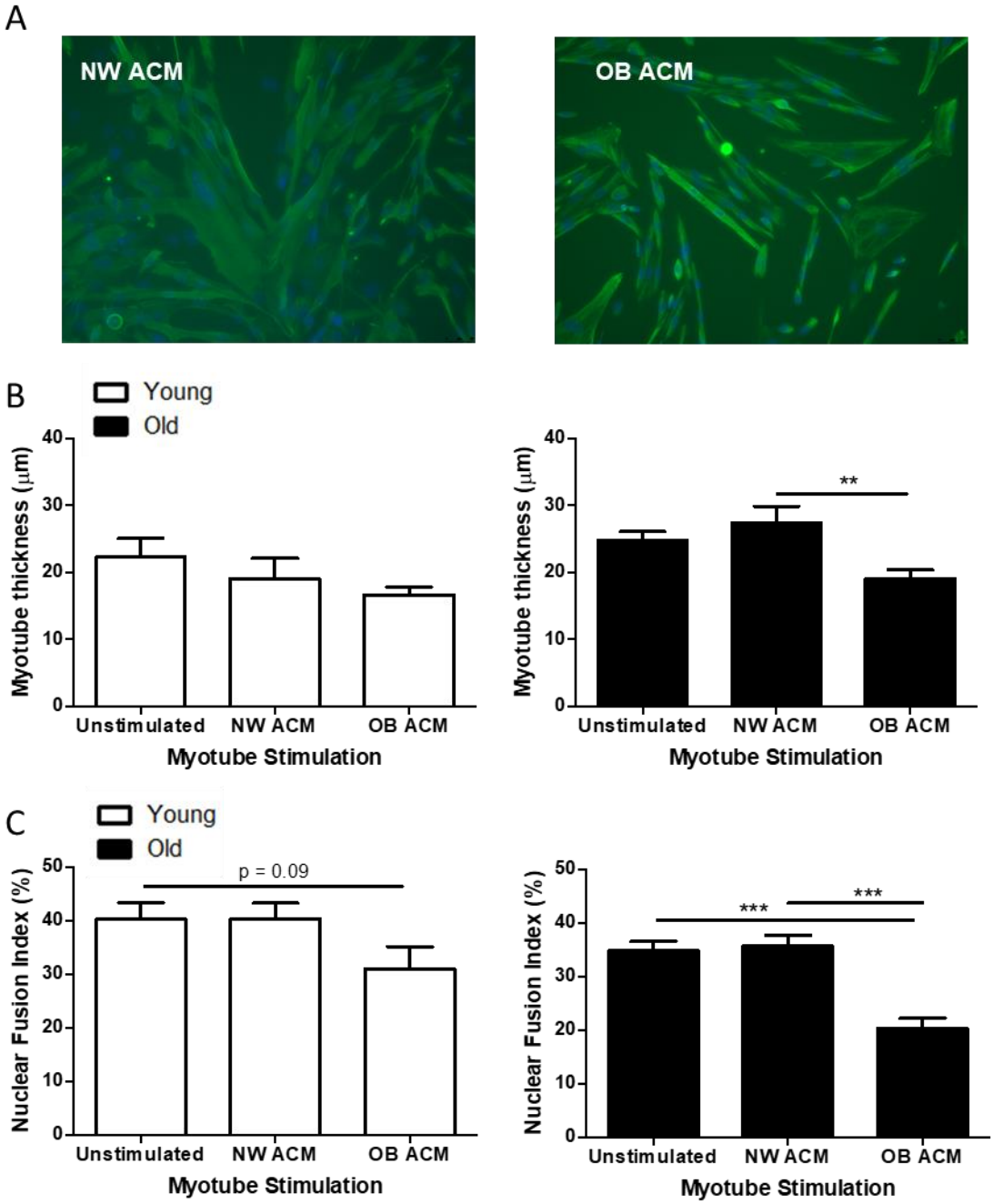

Figure 3.5. Obese adipose conditioned medium stimulation of differentiating primary human myoblasts inhibits myotube formation. A) Representative images at 20x magnification. **B)** Myotube thickness data represent the mean ± SEM of n = 3 biological replicates. Each biological replicate comprises 150 total measurements taken at 63x magnification from 30 myotubes per treatment condition. **C)** Nuclear fusion index data are expressed as mean ± SEM values of n = 3 biological replicates. Each biological replicate comprises 15 images taken at 20x magnification. **p < 0.01, ***p < 0.001 by Mann-Whitney U test with post-hoc Holm's sequential Bonferroni adjustment.

3.3.3 The Effect of Adipose Conditioned Medium on the Mitochondrial Metabolism of Primary Human Myotubes

Basal respiration (17 ± 8 %, p = 0.0003), maximal respiration (17 ± 6 %, p = 0.01), ATP production (9 ± 8 %, p = 0.0005), and spare capacity (22 ± 6 %, p = 0.0003), were significantly elevated by OB ACM compared to NW ACM; the NW values did not differ from unstimulated controls. (Fig. 3.6). OW ACM similarly increased spare capacity, but no other outcome measure was altered by OW ACM.

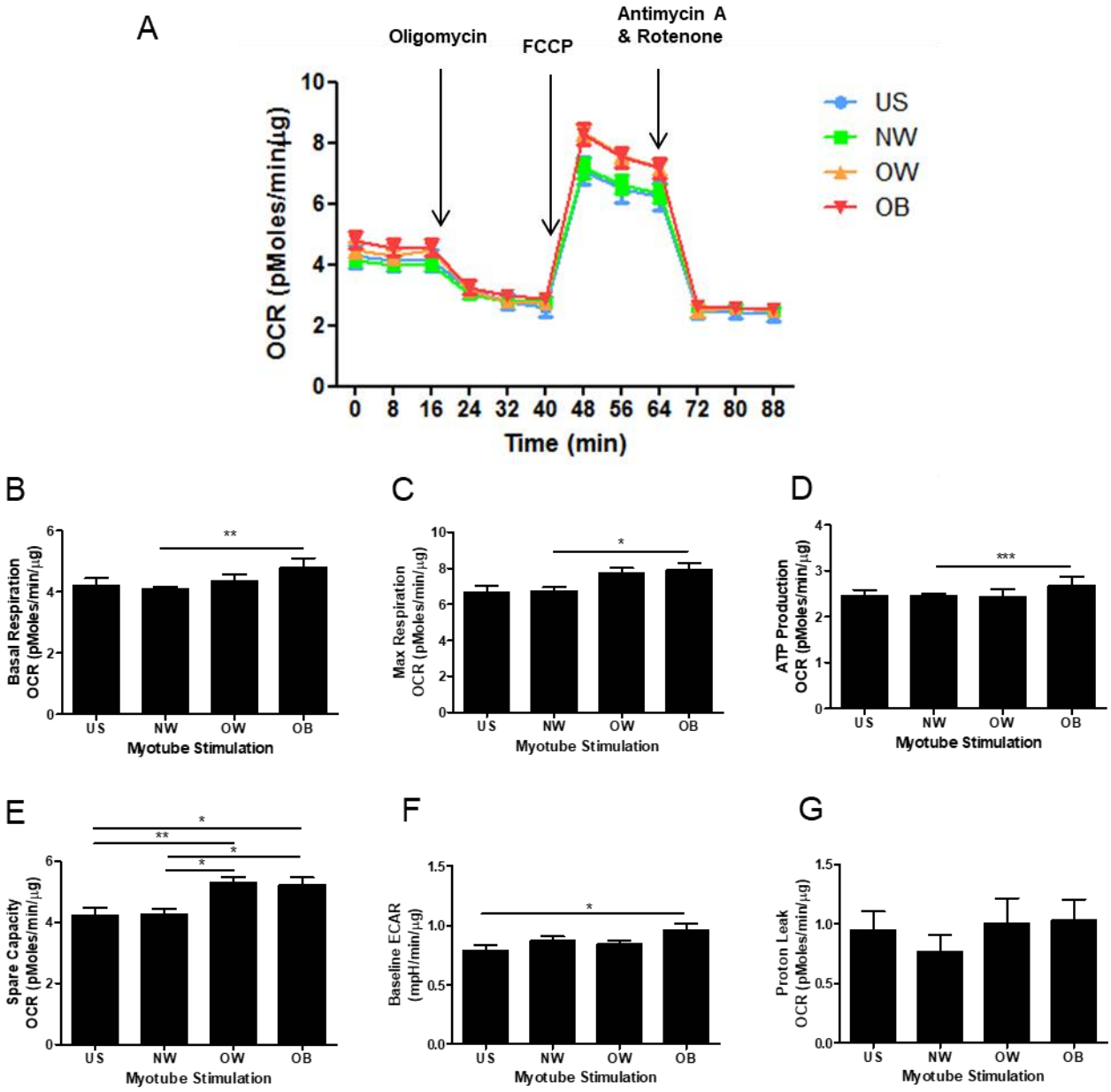

Figure 3.6. Adipose conditioned medium from obese subjects is a metabolic stressor of primary human myotubes. Myotubes derived from commercially available primary human skeletal myoblasts were stimulated for 24 h with conditioned medium derived from the adipose tissue of normal weight (NW, n = 5), overweight (OW, n = 7) and obese (OB, n = 7) individuals. An XF Mito Stress Test was carried out on the Seahorse XFe96 analyser. Data are expressed as mean ± SEM values of n = 4 independent experiments. Each independent experiment comprises data from quadruplicate measurements. $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ by Mann-Whitney U test with post-hoc Holm's sequential Bonferroni adjustment.

3.4 Discussion

3.4.1 The Differential Expression of Adipokines in Lean and Non-Lean Subcutaneous Adipose Conditioned Medium

Resistin and Serpin E1 ACM concentrations were significantly elevated in the OW/OB group compared to their NW counterparts, with FABP4 concentrations being diminished ($p = 0.06$). Resistin is a pro-inflammatory adipokine that is produced predominantly by monocytes and macrophages in humans, with a smaller proportion being produced by adipocytes (287). However, given the importance of adipose tissue M1 macrophage accumulation in ageing and obesity (232,234–236), adipose secretion of resistin may be of some consequence in sarcopenic obesity. Plasma and serum resistin concentrations have been positively correlated with BMI (289,290). Indeed, previous work from our group, using the same patient population from which ACM was generated, (Philp AM, PhD thesis) demonstrated that serum resistin concentrations in the OW and OB are significantly elevated compared to NW individuals (NW, 9818 ± 7325 pg/mL; OW/OB, 13041 ± 9013 pg/mL; $p = 0.03$).

Few studies describe the effect of resistin on sarcopenic muscle. Plasma resistin concentrations have been reported to have an inverse relationship with quadriceps torque in old (69-81 yr), but not in young (18-30 yr), participants (291). C2C12 myoblast proliferation is increased by the transfection of a human resistin eukaryotic expression vector, and such transfection reduces the expression of desmin and results in thinner myotubes (292). We are unaware of any studies that have used primary human myotubes, coupled with exogenous resistin to investigate the effects of this adipokine on skeletal

muscle. Notably, human resistin displays only 59 % amino acid sequence homology to murine resistin. Thus, extrapolation of the effects of murine resistin to human physiology are particularly problematic (293).

Fatty acid binding proteins, including FABP4, are cytosolic proteins that bind hydrophobic molecules and act as lipid chaperones to facilitate fatty acid metabolism (360). ACM concentrations of FABP4 tended ($p = 0.06$) to diminish in non-lean ACM. Previous work from our lab (Philp AM, PhD thesis) measured the serum adipokine concentrations of 174 patients recruited to the same study from which the SAT was sourced for ACM generation; 17 of the 45 SAT ACM donors were included in the serum analyses. FABP4 serum concentrations were significantly elevated in non-lean individuals. This paradox between the diminished expression of FABP4 in non-lean adipose tissue and its elevated serum concentration in the same population has been described in the literature and may perhaps be explained by a significantly higher expression of FABP4 in the hepatic tissue of obese individuals (361). Elevated serum FABP4 is well established as a predictor of pre-eclampsia (362), gestational diabetes mellitus (363) and cardiovascular disease (364,365). However, circulatory FABP4 has not been established as being mechanistically important in the pathogenesis of such conditions and may simply act as a biomarker for dysfunction within individual tissues.

Serpin E1 (plasminogen activator inhibitor-1) – concentrations of which were elevated in non-lean ACM – is a protease inhibitor that inhibits the activation of plasminogen via tissue plasminogen activator (tPA) and urokinase (uPA) (366,367). The plasminogen activation system facilitates the remodelling of the skeletal muscle extracellular matrix

(368). Serpin E1 is an acute phase reactant of which macrophages and adipocytes are prolific producers (366). It is therefore unsurprising that non-lean adipose tissue displays increased serpin E1 secretion. Serpin E1 has been implicated in the development of atherosclerosis and favourable tumour microenvironments (366). Extravascular Serpin E1 can alter extracellular matrix deposition as well as activate intracellular signalling pathways (e.g. the NFκB and ERK pathways) via interaction with urokinase plasminogen activator receptor (uPAR) and low-density lipoprotein-like receptor-1 (LRP-1) receptors (366,369,370). Thus, it is biologically plausible that adipose-derived serpin E1 could exert biological effects in distal tissues, including skeletal muscle. Indeed, it is well established that activation of plasminogen supports skeletal muscle regeneration. uPA-/- and plasminogen -/- mice display severe defects of skeletal muscle regeneration; tPA-/- mice are unaffected, therefore uPA-mediated plasminogen activation appears to be important in skeletal muscle regeneration (371,372). In mice (male C57BL mice with a heterozygous mutation in the insulin II gene; Akita mice), with systemically elevated serpin E1, the serpin E1 inhibitor PAI-039 rescues skeletal muscle regeneration in response to cardiotoxin injury (373). Pertinently, the SC of human participants with Duchenne muscular Dystrophy secrete more serpin E1 than healthy control cells (374). Thus, there is some evidence to suggest that the elevated secretion of serpin E1 by non-lean adipose tissue could have a detrimental effect on skeletal muscle myogenesis.

The composition of the ACM generated for this work is representative of adipokine secretion by a fixed weight of adipose tissue. However, OB and OW individuals have a greater adipose tissue mass than their NW counterparts thus the effect of secreted factors from obese adipose tissue on skeletal muscle could be under-represented in our ACM *in*

vitro model. Furthermore, it is important to note that BMI is an imperfect, if convenient surrogate for adiposity, that is useful in predicting risk (e.g. of cardiovascular disease (375,376)) at a population level, but is less useful for individuals, e.g. elite rugby union forwards may often be classified as OB, despite having low body fat percentages (377). However, dissection studies of elderly cadavers have suggested that about 80 % of the variance in total adiposity can be predicted by BMI (378). The predictive value of BMI for SAT mass is lower, at 61 % for women and 78 % for men (378). Such values are consistent with DEXA validations of BMI as a marker of adiposity (379). Therefore, for a given adipokine secretion profile and a given BMI classification, an individual with a low body fat percentage will have a lower systemic burden of adipose-derived inflammation than an individual with a higher body fat percentage. This is an important consideration. Of the adipokines that we measured in ACM, only resistin and serpin E1 were differentially secreted by a fixed mass of adipose tissue between lean and non-lean individuals. However, these differences are likely to be magnified both locally and systemically by increased adiposity in the non-lean. For this reason, our observations of ACM composition do not preclude other adipokines from playing an important role in the aetiology of sarcopenia or indeed other pathologies. For example, no difference in IL-6 ACM content was observed between lean and non-lean participants. However, systemic concentrations of IL-6 increase with age (264) and despite low concentrations of IL-6 appearing to support skeletal muscle hypertrophy (266,268,380), higher concentrations of IL-6 induce skeletal muscle atrophy (270,271). Thus, adipokines that were not observed to be differentially expressed by lean and non-lean adipose tissue may still be important contributors to the aetiology of sarcopenia.

Adipose tissue is located primarily in two anatomical depots, SAT and VAT. In this study, we were limited to using SAT for the generation of ACM. VAT is considered to be the more prolific secretor of adipokines (41,276). Adipocyte hypertrophy (201,202) and macrophage accumulation (234,240) – which drive AT inflammation – are more prominent in VAT than SAT. VAT is also more often associated with an increased risk of cardiovascular disease and the metabolic syndrome (39,40). VAT mass has frequently been shown to have a substantial negative correlation with skeletal muscle mass (29,258), and VAT area can predict 20 % of the variance in appendicular lean soft tissue area after 2 yr (259). Indeed, Pellegrinelli et al. generated conditioned medium from human SAT and VAT adipocytes. Although they do not describe the composition of these media in sufficient detail, it is evident that OB VAT conditioned medium contained greater concentrations of adipokines such as IL-6, granulocyte colony-stimulating factor (G-CSF) and IL-7 than OB SAT conditioned medium (47).

However, the importance of VAT as a source of inflammation does not preclude SAT from playing a prominent role in inflammation-mediated sarcopenia. Negative correlations have also been described between total AT mass (28), SAT mass (258) and skeletal muscle mass. SAT mass does not appear to increase with age in men for a given BMI (44), however, when younger and older cohorts are not BMI matched, an absolute gain of SAT in conjunction with increased body weight and BMI is evident with advancing age (45). Importantly, VAT represents a small proportion of total AT mass. Thus, SAT is likely to also be an important contributor to the systemic inflammatory burden.

IMAT mass also increases with age (42,120). Given the proximity of IMAT to skeletal

muscle, it may be an important local contributor to sarcopenic obesity. However, little is known about adipokine secretion by IMAT due to the technical difficulties associated with its biopsy in adequate quantities for study. In frail elderly, IMAT accumulation positively correlates with IL-6 protein expression within the skeletal muscle (120). Surprisingly little is known about the mechanisms underlying the development of IMAT. A population of skeletal muscle non-myogenic progenitor cells, widely termed fibro-adipogenic progenitor cells (FAP) have been identified as one possible source of adipocytes within skeletal muscle (381). FAP were simultaneously identified in mice by two research groups as Sca-1^+ $CD34^+$ $PDGFR\alpha^+$ cells with both adipogenic and fibrogenic, but not myogenic potential (382,383). More recently, FAP have been identified in human skeletal muscle (381). Notably, adipocytes formed by FAP display a disordered metabolism compared to subcutaneous adipose stroma cell-derived adipocytes e.g. they are insensitive to insulin-induced glucose uptake (384). Thus, some evidence exists to suggest that FAP-derived adipose tissue differs from SAT. Should FAP be definitively identified as contributors to IMAT, their *in vitro* culture may offer a method of profiling the IMAT secretome.

3.4.2 The Composition of Adipose Conditioned Medium is Highly Variable, Irrespective of Body Mass Index

One of the observations from our profiling of the cytokine content of ACM is the degree of variability that exists within individuals and between individuals. It is evident in Fig. 3.2 that individual NW, OW and OB ACM samples display considerable deviation of their adipokine content from the NW group mean. This visualisation also highlights that the

degree of such variability is less in NW ACM. This phenomenon was quantified by the design of the AVS (Appendix Fig. 3.1). The AVS clearly illustrates that the OW and OB ACM are on average significantly different in their constituents than NW ACM. Furthermore, a plot of OB ACM adipokine concentrations, in which four OB ACM with similar resistin concentrations are highlighted (Appendix Fig. 3.1) illustrates that such clustering for one adipokine is not predictive of those samples containing similar concentrations of other adipokines. Thus, while BMI clearly alters the composition of the milieu secreted by adipose tissue, the degree to which particular adipokines are affected is unpredictable.

Here, the AVS score is representative of 21 adipokines that we measured using multiplex magnetic-bead human cytokine assays. Proteomics approaches have identified hundreds of potential adipokines that are expressed by human subcutaneous adipocytes (34,385). Thus, the potential for adipose tissue secretome variability between individuals is substantial. Most proteomic profiling has thus far utilised primary human adipocytes (34,385,386) or adipose tissue (387), rather than adipose tissue conditioned media and has thus been limited to predicting whether individual proteins are likely to be secreted by adipose tissue.

As noted previously, Pellegrinelli et al. examined the effect of an inflammatory milieu secreted by human SAT and VAT on myotubes, but used adipocytes alone, rather than adipose tissue to generate conditioned medium (47). The results of their multiplex cytokine analysis of those conditioned media is provided in insufficient detail to draw meaningful conclusions as to the differences between their NW and OB subcutaneous adipocyte conditioned medium (47). Thus, although we quantified a limited panel of

adipokines in our ACM, it represents a substantial contribution to our knowledge of the adipose tissue secretome and how it is altered by obesity. This work will benefit from expansion. First, by expanding the numbers of participants, particularly in the NW group. Adipose tissue was obtained from patients undergoing total hip and knee replacement surgery for osteoarthritis. Osteoarthritis is more common in non-lean individuals (388). Thus, our recruitment in the NW category lagged behind that in the OW and OB categories over the lifetime of this project. Second, by carrying out metabolic phenotyping of the participants to ascertain whether measures of insulin resistance better explain the variability in SAT adipokine secretion than BMI. Finally, this work should be developed by the use of proteomic approaches to expand on our adipokine profiling of ACM.

Excessive exogenous delivery of free fatty acids can have detrimental effects on skeletal muscle, including insulin resistance and altered skeletal muscle lipid partitioning (241,389–391). Thus, the multiplex magnetic bead-based immunoassays of ACM were supplemented by colourimetric assays designed to quantify free fatty acids, total cholesterol and free cholesterol. No significant difference in their ACM concentrations was observed. It is again evident from these data that we recruited far fewer individuals in the NW category than in the OW and OB categories. Increasing the sample size in the NW category – which will be possible in our laboratory in the coming years – would increase the statistical power of such assays. Furthermore, the assays detailed here detect total fatty acid and cholesterol content of the ACM. It is possible that the fatty acid and cholesterol species present in ACM differ between lean and non-lean individuals. Lipidomic profiling of such ACM, in addition to the proteomic work proposed, would be a worthwhile endeavour.

3.4.3 Obese Adipose Conditioned Medium has a Detrimental Effect on the Formation of Elderly Myotubes

Primary human myogenic cultures were differentiated to form myotubes in the presence of ACM. Six ACM were used in this work, three NW and three OB. Adipose tissue donor participants were those undergoing joint replacement surgery; the skeletal muscle donor participants were healthy recruits. The timing of subject recruitment in those studies precluded an experimental design in which each primary myogenic culture was treated with a panel of the same NW and OB ACM. Thus, each young myogenic culture donor was paired with an elderly donor, and those cultures were treated with the same NW and OB ACM.

Myotubes from elderly participants that were stimulated with OB ACM were 30 % thinner than their NW ACM counterparts, and the NFI of elderly myotubes was diminished by more than 40 % (Fig 3.5, B). The same ACM samples had no significant effect on the formation of myotubes by myogenic cultures derived from young participants.

The ACM used were chosen primarily because there was sufficient ACM available to conduct the experiment. It is evident from Table 3.3 that overall the OB ACM had higher IL-6, leptin, resistin and visfatin concentrations as well as lower FABP4 concentrations than their NW counterparts. It is biologically plausible that these differences in ACM composition, acting in concert could have a profound effect on myotube development. The discrepancy in leptin concentration between these NW and OB ACM is not representative of the lean and non-lean populations as a whole (Table 3.2). It is also possible that other unmeasured factors – some perhaps undiscovered – within OB ACM contributed to the considerable declines in MTT and NFI observed in elderly cultures. Despite the variability

in ACM composition, these effect sizes are large, with relatively small SEM values observed for the three myogenic cultures in each age group (~5-6%). While three biological replicates were sufficient to detect these changes in elderly myotubes, it is possible that our assay is underpowered to detect lesser, but none the less real and physiologically important decreases in MTT and NFI in young cultures. Ways in which the assay might be improved are considered in Chapter 7.

Our finding that OB ACM significantly reduced MTT and NFI in elderly myotubes, whereas young myotubes treated with the same ACM were not affected to the same extent is both remarkable and novel. Previous work in this area has shown that conditioned medium from SAT adipocytes derived from lean individuals does not alter the MTT or NFI of myotubes in myogenic cultures isolated from a neonate (47). In the same study, conditioned medium from VAT adipocytes significantly diminished MTT and NFI. However, the SVF of adipose tissue, which includes preadipocytes and macrophages, is a more prolific secretor of pro-inflammatory cytokines than mature adipocytes (46); the work presented here considers the inflammatory milieu secreted by adipose tissue as a whole. In the 3D adipocyte and myotube co-culture system used by Pellegrinelli et al., they observed that VAT adipocytes modified myokine secretion by myotubes, creating a unique inflammatory milieu that was more than the sum of its adipocyte and myotube parts (47). In future work, quantification of adipokine concentrations in ACM after 24 h in culture with myotubes from young and old participants might yield additional useful information about the inflammatory environment that detrimentally affects myotube formation.

The mechanisms underlying the differential responses of young and elderly myotubes to OB ACM were not explored in this study, yet plausible avenues of enquiry exist. Primary

human myogenic cultures are known to retain some of the characteristics of their donors (76–78). Whether the differentiation of primary human myogenic cultures diminishes with age is controversial (146,147), although culture of myoblasts from older individuals in autologous serum has been reported to adversely affect their differentiation (148). Definitively establishing whether such a difference in differentiation capacity exists under physiologically relevant culture conditions is necessary, only then can a detailed examination of the molecular differentiation program (e.g. myogenic regulatory factor expression) be made to elucidate the mechanisms that underlie an age-related decline in differentiation capacity in the presence of OB ACM.

Differential expression of cytokine receptors between young and elderly individuals has been widely described, including in the brain (392), spleen (392) and NK cell population (393). Indeed, in a later chapter of this thesis (Chapter 6), diminished expression of the IL-15 receptor IL-2Rβ is described in elderly skeletal muscle. We are unaware of any comprehensive profile of cytokine receptor gene or protein expression made in aged human skeletal muscle. It is possible that altered cytokine receptor expression levels leave elderly myogenic cultures more susceptible to the detrimental effects of OB ACM on culture differentiation.

3.4.4. Obese Adipose Conditioned Medium Increases Myotube Oxidative Metabolism Without Altering Glycolytic Activity

24 h stimulation of myotubes with OB ACM (n = 7) increased basal respiration, maximal respiration, ATP production and spare capacity compared to NW ACM (n = 5). Baseline

extracellular acidification rate (ECAR) — a surrogate measurement of glycolytic activity – did not differ with BMI classification, although ECAR was slightly elevated by OB ACM compared to unstimulated myotubes. Thus, the increase in oxygen consumption observed in myotubes stimulated with OB ACM is unlikely to be due to a high rate of glycolysis in the presence of an excess of oxygen (the 'Warburg effect') (394,395). Thus, OB ACM appears to increase the energetic demand of primary human myotubes, with that demand being fulfilled by oxidative metabolism. The mechanism by which OB ACM increases myotube energetic demand remains to be determined.

It is possible that the inflammatory milieu of OB ACM may increase muscle protein turnover and indeed alter the balance between muscle protein synthesis and breakdown. For example, evidence exists to suggest that TNFα activates muscle catabolic pathways. *In vitro*, stimulation of C2C12 myotubes with rTNFα induces myotube atrophy and increases the expression of the ubiquitin E3 ligases MAFbx and MURF-1, which target proteins for proteasomal degradation (65,278). *In vivo,* rTNFα administration (IV, 100 μg/kg) to rats increases skeletal muscle ubiquitin gene expression, as well as increasing the ubiquitination of skeletal muscle proteins (279,280). However, a study of 4 h human rTNFα infusion in healthy young men – which raised plasma TNFα concentrations to ~17 pg/mL (similar to our most concentrated ACM) – did not observe any alterations in skeletal muscle protein turnover (396). A similar study of recombinant IL-6 infusion in healthy men achieved plasma concentrations of 140 pg/ml, far lower than the IL-6 content of ACM (Table 3.2). A 50 % decrease in muscle protein turnover was observed, with a small increase in net muscle protein breakdown (397). Little evidence exists to suggest what effect other adipokines, e.g. resistin might have on skeletal muscle protein turnover.

Primary human myotubes represent a useful *in vitro* model for the preliminary explorations of such effects. L-[2,6-^{3}H] phenylalanine might be used to measure muscle protein synthesis and breakdown in the presence of ACM and individual recombinant cytokines (67,398). It is anticipated that such studies will be possible in our lab, following the generation of additional ACM.

Finally, although the increased basal respiration in OB ACM-treated myotubes was accompanied by an increase in ATP production, the presence of energy sinks in such cells, e.g. futile substrate cycles such as the one between fructose-6-phosphate and fructose 1,6-biphosphate that is known to be induced by TNFα (399) cannot be discounted.

24 h stimulation of rat L6 myotubes with resistin, concentrations of which were increased in OB ACM has been found to inhibit glucose uptake and impair glycogen synthesis (308,309). Resistin has also been observed to inhibit palmitate uptake by L6 myotubes (310). It is therefore unlikely that resistin, (should it suppress both glucose and fatty acid uptake in human myotubes – a considerable assumption) is in large part responsible for the increased oxidative metabolic activity observed in OB ACM stimulated cells. However, this assumes that primary human myotubes behave similarly to an immortalised rat cell line. Furthermore, the concentrations of resistin used in such studies were up to 250 times greater than those found in ACM. The metabolic effects of resistin on human myotubes are explored in Chapter 5. Other prominent components of the adipose inflammatory milieu could also influence myotube metabolism in those ACM with elevated concentrations. For example, TNFα and IL-1β increase glucose uptake by L6 myotubes

(400); a separate study found that TNFα increased L6 myotube glycolytic activity. IL-6 infusion in human participants increases whole-body and skeletal muscle lipolysis, without affecting glucose turnover (316–318). To summarise, many adipokines are known to alter myotube and skeletal muscle metabolism. However, the variability in the composition of adipose inflammatory milieux is such that discussion of which adipokines are most consequential in producing the metabolic changes observed with OB ACM stimulation falls within the realms of informed speculation.

3.5 Limitations and Future Directions

The limitations of and the opportunities for development of this work have been outlined above and are summarised here. Firstly, recruitment of NW participants for SAT biopsy was limited. Recruitment of patients undergoing joint replacement surgery is ongoing. Thus, the potential exists for our laboratory to generate additional NW ACM in the future. Furthermore, while myogenic cultures from three individuals were sufficient to detect a significant OB ACM-induced decrease in MTT and NFI in elderly cultures, it is possible that smaller, but important decreases were not detected in cultures derived from the young. It is evident in subsequent chapters of this thesis that three biological replicates are sufficient for such assays when recombinant cytokine stimulations are used, but ACM, perhaps due to the variability in its adipokine composition requires the power of additional replicates. This work establishes a statistical precedent (e.g. standard deviations) for the planning of future studies.

Resistin and serpin E1 are identified here as adipokines that are differentially secreted by the adipose tissue of lean and non-lean individuals. It is clear from the discussion above

that both may influence skeletal muscle mass. There is also limited evidence to suggest that resistin might alter skeletal muscle metabolism. Further exploration of both adipokines in this regard is warranted. Resistin has emerged as the adipokine with the potential to be of most consequence in the aetiology of sarcopenia. Its effects in skeletal muscle are therefore considered further in Chapters 4 and 5 of this thesis. The inflammatory milieux secreted by VAT and IMAT are of great interest in understanding the pathogenesis sarcopenic obesity. Given the prolific inflammatory profile of VAT and the proximity of IMAT to skeletal muscle, these milieux should be comprehensively characterised.

The variability in ACM adipokine concentrations, irrespective of BMI is notable. Comprehensive proteomic and lipidomic profiling of SAT, VAT and IMAT ACM with respect to BMI/adiposity would, therefore, be of great utility to researchers interested in the mechanisms underlying sarcopenic obesity.

This chapter has presented an overview of the effects of ACM on myotube oxidative metabolism. There remains a need to determine which adipokines are most consequential in driving the increase in such metabolism by OB ACM. The possibility that OB ACM causes an increase in muscle protein turnover should be considered.

Finally, work such as ours would benefit from establishing the most physiologically relevant culture conditions for human primary myogenic cultures. Subsequently, the issue of whether myogenic cultures derived from older individuals differ from those of their younger counterparts might be definitively established.

3.6 Conclusions

This chapter has addressed its stated objectives of

- Quantifying the secretion of prominent known adipokines by the adipose tissue of elderly participants with respect to BMI.
- Determining whether these inflammatory milieux alter the development of myotubes in primary myogenic cultures derived from young and old participants.
- Ascertaining whether the inflammatory milieux alter myotube oxidative metabolism.
- Identifying candidate adipokine/s for further study which may be most consequential in the pathophysiology of sarcopenic obesity.

The secretion of resistin was significantly increased in non-lean adipose tissue. It is evident from the preceding discussion that that resistin might have detrimental myogenic and metabolic actions in skeletal muscle. Thus, subsequent chapters examine these actions in greater detail.

Chapter 4

Resistin and *In Vitro* Human Myogenesis

4.1 Introduction

It was established in Chapter 3 that OB ACM has a detrimental effect on the differentiation of myogenic cultures derived from elderly participants. It was further established that resistin is differentially secreted by the adipose tissue of lean and non-lean individuals and thus represents a plausible candidate for mediating – at least in part – these anti-myogenic effects. Notably, the OB ACM used in these experiments had elevated concentrations of visfatin and leptin compared to the NW ACM that were used (Table 3.3); this was not representative of the NW and OB populations as a whole (Table 3.2). Furthermore, visfatin and leptin are understudied with respect to their effects on myogenesis. Thus, we identified resistin, leptin and visfatin for further study in this regard. NFκB pathway signalling (Fig. 4.1) has in the past, been implicated as both a positive and negative regulator of myogenesis (401). Genetic approaches to this problem have now clearly established the classical NFκB pathway as a negative regulator of myogenesis. Myotube formation has been demonstrated to be enhanced in primary cultures derived from p65 knockout ($p65^{-/-}$) mice (402) and in mice with the deletion of one NFκB p65 allele ($p65^{+/-}$) (403). $p65^{-/-}$ mice have also displayed a 76 % increase in tibialis anterior fibre number (402). Furthermore, IKKβ-deficient mice are protected against denervation-induced skeletal muscle atrophy (3). Resistin has been shown to activate NFκB signalling in the human liver cancer cell line HepG2 (404), human coronary artery endothelial cells (405) and human macrophages (406). Thus, we hypothesised that resistin might impair primary human myogenesis by activating NFκB signalling.

Therefore, the objectives of this chapter were as follows:

1. To determine whether resistin, leptin and visfatin alter primary human myogenesis *in vitro*, and to establish whether they are – at least in part – responsible for the detrimental effects of OB ACM on primary human myogenesis.
2. To determine whether resistin activates classical NFκB pathway signalling in primary human myogenic cultures.
3. To ascertain if such NFκB pathway signalling mediates any detrimental effect of resistin on myogenesis.

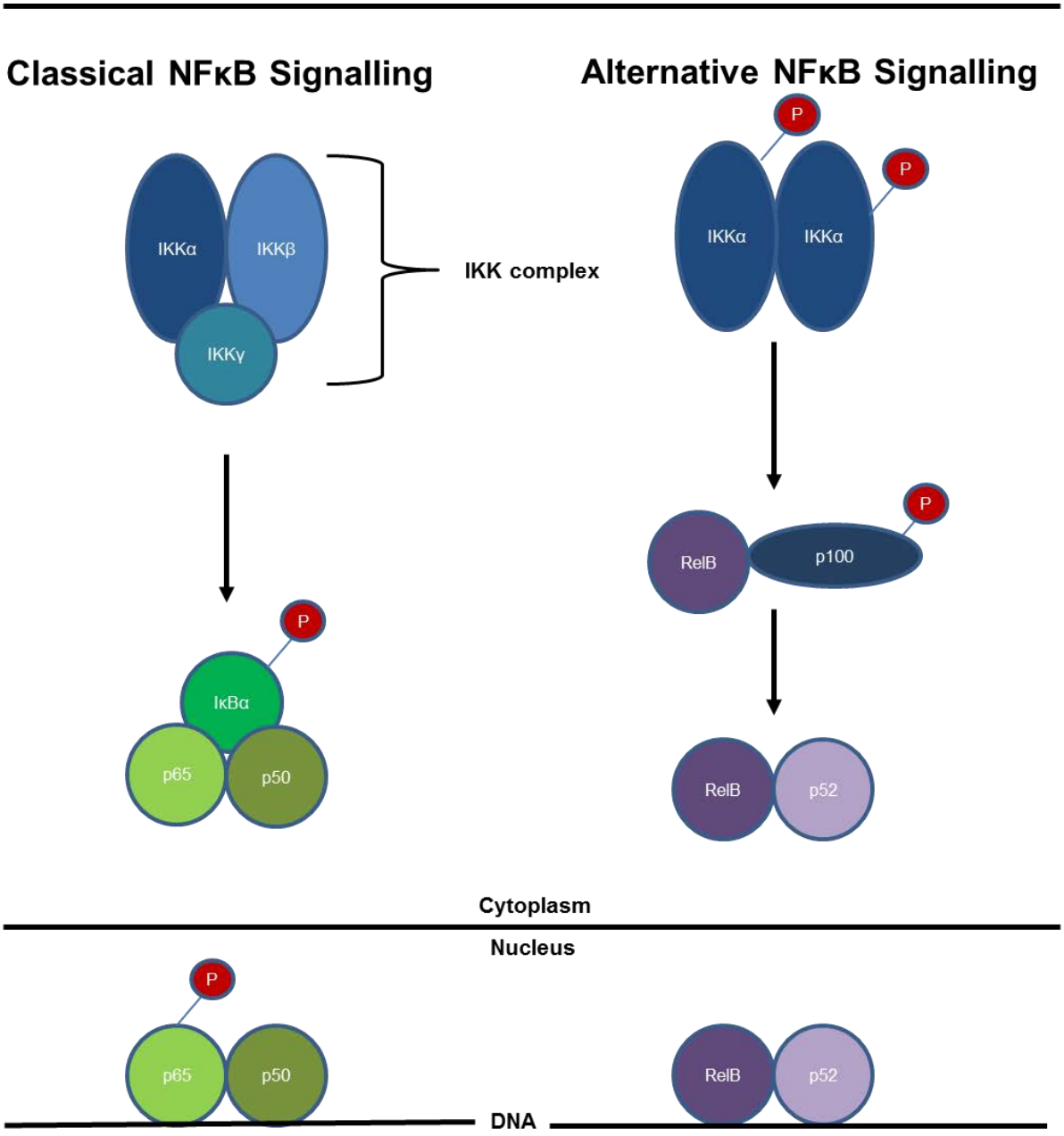

Figure 4.1. Principal NFκB signalling pathways. Classical (canonical) signalling: Upon ligand binding to a cell surface receptor, the IKK complex is recruited and phosphorylates IκBα. IκBα is subsequently degraded, leaving p65 and p50 transcription factors free to translocate to the nucleus. **Alternative (non-canonical) signalling:** Ligand binding phosphorylates and activates the IKKα complex which in turn activates p100, resulting in its processing to p52 and the liberation of the p52/RelB heterodimer.

4.2 Methods

Subconfluent myoblasts from young (n =3) and elderly (n = 3) participants were switched to differentiation media containing recombinant leptin (0 ng/mL or 5 ng/mL). Media were renewed every 2 d. At 8 d, cultures were fixed, IF stained for desmin and with DAPI, imaged on an epifluorescence microscope and MTT and NFI were quantified as previously described. A concentration of 5 ng/mL leptin was chosen as it represents the upper end of the concentrations observed in ACM (Fig. 3.1). The experiment was repeated with recombinant resistin and visfatin. For much of the mechanistic work that follows these experiments, the primary myogenic cultures that we isolated from skeletal muscle biopsies are supplemented by commercially available primary human myoblasts. We first sought to establish that the myogenic responses of these cells under OB ACM and resistin stimulation conditions reflect the responses that we observed in our in-house cultures. To that end, commercially available primary human myoblasts from a 21 yr old female were differentiated in media containing 0 ng/mL or 5 ng/mL recombinant human resistin or in OB ACM. Three independent experiments were conducted, using genetically identical myotubes that had been derived from three different manufacturer vials and that had been cultured separately. The OB ACM used were the same as those detailed in Chapter 3 (Table 3.3). To confirm the anti-myogenic role of resistin in ACM, resistin was immunoprecipitated from OB ACM using resistin antibody-agarose bead conjugates. IgG isotype antibody-agarose bead conjugates were used on the same samples as a control. Subconfluent commercially available myoblasts were differentiated in these ACM as previously described. Resistin is known to activate classical NFκB signalling in other cell types (404–406). Therefore, similar signalling activity was examined in our primary human

myotubes. Myogenic cultures were differentiated for 48 h in the presence of 5 ng/mL recombinant resistin, with or without the IKKβ inhibitor 5-(p-Fluorophenyl)-2-ureido]thiophene-3-carboxamide (TPCA-1) and serine 536 phosphorylation of p65 (p-p65) was measured by immunoblotting. Myogenic cultures were also differentiated for 8 d (in media containing 0 ng/mL or 5 ng/mL recombinant resistin ± 40 nM TPCA-1) to examine the effect of such conditions on MTT and NFI.

4.3 Results

4.3.1 The Effect of Recombinant Adipokines on the Differentiation of Primary Human Myotubes from Young and Old Participants

Recombinant leptin reduced MTT (23 ± 6 % p = 0.04) in old myogenic cultures only (Fig. 4.2, A). NFI was unaffected in either age group (Fig. 4.2, B).

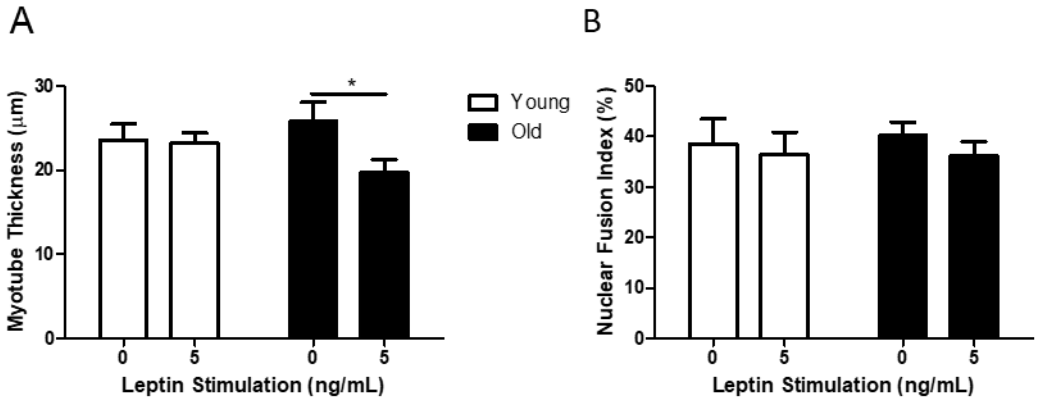

Figure 4.2. Recombinant leptin reduces myotube thickness in myotubes derived from elderly subjects. A) Myotube thickness data represent the mean ± SEM of n = 3 biological replicates. Each biological replicate comprises 150 total measurements taken at 63x magnification from 30 myotubes per treatment condition. **B)** Nuclear fusion index data are expressed as mean ± SEM values of n = 3 biological replicates. Each biological replicate comprises 15 images taken at 20x magnification. * $p < 0.05$ by unpaired t test.

Visfatin did not alter MTT or NFI (Fig. 4.3).

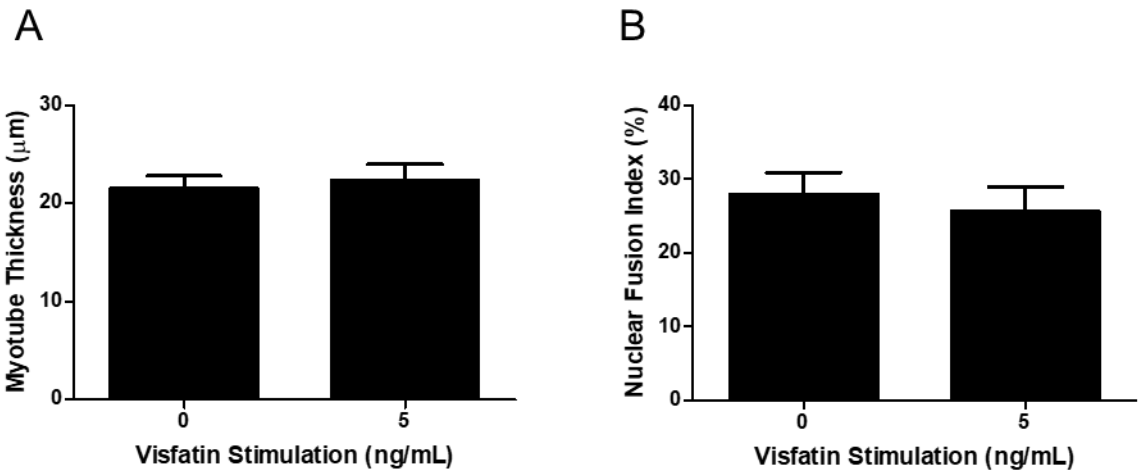

Figure 4.3. Recombinant visfatin does not alter myotube development. A) Myotube thickness data represent the mean ± SEM of n = 3 biological replicates. Each biological replicate comprises 150 total measurements taken at 63x magnification from 30 myotubes per treatment condition. **B)** Nuclear fusion index data is expressed as mean ± SEM values of n = 3 biological replicates. Each biological replicate comprises 15 images taken at 20x magnification.

Resistin significantly reduced MTT in both young (18 ± 5 %, p = 0.04) and old (24 ± 6 %, p = 0.04) myogenic cultures (Fig. 4.4, A). NFI was significantly diminished in old cultures only (25 ± 13 %, p = 0.0003) (Fig. 4.4, B).

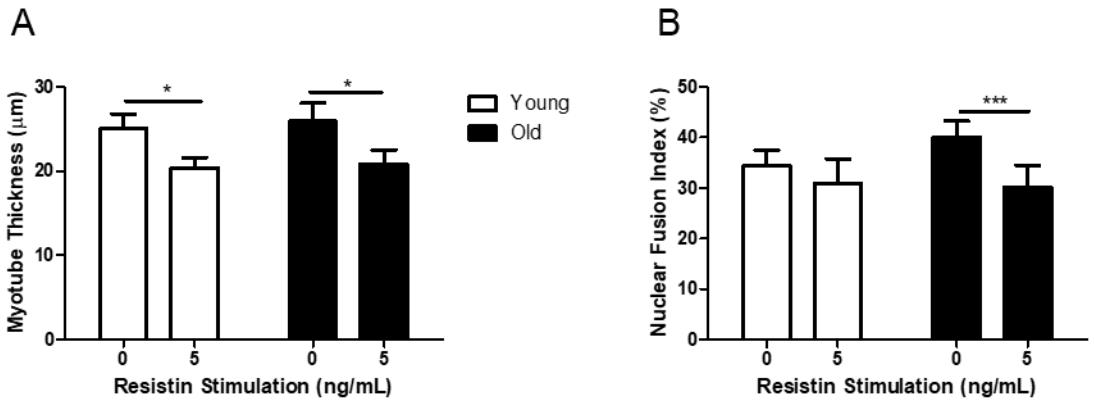

Figure 4.4. Recombinant resistin impairs myotube formation in myotubes derived from young and elderly subjects. A) Myotube thickness data represent the mean ± SEM of n = 3 biological replicates. Each biological replicate comprises 150 total measurements taken at 63x magnification from 30 myotubes per treatment condition. **B)** Nuclear fusion index data are expressed as mean ± SEM values of n = 3 biological replicates. Each biological replicate comprises 15 images taken at 20x magnification. *p < 0.05, ***p < 0.001 by unpaired t test.

4.3.2 Resistin and Obese Adipose Conditioned Media are also Detrimental to the Differentiation of Commercially Available Primary Human Myoblasts

MTT was significantly reduced by both resistin (26 ± 6 %, p = 0.04) and OB ACM (26 ± 4 %, p = 0.02) compared to unstimulated control myotubes (Fig. 4.5, A). Additionally, NFI was significantly diminished by resistin (23 ± 6 %, p = 0.01) and OB ACM (73 ± 13 %, p = 0.0008) stimulation (Fig. 4.5, B).

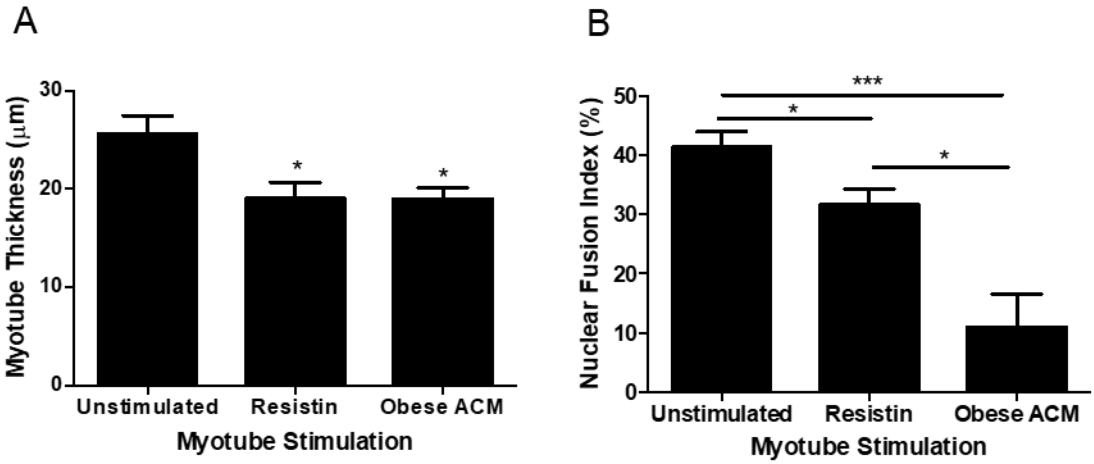

Figure 4.5. In commercially available primary human skeletal myoblasts, myogenesis is impaired by stimulation with recombinant resistin and obese adipose conditioned medium. A) Myotube thickness data represents the mean ± SEM of n = 3 independent experiments. Each independent experiment comprises 150 total measurements taken at 63x magnification from 30 myotubes per treatment condition. *p < 0.05 vs. unstimulated control by Mann-Whitney U test with post-hoc Holm's sequential Bonferroni adjustment. **B)** Nuclear fusion index data are expressed as mean ± SEM values of n = 3 independent experiments. Each independent experiment comprises 15 images taken at 20x magnification. *p < 0.05, ***p < 0.001 by Mann-Whitney U test with post-hoc Holm's sequential Bonferroni adjustment.

4.3.3 Immunoprecipitation of Resistin from Obese Adipose Conditioned Medium Improves Myogenesis

The success of the immunoprecipitation of resistin from ACM was confirmed by elution of antibody-antigen complexes from the agarose beads, and detection of resistin in these elutes by immunoblotting (Fig. 4.6, A). All control elutes were negative for resistin. To further confirm the success of the immunoprecipitation, ACM resistin concentrations were quantified by an ELISA which employed a different anti-resistin antibody than the immunoprecipitation procedure. Resistin concentrations in OB ACM were diminished following resistin immunoprecipitation (Fig. 4.6, B). In these commercially available cells, NFI was previously shown to be substantially inhibited by OB ACM (Fig. 4.5, B). Thus, NFI

was chosen as a reliable outcome to determine whether a reduction in OB ACM resistin concentration rescues myogenesis. Resistin immunoprecipitation from OB ACM increased NFI by 60 ± 16 % ($p = 0.03$; Fig. 4.6, C). Linear regression analysis of resistin concentration and NFI indicates a possible negative association between the two, but this was not statistically significant in this small data set (Fig. 4.6, D).

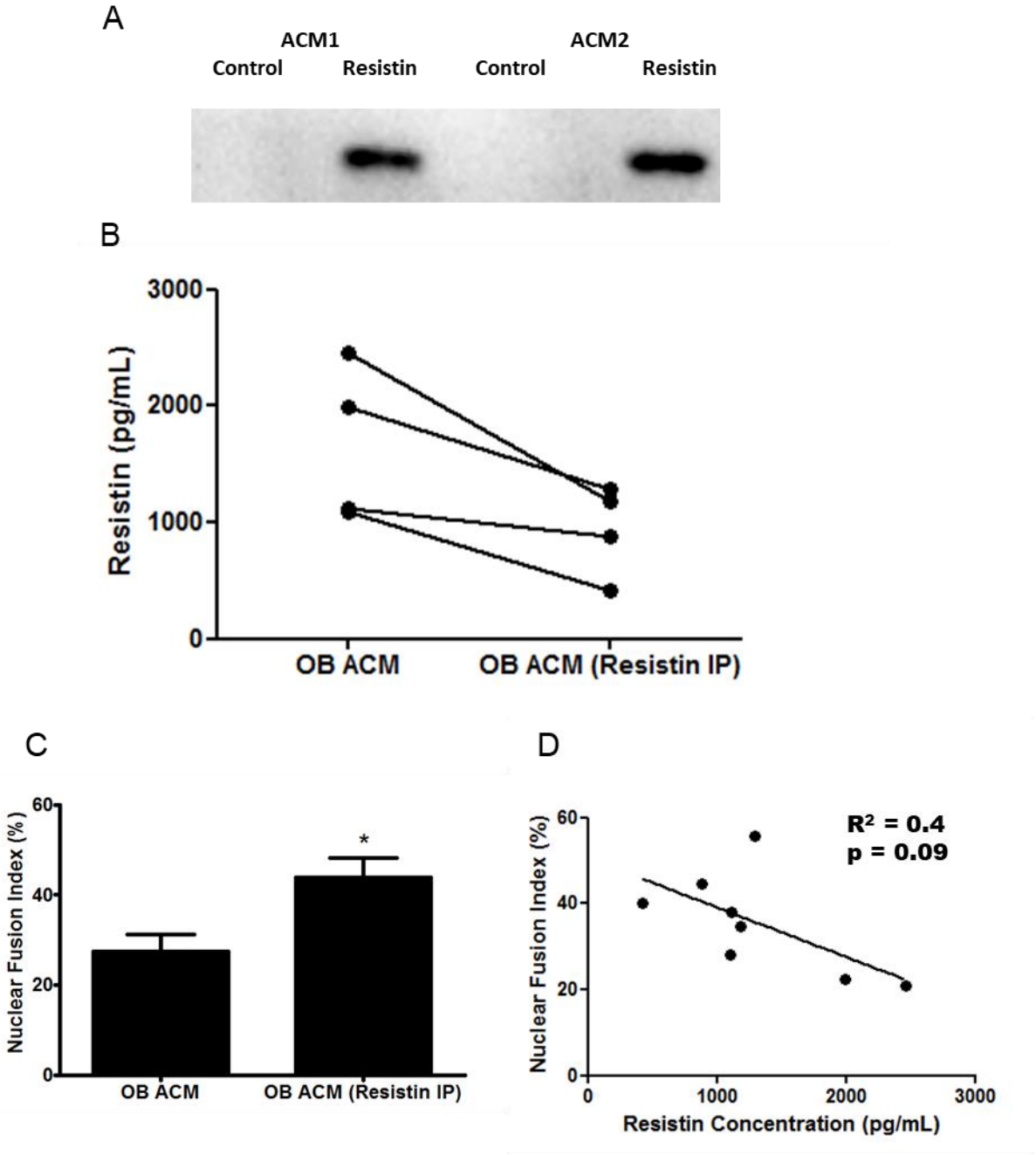

Figure 4.6. Immunoprecipitation of resistin from obese adipose conditioned medium (OB ACM) improves myogenesis. A) The antibody-antigen complexes were eluted from the agarose beads and resistin was detected in the samples by immunoblotting. **B)** Resistin was quantified in the ACM by ELISA. **C)** Subconfluent, commercially available primary human skeletal myoblasts from a female aged 21 yr were switched differentiation media (OB ACM; n = 4 or OB ACM – resistin IP, n = 4). Media were renewed every 2 d. At 8 d, myotubes were fixed, immunofluorescence stained for desmin and with DAPI and imaged on an epifluorescence microscope. Nuclear fusion index data are expressed as mean ± SEM values of n = 3 independent experiments. Each independent experiment comprises 15 images taken at 20x magnification. *$p < 0.05$ vs OB ACM by unpaired t test. **D)** Linear regression of ACM resistin concentration and nuclear fusion index.

It was confirmed that classical NFκB signalling was suppressed during myogenesis (Fig. 4.7, A). Myogenic cultures differentiated for 48 h in the presence of 5 ng/mL recombinant resistin displayed a significant increase in serine 536 phosphorylation of p65 (p-p65); such phosphorylation was inhibited by the presence of the IKKβ inhibitor 5-(p-Fluorophenyl)-2-ureido]thiophene-3-carboxamide (TPCA-1) (Fig. 4.7 B, C). An uncropped version of the blot presented in Fig. 4.7, B is included in Appendix Fig. 4.1 to demonstrate the detection of a distinct dominant band at the appropriate molecular weight (~ 65 kDa).

Having established that resistin activates the classical NFκB signalling pathway during myogenesis, the ability of TPCA-1 to rescue myotubes from the anti-myogenic actions of resistin was explored. As before (Fig. 4.4, 4.5), 8 d resistin stimulation of differentiating myogenic cultures significantly diminished MTT and NFI (Fig. 4.8), a phenomenon that was completely reversed by co-incubation with TPCA-1. Activation of the classical NFκB signalling pathway is often associated with increased expression of the E3 ubiquitin ligases *MAFbx* and *MURF-1* (407,408); indeed, recombinant resistin stimulation of differentiating myogenic cultures induced a variable increase in their expression – this was reversed by the presence of TPCA-1 (Fig. 4.8).

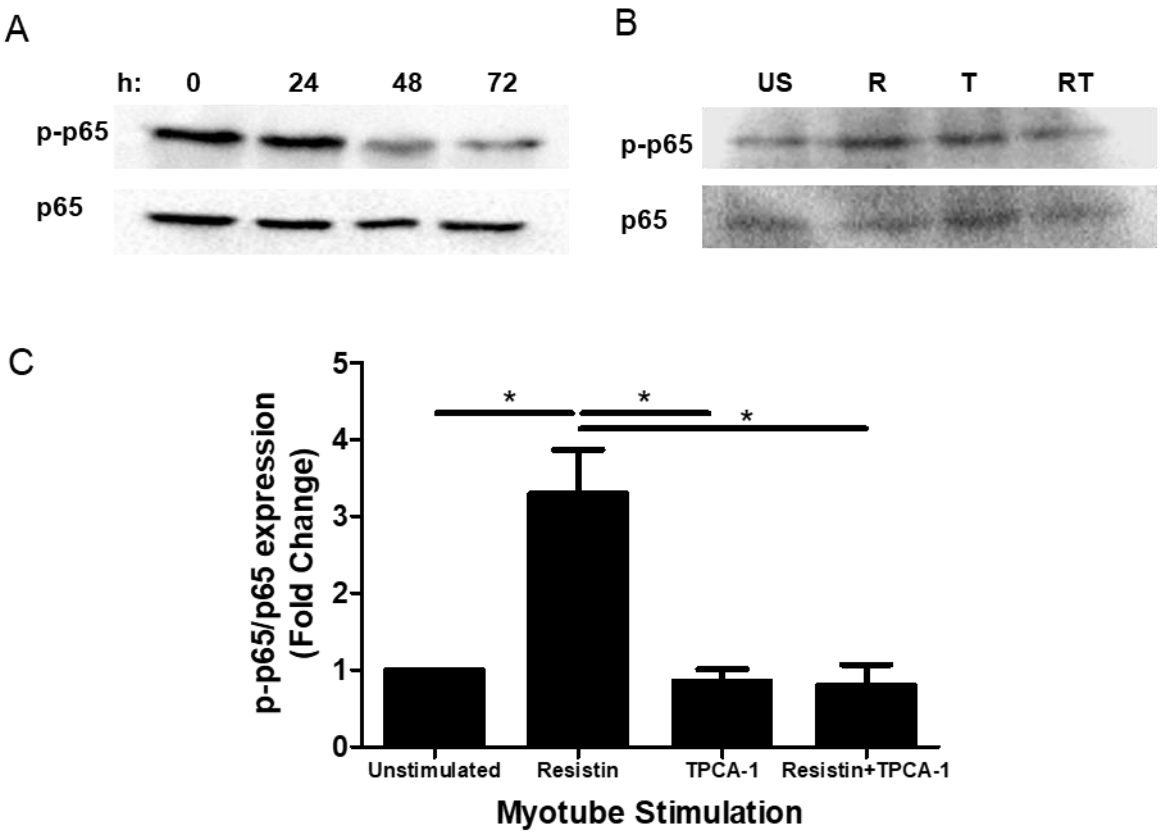

Figure 4.7. Recombinant resistin activation of the classical NFκB pathway in differentiating myotubes is reversed by the IKK2 inhibitor TPCA-1. A, B. C) p-p65 (serine 536) protein expression during early differentiation. US = unstimulated, R = resistin, T = TPCA-1, RT = resistin + TPCA-1. Data are expressed as mean ± SEM values of n = 4 independent experiments. *$p < 0.05$ by one-way ANOVA with post-hoc Bonferroni correction.

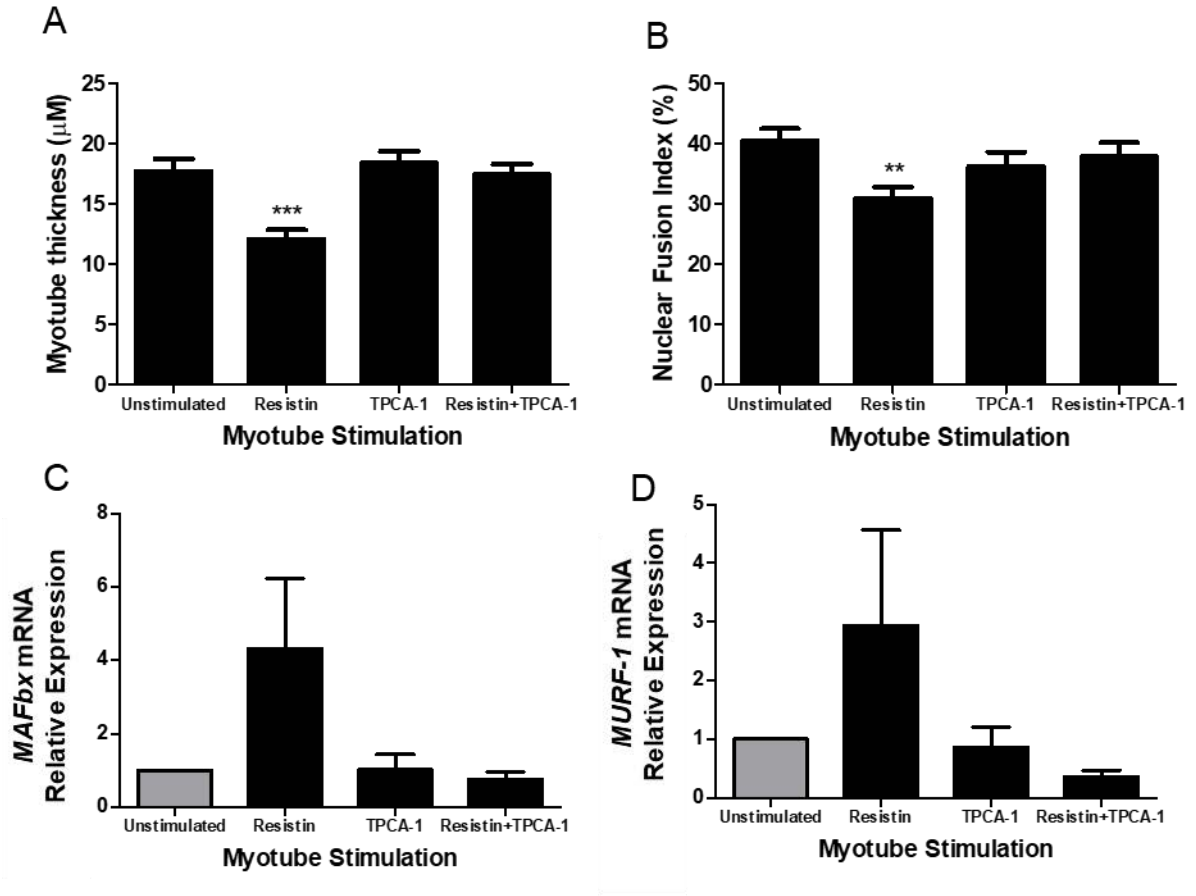

Figure 4.8. The NFκB inhibitor TPCA-1 rescues myogenesis in primary human myogenic cultures that have been treated with recombinant resistin. A) Myotube thickness data represents the mean ± SEM of n = 3 independent experiments. Each independent experiment comprises 150 total measurements taken at 63x magnification from 30 myotubes per treatment condition. **B)** Nuclear fusion index data are expressed as mean ± SEM values of n = 3 independent experiments. Each independent experiment comprises 15 images taken at 20x magnification. **p < 0.01, ***p < 0.001 vs unstimulated control by Mann-Whitney U test with post-hoc Holm's sequential Bonferroni adjustment. **C,D)** RT-qPCR measurement of MAFbx and MURF-1 gene expression. Data are expressed as mean ± SEM values of n = 3 independent experiments. Each independent experiment comprises data from triplicate culture wells, with each being assayed in triplicate.

4.4 Discussion

4.4.1 The Effect of Recombinant Adipokines on *In Vitro* Human Myogenesis.

In Chapter 3 of this thesis, resistin was identified as a potentially important mediator of the anti-myogenic effects of OB ACM. Having established a clear rationale for examining the myogenic effects of resistin, it was demonstrated in this chapter that exogenous recombinant resistin – at the concentrations found in OB ACM – does indeed inhibit myogenesis. 5 ng/mL recombinant resistin significantly and similarly reduced MTT in both young and old myogenic cultures; NFI was significantly diminished in old cultures only (Fig. 4.4). Furthermore, immunoprecipitation of resistin from OB ACM rescued myogenesis (Fig. 4.6). NFI had previously been shown to be substantially inhibited by OB ACM in these cells (Fig. 4.5, B).Thus, NFI was chosen as a reliable outcome to determine whether a reduction in OB ACM resistin concentration rescues myogenesis. Indeed, a substantial (R^2 = 0.4), but not statistically significant (p = 0.09) negative relationship was observed between ACM resistin concentration and NFI (Fig. 4.5).

Despite the considerable variability in OB ACM adipokine composition described in Chapter 3, resistin is an adipokine that is enriched in the adipose inflammatory milieu of many OB individuals (Fig. 3.1), and it has a demonstrable negative effect on myogenesis. The dearth of evidence regarding the effect of resistin on skeletal muscle growth, maintenance and regeneration has previously been noted (Sections 1.7.4, 3.3.1). However, our results concur broadly with the few relevant studies that exist. One study showed that plasma resistin concentrations had an inverse relationship with quadriceps torque, but not handgrip strength in old (69-81 yr), but not in young (18-30 yr), participants (291). In a separate study, C2C12 myoblast proliferation was increased by the transfection

of a human resistin eukaryotic expression vector, and such transfection reduced the expression of desmin and resulted in thinner myotubes (292). Our use of a human *in vitro* model is of particular importance, given that human resistin displays poor amino acid sequence homology to murine resistin (293).

The OB ACM used to differentiate myoblasts (described in the previous chapter, Section 3.2.3) had elevated concentrations of visfatin and leptin compared to the NW ACM that were used (Table 3.3); this was not representative of the NW and OB populations as a whole (Table 3.2). Additionally, visfatin and leptin are understudied with respect to their effects on myogenesis. Thus, it was decided that further study of their myogenic effects was warranted. 5 ng/mL recombinant leptin reduced MTT in old myogenic cultures only, NFI was not affected (Fig. 4.1). 5 ng/mL recombinant visfatin did not alter myogenesis (Fig. 4.2). It is therefore likely that leptin contributed to the anti-myogenic effects of OB ACM in the experiment described in Section 3.2.3. The known effects of leptin on skeletal muscle growth, maintenance and regeneration were reviewed in the first chapter of this thesis (Section 1.7.5). It was established that while large alterations of systemic leptin concentrations in mice suggest that the adipokine has a positive relationship with skeletal muscle mass (295,296), studies in human participants suggest that large hypertrophic or atrophic effects are not seen with plasma leptin fluctuations within the physiological range. For example, serum leptin concentrations have been positively correlated with FFM in men, but not in women (297). However, men in the highest quintile for FFM also displayed the highest BMI and fat mass; the anti-gravity overload stimulus of their fat mass is an important confounder. In contrast, studies of late middle-aged and elderly participants, have noted significant, if small (β = ~ -0.2) negative associations between plasma leptin

and skeletal muscle mass (298,299). Our finding that 5 ng/mL recombinant leptin reduced MTT in cultures derived from elderly participants compliments these observations.

We utilised commercially available primary human myoblasts from a 21 yr old female for some of our mechanistic work and first established that the myogenic responses of these cells under OB ACM and resistin stimulation conditions reflected the responses that we observed in our in-house cultures (Fig. 4.5). It is notable that both resistin and OB ACM reduced the NFI of such cultures, a phenomenon that was not observed in our in-house young myogenic cultures (Fig. 3.5, Fig. 4.4). The lack of biological variability in experiments conducted with primary cells from one individual is likely to be responsible for this observation; although myogenic cultures isolated from young skeletal muscle are overall clearly less susceptible to the effects of OB ACM and resistin on NFI (Fig. 3.5, Fig. 4.4), individual variability in this response is to be expected.

4.4.2 Resistin Inhibits Human Myogenesis by Activating the Classical NFκB Signalling Pathway

Having established the effects of resistin on the differentiation of primary human myogenic cultures, a mechanism of its action was sought. Mouse receptor tyrosine kinase-like orphan receptor (ROR) (409) and decorin (410) are thought to be receptors for murine resistin. Toll-like receptor 4 (TLR4) has been suggested as a receptor for human resistin (411), and TLR4 are found in primary human myogenic cultures (412). Adenylyl cyclase-associated protein 1 (CAP1) is also a known receptor for human resistin (413), but only CAP2 is expressed in human skeletal muscle (414). While the receptor/s for resistin in skeletal muscle remain to be definitively identified, it is clear that resistin activates NFκB

signaling widely, such activation having been demonstrated in the human liver cancer cell line HepG2 (404), human coronary artery endothelial cells (405) and human macrophages (406). Indeed, bioinformatic analysis in HepG2 cells suggested that resistin might also activate the 5' AMP-activated protein kinase (AMPK) and extracellular signal-regulated kinases 1 and 2 (ERK 1/2) signalling pathways, but molecular investigations determined that it signalled via the NFκB pathway alone (404). The canonical wnt signalling pathway is an important additional pathway that supports myogenesis (415–418), but work in primary human osteoblasts in our lab demonstrated that resistin promotes its activation rather than inhibition (419). Such activation would be expected to facilitate myogenesis. Thus, activation of the classical NFκB pathway by resistin remains the most plausible mechanism by which it might inhibit myogenesis.

Until recently, it was unclear whether activation of the classical NFκB pathway is a positive or a negative regulator of myogenesis (401). Many early studies relied upon electrophoretic mobility gel shift assays (EMSA), which are subject to considerable technical variability between labs (401,420,421). Additionally, NFκB activity appears to be sensitive to culture conditions, with C2C12 cultures displaying transient NFκB activity upon their first exposure to differentiation medium (422). More recently, genetic approaches have definitively established the classical NFκB pathway as a negative regulator of myogenesis. Myogenesis has been shown to be enhanced in $p65^{-/-}$ myoblasts; in the same study, 4 wk old $p65^{-/-}$ mice displayed a 76 % increase in tibialis anterior fibre number, although mean fibre diameter was reduced by 39 % (402). In another study, IKKβ-deficient mice were protected against denervation-induced skeletal muscle atrophy (3).

Furthermore, inhibition of classical NFκB pathway signalling with IKKβ inhibitor IV enhanced the myogenic differentiation of primary murine cultures from wild-type mice (403). A recent study used a bi-allelic Cre-lox transgenic mouse in which NFκB can be activated specifically in muscle SC following administration of tamoxifen (423). It demonstrated that NFκB activation in the SC of aged mice inhibits skeletal muscle regeneration in response to cryoinjury.

In our primary human myogenic cultures, classical NFκB pathway activity (as measured by p65 phosphorylation) was diminished at 48 h post-differentiation (Fig. 4.7, A); this concurs with previous observations made in C2C12 cultures (402). The addition of 5 ng/mL recombinant resistin to such cultures resulted in persistent p65 phosphorylation at 48 h, a phenomenon that was reversed by the addition of 40 nM TPCA-1 (Fig. 4.7, B). TPCA-1 alone did not further diminish p-p65 expression, likely due to the small magnitude of its expression in differentiating myotubes beyond 24 h (Fig. 4.7, A).

This work has focused on identifying a central cell signalling pathway that is responsible for resistin-induced inhibition of myogenesis. We present compelling evidence that the classical NFκB pathway is such a pathway. What remains to be definitively established is which NFκB target genes mediate this inhibition. Activation of the classical NFκB signalling pathway is often associated with increased expression of the E3 ubiquitin ligases *MAFbx* and *MURF-1* (407,408); these genes are binding targets of the p50 subunit of the classical pathway and its transcriptional co-activator Bcl-3 (408,424). Given that MAFbx and MURF-1 are well documented as mediators of mature myofibre atrophy, and have also been specifically implicated in the inhibition of myogenesis (425,426), they were chosen

as NFκB target genes for further molecular corroboration of our observations of improved MTT and NFI. Resistin induced a variable and non-significant increase (across three independent experiments) in MAFbx and MURF-1 expression (Fig. 4.8); this was eliminated by co-stimulation of myogenic cultures with resistin and TPCA-1.

Cyclin D1 is a transcriptional target of NFκB and is a known inhibitor of myogenesis (403,427,428). Furthermore, p65 directly interacts with Cyclin D1 and is thought to stabilise its expression (429). Indeed, we observed a small increase in total p65 expression in myoblasts that were stimulated with resistin for 24 h (Appendix Fig. 4.2) suggesting that exposure to resistin prior to differentiation might leave such myoblasts unprepared for successful differentiation. The transcription factor, Ying-Yang 1 (YY1) is regulated by p65 and acts as a transcriptional repressor of many genes (e.g. troponins, myosin heavy chains and α-actin) that are key to mature myofibre development (430). p65 and YY1 are also known to interact with the microRNA miR-29. miR-29 expression enhances myogenesis (431) such expression is repressed by YY1 (432). At the onset of differentiation, the decrease in classical NFκB pathway activity releases miR-29 expression from the inhibitory effects of YY1. miR-29 also exerts negative feedback on YY1 via binding to its 3'UTR (432). The effect of NFκB activity on growth factor and cytokine expression by myotubes and myofibres must also be considered. For example, skeletal muscle regeneration is improved in $p65^{+/-}$ mice post cardiotoxin injury, compared to wild-type controls. Skeletal muscle hepatocyte growth factor (HGF) expression was found to be elevated during such regeneration and silencing this expression with a small hairpin RNA targeting HGF eliminated the improvement in regeneration conferred by the

$p65^{+/-}$ genotype (433). Delineating the downstream mediators of classical NFκB pathway activity in the inhibition of myogenesis will be crucial to the development of pharmaceutical interventions to enhance skeletal muscle regeneration not just in instances of catastrophic skeletal muscle injury, but also in ageing skeletal muscle. The relevance of SC activation and myogenesis to skeletal muscle growth and maintenance over the lifespan was considered in Chapter 1 and is considered again in Chapter 7.

NFκB signalling is central to many cellular processes, including immunity, cell proliferation and apoptosis as well as embryonic and neuronal development (434). Thus, many existing pharmacological agents that are known to inhibit the NFκB pathway have significant side effects, such that they are unsuitable for the prevention of sarcopenic obesity. Thalidomide is one such drug. When used as a chemotherapeutic agent it causes thrombo-embolic events, peripheral neuropathy and increases infection rates (435). Similarly, the 26S proteasome inhibitor bortezomib – which inhibits NFκB by preventing the degradation of IκBα – also has serious side effects, including peripheral neuropathy, thrombocytopenia and reactivation of herpes zoster (435,436).

However, there exists one drug class with few side effects that may prevent sarcopenia by inhibiting the NFκB pathway. Non-steroidal anti-inflammatory drugs (NSAIDs) are known principally as cyclooxygenase (COX) inhibitors (437). However, NSAIDs are also known to inhibit the classical NFκB pathway by suppressing IκBα degradation (438), a mechanism that might be expected to facilitate myogenic adaptations to exercise. Some studies have found that NSAIDs impair SC proliferation responses to exercise in young healthy participants (439,440) and that low doses of ibuprofen, coupled with short-term

resistance training (6 wk) had no beneficial effect on skeletal muscle mass (441). However, in a more recent study, more severe skeletal muscle injury was induced by electrical stimulation and NSAIDs promoted SC activation (442). Furthermore, standard doses of paracetamol (4 g/day) and ibuprofen (1.2 g/day) appear to significantly increase muscle volume and strength acquisition during a 12 wk resistance training program in elderly (443). Thus, it may be that cost-effective pharmaceutical interventions for the amelioration of NFκB-induced loss of skeletal muscle mass already exist; ibuprofen used in conjunction with a proton pump inhibitor to prevent NSAID-induced upper gastrointestinal mucosal damage (444) might represent a good starting point.

Our work used TPCA-1, an inhibitor of IKKβ (IC_{50} = 18 nM) that is also capable of inhibiting IKKα and thus the alternative NFκB pathway at much higher concentrations (IC_{50} = 400 nM) (445). The concentration of TPCA-1 used in this work (40 nM) was chosen to produce a robust inhibition of IKKβ and is 10-fold lower than the concentration that would produce any consequential inhibition of IKKα. In any case, the alternative NFκB pathway (Fig. 4.1) does not appear to regulate myogenesis. IKKα expression is induced late in the myogenic program and myogenesis is normal in primary murine myoblasts lacking IKKα (401,402). However, IKKα does appear to facilitate mitochondrial biogenesis in differentiating myotubes and is essential for the survival of mature myotubes under starvation conditions (402).

4.5 Limitations and Future Directions

Importantly, this work has established that resistin activates classical NFκB pathway signalling and that reversing this inhibition ameliorates the deleterious effects of resistin

on *in vitro* primary human myogenesis. However, the receptor/s by which resistin initiates such signalling remain to be definitively established. Furthermore, the significance and interdependence of the NFκB target genes which mediate its inhibition of myogenesis are not fully understood. It appears that NFκB interactions with YY1 and cyclin D1 might play a central role, with the expression of many genes that are key to mature myofibre maintenance and development (e.g. troponins, myosin heavy chains, α-actin, MAFbx, MURF-1) known to also be affected. The delineation of these events is crucial to our wider understanding of the myogenic program, not just that under conditions of resistin stimulation.

Given that standard doses of paracetamol and ibuprofen can enhance strength and muscle volume acquisition during a 12 wk resistance training programme, it would be valuable to confirm their pro-myogenic utility and to determine whether such an intervention is effective and safe in a population with sarcopenic obesity.

4.6 Conclusions

This chapter has established that:

- Resistin and leptin have a deleterious effect on *in vitro* human myogenesis.
- Resistin – at least in part – mediates the detrimental effects of OB ACM on primary human myogenesis.
- Resistin activates classical NFκB pathway signalling in primary human myogenic cultures.
- Such NFκB pathway signalling mediates the detrimental effect of resistin on

skeletal myogenesis.

Chapter 5

Resistin and *In Vitro* Human Myotube Metabolism

5.1 Introduction

The preceding data chapters have established a rationale for the study of resistin with respect to ageing skeletal muscle. In Chapter 4, an anti-myogenic effect of resistin was established, and the classical NFκB pathway was identified as a central signalling pathway through which resistin acts to exert these effects. The interdependence of skeletal muscle mass, function and metabolism in ageing was established in the introductory chapter of this thesis (e.g. see Sections 1.6.3.2 and 1.7.7).

Resistin is understudied regarding its metabolic effects on skeletal muscle. In one study, resistin inhibited glucose uptake and impaired glycogen synthesis during a 24 h stimulation of rat L6 myotubes (308,309). Another study found that resistin inhibited palmitate uptake by L6 myotubes (310). However, such studies are few, limited in scope and it is unknown whether primary human myotubes or human skeletal muscle behave similarly to immortalised rodent cell lines. In completing the experiments described in Chapter 4, the appearance of prominent intramyocellular structures – which were thought to be lipid droplets – was noted in myotubes formed under resistin stimulation conditions. This observation appeared to agree with reports of lipid accumulation in resistin-stimulated human macrophages (446) and of hepatic steatosis in resistin-treated mice (404). Thus, confirmation of such lipid accumulation in our myotubes, as well as quantification of their fatty acid oxidative capacity, was identified as an experimental priority for subsequent studies investigating the effect of resistin on myotube metabolism.

The rationale for hypothesising that resistin exerted its anti-myogenic effect via activation of the classical NFκB signalling pathway (Chapter 4) was clear; resistin had been shown to activate NFκB signalling in other human cell types, and such signalling is now well-

established as anti-myogenic. It is less clear whether such signalling might mediate any metabolic effects of resistin on primary human myotubes.

Therefore, the objectives of this chapter were as follows:

1. To determine whether resistin alters oxidative metabolism in primary human myotubes.
2. To investigate whether resistin stimulation of developing myotubes promotes the accumulation of intramyocellular lipids.
3. To quantify the free fatty acid oxidative capacity of resistin-stimulated myotubes.
4. To ascertain if inhibition of NFκB pathway signalling reverses any metabolic alterations induced by resistin.

5.2 Methods

To screen for a metabolic effect of resistin on primary human myotubes, differentiated myotubes were maintained for 24 h in differentiation media supplemented with or without 5 ng/mL recombinant resistin. At 24 h myotubes were stained with Mitotracker® Green FM, which stains all mitochondria and Mitotracker® Orange CM-H2TMRos which accumulates in mitochondria in a membrane-potential dependent fashion. 15 immunofluorescent images at 20x magnification were captured from each independent experiment (n = 3 independent experiments). The mean fluorescence intensity of the green and red channels of each image was quantified using Image J software. We used

a Seahorse® XFe96 analyser, in conjunction with an XF Mito Stress Test to more fully characterise the effects of resistin on myotube mitochondrial metabolism. Commercially available primary human myoblasts from a 21 yr old female were used, and four independent experiments were conducted, using genetically identical myotubes that had been derived from four different manufacturer vials and were cultured separately. Firstly, fully differentiated myotubes were stimulated for 24 h with differentiation media (containing 0 ng/mL or 5 ng/mL recombinant resistin ± 40 nM TPCA-1). It was established in Chapter 4 that resistin signals via the classical NFκB pathway and that inhibiting this pathway with the IKKβ inhibitor TPCA-1 reverses the detrimental effects of resistin on *in vitro* human myogenesis. Given that TPCA-1 restores normal myogenesis, we sought to establish whether it could also normalise any metabolic effects of resistin in such myotubes. The Seahorse® XF Mito Stress Test was repeated on myotubes that had been differentiated for 8 d in the presence of 0 ng/mL or 5 ng/mL recombinant resistin ± 40 nM TPCA-1.

In completing the work described in Chapter 4, we observed the accumulation of intramyocellular structures in resistin-stimulated myotubes. We hypothesised that these were lipid droplets. Here, we sought to confirm this. As before, myogenic cultures (n = 3 independent experiments) were differentiated for 8 d in the presence of 0 ng/mL or 5 ng/mL recombinant resistin ± 40 nM TPCA-1. The resulting myotubes were stained with Oil Red O, imaged, and the staining was quantified using Image J software.

Having observed considerable myotube lipid accumulation under resistin stimulation conditions, we sought to quantify the capacity of such myotubes to oxidise endogenous and exogenous fatty acids using an XF Palmitate-BSA FAO Substrate Mitochondrial Stress Test, carried out on the Seahorse XFe96 analyser.

5.3 Results

5.3.1 Mitotracker® Orange CM-H2TMRos Staining of Mitochondria in Resistin-Stimulated Myotubes

Mitotracker® Green FM intensity was not altered by resistin stimulation, indicating that the total number of mitochondria did not differ between stimulation conditions. Mitotracker® Orange intensity was reduced by 11 ± 3 % in resistin-stimulated myotubes ($p = 0.09$ by unpaired t-test). When the Mitotracker® Orange intensity in each image was corrected for the corresponding Mitotracker® Green FM intensity (a surrogate for total mitochondrial mass), the reduction in Mitotracker® Orange intensity (10 ± 3 %) approached significance ($p = 0.06$ by unpaired t-test), indicative of a reduction in mitochondrial activity.

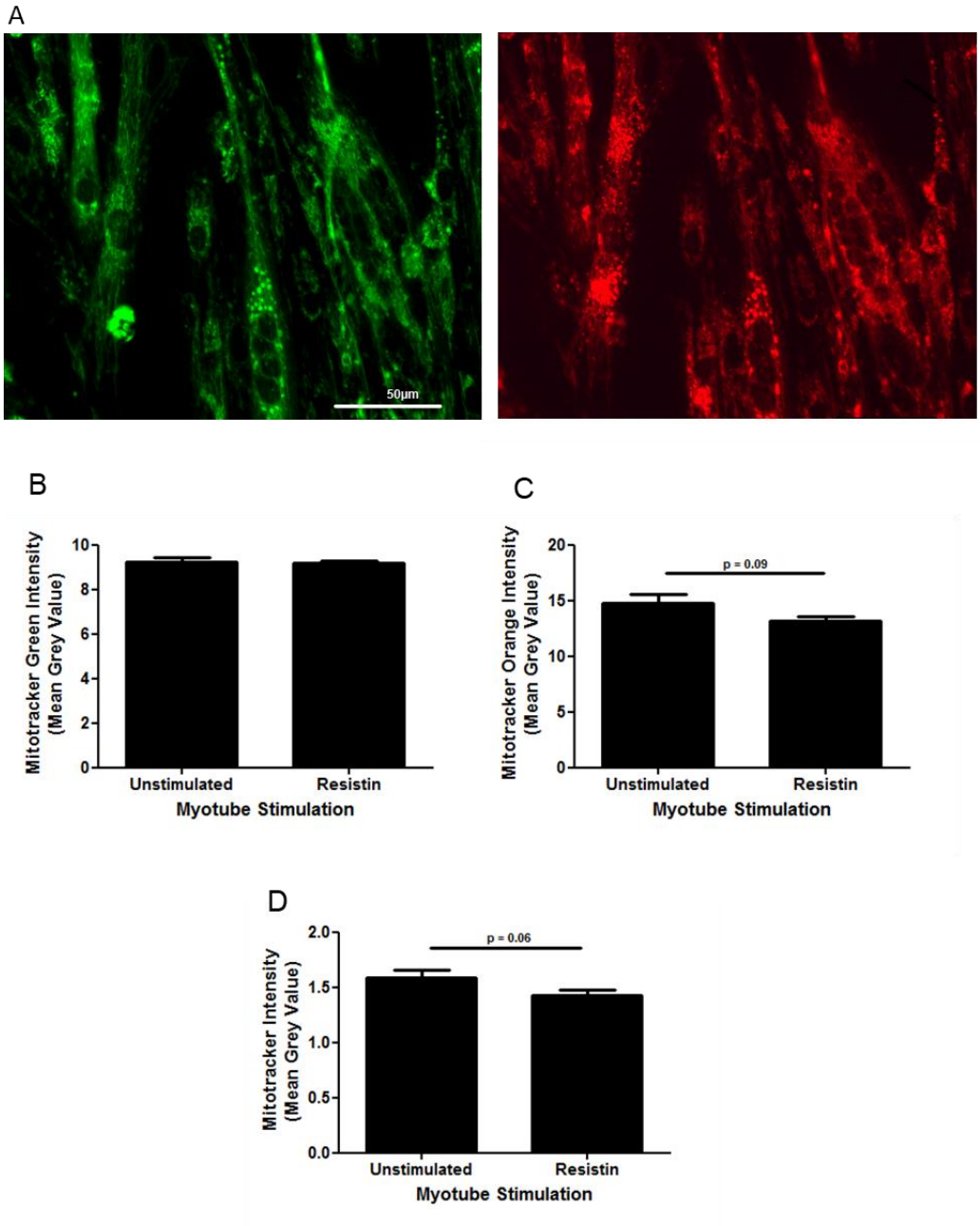

Figure 5.1. 24 h Recombinant resistin stimulation of myotubes causes a decline in mitochondrial membrane potential that approaches statistical significance. Commercially available 8 d differentiated myotubes were maintained for 24 h in differentiation media (containing 0 ng/mL or 5 ng/mL recombinant resistin). At 24 h, myotubes were stained with 100 nM Mitotracker® Green FM and 200 nM Mitotracker® Orange CM-H2TMRos. Myotubes were imaged immediately on an epifluorescence microscope and the mean grey intensity value of the green and red channels was quantified using Image J software. **A)** Example Mitotracker® images. **B-D)** Mitotracker intensity. Data are expressed as mean ± SEM values of 45 images at 20x magnification taken from n = 3 independent experiments.

5.3.2 Resistin is a Metabolic Stressor of Primary Human Myotubes

Basal respiration, maximal respiration, ATP production, spare capacity and baseline ECAR measurements were unaffected by any 24 h stimulation condition (Fig. 5.2, A-F). However, proton leak was significantly increased (26 ± 14 %, $p = 0.03$) by resistin stimulation. Furthermore, this phenomenon was reversed by co-stimulation with TPCA-1 (Fig. 5.2, G).

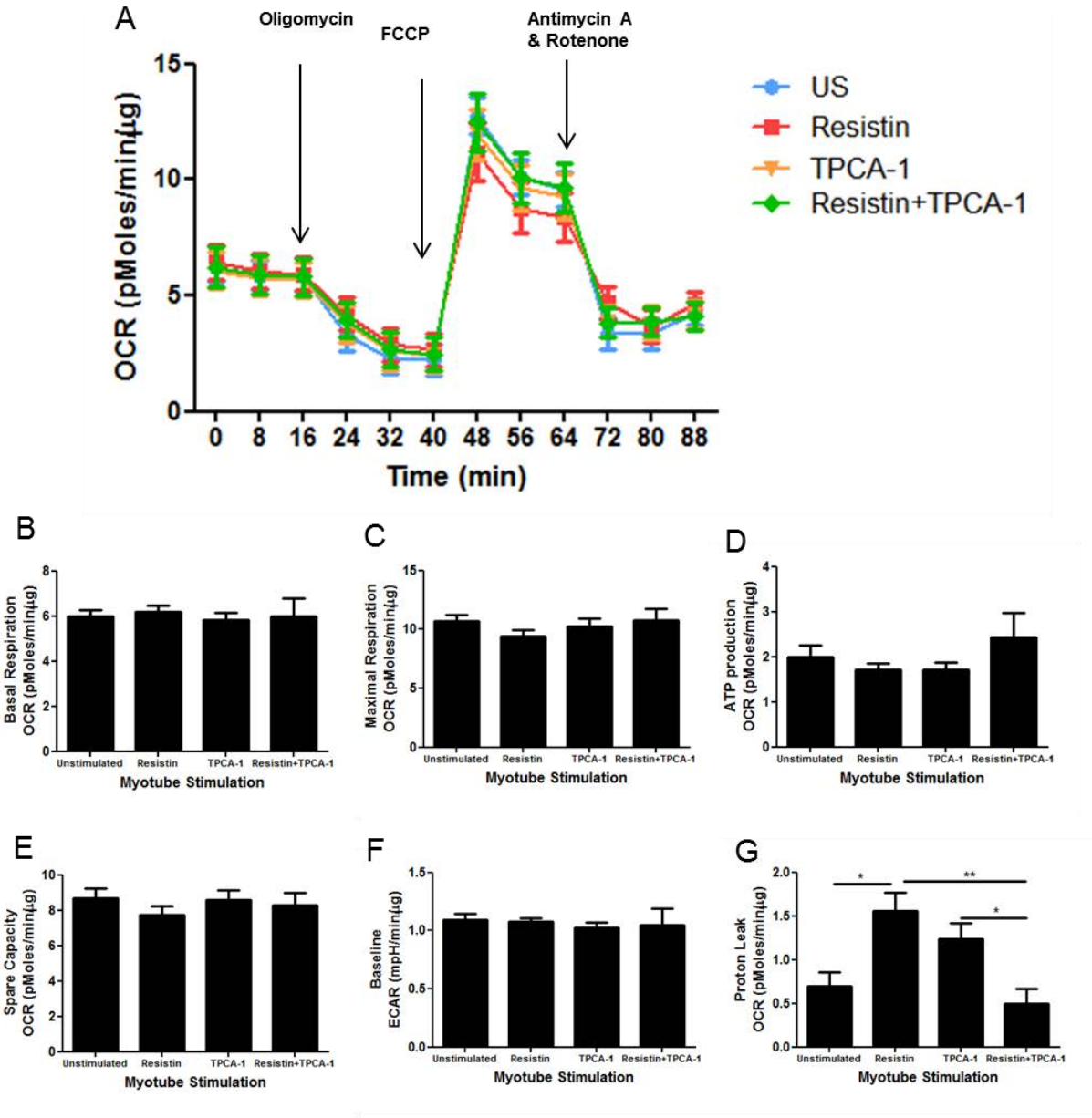

Figure 5.2. 24h recombinant resistin stimulation of myotubes causes an increase in proton leak that is prevented by TPCA-1. Commercially available 8 d differentiated myotubes were maintained for 24 h in differentiation media (containing 0 ng/mL or 5 ng/mL recombinant resistin ± 40 nM TPCA-1). An XF Mito Stress Test was carried out on the Seahorse XFe96 analyser. Data are expressed as mean ± SEM values of n = 4 independent experiments. Each independent experiment comprises data from quadruplicate measurements. $p < 0.05$, ** $p < 0.01$ by one-way ANOVA with post-hoc Bonferroni correction.

Longer-term (8 d) stimulation of developing myotubes induced more profound metabolic changes than those described above (Fig. 5.3 A, B). Resistin increased basal respiration (36 ± 7 %, $p < 0.0001$) and ATP production (114 ± 12 %, $p < 0.001$) and on average increased proton leak (91 ± 21 %), although this did not reach statistical significance ($p = 0.055$); Maximal respiration, spare capacity and baseline ECAR were unaffected (Fig. 5.3, C-H). In all instances, co-stimulation of the cultures with TPCA-1 reversed these effects. Notably, TPCA-1 independently increased ATP production compared to unstimulated controls (Fig. 5.3, E), but no other outcome was significantly altered. Fig. 5.3, B visualises the relative contribution of mitochondrial and non-mitochondrial oxygen consumption as well as that of proton leak, to the baseline OCR. Non-mitochondrial respiration represented 46 ± 1 % of OCR in unstimulated myotubes, with mitochondrial respiration being responsible for 45 ± 1 % of OCR. Resistin stimulation increased basal OCR (see above) but did not substantially alter the relative contributions of mitochondrial and non-mitochondrial respiration (Fig. 5.3, B).

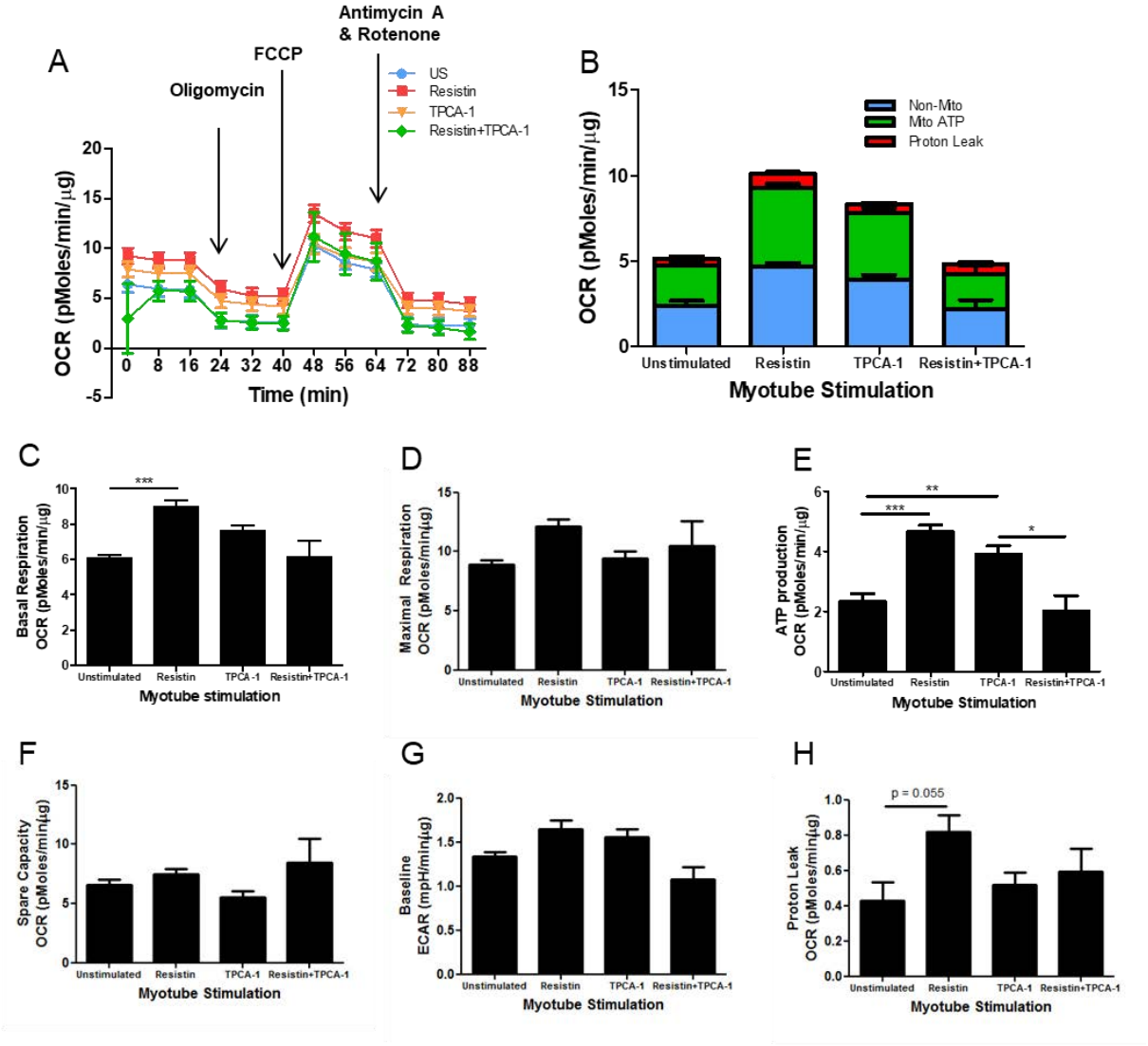

Figure 5.3. Resistin is a metabolic stressor of developing myotubes. Subconfluent myoblasts were switched differentiation media (containing 0 ng/mL or 5 ng/mL recombinant resistin ± 40 nM TPCA-1) for 8d. Media were renewed every 2 d. An XF Mito Stress Test was carried out on the Seahorse XFe96 analyser. Data are expressed as mean ± SEM values of n = 4 independent experiments. Each independent experiment comprises data from quadruplicate measurements. $p < 0.05$, ** $p < 0.01$ $p < 0.001$ by Mann Whitney U test with post-hoc Holm's sequential Bonferroni adjustment. Panel B visualises the relative contribution of mitochondrial oxygen consumption, non-mitochondrial oxygen consumption and proton leak to the total baseline oxygen consumption rate (OCR).

5.3.3 Resistin Promotes Myotube Lipid Accumulation

Resistin significantly increased both the Oil Red O area in each field of view (57 ± 16 %, p = 0.004; Fig. 5.4, A) and the average Oil Red O particle size (36 ± 9 %, p = 0.0003; Fig. 5.4, B). Co-stimulation of the myogenic cultures with resistin and TPCA-1 reversed this phenomenon (Fig. 5.4).

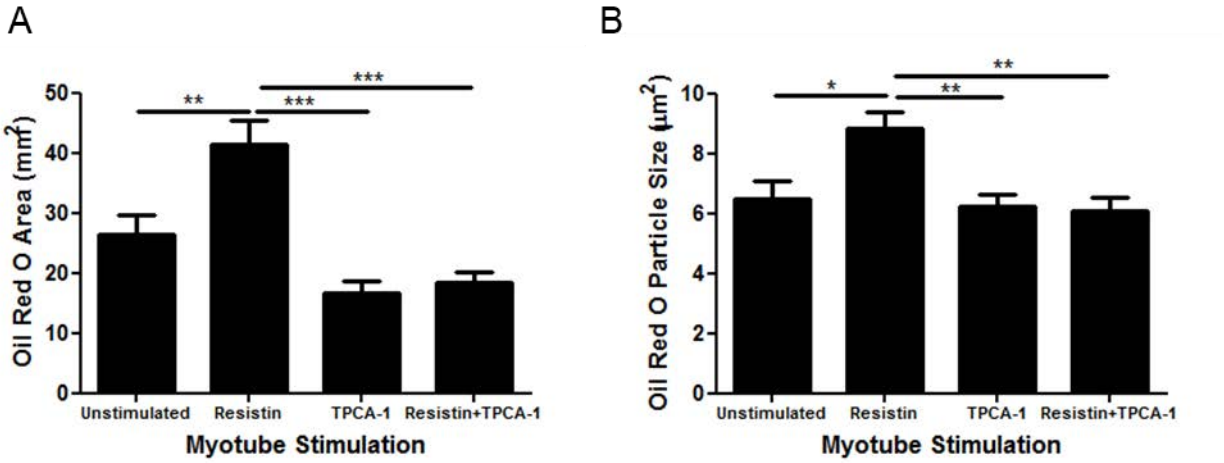

Figure 5.4. Recombinant resistin stimulation of developing myotubes from commercially available primary human skeletal myoblasts causes an accumulation of intracellular lipid that is prevented by TPCA-1. Subconfluent myoblasts were switched differentiation media (0 ng/mL or 5 ng/mL recombinant resistin ± 40 nM TPCA-1) for 8d. Media were renewed every 2 d. **A)** Oil Red O area. **B)** Oil Red O particle size. Data are expressed as mean ± SEM values of n = 3 independent experiments. Each independent experiment replicate comprises data from 15 images taken at 20x magnification. $p < 0.05$, ** $p < 0.01$, ***$p < 0.001$ by Mann Whitney U test with post-hoc Holm's sequential Bonferroni adjustment

5.3.4 Resistin Increases the Capacity of Primary Human Myotubes to Oxidise Fatty Acids, but Inhibition of the Classical NFκB Pathway Does Not Reverse This Phenomenon

Endogenous fatty acid oxidation was not detected in unstimulated myotubes. Resistin significantly increased both endogenous and exogenous fatty acid oxidation (Fig. 5.5). Surprisingly, however, TPCA-1 stimulation alone also enhanced exogenous fatty acid oxidation. Furthermore, co-stimulation of developing myotubes with resistin and TPCA-1 did not reverse the effects of resistin alone (Fig. 5.5).

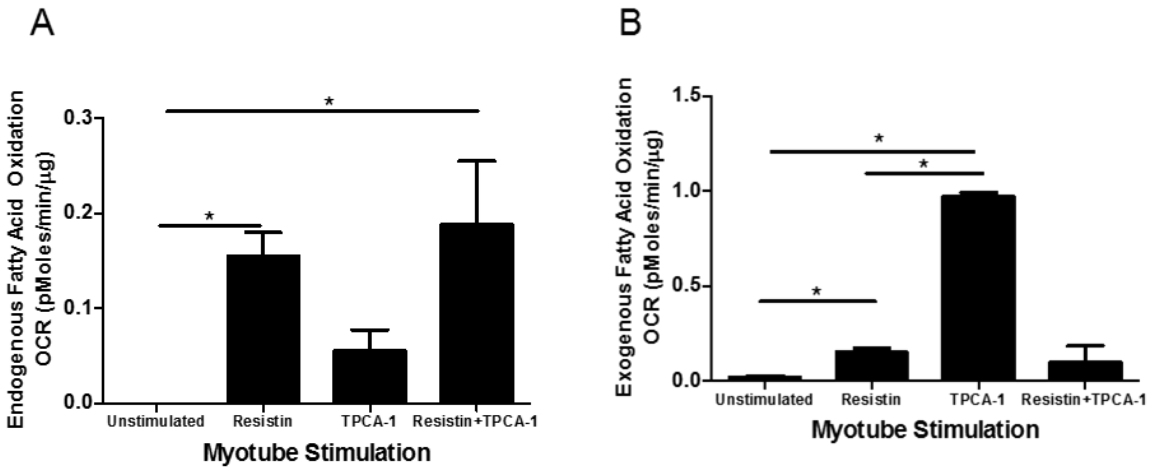

Figure 5.5. Recombinant resistin stimulation of developing myotubes from commercially available primary human skeletal myoblasts causes an increase in fatty acid oxidation that is not reversed by TPCA-1. Subconfluent myoblasts were switched differentiation media (0 ng/mL or 5 ng/mL recombinant resistin ± 40 nM TPCA-1). Media were renewed every 2 d. At 8 d an XF Palmitate-BSA FAO Substrate Mito Stress Test was carried out on the Seahorse XFe96 analyser. Data are expressed as mean ± SEM values of quadruplicate measurements. *p < 0.05 by Mann Whitney U test with post-hoc Holm's sequential Bonferroni adjustment.

5.3.5 Resistin Does Not Induce Substantial Changes in Metabolic Gene Expression

Despite the profound changes in oxidative metabolism (Section 5.2.2) and lipid accumulation (Section 5.2.3) induced by myotube differentiation in the presence of resistin, no significant alterations in metabolic gene expression were observed (Fig. 5.6). A non-significant (0.66-fold, p = 0.09) decrease in *GLUT4* expression was found (Fig. 5.6,

A). The rationale for quantifying each of the genes presented is detailed in the discussion section of this chapter.

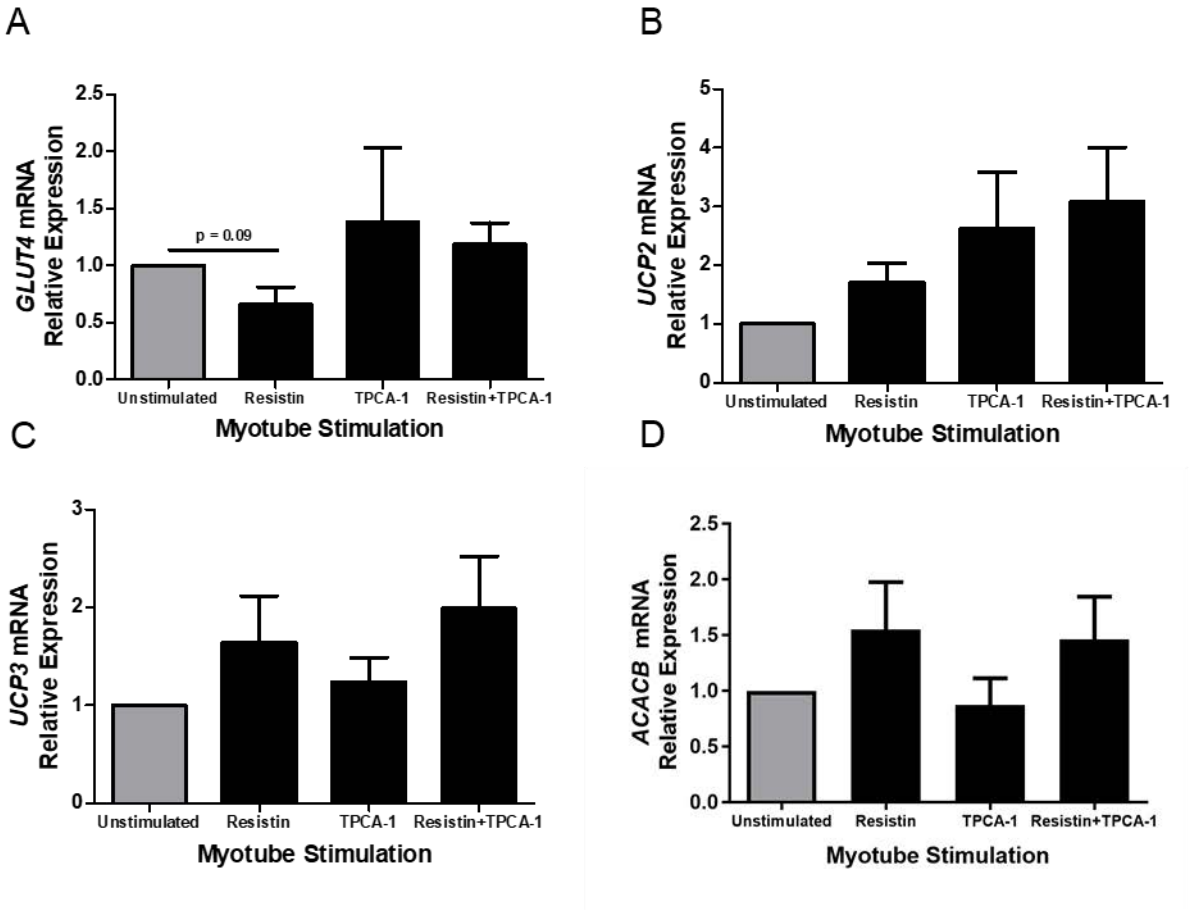

Figure 5.6. Recombinant resistin stimulation of developing myotubes does not induce any notable changes in metabolic gene expression. Subconfluent commercially available myoblasts were switched differentiation media for 8 d (containing 0 ng/mL or 5 ng/mL recombinant resistin ± 40 nM TPCA-1). Media were renewed every 2 d. Data are expressed as mean ± SEM values of n = 3 independent experiments. Each independent experiment comprises data from triplicate culture wells, with each being assayed in triplicate. $p < 0.05$ for Log_{10} transformed data vs. unstimulated condition by Wilcoxon signed-rank test.

5.4 Discussion

5.4.1 In Myotubes, Resistin Promotes Enhanced ATP Production, Increased Fatty Acid Oxidation and Lipid Accumulation

In Chapter 3, resistin was established as an adipokine that has a detrimental effect on primary human myogenesis *in vitro*.; a clear rationale for its prioritisation for such study was established in Chapter 2. Resistin is also understudied regarding its metabolic effects on skeletal muscle. This chapter establishes that resistin has profound metabolic effects on primary human myotubes *in vitro.* It characterises some aspects of this dysfunction, and although important questions remain as to how and why such metabolic adjustments evolve, this work is distinct in illuminating the breadth of such dysfunction and in doing so in primary human cultures.

Staining of myotubes with Mitotracker® Green FM and Mitotracker® Orange CM-H2TMRos was employed as a method for detecting differences in mitochondrial mass or mitochondrial membrane potential following 24 h resistin stimulation. Mitotracker® Green FM staining indicated that the total number of mitochondria did not differ between myotubes stimulated with or without resistin. When the Mitotracker® Orange intensity in each image was corrected for the corresponding Mitotracker® Green FM intensity, the reduction in Mitotracker® Orange intensity in resistin-stimulated myotubes approached significance. This finding, in addition to pilot data from the first independent experiments detailed in Fig. 5.2 and Fig. 5.3 confirmed to us the utility of pursuing the study of the metabolic effect of resistin on myotubes *in vitro*.

Indeed, short-term (24 h) resistin stimulation of fully differentiated myotubes (Fig. 5.2, G) and longer-term (8 d) stimulation of myogenic cultures during differentiation (Fig. 5.3, H;

p = 0.055) induced an increase in proton leak (although this did not quite reach statistical significance in the 8 d stimulated cultures). The IKKβ inhibitor TPCA-1 prevented this enhanced proton leak. These findings are in accordance with the apparent resistin-induced fall in mitochondrial membrane potential indicated by reduced Mitotracker® Orange staining intensity. 24 h resistin stimulation of myotubes did not induce any changes in the other outcomes measured by the Seahorse® XF Mito Stress Test, with more profound metabolic changes being observed in myotubes formed for 8 d under such stimulation conditions (Fig. 5.3). In addition to proton leak, resistin induced a marked increase in basal respiration and ATP production; co-stimulation of the cultures with TPCA-1 reversed these effects. Such resistin stimulation of developing myotubes also promoted lipid accumulation (Fig. 5.4), a phenomenon that again, was reversed by the presence of TPCA-1. Both endogenous and exogenous fatty acid oxidation were enhanced in such lipid-laden myotubes (Fig. 5.5). Surprisingly, however, TPCA-1 stimulation alone also enhanced exogenous fatty acid oxidation, and co-stimulation with resistin and TPCA-1 did not reverse the effects of resistin alone. Thus, while inhibition of resistin signalling via the classical NFκB pathway normalised myotube basal respiration, ATP production, proton leak and lipid content, it did not do so by altering fatty acid oxidation.

5.4.2 Resistin as a Metabolic Stressor of Myotubes – Possible Mechanisms of action

With resistin stimulation of myotubes, the metabolic picture that we describe is one of increased ATP production, increased basal OCR, increased fatty acid oxidation and lipid

accumulation. The data presented in Fig. 5.5, B demonstrate that resistin-stimulated myotubes display an enhanced capacity to oxidise exogenous fatty acids. The notion that enhancement of fatty acid uptake is largely responsible for resistin-induced myotube lipid accumulation is supported by the small contribution of de novo lipogenesis to skeletal muscle lipid storage (447–449) coupled with the large (~ 60 %) increase in myotube lipid accumulation described here (Fig. 5.4). The combination of resistin and TPCA-1 did not alter either endogenous or exogenous fatty acid oxidation, compared to stimulation with resistin alone, suggesting that the normalisation of myotube lipid content by TPCA-1 is not due to an enhancement of myotube fatty acid oxidative capacity. It may be that inhibition of the classical NFκB pathway by TPCA-1 reduces myotube fatty acid uptake, thus allowing a larger proportion of such fatty acids to be oxidised.

Indeed, some evidence exists to support this hypothesis. Resistin stimulation of human macrophages has been shown to promote intracellular lipid accumulation, with a concomitant increase in the cell surface expression of the fatty acid transporter CD36 (446). A search of the ArrayExpress database (450) yielded microarray data from a study (E-GEOD-64060) of mice lacking brown adipose tissue (BAT) CD36 expression. Resistin gene expression was detected only in CD36 null mice; in the context of the other studies discussed here, this may represent a compensatory response to the absence of CD36 expression. Evidence that fatty acid transporters such as CD36 and the fatty acid transport proteins (FATPs) are direct transcriptional targets of NFκB is lacking. However, some evidence of their interdependence does exist. In mice with hepatic steatosis, NFκB gene expression correlated closely with the expression of FATP genes ($R^2 = 0.77$, $p < 0.01$) (451). Another study described a substantial reduction in NFκB expression in human

macrophages following inhibition of CD36 activity with Sulfo-N-succinimidyl oleate (452). It is unclear from the manuscript which NFκB subunit was detected by the antibody used. The possibility that enhanced fatty acid uptake might itself activate NFκB transcriptional activity must also be considered and indeed, this has been demonstrated in L6 myotubes (453). Thus, while the promotion of fatty acid uptake via resistin-mediated NFκB activation is plausible, the current evidence is tenuous and requires further study. Such a mechanism would, however, explain our observation that co-stimulation of myogenic cultures with resistin and TPCA-1 prevents lipid accumulation without altering fatty acid oxidation rates. The substantial enhancement of exogenous fatty acid oxidation by TPCA-1 alone, while not necessarily indicative of increased fatty acid uptake, represents an additional reason for caution in considering this hypothesis.

Small amounts of de novo lipogenesis occur in skeletal muscle, yet such lipogenesis might be of consequence in resistin-stimulated myotubes. Resistin is certainly capable of inducing lipogenesis in the liver. Resistin-null Ob/Ob mice have been reported to be protected from hepatic steatosis and displayed decreased hepatic expression of enzymes involved in de novo lipogenesis, including acetyl-coA carboxylase (ACC) and fatty acid synthase (FAS) (454). In our myotubes, resistin stimulation increased the expression of the ACC gene *ACACB* 1.5-fold (Fig. 5.6, D), but this did not reach statistical significance. Other work has described the stimulation of the human HepG2 hepatocyte cell line with resistin (50 ng/mL). Increased expression of the master regulators of fatty acid synthesis – the sterol regulatory element-binding proteins (SREBP1, SREBP2) was observed (455). SREBP1 and SREBP 2 are well-established promotors of lipogenic gene expression (456) and indeed, resistin also promoted the expression of ACC, Stearoyl-CoA desaturase

(SCD, the rate-limiting enzyme in monounsaturated fatty acid synthesis) and diglyceride acyltransferase (DGAT1, which catalyses the formation of triglycerides) in the HepG2 cells (455). In mice, the SREBP1 gene contains an NFκB promotor sequence (GGGRNNYYCC; (457)), to which NFκB binds (458) and NFκB may act in concert with SP1 to promote SREBP1 gene expression and thus lipogenesis (459). This particular murine NFκB promotor sequence is not conserved at the same position in humans (458), but a search for the GGGRNNYYCC sequence in the promotor regions of human SREBP1, SREBP2 (Ensembl release 89; (460)) yielded possible NFκB promotor sequences within close proximity to the first SREBP exon (SREBP1: -91 bp, 5'-GGGGTCCCC-3'; SREBP2: +11 bp, 5'- GGGAATCCC-3'). The database of transcriptional start sites (DBTSS) contains tissue-specific transcriptional start site positions for human genes (461,462). The true transcriptional start site for human skeletal muscle SREBP2 is 15 bp distal to the NFκB promotor sequence (Appendix Figure 5.1), and thus the NFκB promotor sequence that we identified at +11 bp lies immediately upstream of the first SREBP2 exon, rather than within it. Thus, while direct evidence of NFκB binding and activation of human SREBP expression is lacking, it represents a plausible mechanism by which resistin might activate lipogenesis and warrants further exploration.

Indeed, enhanced fatty acid uptake, fatty acid oxidation and lipogenesis might co-exist in resistin-stimulated myotubes. If resistin is a potent direct activator of both fatty acid uptake and lipogenesis in skeletal muscle, such processes might also exert positive feedback upon one another. Enhanced fatty acid uptake and thus substrate oversupply to the TCA cycle could reasonably be expected to produce an excess of mitochondrial citrate, which

upon transport to the cytoplasm would allosterically activate ACC, thus facilitating malonyl-CoA production. Malonyl-CoA acts as the substrate for fatty acid synthesis, thus renewing the fatty acid pool and mitochondrial substrate oversupply. This positive feedback, coupled with direct activation of the lipogenic program by resistin might amplify the usual small contribution of de novo lipogenesis to skeletal muscle lipid content (Fig. 5.7). Similar futile substrate cycling between de novo lipogenesis and fatty acid oxidation had been demonstrated with leptin stimulation of *ex vivo* murine skeletal muscle and is essential for leptin to stimulate thermogenesis in such muscle (463). The synthesis of one molecule of palmitate in skeletal muscle would theoretically cost 14 molecules of ATP (448), and thus should facilitate the dissipation of intracellular lipid as has been suggested with regard to leptin (448,464). Indeed, we stimulated developing myotubes for 8 d with 5 ng/mL leptin and demonstrated a substantial decrease in myotube lipid content (Appendix Figure 5.2). However, if resistin is a direct activator of both fatty acid uptake and lipogenesis in skeletal muscle, enhanced fatty acid oxidation (Fig. 5.5) coupled with such futile substrate cycling might be insufficient to dissipate the intramyocellular fatty acid burden. Inhibition of carnitine palmitoyltransferase 1 (CPT-1, which is rate-limiting for long-chain fatty acid transport into mitochondria) by malonyl CoA might be expected to prevent such a substrate cycle but the absolute requirement for both de novo lipogenesis and fatty acid oxidation to facilitate leptin-induced skeletal muscle thermogenesis suggests that this is not necessarily the case (463). Indeed, it has been noted that malonyl-CoA independent regulation of CPT-1 has tended to be overlooked (465) and fatty acids can directly induce CPT-1 gene expression (466–468).

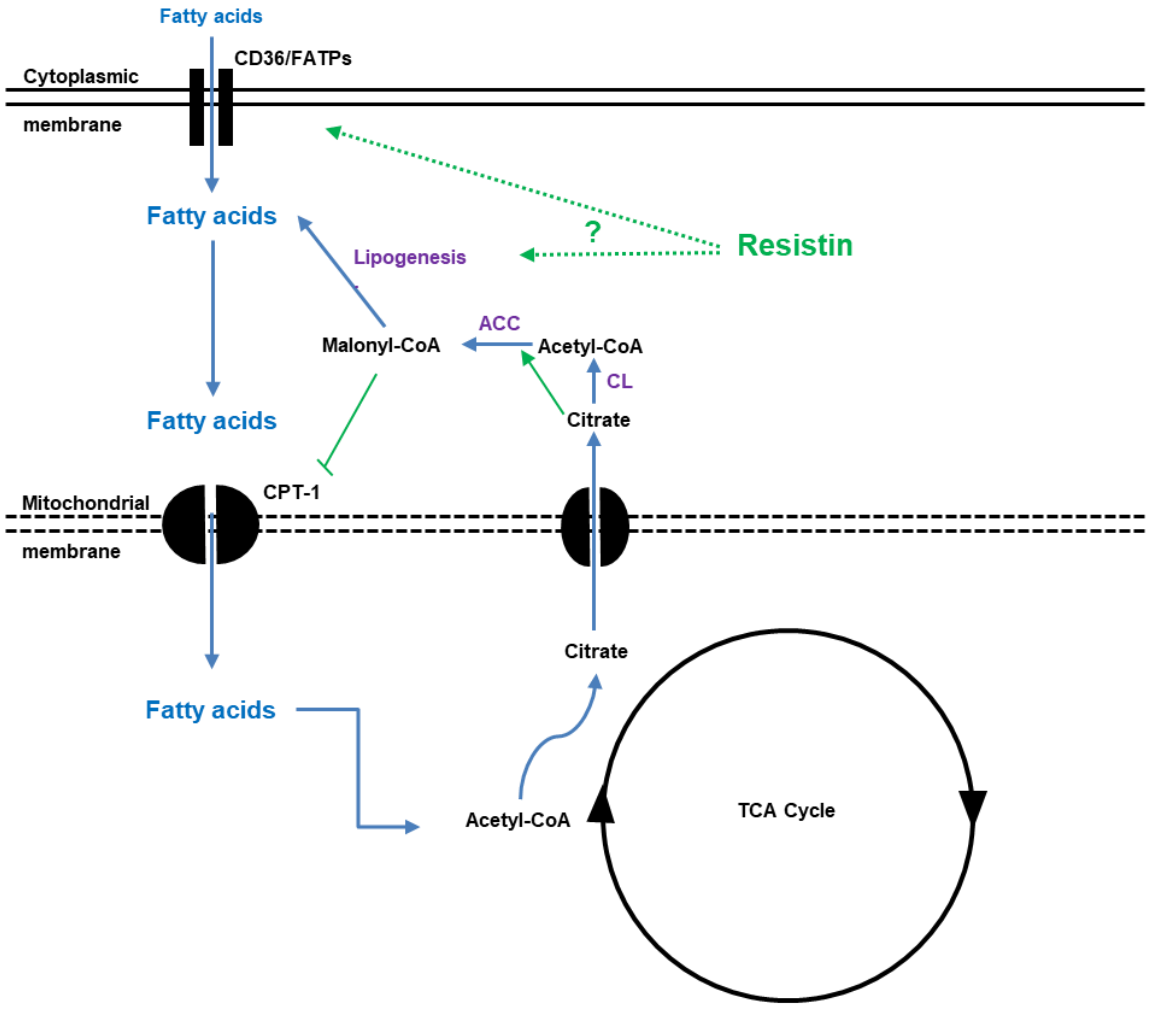

Figure 5.7. A possible energy-dissipating 'futile' substrate cycle between de novo lipogenesis and lipid oxidation. If resistin is – as the existing literature and our results suggest – a direct activator of both fatty acid uptake and lipogenesis in skeletal muscle, such processes might exert positive feedback upon one another. Enhanced fatty acid uptake, and thus substrate oversupply to the TCA cycle could reasonably be expected to produce an excess of mitochondrial citrate, which upon transport to the cytoplasm would allosterically activate acetyl CoA carboxylase (ACC), thus facilitating malonyl-CoA production. Malonyl-CoA acts as the substrate for fatty acid synthesis, and thus may renew the fatty acid pool and promote mitochondrial substrate oversupply. Malonyl CoA might be expected to inhibit carnitine palmitoyl transferase 1 (CPT-1) to prevent such a substrate cycle, but the absolute requirement for both de novo lipogenesis and fatty acid oxidation to facilitate leptin-induced skeletal muscle thermogenesis suggests that this is not the case. Adapted from Solinas et al. FEBS Lett. 2004 Nov 19;577(3):539-44.

In addition to the above, resistin-stimulated (24 h and 8 d) myotubes displayed an increase in proton leak, and this phenomenon was reversed by co-stimulation with TPCA-1 (Fig.

5.2, 5.3). Proton translocation across the inner mitochondrial membrane via routes other than the ATP synthase complex uncouples oxidative phosphorylation from respiration and is termed proton leak. Proton leak is principally facilitated by the adenine nucleotide translocase (ANT) and uncoupling proteins (UCP). ANT activity is induced by fatty acids (469,470), and this phenomenon appears to preserve skeletal muscle insulin sensitivity in the presence of excess fatty acids (471). Controversies still exist regarding the mechanism of proton transport by UCP, although such activity is undoubtedly promoted by fatty acids and may occur via their acute activation of UCP or via their chronic alteration of UCP expression (472). The UCP2 and UCP3 genes are expressed in skeletal muscle, although these isoforms are understudied in contrast with UCP1 and their importance to mitochondrial bioenergetics remains unclear (472). Detailed discussion of such controversies in the study of UCP biology is beyond the scope of this thesis (see (472) for a recent review). UCP2 and UCP3 gene expression was not altered by resistin (Fig. 5.6, B,C), although the observation that resistin is capable of inducing proton leak in myotubes at 24 h (Fig. 5.2) would suggest any contribution by ANT or UCP to such proton leak takes place via their activation rather alteration of their expression. Future work should focus on establishing whether resistin's effects on myotube fatty acid metabolism is responsible for ANT and UCP activation and thus enhanced proton leak. Such proton leak may be a further adaptation intended to dissipate the intramyocellular fatty acid burden in resistin-stimulated myotubes.

The apparent decrease in *GLUT4* expression (Fig. 5.6, A) in resistin-stimulated myotubes is broadly in keeping with the general principle of the glucose-fatty acid cycle proposed by Randle et al. (473) i.e. that use of one nutrient (fatty acids) inhibits the use of another

(glucose). However, an integrated examination of glucose and fatty acid uptake and oxidation will be necessary in resistin-stimulated myotubes to properly delineate any alterations in metabolic fuel utilisation. Any future work designed to further detail the metabolic effects of resistin in myotubes should consider the possibility that ROS overproduction might contribute to proton leak. ROS activate UCPs and ANT (474–476), and activation of such proton leak appears to protect against further ROS formation. For example, UCP3$^{-/-}$ mice produce an excess of skeletal muscle ROS (477,478) and 26 weeks of high-fat feeding in such mice lowers mitochondrial capacity, suggesting that UCP3 may play a role in compensating for conditions of chronic free fatty acid excess (479).

5.5 Limitations and Future Directions

This chapter has described substantial and important effects of resistin on young primary human myotubes *in vitro*. The picture that has emerged is one of resistin as a metabolic stressor of myotubes, specifically one of increased ATP production, basal OCR, fatty acid oxidation, proton leak and lipid accumulation. While this work is novel and represents an advancement in our knowledge of the metabolic effects of resistin on human myocytes, it has several important limitations and highlights important areas of future investigation. First, the measurement of glucose and fatty acid uptake, as well as their oxidation, would establish a clearer picture of metabolic fuel utilisation in such myotubes. Second, it is plausible that resistin might stimulate skeletal muscle lipogenesis and therefore this too must be quantified. Third, the role of the classical NFκB pathway in mediating any resistin-induced increases in fatty acid uptake and lipogenesis should be explored; specifically,

any induction of the expression of fatty acid transporters and lipogenic enzymes should be considered. Demonstration of NFκB binding to the human SREBP NFκB promoter sequences could form part of this work. Finally, it is necessary to establish whether resistin activates ANT and UCP to enhance proton leak. Addressing such issues will allow a complete modelling of resistin's metabolic effects on human myocytes.

5.6 Conclusions

1. Resistin increases ATP production, basal OCR, fatty acid oxidation, proton leak and lipid accumulation in primary human myotubes.
2. Inhibiting NFκB activity reverses the above effects, with the exception of the changes in fatty acid oxidation.
3. The possibility that resistin directly activates myocyte fatty acid uptake and de novo lipogenesis should be considered, along with the hypothesis that a futile substrate cycle between fatty acid oxidation and de novo lipogenesis might be insufficient to dissipate the considerable intramyocellular fatty acid burden.

Chapter 6

IL-15 and *in vitro* Human Myogenesis

6.1 Introduction

Sarcopenia is a major contributor to frailty and disability in the elderly (9,10). Importantly, recent studies in the mouse suggest that IL-15, a 14 kDa four-helix bundle cytokine may play a central role in the growth and maintenance of skeletal muscle (320–322,480). However, to date, no studies have examined the expression or functional role of IL-15 in human skeletal muscle tissue or primary myogenic cells.

Stimulation of murine C2C12 myoblasts with rIL-15 increases myoblast proliferation and MHC expression, promoting enhanced myogenesis and the development of larger myotubes (49). Furthermore, *ex-vivo* stimulation of rat extensor digitorum longus muscle with rIL-15 decreased skeletal muscle proteolytic rate (342), suggesting an anti-atrophic effect of IL-15 on muscle tissue. Indeed, it is suggested that IL-15 may play an important key role in the maintenance of muscle mass in the presence of atrophic stimuli. For example, rIL-15 ameliorated the induction of protease (cathepsin L) activity in TNFα and dexamethasone stimulated C2C12 myotubes (50). Furthermore, in an experimentally-induced sepsis mouse model, rIL-15 reduced the mRNA expression of the E3 ligases MAFbx and MuRF-1 which ubiquitinate and target proteins for proteasomal degradation (50). rIL-I5 treatment of Yoshida AH-130 ascites hepatoma rats decreased skeletal muscle protein degradation 8-fold and significantly limited loss of body mass as well as soleus and tibalis muscle mass (481). It has been proposed that IL-15 is a compensatory factor, expressed in skeletal muscle to mitigate conditions promoting skeletal muscle atrophy (51).

Importantly, current evidence from *in vivo* human studies points to a crucial role for

myogenesis in adult skeletal muscle maintenance, hypertrophy and remodelling in response to disuse atrophy as well as injurious and non-injurious exercise (482). Therefore, in this study, we used a model of adult human myoblast differentiation into myotubes to determine the effect of IL-15 on myogenesis. We further sought to establish whether the purported myogenic effects of IL-15 are conserved in elderly human skeletal muscle, since IL-15 signalling may represent an important pathway for the identification and development of therapeutic approaches designed to preserve the loss of skeletal muscle mass and quality in ageing. Given the propensity of the elderly to develop sarcopenia, we hypothesised that myogenic cultures derived from the skeletal muscle of elderly individuals would be more resistant to the pro-myogenic and hypertrophic effects of IL-15 than those of young individuals. Finally, we sought to examine whether IL-15 could protect primary human myotube development from the deleterious effects of TNFα, a pro-inflammatory cytokine implicated in the pathogenesis of sarcopenia (483) and cachexia in chronic illness (22).

6.2 Methods

To examine the effect of IL-15 on human myotube development, myoblasts from young participants were differentiated for 8 d in the presence of rIL-15 at 1, 25 and 100 ng/mL. The resulting myotubes were fixed, IF stained for desmin and counterstained with DAPI. Plates were imaged and myogenesis quantified by determining MTT and NFI.

The mRNA expression of known mediators of myogenesis (namely myomaker, and the myogenic transcription factors MyoD, myogenin and Myf5) were measured by RT-qPCR in undifferentiated myoblasts and in myoblasts during their differentiation into myotubes.

In a separate experiment, myoblasts at 90% confluence were switched to differentiation medium, or to differentiation media containing rIL-15 (at 1 ng/mL or 25 ng/mL). After 8 d of differentiation, myogenic regulatory factor gene expression was quantified by RT-qPCR. Next, we sought to determine whether myogenic cultures derived from elderly participants were sensitive to the pro-myogenic effects of rIL-15 and whether rIL-15 was capable of reversing the deleterious effects of recombinant TNFα (rTNFα) on myogenesis using an rTNFα concentration representative of those found systemically in cachexia (484,485). Differentiating myoblasts from young and old participants were stimulated with either rTNFα (1 ng/mL) alone, rIL-15 (25 ng/ml) ± rTNFα (1 ng/mL) or were left unstimulated (controls) over 8 d. Media were renewed every 2 d. The resulting myotubes were fixed, IF stained and imaged as before.

The expression of myogenic regulatory factors (MyoD, MyoG), the E3 ubiquitin ligases (MAFbx, MURF-1) and the acticity of pro-myogenic signalling nodes (Akt, ERK) were quantified by immunoblotting in in both undifferentiated myoblasts and in differentiating (48 h) cultures.

Cognisant of the suggestion that IL-15 expression increases in order to compensate for conditions that promote skeletal muscle loss (51), we quantified circulatory levels of IL-15 by ELISA. We also quantified the gene expression of IL-15 and its receptors by RT-qPCR in young and old skeletal muscle tissue derived from skeletal muscle biopsies. We sought to establish whether an inflammatory stimulus could induce IL-15 secretion and expression in primary human myotubes. To that end, 8 d differentiated myotubes were stimulated with 20 ng/mL TNFα and the concentration of IL-15 in culture supernatants was

measured by ELISA at 24 h. Furthermore, the myotube expression of *IL15, IL15RA* and *IL2RB* were quantifed in such cultures by RT-qPCR.

We then investigated whether disruption of IL15 binding to its receptor would alter myogenesis in the presence of TNFα. We employed an antibody against the IL-15 receptor binding subunit, IL-15rα. Myoblasts from elderly participants were differentiated for 8 d in the presence of IL-15 (25 ng/mL) or TNFα (1 ng/mL) with the further addition of an IL-15rα neutralising antibody (1μg/mL) or an IgG isotype control (1 μg/mL). Media were renewed every 2 d. MTT and NFI were quantified as previously described.

6.3 Results

6.3.1 Effect of IL-15 on Human Myotube Development and Myogenic Gene Expression

rIL-15 (100 ng/mL) significantly increased the MTT of differentiated myotubes by 22 ± 5 % ($p < 0.01$) (Fig. 6.1 A, B). Stimulation of differentiating myotubes for 8 d with either 25 ng/mL or 100 ng/mL rIL-15 also enhanced the NFI (35 ± 4 %, $p < 0.0001$; 45 ± 7 %, $p < 0.0001$ at 25 and 100 ng/mL respectively), compared to unstimulated controls (Fig. 6.1, C). Furthermore, the average number of myonuclei in each myotube was enhanced by rIL-15 stimulation (114 ± 20 %, $p < 0.0001$; 128 ± 27 %, $p < 0.0001$ at 25 and 100 ng/mL respectively) (Fig 6.1, D).

Stimulation of myoblasts with rIL-15 (25 ng/mL) induced a small (1.3-fold) but highly significant ($p < 0.0001$) increase in the expression of myomaker, a cell membrane protein that is essential for myoblast fusion (Fig. 6.1, E). Notably, this alteration in myomaker expression was not accompanied by changes in the expression of either *MYF5, MYOG*

or *MYOD1* expression (Appendix Fig. 6.1).

rIL-15 increased the gene expression of *MYOG* (1.45-fold, $p = 0.09$; 2.5-fold, $p < 0.0001$ at 1 and 25 ng/mL respectively) *and MYOD* (2.36 fold, $p = 0.02$; 3.32-fold, $p < 0.05$ at 1 and 25 ng/mL respectively) in differentiated myotubes (Fig. 6.1, F, G).

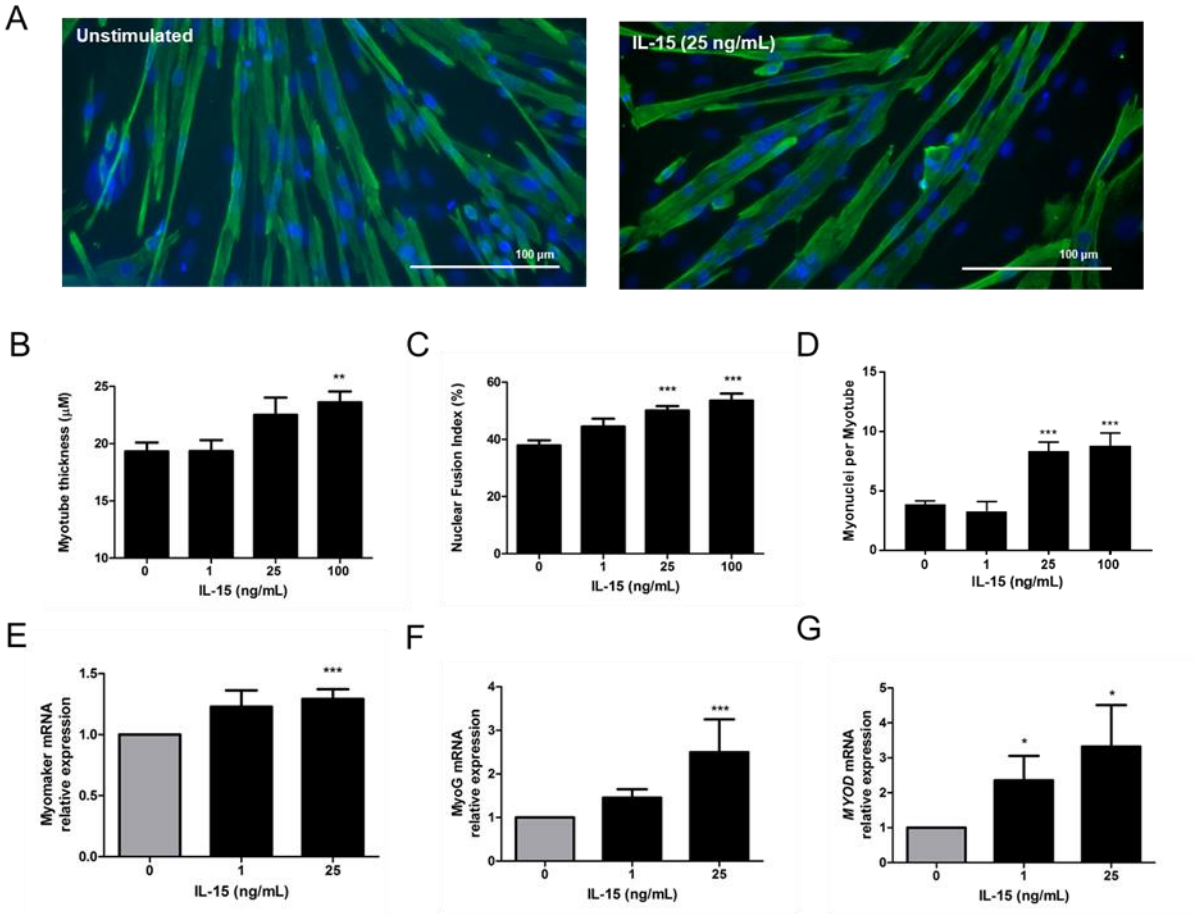

Figure 6.1. Recombinant IL-15 stimulation of differentiating primary human myoblasts enhances myotube formation and promotes myogenic gene expression. A) Representative images at 20x magnification. **B)** Myotube thickness data represents the mean ± SEM of n = 3 biological replicates. Each biological replicate comprises 150 total measurements taken at 63x magnification from 30 myotubes per treatment condition. **p < 0.01 vs. unstimulated (0 ng/mL) condition by Mann-Whitney U test with post-hoc Holm's sequential Bonferroni adjustment. **C)** Nuclear fusion index data are expressed as mean ± SEM values of n = 3 biological replicates. Each biological replicate comprises 15 images taken at 20x magnification. ***p < 0.001 vs. unstimulated (0 ng/mL) condition by one-way ANOVA with post-hoc Bonferroni correction. **D)** Number of myonuclei per myotube is expressed as mean ± SEM values from 15 images taken at 20x magnification from 3 biological replicates. **E)** Myoblast Myomaker gene expression **F, G)** Myotube MYOG and MYOD gene expression. *p < 0.05, ***p < 0.001 vs. unstimulated condition by Mann-Whitney U test with post-hoc Holm's sequential Bonferroni adjustment. Data expressed as mean ± SEM. 3 technical replicates (each assayed in triplicate) per biological replicate, 3 biological replicates.

6.3.2 Recombinant IL-15 Stimulation of Differentiating Primary Human Myoblasts Partially Reverses TNFα-induced Inhibition of Myogenic Differentiation in Young and Old Myotubes

rIL-15 increased the MTT of myotubes from elderly donors (22 ± 5 %, p = 0.02), indicating that they are sensitive to the hypertrophic effects of IL-15 that we observed in the young (Fig. 6.2, B). In young myotubes, rTNFα alone induced a 30 ± 5 % decrease in myotube thickness ($p < 0.0001$) compared to unstimulated control myotubes. However, co-incubation of differentiating myoblasts with rIL-15 and rTNFα partially reversed this effect, limiting loss of MTT to 11 ± 6 % of the control, a significant improvement compared to the rTNFα alone condition (p = 0.04). In old myotubes, the decrease in MTT induced by TNFα was not significant, however, myotubes co-stimulated with TNFα and rIL-15 condition were significantly thicker (29 ± 7 %, P = 0.001) than their TNFα-stimulated counterparts (Fig. 6.2, B).

rIL-15 alone did not significantly increase the NFI in old myotubes or in this cohort of young myotubes. However, in both age groups co-incubation of differentiating myoblasts with rIL-15 and rTNFα completely reversed TNFα-induced reductions in NFI, resulting in NFI values which did not differ significantly from the control (Fig. 6.2, C). rIL-15 stimulation of proliferating myoblasts had no effect on their proliferation or survival rates (Appendix Fig. 6.2). Furthermore, in differentiated cultures the number of nuclei per field of view did not differ significantly between stimulation conditions, suggesting that their effects on MTT and NFI were not due to differences in cell survival, proliferation or errors in cell seeding density (Appendix Fig. 6.2). As before (Fig. 6.1), 25 ng/mL rIL-15 induced a substantial and significant increase in the average number of myonuclei in each myotube. However,

myotubes formed in the presence of rTNFα did not differ from unstimulated controls in their myonuclear number, irrespective of whether they were co-stimulated with rIL-15 (Appendix Fig. 6.2).

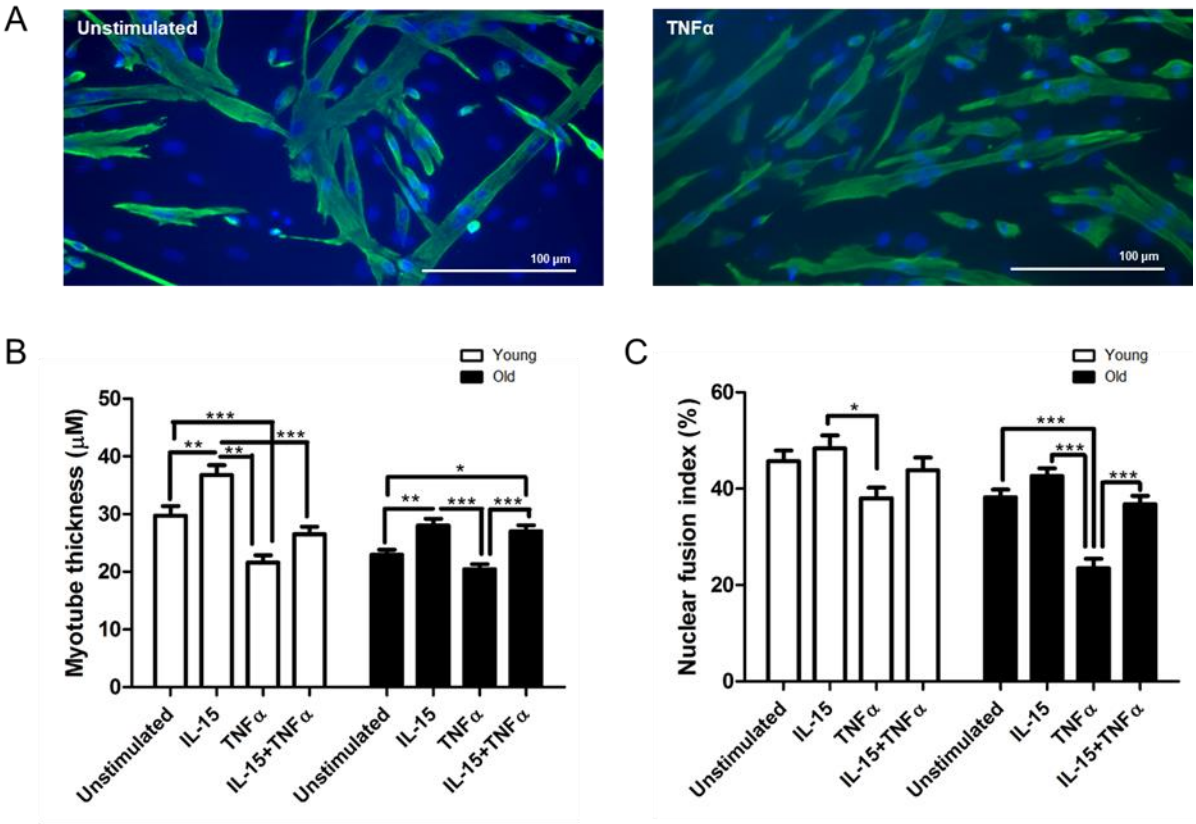

Figure 6.2. Recombinant IL-15 stimulation of differentiating primary human myoblasts partially reverses TNFα-induced inhibition of myogenic differentiation of cells from both young and old individuals. Subconfluent myoblasts were switched to differentiation medium containing the indicated recombinant cytokines (IL-15, 25 ng/mL; TNFα, 1 ng/mL) for 8 d. Media were renewed every 2 d. Myotubes were fixed, immunofluorescence stained for desmin and DAPI and imaged on an epifluorescence microscope. **A)** Representative images at 20x magnification. **B)** Myotube thickness data represents the mean ± SEM of n = 3 biological replicates. Each biological replicate comprises 150 total measurements taken at 63x magnification from 30 myotubes per treatment condition. **C)** Nuclear fusion index data are expressed as mean ± SEM values of n = 3 biological replicates. Each biological replicate comprises 15 images taken at 20x magnification. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. unstimulated condition by 2-way ANOVA with post-hoc Bonferroni correction.

Having observed pro-myogenic effects of rIL-15 both in the presence and the absence of a pro-inflammatory (rTNFα) stimulus, we sought further molecular corroboration for our observations in both undifferentiated myoblasts and in differentiating cultures. Subconfluent myoblasts were switched to growth medium containing the recombinant

cytokines (IL-15, 25 ng/mL; TNFα, 1 ng/mL) or were left unstimulated for 2 d. Immunoblotting demonstrated that rIL-15 induced the expression of total ERK 1/2 (p44/42 MAPK), phosphorylated-ERK (Thr202/Tyr204) and total Akt. A modest induction of total ERK and phosphorylated ERK expression was evident in cultures that were co-stimulated with rIL-15 and TNFα (Fig. 6.3, A). No changes in MyoD, MAFbx or MURF-1 expression were observed and myogenin was not detected in these undifferentiated cultures (Fig. 6.3, A).

Secondly, subconfluent myoblasts were switched to differentiation medium containing the recombinant cytokines or were left unstimulated for 2 d, at which time myotubes begin to form in our cultures. Similar to the undifferentiated cultures, stimulation with rIL-15 induced an increase in total ERK and phosphorylated ERK expression (Fig. 6.3, B). As expected, myogenin protein could be detected in these differentiated cultures, and its expression was increased in cultures stimulated with either rIL-15 or rTNFα, compared to unstimulated myotubes (Fig. 6.3, B). In addition, rIL-15 induced an increase in MHC protein content (Appendix Fig. 6.3), despite not significantly increasing total protein synthesis (Appendix Fig. 6.3), as measured using the non-radioactive surface sensing of translation (SUnSET) method (486,487).

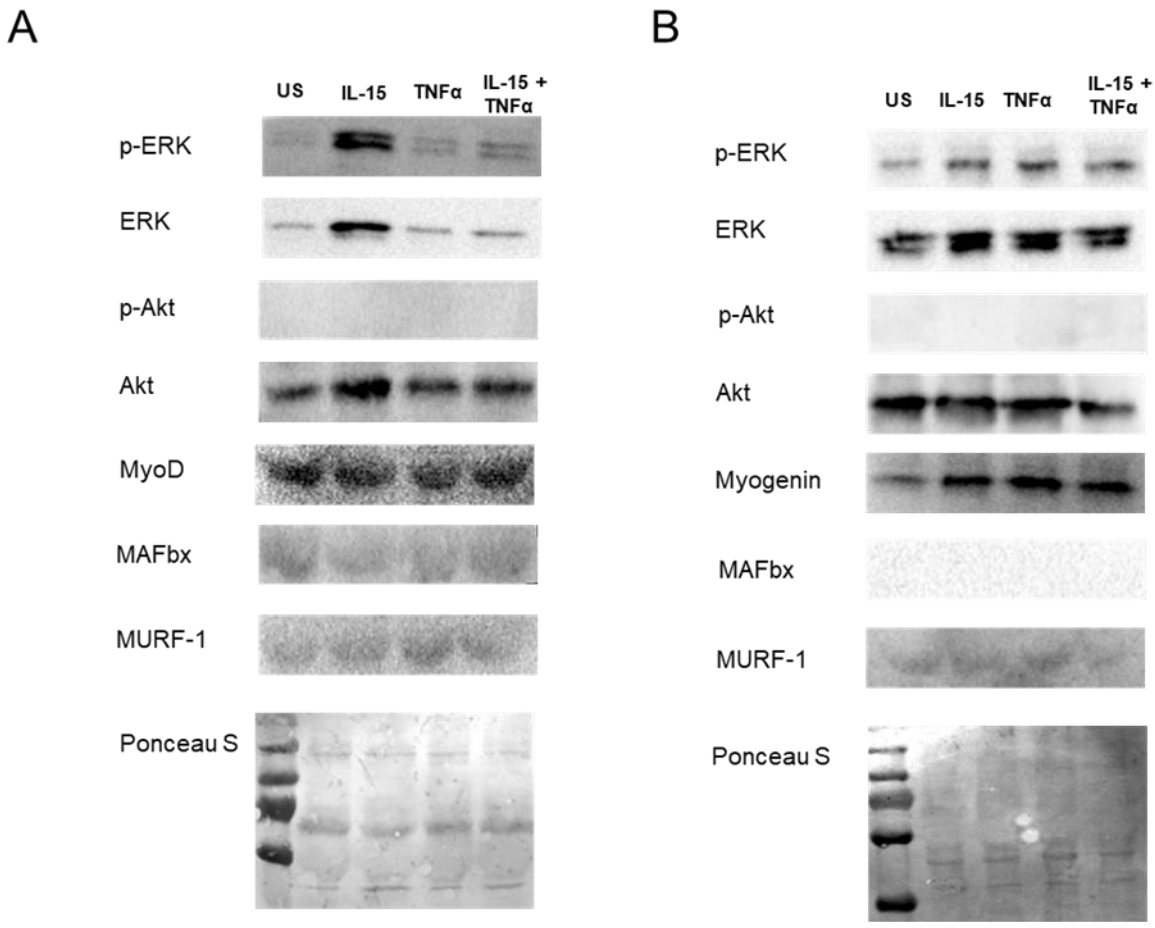

Figure 6.3. Recombinant IL-15 stimulation of primary human myogenic cultures induces molecular changes that suggest it has pro-myogenic actions. A) Commercially available subconfluent myoblasts from a female aged 21 yr were switched to growth medium containing the indicated recombinant cytokines (IL-15, 25 ng/mL; TNFα, 1 ng/mL) for 2 d. The expression of the indicated proteins was determined by immunoblotting. Full-length blots are presented in Appendix Figure 6.5. **B)** Commercially available subconfluent myoblasts from a female aged 21 yr were switched to differentiation medium containing the indicated recombinant cytokines (IL-15, 25 ng/mL; TNFα, 1 ng/mL) for 2 d. The expression of the indicated proteins was determined by immunoblotting. Full-length blots are presented in Appendix Figure 6.1. Immunoblots are representative of duplicate independent experiments.

6.3.3 Elderly Individuals Display Increased Skeletal Muscle IL-15 Expression and Increased Plasma IL-15 Concentrations

Skeletal muscle expression of *IL15* was 2-fold higher (Fig. 6.4, A) in old participants, compared to young participants. Furthermore, circulatory concentrations of IL-15 were significantly greater in older participants, being ~ 50 % higher ($p = 0.0006$) in the old compared to the young (Fig. 6.4, D). However, we also found that the IL-15 receptor signalling subunit *IL2RB* was decreased by ~ 80 % in old skeletal muscle, compared to young, with no change in the expression of *IL15RA* (Fig, 6.4, B, C).

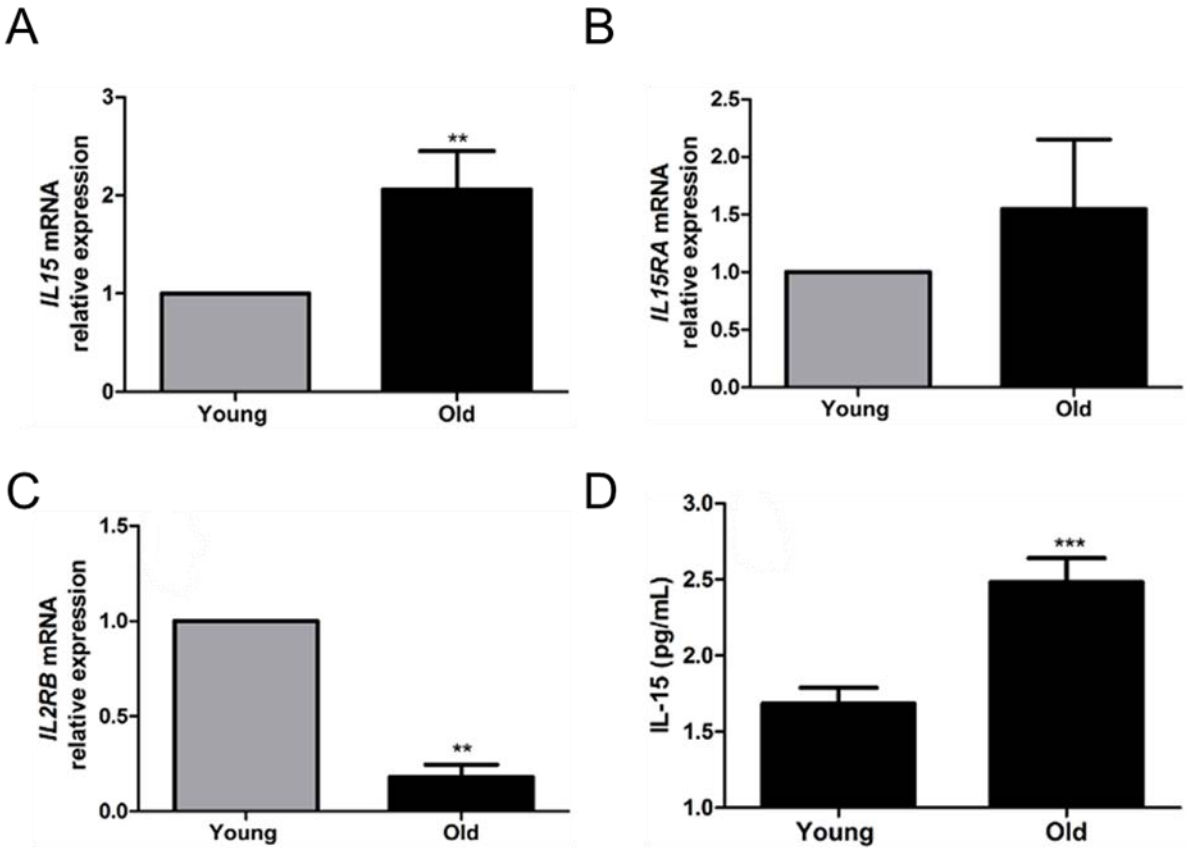

Figure 6.4. Elderly individuals display increased skeletal muscle IL-15 expression and increased plasma IL-15 concentrations. A, B, C) *IL15*, *IL15RA* and *IL2RB* gene expression were quantified in skeletal muscle tissue by RT-PCR. **p < 0.01, vs. unstimulated condition by Mann-Whitney U test, N = 5. **D)** Plasma IL-15 concentrations were assayed by ELISA. ***p < 0.001 vs. the unstimulated condition by t-test. Data expressed as mean ± SEM, N = 10.

6.3.4 TNFα Induces IL-15 Expression in and its Secretion from Primary Human Myotubes

Stimulation of primary human myotubes from young participants with 20 ng/mL rTNFα for 24 h induced a 19-fold increase in myotube *IL15* expression (p= 0.0004) (Fig. 6.5, A). Myotubes were found to express both *IL15RA* and *IL2RB*, and 24 h rTNFα stimulation significantly induced the expression of both *IL15RA* (10-fold; p = 0.0014), and *IL2RB* (126-

fold; p = 0.01), relative to non-stimulated control myotubes (Fig. 6.5, B, C). We also observed a corresponding 4-fold (p = 0.0006) increase in culture supernatant IL-15 concentration (Fig. 6.5, D), indicating a significant increase in IL-15 secretion from rTNFα stimulated human myotubes.

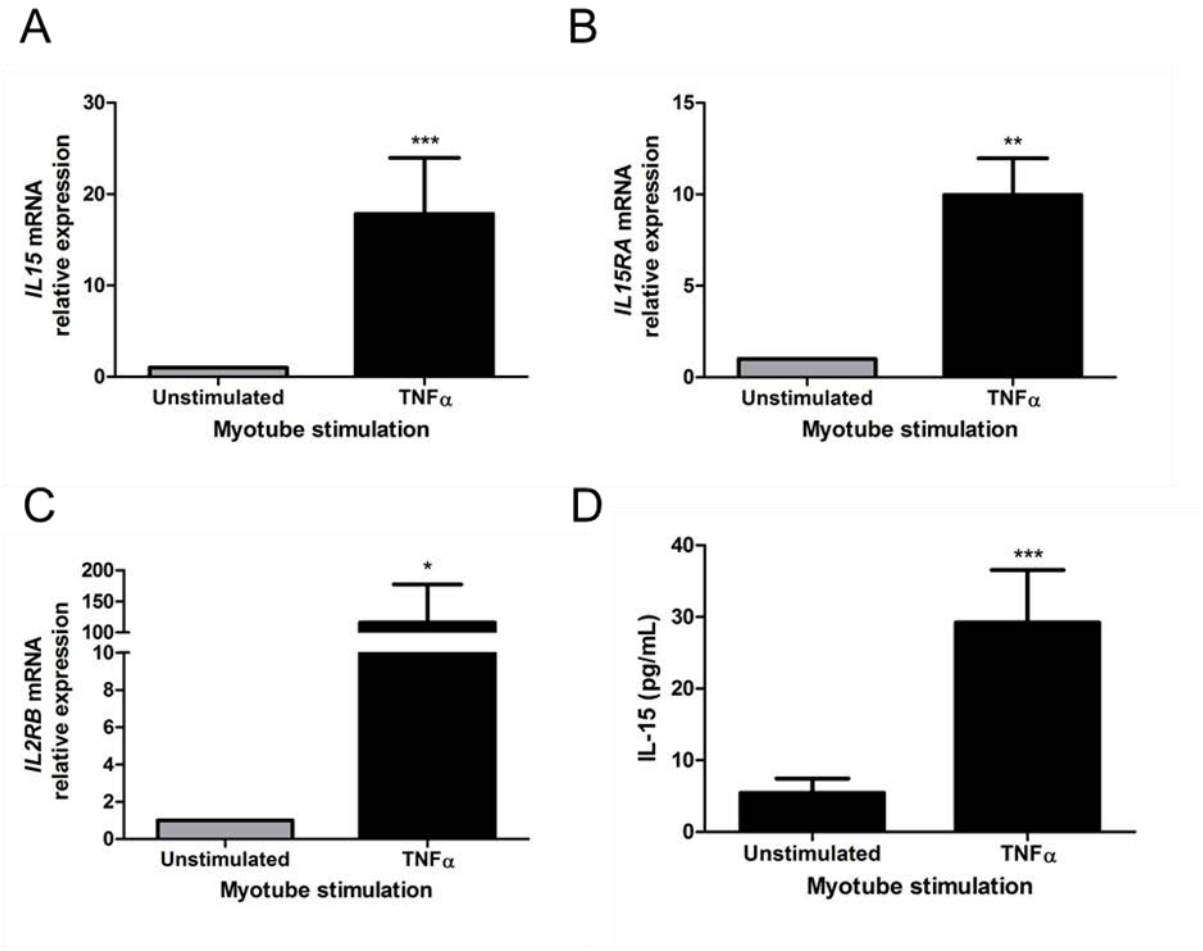

Figure 6.5. Stimulation of primary human myotubes with recombinant TNFα induces IL-15/L-15 receptor gene expression and the secretion of IL-15. Primary human myotubes were stimulated with recombinant TNFα (20 ng/mL) for 24 hours. **A, B, C)** *IL15*, *IL15RA* and *IL2RB* gene expression were quantified by RT-PCR. $^{**}p < 0.01$, $^{***}p < 0.001$ vs. unstimulated condition by Mann-Whitney U test. 3 technical replicates (each assayed in triplicate) per biological replicate, 3 biological replicates. **D)** Supernatant IL-15 concentrations were assayed by ELISA. $^{***}p < 0.001$ vs. unstimulated condition by one-way ANOVA with post-hoc Bonferroni correction. Data expressed as mean ± SEM; 5 technical replications per biological replicate, 3 biological replicates.

6.3.5 Antibody Neutralisation of IL-15rα Eliminates the Myogenic Effects of IL-15 and Enhances the Detrimental Effects of TNFα on Myotube Development

The presence of the IL-15rα neutralising antibody significantly reduced the MTT of

otherwise untreated (24 ± 4%, p = 0.003), IL-15 stimulated (35 ± 3%, p = < 0.0001) and TNFα stimulated (17 ± 2%, p = 0.014) myotubes compared to their IgG isotype treated controls (Fig. 6.6, A). The IL-15rα neutralising antibody had a similar effect on NFI, although the numerically considerable reduction in NFI observed in TNFα stimulated myotubes (56 ± 21 %, p = 0.16) treated with a IL-15rα neutralising antibody did not reach significance (Fig. 6.6, B). The average number of myonuclei in each myotube was not significantly altered by any treatment condition (Appendix Fig 6.4).

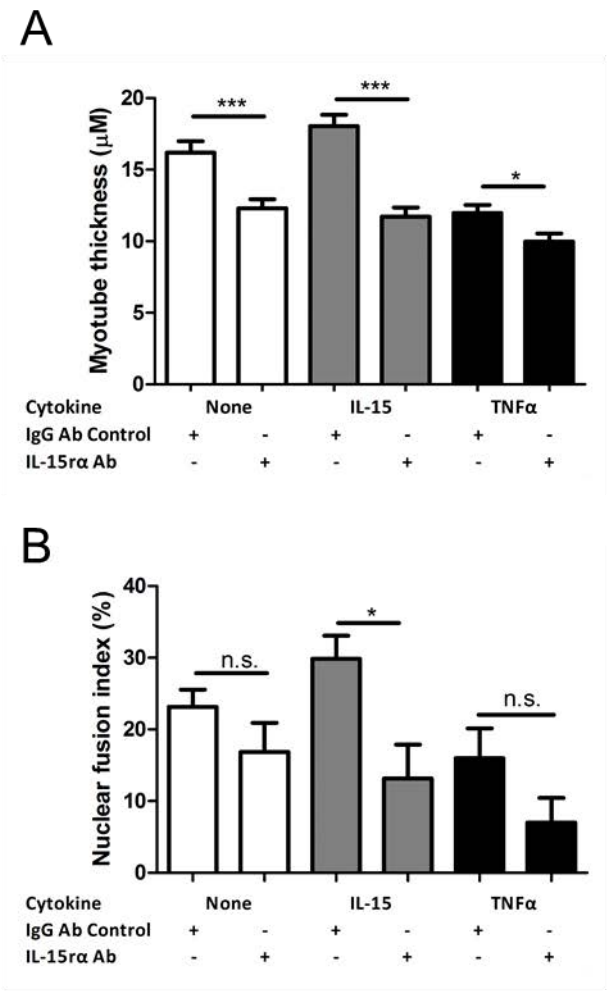

Figure 6.6. Antibody neutralisation of IL-15rα eliminates the myogenic effects of IL-15 and enhances the detrimental effects of TNFα on myotube development. Subconfluent myoblasts were switched to differentiation medium containing the indicated recombinant cytokines and antibodies (IL-15, 25 ng/mL; TNFα, 1 ng/mL; IL-15rα antibody, 1μg/mL; IgG1 antibody, 1μg/mL) for 8 d. **A)** Myotube thickness data represents the mean ± SEM of n = 3 biological replicates. Each biological replicate comprises 150 total measurements taken at 63x magnification from 30 myotubes per treatment condition. **B)** Nuclear fusion index data are expressed as mean ± SEM values of n = 3 biological replicates. Each biological replicate comprises 15 images taken at 20x magnification. * $p < 0.05$, ***$p < 0.001$ vs. IgG control condition by t-test.

6.4 Discussion

We have demonstrated for the first time that IL-15 enhances human myotube development and can partially protect against the deleterious effects of TNFα on myogenesis. Secretion of IL-15 by primary human myotubes derived from patients with polymyositis and dermatomyositis has been previously described (489). Here we show for the first time that myotubes derived from the skeletal muscle of healthy participants also secrete IL-15. These data are a significant advancement on previous *in vitro* work describing the effects of IL-15 on skeletal muscle growth and maintenance as we have used primary human myogenic cultures, rather than an immortalised murine cell line.

6.4.1 IL-15 Promotes *in vitro* Human Myotube Development, Likely by Enhancing the Myogenic Programme

Our studies were primarily designed to firstly examine the functional effect of IL-15 in human myogenesis and revealed that IL15 had a marked effect on increasing MTT, NFI and the number of myonuclei per myotube (Fig. 6.1). The magnitude of these changes (a 22 % increase in MTT at the 100 ng/mL dose of rIL-15) is considerable when placed in an integrated physiological context. A previous study in humans has shown that even a small reduction in quadriceps lean mass following limb immobilisation (5 % over two weeks) is accompanied by a significant decline in isometric strength (490). Furthermore, 12 weeks of progressive resistance training in older men increases MHC I fibre diameter by 20 %, a change which is associated with a 55 % increase in maximal isometric force production by individual muscle fibres (491). We also observed that myotubes were 19 % thinner in

our cultures derived from elderly participants compared to young participants.

The IL-15-mediated increase in MTT is unlikely to be due to induction of myoblast hyperplasia as IL-15 did not affect either the proliferation or viability of subconfluent myoblasts. This is in agreement with previous observations that IL-15 has no effect on proliferation, as measured by [^{3}H]thymidine incorporation in C2C12 myoblasts (49). Instead, the hypertrophic mechanism appears to be via an enhancement of myoblast fusion and the myogenic program, since we observed a > 30 % increase in the NFI and a greater than 2-fold increase in the number of myonuclei in myotubes formed under IL-15 stimulation conditions. This notion of enhanced myogenesis is supported by our finding of total Akt induction in IL-15-stimulated confluent myoblasts that were ready for myogenic differentiation. Induction of total Akt expression (in particular that of Akt2) is essential for full myotube development (492). Furthermore, we observed an increase in total ERK 1/2 and phosphorylated-ERK expression in IL-15 stimulated myoblasts. Myogenic cultures appear to have a biphasic requirement for ERK1/2 activity; ERK 1/2 appears to inhibit differentiation in proliferating cultures, but its activity facilitates myotube formation in differentiating cultures (493). Given that these myoblasts had reached a confluence sufficient for myogenic differentiation, it is reasonable to hypothesise that ERK 1/2 activation by IL-15 might promote their fusion. This requires further study.

Furthermore, IL-15 induced an increase in myomaker gene expression in confluent myoblasts that were ready for myogenic differentiation. Myomaker is a recently discovered muscle-specific cell membrane protein that has been reported to be essential for myoblast fusion and muscle regeneration (494,495). Most recently, myomaker dependent myoblast

fusion to existing myofibres has been shown to be essential for overload-induced hypertrophy to occur in mice (496). The finding that myomaker expression increased in IL-15 stimulated myoblasts represents a potentially novel mechanism of myogenic action for IL-15 in human skeletal muscle. In chicken myoblasts, the MRFs MYOG and MYOD have been shown to bind to an E-box near the myomaker transcription start site, thus inducing myomaker expression (497). Indeed, IL-15 induced an increase in the expression of both *MYOD* and *MYOG* in differentiated myotubes.

6.4.2 The Myogenic Actions of IL-15 are Retained in Myogenic Cultures Derived from Elderly Participants and Autocrine IL-15 Secretion from Such Myotubes Supports Myogenesis

Ageing is associated with a loss of skeletal muscle mass and quality – sarcopenia (6). Given that our myotubes from elderly participants formed less well than those from their young counterparts, we hypothesised that elderly cultures might be less sensitive to the myogenic actions of IL-15. However, we observed no difference in the functional responsiveness to exogenous rIL-15 stimulation between old and young myotubes, demonstrating that IL-15 signalling is not functionally impaired in the elderly. Despite observing no difference in the functional responsiveness to exogenous rIL-15 stimulation between young and old myotubes we did observe that the skeletal muscle tissue expression of the signalling subunit of the IL-15 receptor (*IL2RB)* was found to be significantly reduced in the elderly, compared to the young participants. However, the expression of the IL-15 receptor binding subunit, *IL15RA* was maintained. Given its high affinity for IL-15 compared to the signalling subunit, maintenance of *IL15RA* expression

may compensate for the reduction in *IL2RB* expression.

Importantly, evidence from *in vivo* human studies suggest an important role for myogenesis in adult skeletal muscle (482); this is discussed as part of a wider evaluation of the physiological relevance of our *in vitro* myogenic model in Chapter 7. Therefore, our finding that IL-15 also promotes myoblast fusion in developing primary human myofibres derived from old participants is particularly pertinent. Furthermore, one purpose of neutralising the high-affinity IL-15 receptor binding subunit, IL-15rα in elderly myotubes was to confirm that the pro-myogenic activity of IL-15 in elderly individuals is not limited to the pharmacological doses of the recombinant cytokine used by ourselves and others (50,52). Our neutralisation of the receptor was successful; it completely inhibited the myogenic actions of exogenous rIL-15 (Fig. 6.6). In otherwise unstimulated elderly myotube cultures, IL-15rα neutralisation had a deleterious effect on myogenesis, suggesting that autocrine IL-15 secretion in elderly myotubes is crucial to support optimal myotube formation.

6.4.3 IL-15 can Ameliorate the Detrimental Effects of TNFα on Myogenic Differentiation

Given the emerging importance of inflammation in the aetiology of sarcopenia (483), and its pathological role in cachexia (22) it is remarkable and of potential clinical importance that IL-15 proved capable of ameliorating the deleterious effects of TNFα on primary human myotube development, regardless of culture donor age (Fig. 6.2). TNFα is known to induce myotube atrophy (65) and its plasma levels increase with age, independent of

BMI (264). Given the positive myogenic actions of IL-15, we hypothesise that the observed TNFα-mediated induction of IL-15 secretion and expression of *IL15* and its receptor subunits *IL15RA* and *IL2RB* (Fig. 6.4), may represent a homeostatic mechanism, whereby IL-15 is induced to facilitate skeletal muscle maintenance in the presence of inflammation e.g. in inflammaging or in chronic clinical conditions that are associated with cachexia (498). Indeed, we observed that MTT was decreased to an even greater degree by TNFα in the presence of a neutralising IL-15rα antibody (Fig. 6.6, A), supporting the notion that IL-15 serves as compensatory factor in highly inflammatory environments. The induction of IL-15 secretion from TNFα stimulated myofibres might also, in part, explain why concentrations of TNFα in the normal physiological range have been reported to support myotube formation (282). It is important to note that despite clearly demonstrating the pro-myogenic actions of IL-15, we have not fully established the molecular mechanism by which IL-15 ameliorates the detrimental effects of TNFα on myogenesis. It may be that that IL-15 remains pro-myogenic in the presence of TNFα. However, our molecular signalling analysis showed that the IL-15-mediated induction of pERK and of total AKT was markedly diminished by the presence of TNFα (Fig. 6.3, A, B). Furthermore, unlike with IL-15 stimulation alone, co-stimulation of differentiating myotubes with IL-15 and TNFα did not increase the number of myonuclei per myotube (Appendix Fig. 6.2, D, E).

6.5 Conclusion

We have provided evidence for the first time that IL-15 enhances *in vitro* human myogenesis and can partially protect against the deleterious effects of TNFα on human myotube development. Targeting the IL-15 signalling pathway may therefore be an

effective therapeutic approach in combating skeletal muscle atrophy, particularly in inflammatory disease states.

Chapter 7

General Discussion

7.1 Background

Extensive restatement of background information that is presented in previous chapters is not intended here. However, a summary of the broader scientific landscape that lead to the conception of this work is necessary.

Sarcopenia is a complex, multifactorial phenomenon which dietary interventions and physical activity do not completely reverse. Globally, 5-10 % of those over 65 yr meet the criteria for its formal diagnosis and its prevalence increases with advancing age (7). Sarcopenia and sarcopenic obesity are associated with an increased risk of cardiovascular, metabolic and musculoskeletal diseases as well as poorer quality of life scores and an increased incidence of falls and fractures (9–12). Sarcopenia increases all-cause mortality 4-fold (13). The ageing global population will require considerable increases in age-associated healthcare spending (14) and sarcopenic individuals are considerably more expensive in this regard than their non-sarcopenic counterparts (15). There are few effective therapeutic interventions available for the prevention or reversal of sarcopenia.

In sarcopenia, the nature of the decline in physical performance, as well as the changes in myofibre size, type and number are well understood. It is the nature of the age-associated cellular and molecular changes that underpin such aspects of sarcopenia pathophysiology that are incompletely understood (e.g. changes in the function of SC, mitochondria and the neuromuscular junction). Indeed, the interdependence of these cellular or molecular changes has not been fully elicited, and it is still unclear which of these events might be primary in the development of sarcopenia. We therefore consider it important that each of these aetiological factors continues to be characterised.

Inflammation is well established as a major contributor the cachexia of chronic illness (22). Ageing is often accompanied by chronic low-grade inflammation, referred to as 'inflammaging' (23). Adipose tissue – which accumulates with age – is a prolific secretor of pro-inflammatory adipokines (34). Total adipose tissue mass and VAT mass are negatively correlated with skeletal muscle mass (28,29). High systemic levels of pro-inflammatory cytokines (e.g. TNFα and IL-6) are positively associated with reduced skeletal muscle mass in elderly populations (25–27). The effects of many adipokines on skeletal muscle mass maintenance are unknown and indeed, at the outset of this work there existed a need to identify which adipokines are most important in this respect, particularly in non-lean elderly who are subject to the greatest systemic adipokine burden. The need to identify more avenues for the development of pharmacological therapeutic interventions in sarcopenia was foremost in our minds at the conception of this project. To that end, our attention was drawn to IL-15 as a myokine that is known to promote the formation of C2C12 myotubes in culture and can prevent the loss of skeletal muscle mass in mice exposed to systemic inflammation (49,50). We noted with interest – given the inflammatory burden of ageing and its proposed contribution to sarcopenia – that it has been asserted that IL-15 is expressed in skeletal muscle in response to stimuli that promote loss of muscle mass, to mitigate such loss (51). However, the effect of IL-15 on human myogenesis, as well as myofibre size maintenance and hypertrophy was unexplored. Furthermore, given the high concentrations of recombinant IL-15 used in murine studies, it was unclear to us whether autocrine skeletal muscle IL-15 secretion is necessary for effective myogenesis.

7.2 Summary of the Hypotheses and Aims of this Thesis

The two core hypotheses that were generated at the conception of this thesis were:

1. That there exist understudied adipokine constituents of the adipose inflammatory milieu that are differentially secreted by lean and non-lean adipose tissue and that the enhanced secretion of these adipokines is detrimental to myogenesis and in part responsible for sarcopenia.
2. That the pro-myogenic and hypertrophic actions of IL-15 would be retained in human myogenic cultures, but that cultures derived from the skeletal muscle of elderly individuals would be more resistant to these effects than those of young individuals.

The following aims were therefore identified:

1. To determine the effect of the adipose inflammatory milieux secreted by lean and non-lean adipose tissue on primary human myogenesis and to identify understudied adipokine constituents that may be consequential in mediating such effects (Chapter 3).
2. To identify in these adipose inflammatory milieux a candidate adipokine of apparent myogenic consequence and detail its myogenic actions and the mechanism of such action (Chapter 4).
3. To consider whether the candidate adipokine has metabolic effects on primary human myocytes (Chapter 5).
4. To detail the myogenic actions of IL-15 in primary human myogenic cultures derived from young and old participants and to established whether its purported pro-

myogenic and hypertrophic actions can be sustained in a pro-inflammatory environment (Chapter 6).

7.3 Summary of Results

In Chapter 3, we demonstrate that OB ACM, but not NW ACM has a detrimental effect on *in vitro* myotube formation and that myogenic cultures from elderly individuals are more substantially affected by the anti-myogenic effects of OB ACM. This chapter also details our quantification of ACM constituent adipokines. This work allowed us to identify resistin as an adipokine that is significantly enriched within non-lean ACM. We hypothesised that resistin might be in part responsible for the anti-myogenic actions of OB ACM, and mindful of the fact that resistin is understudied in this regard, we identified it as an adipokine which warranted further study.

In Chapter 4, we demonstrate that recombinant resistin – at concentrations that are found in OB ACM – has a detrimental effect on *in vitro* human myogenesis. Furthermore, immunoprecipitation of resistin from OB ACM, such that its concentrations were more reflective of those found in NW ACM, restored myogenesis. We hypothesised, based on findings by others in HepG2 (404), human coronary artery endothelial cells (405) and human macrophages (406) that resistin might be activating the classical NFκB pathway in our myogenic cultures. Mindful that such NFκB activity is now well established as an inhibitor of myogenesis (402,499), we further hypothesised that this may – at least in some measure – be the mechanism by which resistin exerts its anti-myogenic actions. Indeed, we demonstrated that this is the case and inhibition of classical NFκB pathway signalling with the IKKβ inhibitor TPCA-1 restored myogenesis in the presence of resistin.

We noted a dearth of studies on the metabolic effects of resistin in human skeletal muscle and questioned whether our myotubes, when formed in the presence of resistin would display any alterations in their oxidative metabolism. Using principally the Seahorse® XFe96 analyser, coupled with Oil Red O staining of myotubes, we demonstrate in Chapter 5 that resistin is a metabolic stressor of myotubes, promoting increased ATP production, basal OCR, fatty acid oxidation, proton leak and lipid accumulation. This chapter, in particular, identifies considerable knowledge gaps and areas for future investigation which are discussed below.

Chapter 6 is somewhat distinct from the other data chapters in this thesis, in that we detail the functional effects of a pro-myogenic myokine. However, like the other work described here its functional effects are considered with respect to their potential implications for ageing skeletal muscle. We show that IL-15 is pro-myogenic in primary human cultures, including those derived from older individuals, that it can mitigate the detrimental effects of an inflammatory environment on myogenesis and that autocrine IL-15 secretion supports myogenesis.

7.4 Limitations

The experimental design employed here – one in which primary human skeletal muscle myoblasts are fully differentiated *in vitro* to myotubes in the presence of a stimulus (ACM or recombinant cytokines) – most closely resembles physiological scenarios in which SC are activated to contribute to regeneration, maintenance and hypertrophy of skeletal muscle fibres. Skeletal muscle regeneration in response to injury is perhaps the most often cited example of SC proliferation and commitment to a myogenic fate (500–502).

However, the situations in which SC are activated are more varied and commonplace than simply that of serious skeletal muscle injury.

The observation that conditional depletion of SC by > 90 % in tamoxifen-treated Pax7-DTA mice (133) results in no difference in the degree of plantaris hypertrophy at 2 wk in response to synergist muscle removal has in the past prompted the conclusion the SC are unimportant for such hypertrophy (134). However, this hypertrophic response plateaus in Pax7-DTA mice beyond two weeks, with vehicle-treated mice displaying a 1.5 times greater increase in muscle size than the SC-depleted counterparts after 8 wk (133). Recent murine evidence challenges the paradigm that SC remain quiescent in their niche until skeletal muscle is exposed to a significant challenge (e.g. eccentric exercise or trauma); 20 % of hindlimb myofibres contained new tdTomato-labelled SC after a 2 wk lineage labelling period (135). In humans, both repetitive eccentric exercise (which may reasonably be considered an extraordinary activity) and typical resistance training (4 sets of 10 repetitions of unilateral leg press and unilateral knee extension at 75 % of one repetition maximum) substantially and significantly increase the number of SC in the G0/G1 phase of the cell cycle after 24 h, suggesting that sizeable cell division had occurred (503,504). It is now becoming evident that SC activation and myoblast fusion play an important role in skeletal muscle hypertrophy, maintenance and regeneration in response to a variety of stimuli over the course of an adult lifespan. Indeed, this notion is further supported by observations that in the elderly, atrophy-prone type IIa fibres display reduced nuclear numbers and the MND are significantly decreased (136). Nuclear number is also significantly diminished in type I fibres (136). Moreover, as with skeletal muscle

performance and size, age-related changes in skeletal muscle SC content are only partially reversible with increased physical activity (155,156,505) (see also Section 1.6.3.1). Primary human myogenic cultures, therefore, offer a useful, *in vitro* model for examining such physiology in the context of adipose-derived inflammation. None the less, this model is imperfect. Myotubes cannot be maintained in culture for prolonged periods of time, and they are immature, displaying distinct differences when compared to skeletal muscle (80,81,83) (see also Section 1.5).

Many of the experiments detailed in this thesis would be enhanced by a parallel examination of the effects of ACM and recombinant cytokines on the atrophy and hypertrophy of fully differentiated myotubes. This would have required access to considerably more skeletal muscle biopsies, and therefore it was not possible to carry out both sets of experiments. We acknowledge that it is not just the effectiveness of the myogenic program that might be altering MTT in our studies. Catabolic or protein synthetic stimuli may have altered MTT, particularly in the latter stages of myotube formation. Secondly, skeletal muscle mass maintenance, hypertrophy and atrophy *in vivo* must be viewed as the product of a complex interplay between factors such as muscle protein synthesis, muscle protein breakdown and myogenesis. Thus, the question as to what effects the adipose inflammatory milieu, resistin and IL-15 have on mature human myofibres remains to be answered and should be considered in parallel with our results to generate a complete picture of their effects in aged skeletal muscle.

None the less, we feel justified in our prioritisation of the *in vitro* myogenic experimental model employed here. The immaturity of myogenic cultures and their resulting myotubes

(80,81,83) (see also Section 1.5) means that in our opinion, they may be more appropriate for the examination of myogenesis, although they undoubtedly retain usefulness as surrogate *in vitro* muscle fibres. Tissue engineering approaches to the problem of developing physiologically relevant *ex-vivo* skeletal muscle surrogates that can be maintained in culture continue to advance (506–509), and such surrogates are likely to be of both experimental and ultimately clinical utility. Had we taken the approach of examining the effects of ACM and recombinant cytokines on the atrophy and hypertrophy of fully differentiated myotubes, we would have been considerably limited by the short time-frame during which the myotubes are fully differentiated but not demonstrating any signs of cell death or atrophy – in our experience a period of about 72 h. While we still consider this work important to carry out, we highlight that the degree of atrophy that might be expected with, for example, physiological concentrations of resistin in such a time-frame might be difficult to accurately and precisely quantify given the limitations of the assay for quantifying MTT and NFI.

For each biological replicate, MTT and NFI measurements made in this thesis comprise a large number of technical replications. Despite this, the 95 % confidence intervals for each subject's MTT measurements represent approximately 10 % of the mean MTT. Thus, the labour-intensive technical replications performed by ourselves and others (47) are necessary to obtain accurate MTT and NFI data for individuals, prior to examining the variability of the response between biological replicates. Ideally, the quantification of MTT and NFI would be carried out by two independent investigators, but the time-consuming nature of the assay in its current form represents a substantial barrier to this. Adobe

Photoshop has been used to quantify myotube surface area; this approach is of considerable advantage in quantifying the area of irregularly shaped myotubes and may increase assay throughput (510). The creation of an automated computer program that is capable of quantifying MTT and NFI in images of desmin (or MHC) and DAPI stained cultures would increase throughput further. Such a program would need a reliable strategy to deal with overlapping myotubes and myonuclei and would need to be validated using observations of the same images by multiple experienced investigators. The author is currently in the early stages of leading a collaborative project which aims to produce such a program to be made available to the research community. If successful, this will be of considerable value to researchers interested in myotube morphology, allowing a greater consistency of results between images and a higher throughput, thus facilitating the recruitment of a greater number of participants for a given experiment.

More specific limitations of the data presented have been considered in the relevant individual data chapters. However, the data in Chapter 5 warrant brief mention again here. These data are novel and represent a considerable advancement of our knowledge of the metabolic effects of resistin on skeletal myofibres. However, it is acknowledged that the interpretation of these data is somewhat speculative and the additional work that has been suggested must be carried out in the future to fully understand the metabolic adaptations/maladaptations that we describe.

Finally, it should be remembered sarcopenia is a multifactorial phenomenon, whose aetiology is incompletely understood. As such, we believe that ours is an important contribution to such understanding, but are mindful that adipose-derived inflammation and the influence of resistin on skeletal myogenesis likely only represents a proportion of its

cause.

7.5 Impact and Future directions

In large part, the impact of the data presented and suggestions for its development has been considered in the relevant data chapters. Here, only those issues of most immediate and broad importance to the musculoskeletal ageing research community are considered together.

Chapter 3 describes subcutaneous adipose inflammatory milieux that are highly variable, irrespective of an individual's BMI. Our characterisation of these milieux used multiplex magnetic bead-based immunoassays of prominent known adipokines. Development of our work should involve comprehensive proteomic and lipidomic profiling of SAT ACM. It may be that such work will identify many more ACM constituents that may be of consequence in the development of sarcopenia and which may represent useful avenues for therapeutic targeting. The inflammatory milieux secreted by VAT and IMAT are also of relevance to our understanding the pathogenesis sarcopenic obesity. Given the prolific inflammatory profile of VAT and the proximity of IMAT to skeletal muscle, these milieux should be comprehensively characterised. As discussed in Section 3.3.1, such characterisations of IMAT are substantially impeded by the difficulties in obtaining IMAT in sufficient quantities for study. Given the practical and ethical difficulties associates with access to viable cadaveric human tissue, recent hypotheses that postulate that FAP cells are a possible source of adipocytes within skeletal muscle (381) may offer the possibility of their *in vitro* culture from skeletal muscle biopsies. Thus, profiling of the IMAT adipocyte secretome might prove possible in the immediate future.

Given the anti-myogenic effects of resistin that we detail in Chapter 4, any future work will need to consider the utility and practicality of inhibiting such actions. For example, the subcutaneous or intravenous administration of monoclonal antibodies or circulating decoy receptors is likely impractical for a population-level programme of sarcopenia prevention. On the other hand, we have shown that resistin exerts its anti-myogenic actions by activating the classical NFκB pathway. In Section 4.3.2 the role of NSAIDs as NFκB inhibitors was discussed. In the elderly, NSAIDs have been shown to increase muscle volume and strength acquisition in response to resistance training (443). Thus, future work should focus on exploring their effectiveness in facilitating the long-term retention of skeletal muscle mass and function in the elderly, while also establishing whether the risk of upper gastrointestinal mucosal damage can reliably and safely be prevented with existing proton pump inhibitors.

In Chapter 6 we show that IL-15 is a pro-myogenic myokine, which can ameliorate the anti-myogenic effects of a pro-inflammatory environment. Inflammation itself (TNFα stimulation) induced IL-15 secretion from myotubes and indeed, the elderly have increased circulating concentrations of IL-15. The molecular mechanisms underpinning these actions of IL-15 remain to be fully elicited. However, we have identified activation of the ERK signalling pathway and induction of myomaker gene expression as areas for further exploration. The outcome of such investigations will dictate what approach, if any, is taken to exploiting the pro-myogenic actions of IL-15 in therapeutic interventions. IL-15 is an important modulator of innate and adaptive immune responses and this will be an important consideration in its therapeutic use (511). Indeed, the first Phase I clinical trial to examine rIL-15 administration to human participants (with metastatic malignancies)

demonstrated considerable efflux of NK and memory CD8 T cells from the circulation and a 10-fold expansion of the NK cell population (512). However, the serum IL-15 concentrations attained with the maximum tolerated dose (0.3μg/kg per day) were greater than 1000 pg/mL, similar to the pharmacological concentrations used in some of our experiments. Given that we have shown that autocrine IL-15 secretion from myotubes supports myogenesis and that the concentrations of IL-15 in the skeletal muscle interstitium are generally less than 20 pg/mL (513), much smaller manipulations of skeletal muscle IL-15 concentrations might have positive benefits in sarcopenia without substantially altering the immune system.

There is a compelling argument to be made that the answer to preventing the deleterious consequences of adipose-derived inflammation lies in preventing the excessive accumulation of adipose tissue. Further, it could reasonably be argued that the answer to this problem is already known; sustained consumption of an energetically sufficient diet will prevent substantial adipose tissue accumulation. If coupled with sufficient physical activity – which is partially effective in preventing and reversing sarcopenia (Section 1.6.4.1) – the phenomenon of sarcopenic obesity might be substantially mitigated. One argument for continuing to pursue additional understanding of the cellular and molecular factors underpinning sarcopenia has already been touched upon in this thesis, namely that physical activity interventions when adhered to diligently are only partially effective in reversing sarcopenia (Section 1.6.3). Furthermore, even if a population had sufficient knowledge of these issues to take personal responsibility for their own dietary and physical activity choices, there now exist considerable societal and environmental impediments to maintaining a healthy weight throughout one's life (for a recent systematic review see

(514)). Moreover, there also exist substantial health and economic costs to society of having an overweight population (515–517). The author advocates addressing these wider societal issues and is a strong proponent of the tenets of public health promotion set out Ottawa Charter (518). However, the continued rise in obesity rates requires a pragmatic multi-faceted approach and thus the continued pursuit of a molecular understanding of obesity-related conditions and the development of pharmaceutical interventions to compliment public heath approaches.

7.6 Concluding Remarks

This thesis addresses its two core hypotheses and in doing so identifies resistin as an adipokine whose secretion is enhanced in non-lean human subcutaneous adipose tissue. Resistin is further identified as having anti-myogenic actions on primary human myogenic cultures and this phenomenon is shown to be mediated by its activation of the classical NFκB pathway. Resistin is also established as a metabolic stressor of *in vitro* primary human myotubes; it increases oxidative metabolism, fatty acid oxidation and promotes intramyocellular lipid accumulation. The myokine IL-15 is shown to support myogenesis, and it is established that IL-15 can mitigate the detrimental effects of an inflammatory environment on myogenesis. Moreover, we show for the first time that these pro-myogenic actions are not limited to the exogenous provision of rIL-15 - autocrine IL-15 secretion by myotubes supports myogenesis.

Chapter 8

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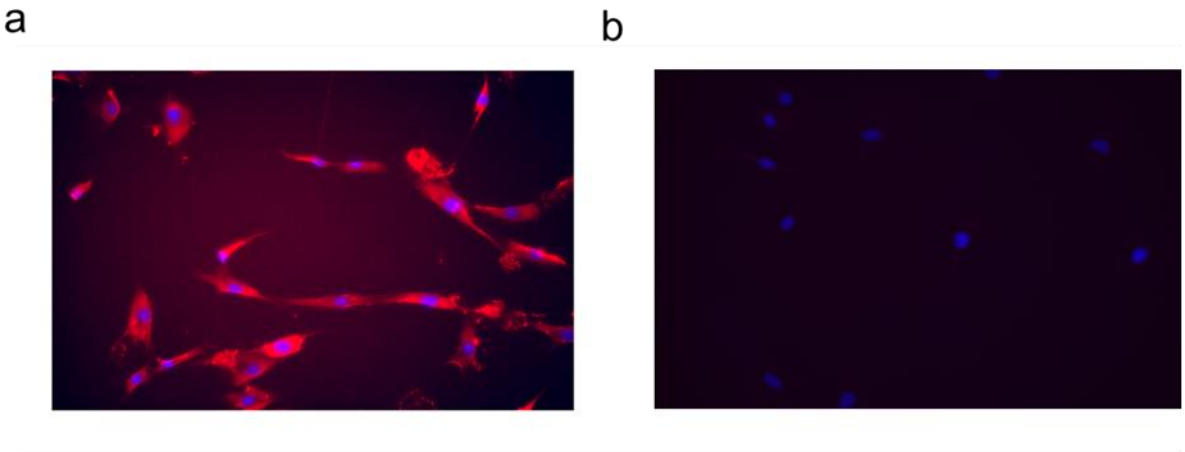
Chapter 9

Appendix

9.1 Appendix to Chapter 2



Appendix Figure. 2.1. Supplementary Figure 7. Primary Human Myoblasts do not express the fibroblast marker TE-7. Myoblasts and synovial fibroblasts were fixed, immunofluorescence stained for TE-7 (red) and with DAPI (blue) and imaged on an epifluorescence microscope. A) Synovial fibroblasts, B) Primary human myoblasts.

Appendix Table 1. Skeletal Muscle Biopsy Participant Characteristics for Each Experiment in this Thesis

Figure	Sex (n, M/F)	Age	BMI	Participants
3.5 Young	3/0	23.8 ± 1.8	24.0 ± 1.0	IS07, IS17, IS18
3.5 Old	2/1	71.3 ± 3.8	22.8 ± 1.2	IS14, IS15, IS16
4.2 Young	2/1	20.8 ± 0.8	23.8 ± 2.5	IS08, IS09, IS10
4.2 Old	2/1	71.3 ± 3.8	22.8 ± 1.2	IS14, IS15, IS16
4.3	2/1	71.3 ± 3.8	22.8 ± 1.2	IS14, IS15, IS16
4.4 Young	2/1	20.8 ± 0.8	23.8 ± 2.5	IS08, IS09, IS10
4.4 Old	2/1	71.3 ± 3.8	22.8 ± 1.2	IS14, IS15, IS16
6.1	1/2	21.7 ± 1.4	20.8 ± 0.9	IS03, IS04, IS05
6.2 Young	1/2	23 ± 0.5	20.4 ± 1.6	IS05, IS06, IS07
6.2 Old	2/1	67.7 ± 0.3	23.7 ± 4.1	IS12, IS13, IS14
6.5	2/1	21.1 ± 1.1	19.5 ± 0.9	IS02, IS03, IS04
6.6	2/1	67.7 ± 0.3	23.7 ± 4.1	IS12, IS13, IS14

Appendix Table 2. Mean Ct Values of Control Conditions for Primers Used in this Thesis.

Gene	Mean Ct Value of Control Conditions
IL15	23.8
IL15RA	16
IL15RB	36.8
MYF5	18.8
MYOD1	23.2
MYOG	25.6
FBX032 (MAFbx)	23.9
TRIM63 (MURF-1)	27.5
PPARG	29.7
PPARD	30.2
ACACB	27.1
PDK1	29.4
UCP2	26.9
UCP3	29.6
SLC2A4 (GLUT4)	31.7
PPARGC1A	29.9
ACTB	15.4
GAPDH	16.5
TMEM8C (Myomaker)	20.7

The efficiency of all primers was established in our lab to be > 95 % under the conditions recommended by Primerdesign (detailed in this thesis). Given this high degree of

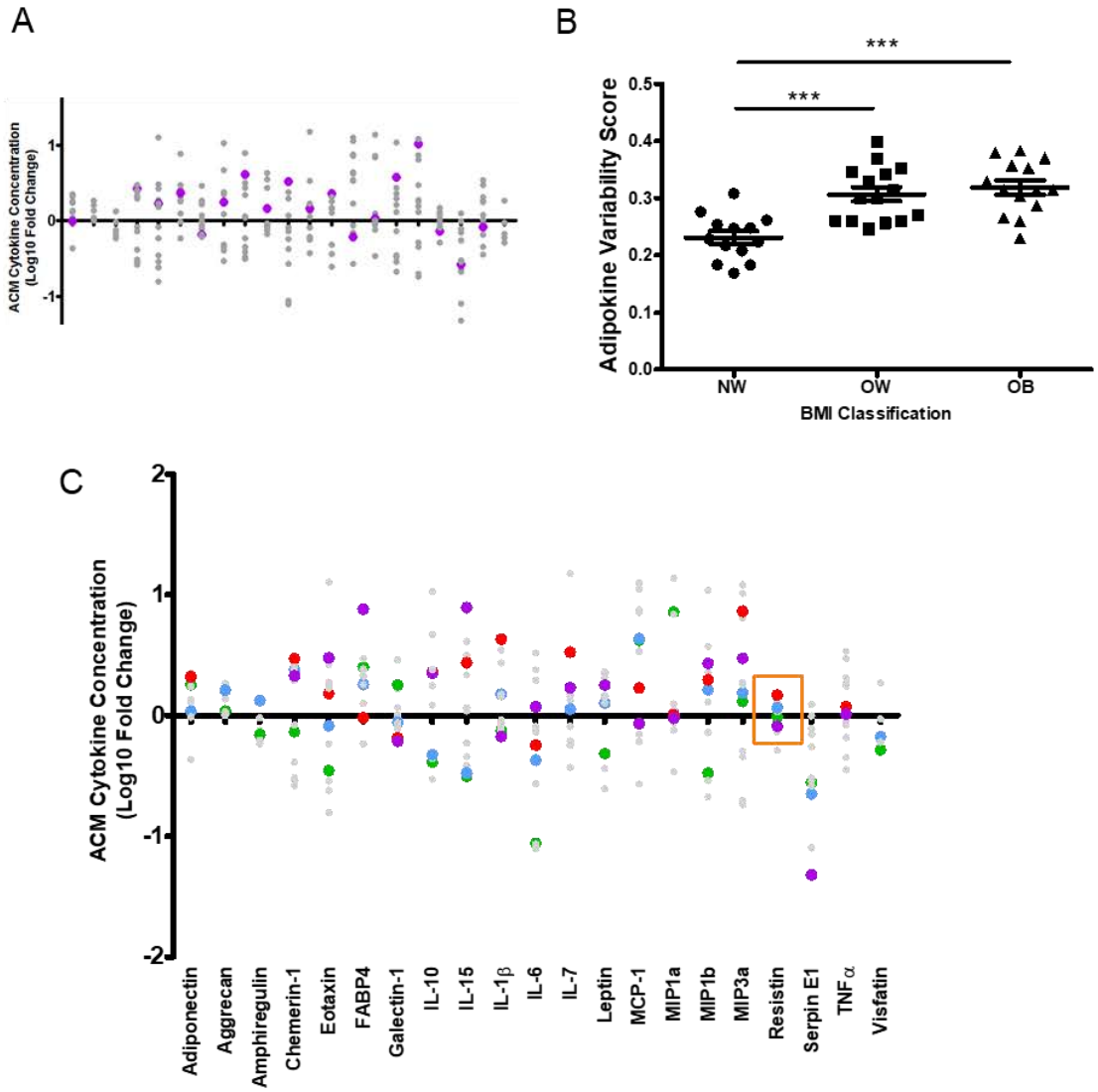
efficiency concordance between primers, efficiency was assumed to be 100% for calculation purposes.

9.2 Appendix to Chapter 3

To calculate the AVS:

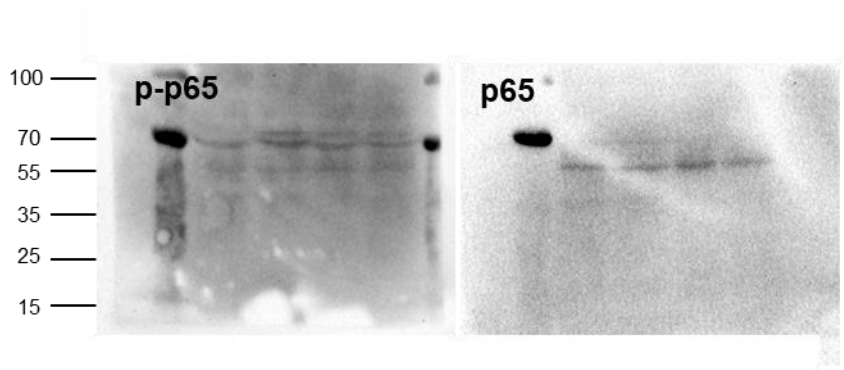
- The mean concentration of each adipokine in NW individuals (n = 13) was calculated.
- For each subject ACM, the concentration of an individual adipokine was expressed as a fold change relative to the NW mean value for that adipokine.
- These fold change values were log10 transformed to achieve a linear scale.
- All negative fold change values were converted to positive numbers.
- For each subject ACM, the average of the values for all adipokines was calculated to attain a subject AVS score.

The AVS score for one OB individual is graphically represented in Appendix Fig. 3.1, A. It may be thought of as the average distance of the log10 fold change adipokine concentrations (purple dots) for that subject from zero. The resulting AVS scores are represented in Appendix Fig. 3.1, B. Both the OW and OB cohorts displayed significantly ($p = 0.0006$, $p = 0.0002$ respectively) more variability in their adipokine secretory milieu compared to the NW cohort, despite the concentrations of few individual adipokines being significantly different from their NW mean values (Chapter 3 Fig. 3.1, Table 3.2). It should not be assumed that the ACM concentration of a given adipokine is predictive of an adipose secretory pattern for all the adipokines that were measured. Four OB subjects with similar resistin ACM concentrations are highlighted (Appendix Fig. 3.1, C). It is evident that similar resistin concentrations in these ACM are not predictive of an adipokine secretion pattern.



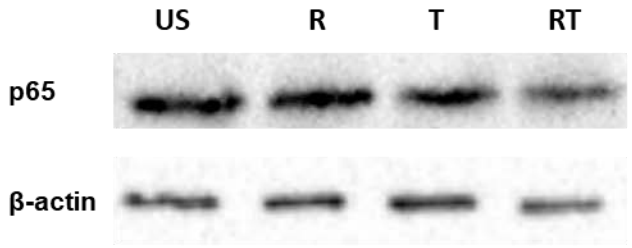
Appendix Figure 3.1. The concentration of selected known adipokines in obese adipose conditioned medium is highly variable within and between individuals. Subcutaneous adipose tissue samples from subjects (aged 45 – 89 yr) of varying BMI were maintained in myoblast differentiation medium for 24 h (1 g adipose tissue per 10 mL medium). The media were aliquoted and stored at -80 °C. The concentrations of known adipokines were determined in normal weight (NW, n = 13), overweight (OW, n = 17) and obese (OB, n = 15) adipose conditioned medium by multiplex magnetic bead-based human cytokine assays. **A)** A graphical representation of the Adipokine Variability Score (AVS). The AVS for each subject (e.g. highlighted in purple) represents the mean distance of the purple dots from zero. **B)** AVS for was calculated for each individual. ***p < 0.001 by Mann Whitney U test with post-hoc Holm's sequential Bonferroni adjustment. **C)** OB ACM data expressed as Log10 of their fold change from the NW mean for each cytokine. The highlighted subjects were chosen as they had similar resistin ACM concentrations. Missing data did not fall within the assay range or were eliminated due to being greater two standard deviations from the mean.

9.3 Appendix to Chapter 4

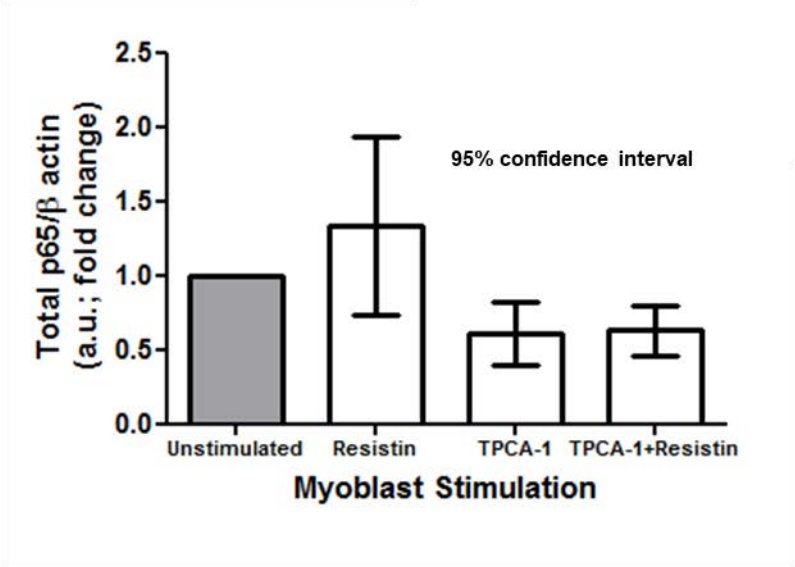


Appendix Figure 4.1. Full p-p65 and p65 immunoblots. Full versions of the cropped immunoblots presented in Fig. 4.7.

A

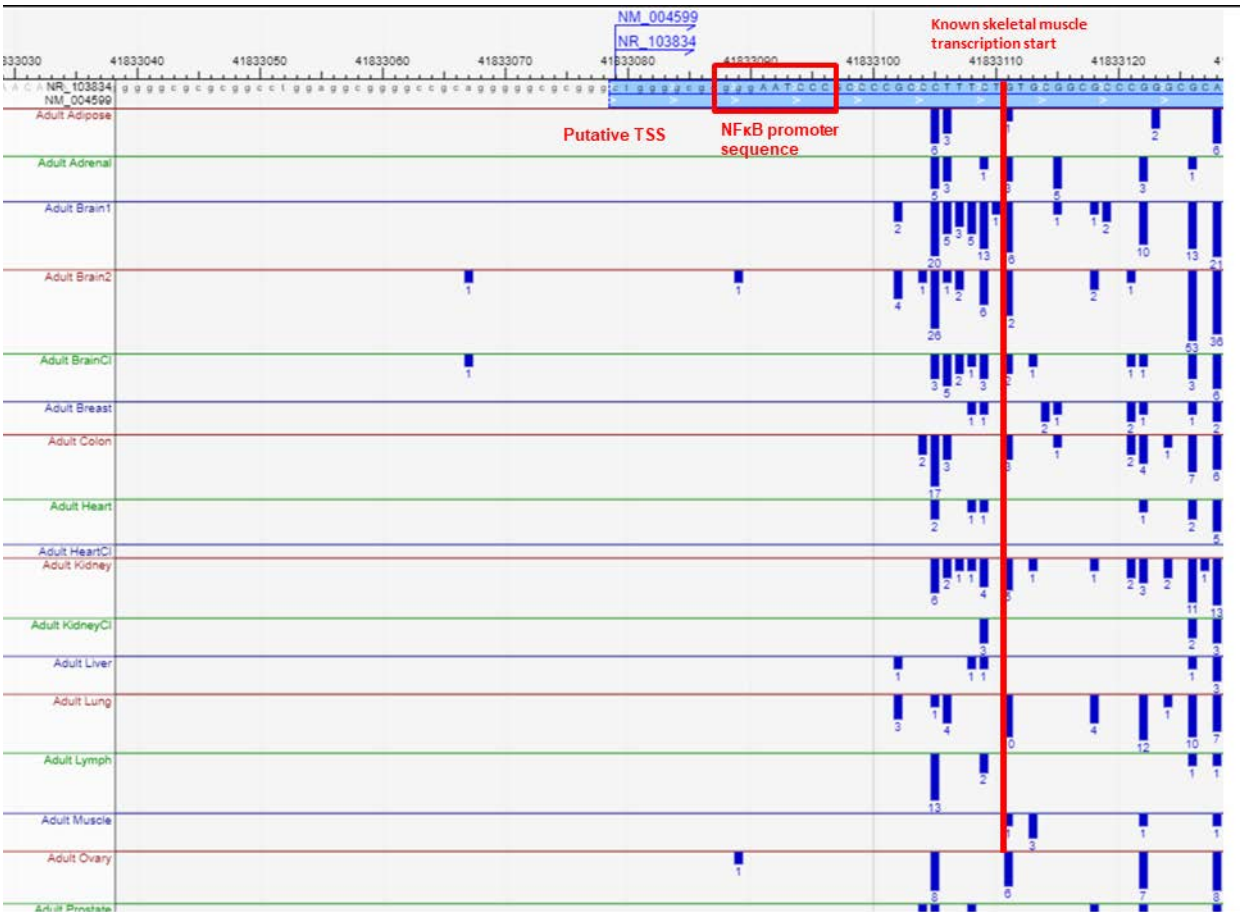


B

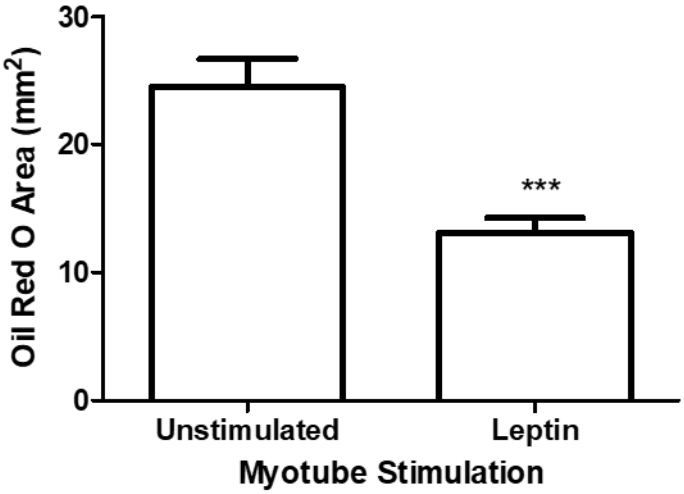


Appendix Figure 4.2. 24 h stimulation with resistin induces a variable increase in total p65 expression by subconfluent myoblasts, which is suppressed by TPCA-1. Subconfluent primary human skeletal myoblasts from a female aged 21 yr were maintained in growth media (containing 0 ng/mL or 5 ng/mL recombinant resistin ± 40 nM TPCA-1) for 24 h. Total p65 and β-actin were detected by immunoblotting. **A)** Representative immunoblots. US = unstimulated, R = resistin, T = TPCA-1, RT = resistin + TPCA-1 **B)** p65 expression, corrected for β-actin expression, expressed as the fold change compared to an unstimulated control. Data are expressed as mean ± 95% confidence interval values of n = 5 independent experiments.

9.4 Appendix to Chapter 5

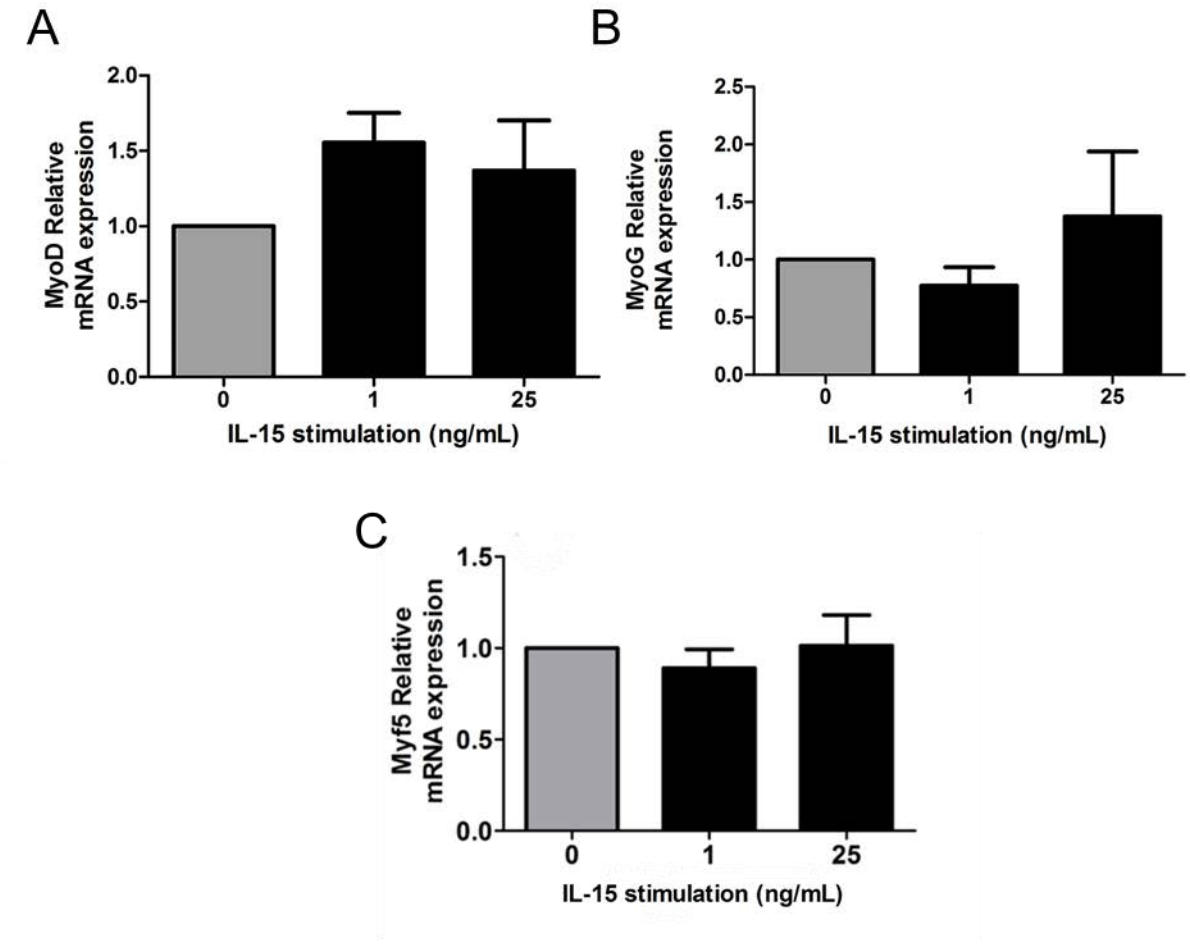


Appendix Figure 5.1. The transcriptional start site for human skeletal muscle SREBP2. Adapted from the database of transcriptional start sites (DBTSS), available from: http://dbtss.hgc.jp/.

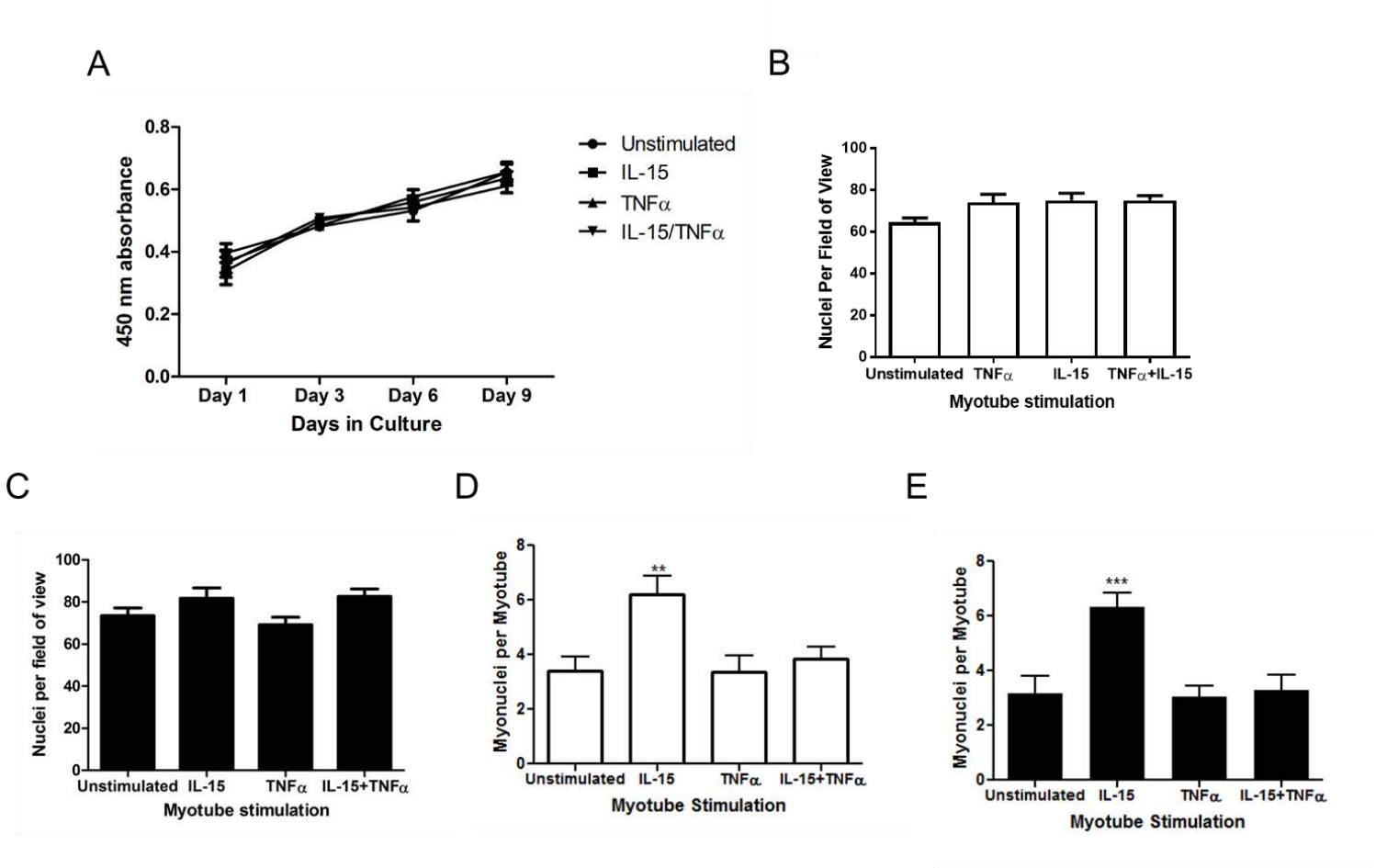


Appendix Figure 5.2. Recombinant leptin stimulation of developing myotubes reduces the accumulation of intracellular lipid. Subconfluent myoblasts from young and elderly subjects were switched differentiation media (containing 0 ng/mL or 5 ng/mL recombinant leptin). Media were renewed every 2 d. At 8 d, myotubes were fixed, permeabilised in 0.2% Triton X and stained with Oil Red O. Myotubes were imaged on a brightfield microscope and the Oil Red O positive area was quantified using Image J software. Data are expressed as mean ± SEM values of n = 3 biological replicates. Each biological replicate comprises data from 15 images taken at 20x magnification.

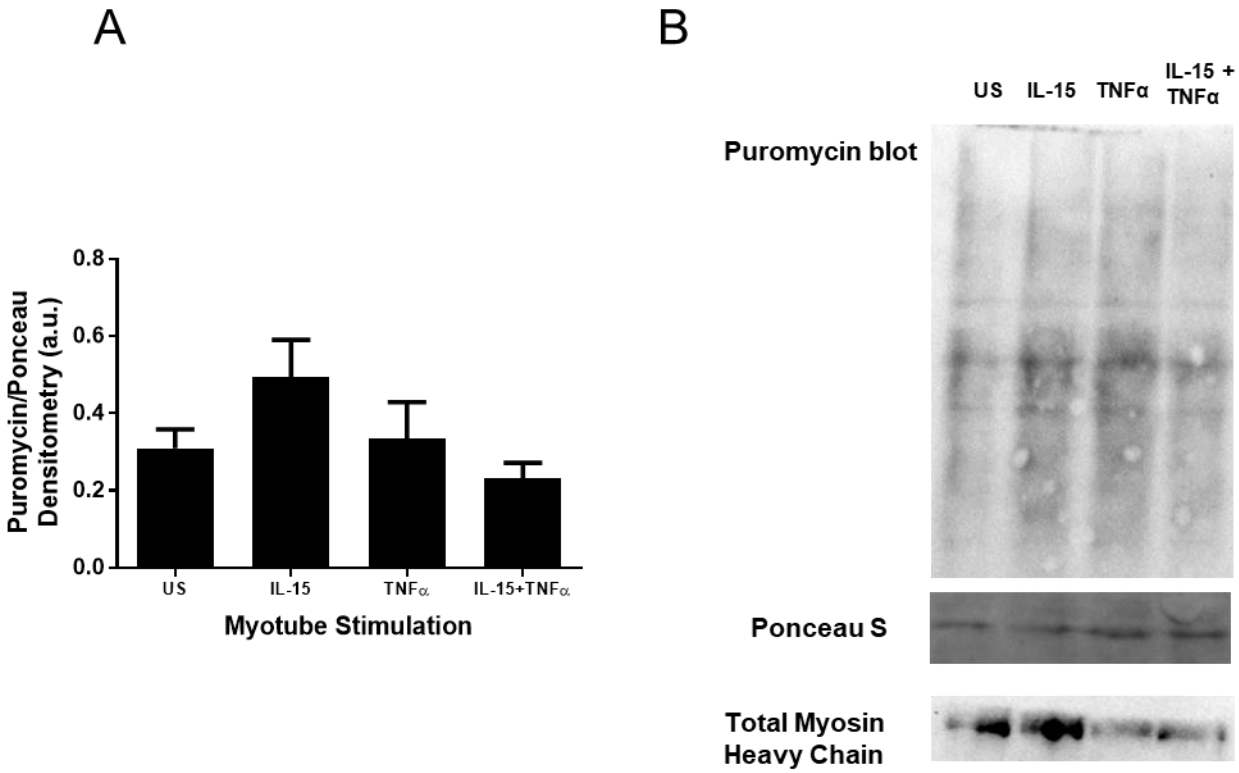
9.5 Appendix to Chapter 6



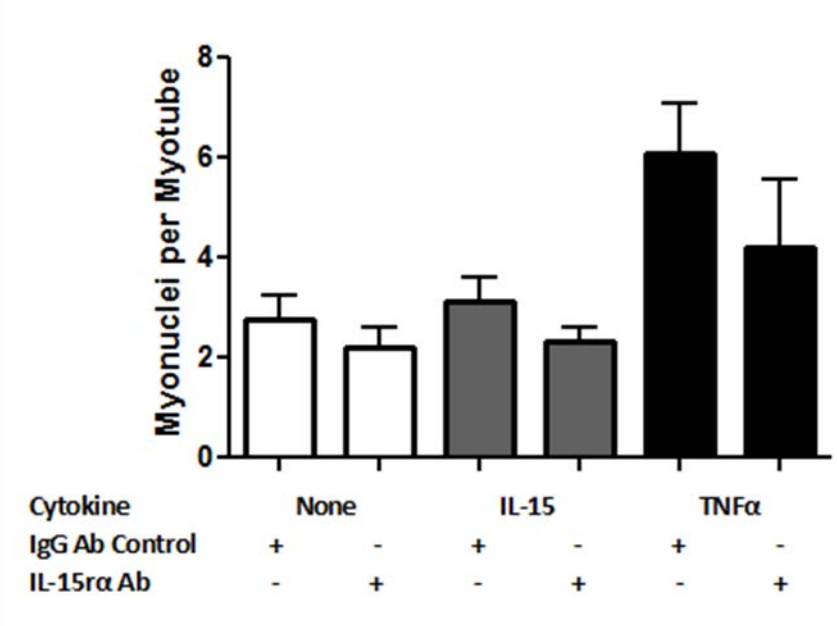
Appendix Figure 6.1. Recombinant IL-15 stimulation of primary human myoblasts does not alter myogenic regulatory factor expression. A, B, C) Primary human myoblasts from young subjects were stimulated with recombinant IL-15 at the indicated concentrations for 8 d. Media were renewed every 2 d. RNA was extracted by the TRIzol® method and *MYOD/MYOG/MYF5* relative gene expression were quantified by SYBR green RT-PCR. Data expressed as mean ± SEM. 3 technical replicates (each assayed in triplicate) per biological replicate, 3 biological replicates.



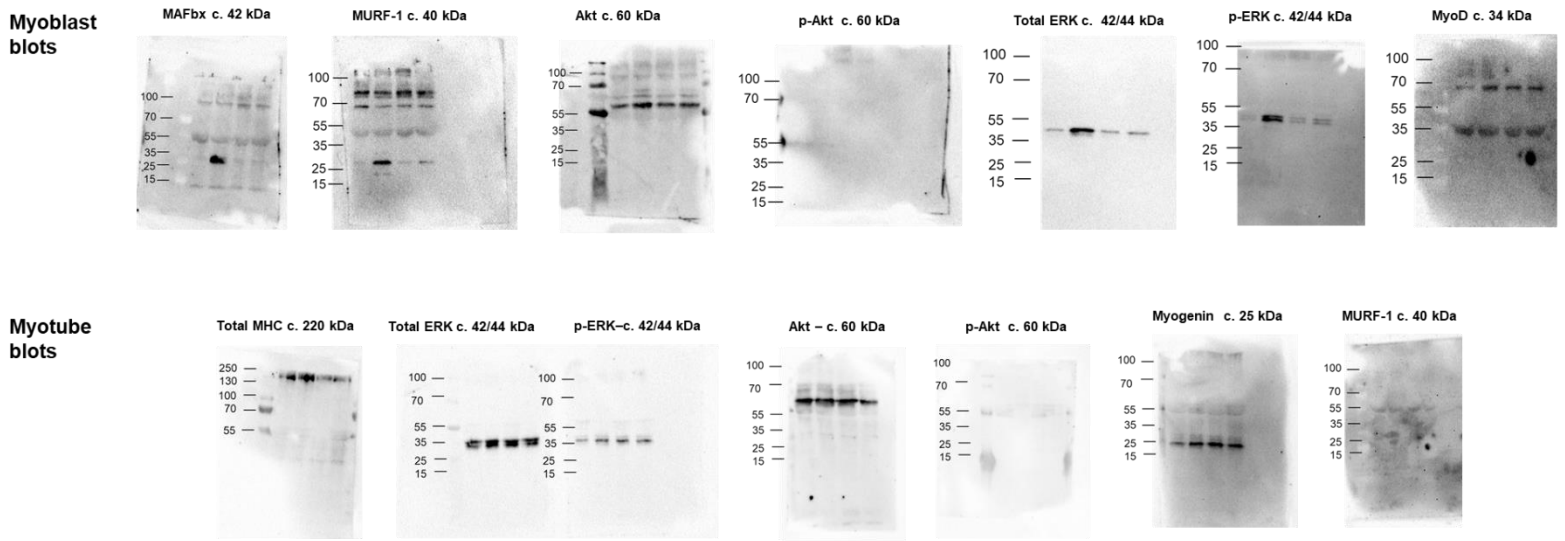
Appendix Figure 6.2. Recombinant TNFα and IL-15 stimulation of primary human myoblasts does not alter their proliferation or survival. A) Primary human myoblasts from young subjects were seeded to 96 well plates and number of viable cells in culture was assayed by colorimetric MTS assay at several time points. Data expressed as mean ± SEM. 4 technical replications per biological replicate, 3 biological replicates. **B-E)** Subconfluent myoblasts from young (white bars) and old (black bars) subjects were switched to differentiation medium containing the indicated recombinant cytokines (IL-15, 25 ng/mL; TNFα, 1 ng/mL) for 8 d. Media were renewed every 2 d. Myotubes were fixed, immunofluorescence stained for desmin and DAPI and imaged on an epifluorescence microscope. Nuclear number data represents the mean ± SEM values from 45 images taken at 20x magnification (from 3 biological replicates). Number of myonuclei per myotube is expressed as mean ± SEM values from 15 images taken at 20x magnification from 3 biological replicates.



Appendix Figure 6.3. Recombinant IL-15 and TNFα stimulation of differentiating primary human myogenic cultures does not substantially alter protein synthesis. Commercially available subconfluent myoblasts from a female aged 21 yr were switched to differentiation medium containing the indicated recombinant cytokines (IL-15, 25 ng/mL; TNFα, 1 ng/mL) for 2 d. **A)** The expression of total myosin heavy chain was determined by immunoblotting. A full-length blot is presented in Appendix Fig. 6.5. **B)** At 2 d, cultures were incubated with 1 uM puromycin for 30 min. The incorporation of puromycin into peptides was quantified by immunoblotting for puromycin. Data are expressed as mean ± SEM, N = 3 independent experiments. A representative immunoblot is also presented.



Appendix Figure. 6.4. Antibody neutralisation of IL-15rα does not alter the number of myonuclei per myotube. Subconfluent myoblasts were switched to differentiation medium containing the indicated recombinant cytokines and antibodies (IL-15, 25 ng/mL; TNFα, 1 ng/mL; IL-15rα antibody, 1µg/mL; IgG1 antibody, 1µg/mL) for 8 d. Media were renewed every 2 d. Myotubes were fixed, immunofluorescence stained for desmin and DAPI and imaged on an epifluorescence microscope. Number of myonuclei per myotube is expressed as mean ± SEM values from 15 images taken at 20x magnification from 3 biological replicates.



Appendix Figure 6.5. Full versions of all cropped immunoblots presented in Chapter 6. Full versions of all myoblast and myotube immunoblots presented in Chapter 6. Stimulation conditions from left to right: unstimulated, IL-15 (25 ng/mL), TNFα (1 ng/mL), IL-15 (25 ng/mL) + TNFα (1 ng/mL).