ACUTE SKELETAL MUSCLE WASTING IN THE CRITICALLY ILL

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Introduction: Critical illness survivors demonstrate skeletal muscle wasting with associated functional impairment. I prospectively characterised this process, and defined the pathogenic roles of altered protein synthesis and degradation.

Methods: Critically ill patients (n=63, 59% male, age 54.7±18.0 years, APACHE II score 23.5±6.5) were recruited ≤24 hours following intensive care admission. Muscle loss trajectory was determined through serial ultrasound measurement of rectus femoris cross-sectional area (RF_{CSA}) and, in a subset, quantification of myofibre area ($Fibre_{CSA}$) and protein/DNA ratio. Histopathological analysis was performed. Muscle protein synthesis and breakdown rates were determined and respective signalling pathways examined.

Results: RF_{CSA} decreased significantly, (-17.7±12.1%, [p<0.001]), underestimating muscle loss determined by $Fibre_{CSA}$ (-10.3±10.9% vs.-17.5±30.2%, p=0.31), or protein/DNA ratio (-10.3±10.9% vs. -29.5±41.5%, p=0.03). Fall in RF_{CSA} was greater in multi- than single-organ failure (-21.5±10.5% vs. -7.2± 9.7%, p <0.0001), even by day 3 (-8.7±16.3% vs. -1.8± 9.6%, p<0.01). Myofibre necrosis occurred in >50% (20/37) of subjects. Protein synthesis was depressed to levels observed in fasted controls (0.035±0.018%/hr vs. 0.039±0.011%/hr, p=0.57), and increased by day 7 (0.076±0.066%/hr, p=0.03) to levels associated with fed controls (0.065±0.018%/hr, p=0.30,) independent of nutritional load. Protein breakdown remained elevated throughout (8.5±5.7 to 10.6±5.7mmol phe/min/IBW, p=0.4).Principal component analysis of intracellular signalling supported a programme of increased breakdown (r=-0.83, p=0.005) and depressed synthesis (r=-.69, p=0.041).

Conclusions: Early rapid skeletal muscle wasting occurs in critical illness, is greatest in those with multi-organ failure, and results from suppression of protein synthesis and increases in catabolism. These effects are independent of feeding and are commonly associated with myonecrosis.

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The work herein was performed by me and Jai Rawal assisted me in the majority of muscle biopsies and in data collection, with the following exceptions:

- 3 patients were biopsied by Jai Rawal and Mark Mcphail
- 8 Haemotoxylin and Eosin Stains were performed by students at the Centre of Human and Aerospace Physiological Sciences
- The CD68 stains was work by Gamanu Ratnnayake
- All qPCR was outsourced to qStandard, (London, UK)

- Whilst the bedside experiments were performed by me, all GCMS work for muscle protein turnover was performed by Anna Selby, Marie Limb and Kenneth Smith.

List of Abbreviations

3MH=	3 Methyl Histidine
4EBP-1=	Eukaryotic Initiation Factor 4E binding protein 1
AKT=	Protein Kinase B
ANOVA=	Analysis of Variance
APACHE II=	Acute Physiology and Chronic Health Evaluation
APTTR=	Activated Partial Thromboplastin Time Ratio
ARDS=	Acute Respiratory Distress Syndrome
ATP=	Adenosine Tri Phospate
AUROC=	Area Under Receiver Operator Curve
BMI=	Body Mass Index
BSA=	Bovine Albumin Serum
$CaCl_2$=	Calcium Chloride
cDNA=	Complementary Deoxyribonucleic Acid
$CoCl_2$=	Cobalt Chloride
COPD-	Chronic Obstructive Pulmonary Disease
CT=	Computer Tomography
CVA=	Cerebrovascular Accident
DAB=	Diaminobenzidine
DAPI=	4'-6-Diamidino-2-phenylindole
DNA=	Deoxyribonucleic Acid
EDTA=	Ethylenediaminetetraacetic acid
EEF2=	Eukaryotic Elongation Factor
EEFs=	Eukaryotic Elongation Factors
EIFs=	Eukaryotic Initiation Factors
EMG=	Electromyogram
ERFS=	Eukaryotic Termination Factors
$Fibre_{CSA}$=	Myofibre Cross Sectional Area
FiO_2=	Fraction of inspired oxygen
FOXO=	Forkhead Box Class O-1
FSR=	Fractional Synthetic Rate
GABA=	Gamma-aminobutyric acid
gDNA	Genomic Deoxyribonucleic Acid
GSK3β=	Glycogen Storage Kinase 3 Beta
H_2O_2=	Hydrogen Peroxide
HCL=	Hydrochloric Acid
HMGCoA=	3-hydroxy-3-methyl-glutaryl-CoA reductase
HRQol=	Health Care Related Quality of Life Questionnaires

IBW=	Ideal Body Weight
ICU=	Intensive Care Unit
ICU-AW=	Intensive Care Unit Acquired Weakness
IGF1-R=	Insulin like Growth Factor 1 Receptor
INR=	International Normalised Ratio
IRS-1=	Insulin Receptor Substrate-1
KIC=	A-ketoisocaproate
LBF=	Limb Blood Flow
LOS=	Length of Stay
LPB=	Limb Protein Breakdown
LPS=	Limb Protein Synthesis
MAC=	Mid Arm Circumference
MAFBx=	Muscle Atrophy F-Box-1
MMT=	Manual Muscle Testing
MOF=	Multi Organ Failure
MPB=	Muscle Protein Breakdown
MPS=	Muscle Protein Synthesis
MRC-SS=	Medical Research Council Sum Score
MRI=	Magnetic Resonance Imaging
mRNA	Messanger Ribonucleic Acid
MTC=	Mid Thigh Circumference
mTOR=	Mammalian Target of Rapamycin
MURF-1=	Muscle Ring Finger protein 1
Myostatin=	Myostatin
NAOH=	Sodium Hydroxide
NCS=	Nerve Conduction Studies
NFκβ=	Nuclear Factor Kappa Beta
NHS=	National Health Service
NICE=	National Institute of Clinical Excellence
NMBA=	Neuromuscular blockade agent
NMDA=	N-methyl D aspartate
NMES=	Neuromusclular Electrical Stimulation
OCT=	Optimal Cutting Temperature Compound
OPD=	Ortho-phenylenediamine
p/f ratio=	PaO_2/FiO_2
P70s6K=	70kDa ribosomal protein S6 kinase
$PaCO_2$=	Partial pressure of carbon dioxide in arterial blood
PaO_2=	Partial pressure of Oxygen in arterial blood
PBS=	Phosphate Buffered Solution
PCA=	Principle Component Analysis
PTEN=	Phosphatase and Tensin homolog
PVDF=	Polyvinylidene fluoride
qPCR=	Quantitative Polymerase Chain Reaction
RF_{CSA}=	Rectus Femoris Cross Sectional Area
RNA=	Ribonucleic Acid

SaO_2=	Oxygen Saturation in arterial blood
SAPS II=	Simplified Acute Physiology Score
SARS=	Severe Acute Respiratory Syndrome
SD=	Standard Deviation
SDS=	Sodium Dodecyl Sulfate
SOF=	Single Organ Failure
SOFA=	Sequential Organ Failure Assessment
TNFα=	Tumour Necrosis Factor Alpha
tRNA	Transfer Ribonucleic Acid
TWEAK=	TNF-related Weak Inducer of Apoptosis
UCL=	University College London
UCLH=	University College London Hospitals
UPP=	Ubiquitin-Proteasome Pathway

Contents

INTRODUCTION

1.1 Critical illness survival: A public health issue

Increasing numbers of patients are admitted to critical care, with this rise projected to continue [1, 2] as new treatments emerge, expectations for care change, and population demographics and patterns of disease alter. Thus, sepsis is the second most common cause of non-ischaemic heart disease related death in the United States, with a rising incidence [3]. The incidence of trauma, a leading cause of injury in the young adult, continues to rise [4]. Meanwhile, recent improvements in the medical care of chronic disease [5, 6] has contributed to the expansion of a cohort of patients with complex interdependent chronic stable disease states [6-8]. The maintenance of such stability is a physiological balancing act, and normally uncomplicated reversible diseases (e.g. infections) may trigger a spiral of physiological decompensation, requiring acute stabilisation-best managed in the critical care unit.

Coupled to such factors is the increase in the ageing population of the world. The current projections are for over 9% of the North American and European population to be over 80 years of age by 2050 [9]. Whilst the relative percentage is projected to be lower in Asia (4.4%), it is worth noting that the absolute numbers will be approximately three times greater [9]. Even the most pessimistic modelling suggests that life expectancies in the developed world will continue to rise despite the increasing burden of chronic disease[10]. Elderly patients are increasingly being admitted to critical care. A multi-centre observational study from Australia and New Zealand places this at increasing by 6% per year [11].

Finally the impact of emergent diseases on the need for critical care cannot be discounted. In the last decade, the world has weathered the Severe Acute Respiratory Syndrome(SARS) outbreak, the H1N1 influenza epidemic, and Avian Flu- all of which have led to unanticipated acute increases in critical care admissions [12]. These may or may not be isolated incidents- hypotheses exist that with

the melting of polar icecaps, older viral strains for which herd immunity has been lost may re-emerge [13]. If climate change proponents are to be believed (and there is almost overwhelming evidence that they should be) then migration and immigration will be a feature of the mid to late 21st century, bringing with them their usual accompaniments of violence and disease [14].

In the face of this increase in demand, critical care mortality is decreasing steadily. Table 1 shows the summary statistics for the England, Northern Ireland and Wales for 2007-2010 from the Intensive Care National Audit and Research Centre (ICNARC), which demonstrate an approximate 1% annual decrease in hospital mortality after critical care unit admission.

Year of admission	2007	2008	2009	2010
Number of admissions	83,116	90,761	77,394	106,479
Critical care unit length of stay (hours), median (IQR) [N]	50 (23, 120) [83,062]	51 (24, 125) [90,754]	52 (24, 124) [77,378]	54 (24, 125) [106,474]
Total acute hospital length of stay (days)*, median (IQR) [N]	13 (6, 28) [79,202]	13 (6, 28) [86,393]	13 (6, 27) [73,625]	13 (6,27) [101,718]
Critical care unit mortality, deaths (%) [N]	14,904 (17.9) [83,115]	15,806 (17.4) [90,760]	13,136 (17.0) [77,390]	17,520(16.5) [106,479]
Acute hospital mortality*, deaths (%) [N]	21,189 (27.1) [78,172]	22,299 (26.1) [85,517]	18,491 (25.4) [72,778]	24,620 (24.4) [100,940]
Duration of advanced respiratory support (calendar days), median (IQR) [N]	1 (0, 3) [37,757]	1 (0, 3) [78,945]	1 (0, 3) [77,021]	0 (0,2) [106,457]
Age, mean (SD) median (IQR) [N]	60.1 (18.8) 64 (48, 75) [83,116]	60.3 (18.7) 64 (49, 75) [90,761]	60.5 (18.5) 64 (49, 75) [77,393]	60.7 (18.4) 64, (49,75) [106,479]
APACHE II score†, mean (SD) median (IQR) [N]	16.7 (7.4) 16 (11, 21) [77,965]	16.3 (7.1) 15 (11, 20) [85,738]	16.2 (7.1) 15 (11, 20) [73,226]	16.0 (7.0) 15 (11,20) [101,047]

*Excluding readmissions to the critical care unit during the hospital stay
†Excluding admissions aged less than 16 years
IQR: interquartile range; N: number of admissions; SD: standard deviation

Table 1: ICNARC summary statistics for 2007-2010

Extrapolating these data to the entirety of the UK, 10,000 more people now survive intensive care every year, in comparison to the previous year. This is likely the result of improvements in several components of critical illness management. Firstly, there now exists a recognition that early intervention results in a decreased severity of disease [15]. Secondly, our understanding and therefore basic management of disease states, such as the Adult Respiratory Distress Syndrome (ARDS) has improved markedly [16-18]. Finally the critical care community has targeted secondary

complications of critical illness as a major contributor to mortality [19-21] - this has been the focus of much research, and alterations in clinical practice.

1.2 The cost of critical care survival

We now know that Intensive Care Unit (ICU) survivors have increased all-cause mortality over the subsequent 5 years [22-29]. One study has followed patients for 15 years, and noted an increased mortality compared to the general population, even at this late stage [30]. Whilst some of this mortality can be attributed to the underlying disease which led to admission or to previously-identified severe co-morbidities, its true aetiology remains unknown. The only risk factors consistently identified are those of prolonged ICU stay [26, 29], ICU delirium [31], poor pre-admission functional status and old age [22, 29, 30]. The latter perhaps resulting from the intrinsically lower physiological reserve of the elderly [32], compounded by their greater burden of chronic stable disease [33-36]. These data are limited, as the majority of critical care studies have not taken long term outcome into consideration, with the focus being on 28 day mortality, or on ICU or hospital outcome [28, 37-41]. Whilst critical illness is therefore associated with substantial post-discharge mortality, a similar increase in morbidity is also recognised.

1.3 Functional impairment post critical illness

In 2003, Herridge *et al.* carried out a landmark study in 104 ARDS survivors, demonstrating persistent functional disability at 1 year [42], later shown to persist to 5 years or beyond [43]. Functional impairment was defined as a below normal score in the SF-36 quality of life questionnaire. Scores in all bar the emotional domain were below those of age- and sex-matched controls. In association, the rate of return to was low, with 49% returning at 1 year, increasing to only 77% at 5 years [42, 43]. Other investigators have made similar observations, with 65% of 117 ARDS patients returning to work at 2 years [22] and 55% 194 general ICU survivors at 1 year [44].

These figures highlight the economic burden of critical care survivors-healthcare utilisation is high, with costs relating to hospital readmission and rehabilitation being equivalent to a third those of the initial illness [23, 26, 45]. It should be noted that the high morbidity and functional impairment post critical illness is not specific to ARDS survivors, but has been seen is survivors of sepsis and trauma [26, 28, 44, 46]. Likewise, such debility is not merely a function of advanced age. Those studied by Herridge [42, 43] were of median age 45 years, in keeping with other studies- Myhren's cohort had a mean age of 49 [44] and Chelluri's a median of 65 [22]. However, increasing numbers of elderly patients are surviving critical care [47], and they may be affected by an even greater short term mortality rate [36, 48], and a high disability rate [49].

All this in turn impacts of the relatives of patients- often their primary caregivers [50]. This was first highlighted in 1994, with loss of income and financial stability as major issues [51]. Two months after a period of mechanical ventilation (mean 13.9 days), 75 % of survivors required the assistance of caregivers, who faced significant mental health and financial impacts [52]. Other studies have corroborated this with caregiver requirements as high as 84% at 1 year [22, 26]. One single centre study in patients (mean age 55) undergoing prolonged ventilation (median 27 days) found that very low numbers (2%) of these patients were discharged home-the remainder entered intermediate or rehabilitation facilities [26]. Studies outside the critical care setting have demonstrated both anergy and delayed wound healing in caregivers of patients suffering from Alzheimer's disease, demonstrating the physiological effects of said burden [53].

Thus, functional debility is common after critical care, and affects all ages. Exercise limitation in outcome studies is widely reported as part of Health Care Related Quality of Life Questionnaires (HRQoL) [23, 24, 42, 44, 54, 55].

Various causal factors for this functional debility have been proposed. Whilst both psychiatric and psychological dysfunction is common after intensive care (short term memory and concentration are both impaired [56, 57], for instance, and Post-Traumatic Stress Disorder is considered common [58]), these do not appear the cause of physical limitation. In Herridge's cohort, the mental component

score was normal by 12 months, and remained so thereafter [43]. This has been seen in larger cohorts of 300 patients, where mental component scores normalise by 6 months [24], implying that any contribution that psychiatric and psychological dysfunction has to functional debility is minor, or that physical dysfunction persists for longer.

Altered pulmonary function has been reported after critical illness, and may contribute to exercise limitation [59]. However, this cannot explain functional limitation: that lung function returns to normal has long been assumed [46], and has now been demonstrated [42, 43]. Similarly, cardiac dysfunction secondary to sepsis is fully and rapidly reversible [60].

Nerve conduction abnormalities have been demonstrated repeatedly in the critically ill patient, and were first described by Bolton in 1984 [61]. Emerging hypotheses for the pathophysiology include acquired channelopathies of voltage gated sodium channels, localising the defect to the muscle membrane [62-64]. In addition, neuronal tissue architecture disruption has been seen in peripheral nerve biopsies [65]. Quantification and description of these functional defects is done by nerve conduction studies (NCS) and electromyography (EMG) [66]. Aside from the technical difficulties of performing and interpreting EMG in the critical care setting, their use in assessing the aetiology or severity of muscle weakness is limited by two factors. Firstly, EMG and NCS abnormalities appear universal in ICU patients and correlate poorly with the severity of muscle function loss [66-70]. Secondly, lack of patient co-operation (for instance, due to sedation or pharmacological paralysis) prevents early detection of neuromuscular abnormalities and thus risk of weakness [71]. Whilst severe neuropathy is debilitating, critical illness neuropathy is much less common than critical illness myopathy [69, 72, 73] and, again, correlates poorly with symptoms of weakness: of the 104 ARDS patients discussed above, 7 had entrapment neuropathies, and none had other evidence of neuropathy despite functional impairment [42].

1.4 Muscle weakness: the major contributor to functional impairment

Critical illness survivors state that muscle wasting and weakness are the greatest problems that they face [7]. Both quality of life questionnaires and 6 minute walk tests have been used to objectively demonstrate that muscle weakness is the primary contributor to functional disability [23, 24, 36, 42-44, 54, 55]. In fact, all patients with functional disability in Herridge *et al.'s* cohort reported muscle weakness as the primary cause of their disability [42, 43], a finding confirmed by other studies [74]. Indeed, rather than having a primary psychological or cardio-respiratory cause, increasing evidence suggests that skeletal muscle dysfunction plays an important role in the pathogenesis of post-critical care debility. Functional disability was objectively demonstrated in the Herridge *et al.'s* study, where 6 minute walk test distances rose to a maximum of 66% predicted [42] at 1 year. These patients had lost 18% of their base line body weight by discharge (more accurately, perhaps, they were at 82% of their baseline weight, with fluctuations after ICU discharge and before hospital discharge being unmeasured), with only 71% of patients recovering their baseline weight at 1 year. Recently a small cohort of survivors underwent cardiopulmonary exercise testing, confirming the major role muscle wasting has in exercise limitation [75]. The commonest limiting symptom was leg fatigue. Cardiopulmonary reserve was evident in most of the patients at peak exercise, defined by the respiratory exchange ratio, breathing reserve and heart rate reserve.

Investigators have examined the association between muscle weakness and clinical outcome measures, and have found muscle weakness to be an independent predictor of mortality [76, 77], associated with increased ventilator dependant time [69, 76, 78] and length of stay [76]. That muscle wasting occurs is not new to clinicians- Asher alluded to this in his treatise on bed rest in 1947 and placed "*beds and graves in the same category*" [79]. Macfarlane *et al.*'s case report is often

quoted as the first case of critical care myopathy (at that stage variably known as acute quadriplegic myopathy) [80].

The National Institute of Clinical Excellence (NICE) has recently issued guidance regarding critical illness rehabilitation [81]. A major weakness in its evidence based guidance is the lack of basic understanding of the pathophysiology of muscle wasting (which the NICE authors acknowledge). NICE strongly recommends that research in this area is prioritised- without understanding the process of muscle wasting; the public health issue of functional debility after critical illness cannot be addressed.

2. Measuring muscle strength in the critically ill

2.1 Manual Muscle Testing

Two forms of a Manual Muscle Testing (MMT) have been described in recent years- The Medical Research Council Sum Score (MRC-sum score) and hand-grip dynamometry. The MRC sum score was first described in 60 patients with Guillain-Barre Syndrome as an assessment tool, as part of the Dutch gamma globulin trial [82, 83]. A 90% inter-rater agreement (defined as <10% change in score) was seen in measurements between blinded observers. In 2002, this technique was deployed in a multi-centred observational study of 95 critically ill patients following 7 days of mechanical ventilation [69]. Patients were expected to respond appropriately to 3 of 5 questions, on 2 consecutive evaluations with a 6 hour interval, to be considered appropriate for MMT. Each limb is scored using the Medical Research Council Strength scale of 0-5 for proximal and distal muscle groups in regards to the muscle groups ability to exert force against resistance (5-4), overcome gravity (3), to move once gravity is eliminated (2) or if only fasciculation (1) or no movement (0) is observed (table 2), and the score for each limb added up (to a maximum of 60). An arbitrary cut off of 48 was used to distinguish weak patients. The incidence of weakness was 25% at awakening (16.6 days from initiation of mechanical ventilation).

MRC sum score	Left	Right	
Shoulder	0-5	0-5	
Elbow	0-5	0-5	
Wrist	0-5	0-5	
Hip	0-5	0-5	
Knee	0-5	0-5	
Ankle	0-5	0-5	
Total	**0-30**	**0-30**	**Total 0-60**

Table 2: Medical Research Council sum score components. Maximum score is 60

Proximal strength was consistently scored lower than distal strength, and the mean MRC sum score was 33.2 (range 8-45) in this group. In 22 patients, neurophysiological studies revealed a sensor-motor axonal peripheral neuropathy, but no neuromuscular blockade. Abnormal EMG activity was seen in 10 patients. Those patients with muscle weakness (as defined by this study) had longer duration of ventilation, specifically post awakening (18.2 (36.3) vs. 7.6 (19.2), p=0.03). Some 67% were followed up at 9 months, at which point all but one had MRC sum score of >48. Despite this promising debut, several concerns exist as regards its clinical and research utility. Whilst excellent inter-rater reliability has been seen in healthy subjects and critical illness survivors, this worsens considerably in the ICU setting [84, 85]. Furthermore, 30-60% of patients have been unable to perform MMT when assessed [69, 86]. Finally MRC-Sum score assessment can detect weakness once it occurs, but is unable to identify those *at risk*. Within those caveats, MRC-sum score testing has its place in clinical trials [87].

Handgrip dynamometry was examined in a multi-centre study of 136 patients. More patients could be examined (79%), and handgrip dynamometry correlated well with MRC-sum score [76].

In summary, the utility of MMT in the early detection of ICU-AW or of those at risk of ICU-AW remains unclear. Both forms of MMT described above have demonstrated the association of ICU-AW with increased length of ventilation, ICU mortality and hospital mortality [69, 76, 78].

2.2 Direct nerve stimulation in the critically ill

Objective muscle strength testing in the critical care setting is fraught with logistical difficulties, and can only be reliably performed using direct nerve stimulation as described by Polkey *et al.* [88]. Whilst distal muscle groups have been tested successfully [89], the relevance of weakness in these groups to subsequent functional disability is unclear. Furthermore, observational work in muscle wasting has shown a differential wasting between proximal anti-gravity muscle and distal muscles [69]. Whilst testing of quadriceps strength in intensive care has been performed successfully [89] the equipment is cumbersome, expensive and needs considerable training to use [90]. The requirement for the patient to be moved to a bespoke chair for measurements to be performed precludes their use in unstable patients. These obstacles are unlikely to be overcome in the foreseeable future, preventing translation into clinically useful tools.

3. Measuring muscle mass in the critically ill patient

3.1 The relationship between muscle mass and strength

Strong correlations have been demonstrated between muscle strength (measured by maximum voluntary isometric contraction) and indices of muscle mass (such as cross sectional area of Rectus Femoris muscle [RF_{CSA}]) in healthy normal subjects, athletes and patients with COPD [91-95]. Correlation have also been demonstrated between RF_{CSA} and physical activity in COPD (as measured by week long tri-axial accelerometry) [96] and endurance in athletes [93]. In the context of the

difficulties described above, measuring changes in muscle mass will likely provide high quality objective data that can be extrapolated to strength and functional disability.

3.2 Non Invasive measurements

3.2.1 Computer Tomography (CT) and Magnetic Resonance Imaging (MRI)

Whilst these modalities are considered to be the gold standard for measurements of muscle mass, their use is limited in the critical care setting. Patient transfer (and its accompanying risks), expense and technical issues (radiation dose in the case of CT; problems with monitoring equipment and ventilator circuits for MRI) preclude their use in larger trials. One study has used computer tomography [97] but the limited number involved (n=8) preclude meaningful interpretation.

3.2.2 Bedside Ultrasound

Using ultrasound in the intensive care setting is appealing as it is cheap, portable and readily available. Rectus Femoris cross sectional area and muscle limb thickness (MLT) have both been used in the past [92, 93, 95, 98-100]. RF_{CSA} correlates well with strength [91-93, 95] and Rectus Femoris muscle volume [94, 101], and has good inter-rater reliability [92, 93], meaning that data derived from cross-sectional area measurements are highly likely to have functional relevance.

3.2.3 Bioimpedance

Bioelectrical impedance analysis is commonly used to determine total body water, which is then used to estimate fat free mass using body weight [102]. Critically ill patients have large fluxes of water, resulting in inaccurate bio-impedance measurements [103, 104]. This, coupled with the lack of reliable weight measures in critical care, renders it impractical.

3.2.4 Anthropomorphic measurements

Whilst these measurements are established in healthy ambulant adults, their place in the critically ill remains uncertain. Complete dissociation has been seen in a study of 9 patients comparing MLT and both upper arm and lower limb circumferences [98]. The same group found similar results in a larger

cohort[100]. Such limited validity may relate in part to difficulties in skin-fold thickness measurement, consequent upon the presence of dependent oedema.

3.2.5 Other methods

In vitro neutron activation analysis has shown significant drops in protein content, corroborating the results of the direct muscle measurements [105-107], but is of limited use given the radiation dose used [108].

4. Invasive Measurements of Muscle Mass

4.1 Muscle Fibre Cross Sectional Area ($Fibre_{CSA}$)

The measurement of skeletal muscle myofibre cross-sectional area ($Fibre_{CSA}$) from histological sections has been used to quantify changes in muscle mass in the critically ill. Numerous case histories and series have shown fibre atrophy, though only in single time point studies [68, 109-113] Only one study has performed serial measurements: Helliwell et *al.* demonstrated mean reductions of $Fibre_{CSA}$ of 3-4% a day [114]. Biopsies in this study were taken at varying time points (hence the expression of results per day), preventing a clear description of the initial phase of muscle wasting in the critically ill. Other more recent studies have also concluded that $Fibre_{CSA}$ decreases, but notably derived this conclusion from comparison with control data rather than longitudinal paired data [115]. Complicating the scarcity of muscle fibre characterisation is the effect of peripheral oedema on the measurements of whole muscle mass: no studies have performed quantification of muscle mass alterations alongside fibre size changes in humans, although a porcine model of sepsis demonstrated increasing $Fibre_{CSA}$ with fluid resuscitation [116]. Biochemical series in critically ill patients have shown high extracellular water levels with normal or low intracellular levels [117-120], corroborating data obtained by isotope dilution [105]. The only direct study examining changes in $Fibre_{CSA}$ in response to fluid challenges demonstrated that oedema does affect $Fibre_{CSA}$ [116], mandating the need for a third modality, preferably one that is independent of fluid content.

4.2 Protein/Deoxyribonucleic Acid ratio (Protein/DNA ratio)

A biochemical method of assessing protein depletion, the Protein/DNA ratio, has been used since the 1950s [121]. This method is based on the assumption that the DNA mass in a cell is constant, and that a fall in Protein:DNA ratio thus infers a reduction in overall cell size/ mass [122]. This approach has been used to demonstrate loss of muscle mass in rodent models of critical illness [123, 124] and in a small cohort of 9 critically ill patients [118]. Importantly, both protein and DNA quantification can occur via fluorescence spectrometry. As the target (protein or DNA) is bound to a detection agent, this technique is unaffected by the water content of the cells.

5. Measuring muscle quality

5.1 Development of myopathy

Whilst the presence of myopathic fibres has been repeatedly noted in the context of muscle wasting following critical illness [65, 111, 112, 115, 125], the time course of their development remains unknown. The majority of histological descriptions relate to single biopsies as a cross-sectional examination of patients with weakness, with few longitudinal studies. Helliwell *et al.* described progressive necrosis, fibre atrophy and regenerating fibres [125]. This is in keeping with aforementioned single time point studies [65, 68, 111, 112, 126]. Loss of thick myosin filaments has been repeatedly described in case series, suggesting muscle architectural disruption [109-112, 127]. Further, with the exception of Helliwell *et al.* [125], the absence of myopathic fibres prior to critical illness had not been established- these being seen in chronic disease [128-131] and senescence [132].

5.2 Presence of necrosis

In the single longitudinal muscle biopsy study, the incidence of necrotising myopathy was 50% [125]. However, the exact time course of this pathology remains unknown (due to the small numbers and non-standardised time points). Clinical correlates also remain unexplored.

5.3 Increasing quantities of connective tissue

A single cohort study noted an increase in connective tissue in myopathic patients [133]. Details of quantification and comparison are not apparent from the manuscript, and a personal communication with the author did not uncover the means of quantification. An increase in collagen has been noted in immobilised muscle[134].

6. Muscle mass regulation-protein homeostasis

Skeletal muscle mass is regulated by a balance between muscle protein synthesis(MPS) and muscle protein breakdown (MPB) [135]. In a 70kg human, approximately 280g of protein is synthesised and degraded each day [136]. The two processes are linked, in a fashion described by Millward as facilitative or adaptive processes, whereby MPS facilitates (allows modulation of muscle mass) and MPB adapts (limiting this modulation) [137]. When exposed to an anabolic stimulus, MPS rises. MPB rises too, but to a lesser amount, resulting in a net synthetic balance. In response to an anti-anabolic stimulus, MPS decreases, and MPB decreases to a lesser degree resulting in a net breakdown (figure 1).

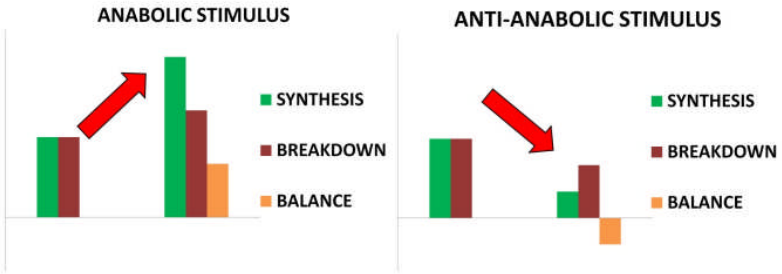


Figure 1: Protein homeostasis in response to anabolic and anti-anabolic stimuli.

The following schematic (figure 2) from Phillips et al [138] demonstrates the daily variation in protein homeostasis in healthy humans, resulting from changes in protein ingestion:

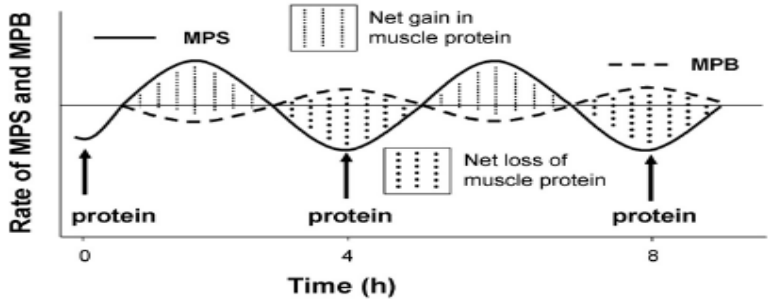


Figure 2: Normal variation in protein homeostasis in response to protein ingestion. MPS=Muscle Protein Synthesis, MPB=Muscle Protein Breakdown.

Within the critical care environment, patients are exposed to a variety of stimuli that affect this balance, the net result of which remains unknown. Four scenarios are possible:

1. MPB is raised significantly, with a small rise in MPS
2. MPB is unchanged and MPS decreases
3. MPB increases and MPS decreases
4. MPB increases and MPS is unchanged.

Whilst scenarios 1 and 2 are in keeping with normal physiology, scenarios 3 and 4 describe a pathological dissociation between MPS and MPB, which hypothetically may occur in the context of severe sepsis or inflammation, perhaps as a result of cellular, mitochondrial or membrane dysfunction [137, 139].

6.1 Factors affecting protein homeostasis in the critically ill

Critically ill patients are exposed to a variety of stimuli which might impact upon muscle turnover, and the combination and intensity of these will vary with the background burden of disease, the nature and severity of the disease state causing admission, the therapies applied, the physiological

response to disease and treatment, and with other patient characteristics/ demographics. This heterogeneity limits the ease with which findings in one specific patient population (e.g. trauma or coronary artery bypass surgery) can be generalised to others [140]. Likewise, chronic respiratory and cardiac disease can both drive skeletal muscle wasting [129, 141] whether or not they are the precipitant for admission to intensive care, or (perhaps) result from it. Immobilisation promotes muscle wasting [142-144], predominantly (in humans) through the suppression of MPS (rather than alterations in MPB) [145]. In clinical observational studies, sepsis and systemic inflammation are reported to act as powerful catalysts of muscle weakness [69, 77]. Although animal models demonstrate that inflammation suppresses MPS and increases MPB, such data in critically ill patients are lacking [146, 147]. However, diverse aetiological factors drive similar physiological derangement which can be quantified in physiological scoring systems and used, with variable success, to predict ICU outcome [148]. Perhaps for this reason, muscle biopsies from critically ill patients demonstrate histopathological similarities across different disease groups [114, 118] suggesting at least some commonality in underlying processes.

6.1.1. Immobilisation

Mechanical ventilation has traditionally been associated with sedation and bed rest, although this has recently been challenged as a universal paradigm [149, 150]. It is established that immobility has a detrimental effect on the musculoskeletal system, both quantitatively and qualitatively (figure 3). Relatively short periods of immobilisation decrease MPS, with no effect on MPB [151]. Furthermore, this altered balance is relatively resistant to programmes in which high dose amino acid feeding is employed [152]. This is in contrast to studies in animals, in which MPB appears the dominant process [142, 153]. Immobilisation has significant effects on peripheral muscle aerobic capacity [154], contractility [143], insulin resistance [155] and architecture [144]. Microvascular dysfunction occurring in severe sepsis is associated with immobilisation and may have an additive effect on reducing MPS [155, 156].

Immobility models have been developed to aid our understanding of this process- immobility (or,

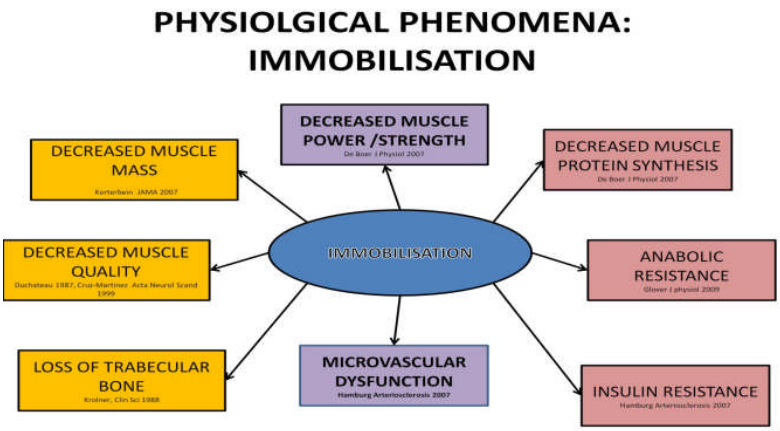


Figure 3: Immobilisation as a dynamic process

Rather, relative immobility) being an issue in research into ageing, chronic diseases and space flight. Four models are commonly used:

6.1.1 Limb Suspension

Commonly achieved in humans using a sling and crutches (occasionally with bespoke shoes), a limb suspension model was used by de Boer *et al.* [151]. In a 23 day programme, muscle mass was seen to fall by 14 days (5.2%). Subjects lost in total of 10% of limb muscle mass by 23 days. Of note, this was not paralleled by continued upregulation of the intracellular signalling molecules driving muscle protein breakdown. Not all muscle activity is suppressed (as determined by EMG), particularly across the knee joint of the suspended limb [157].

6.1.2 Limb Casting

Further immobilisation can be induced by limb casting, preventing knee joint movement. Subjects are usually allowed to continue ambulation on crutches. Whilst numbers of subjects have been relatively small, decreases in muscle mass have been noted in several studies between 10-14 days

[158, 159]. Gibson demonstrated decreased MPS using a limb casting method, albeit over a longer period-6 weeks [145, 160].

6.1.3 Bed Rest and Microgravity

The most commonly used model, bed rest has been seen to cause muscle wasting within 10 days in healthy older adults [154]. However, when a head down position is added (simulating microgravity), Ferrando *et al.* demonstrated loss of muscle mass within 7 days [161]. This combination has been used repeatedly to simulate muscle unloading in space flight- unlike limb casting and limb suspension, bed rest +/- microgravity induces the multisystem effects of immobilisation [157, 161].

6.1.4 Sedation

Sedation remains an unknown factor in immobilisation-related muscle remodelling. Propofol and benzodiazepines positively modulate the inhibitory function of the neurotransmitter gamma-aminobutyric acid (GABA) [162, 163]. GABA facilitates the opening of the voltage gated chloride channels in skeletal muscle, decreasing muscle excitability [163, 164]. Barbiturates and ketamine attenuate the response of excitatory neurotransmitters such as glutamate, decreasing muscle tone by acting on motor associated neurones in the spinal cord via N-methyl D aspartate (NMDA) receptors [163, 165, 166]. Recently, NMDA receptors have been discovered on the post-synaptic endplate in skeletal muscle [167]. Skeletal muscle is believed to require active support from neuronal trophic factors such as neuregulin to maintain mass. Pharmacological attenuation of their transport by sedatives may compound muscle wasting [168, 169]. Thus, continued sedation is likely to have a greater effect on muscle atrophy and weakness than “conscious immobility” in the absence of sedation (figure 4). It is reasonable to postulate that the beneficial effects of minimising sedation

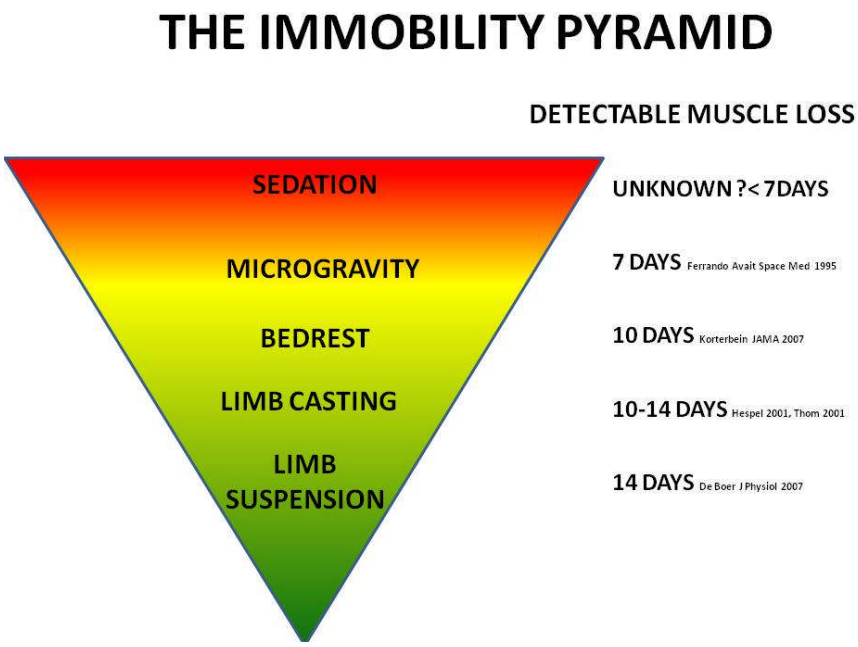


Figure 4: The Immobility Pyramid

[149, 150, 170, 171] might, at least in part, be due to a relative maintenance of muscle mass and function. This hypothesis has yet to be formally examined. There are intrinsic difficulties in separating the effects of sedatives from bed rest in humans, and ethical issues in animal studies. It may be that cell culture work, using human myocytes, is required so as to advance understanding of this issue.

6.1.5 Pharmacological agents

Many drugs have been implicated in the genesis of acute muscle wasting. Over time, the case against aminoglycosides [172] and others have disappeared under scrutiny, from lack of evidence. Two categories remain implicated: neuromuscular blocking agents and glucocorticoids.

6.1.6 Neuromuscular Blocking Agents (NMBAs)

The *non-depolarizing* agents (e.g. Tubocurare, Vecuronium and Atracurium) compete with acetylcholine (ACh) for binding at the nicotinic receptor level of the motor end plate. The *depolarizing* agents (e.g. succinylcholine) act as fixed agonists at these receptors, causing sustained opening of the associated sodium channel, and thus preventing further ACh-driven excitation. Although bolus use of depolarizing NMBAs facilitates endotracheal intubation, data supporting the more prolonged use of non-depolarizing agents are sparse. First described in 1977, reports began to appear of prolonged weakness in patients exposed to a combination of mechanical ventilation, high-dose steroids and NMBAs [80, 173-175]. Based on the mode of action of NMBAs, a series of case reports continued to implicate them as causal [111, 176-182], with over 44 case reports published by 1997 [183]. Consequently, reviews and guidelines have cautioned clinicians of the risks associated with NMBA use [184-197]. There is a certain biological plausibility for the proposed adverse effect: in addition to data reporting inhibition of peripheral nerves myogenesis via the antagonism of trophic factors such as neuregulins [168, 169], animal models of denervation have demonstrated reduced activity of the NaV1.4 sodium channel leading to hypo excitability of muscle [198]. Furthermore, pharmacological denervation has been used repeatedly as a model for muscle atrophy secondary to immobilisation [153, 199].

Table 16 (Appendix) describes the observational studies performed in the last 20 years reporting the association with NMBA use and ICU-AW. It must be highlighted that in addition to the differences in study design (e.g. retrospective and prospective observational studies) the majority of the studies relate to heterogeneous populations, and the frequency of corticosteroid use is high (see below). Furthermore, the methodology to confirm or exclude ICU-AW varies amongst the studies. Despite the strength of concern over NMBA use and its relationship to ICU-AW, only 31% of the 16 studies (3 prospective and 2 retrospective) suggest NMBA use to be associated with ICU-AW. Segredo *et al.* examined 16 patients who received Vecuronium infusions, and demonstrated prolonged

neuromuscular blockade [200]. This is an intrinsically different diagnosis from that of ICU-AW, on the basis of current definitions [71] and time frame; in this study the longest duration of paralysis was 168 hours.

Three studies were performed in patients with severe life threatening asthma, all of whom received high dose corticosteroids [182, 201, 202]. Adnet *eI al.* performed a retrospective cohort study of ventilated asthmatics in 5 centres [202]. Despite the authors' conclusion that the use of NMBAs was the only independent predictor of ICU-AW in a multiple logistic regression analysis, those receiving NMBAS had a greater severity of illness, as demonstrated by the increased use of volatile gas ventilation to manage bronchospasm, which adds a further confounding factor to the appropriateness of the comparison. Douglass *et al.* described 25 consecutive cases of acute asthma requiring mechanical ventilation [182]. Twenty-one patients received Vecuronium, and all received corticosteroids. Whilst the use of Vecuronium *per se* was not associated with myopathy, higher dosages (administered to the most unstable patients) were. Behbehani *et al.* performed a multivariate analysis in a retrospective cohort study over a 10 year period of asthma patients receiving mechanical ventilation in two centres [201]. All 86 patients received intravenous glucocorticoids and 30 patients received NMBAs for a mean of three days. Although 30% of patients receiving NMBAs developed ICU-AW, more than 10% of those who did not receive NMBAs also developed ICU-AW. The expected universal use of glucocorticoids at high dose was again a significant confounding factor. Although this would not wholly explain the differences in prevalence of ICU-AW between those that did and did not receive NMBAs, detailed review of the study design showed other important differences. Specifically, differing total dose of NMBA were used between the two recruiting centres, likely accompanied by a difference in total dose of sedative. These confounding factors could significantly influence the incidence of ICU-AW.

Garnacho-Montero *et al.* investigated a heterogeneous group of 73 critically ill patients with an electrophysiological diagnosis of ICU-AW, and concluded (after multivariate analysis of the data) that NMBA use was an independent risk factor [203]. In addition to the diagnostic limitations of such

electrophysiological tests in patients unable to follow simple commands[68, 69, 133], in a subsequent study by the same group, using the same diagnostic protocol, NMBA use was not demonstrated to be a cause of ICU-AW in 68 heterogeneous critically ill patients [204]. However, an increased use of sedatives *was* observed in those who developed ICU-AW (see above) [204].

In a study investigating the outcome in patients with acute respiratory distress syndrome [87], Papazian and colleagues randomised 340 patients to receive 48 hours of cis-atracurium or placebo. Whilst the primary outcome was 90-day survival, manual muscle testing (MMT) was performed on the day of ICU discharge and again on day 28. There was no difference in the incidence of ICU-AW between groups (29% vs. 32%; p = 0.49). Furthermore, in the subgroup that received glucocorticoids, there was no difference in the frequency of ICU-AW between those that received NMBAs and those that received placebo (37% vs. 30%, p=0.32) [205]. The only other relevant randomised controlled trial involving NMBAs was conducted by Forel *et al.*, albeit without an *a priori* decision to test for ICU-AW [206]. In 36 patients randomised to receive 48 hours of neuromuscular blockade or placebo, no increased incidence of ICU-AW or prolonged ventilation was observed. More recent evidence supports the notion that NMBA use for ≤ 48 hours is not associated with in an increased incidence of ICU-AW or in days of mechanical ventilation [87, 206]. The inference is that short term NMBA administration is not a direct cause of peripheral or respiratory muscle weakness.

The use of neuromuscular blockade has decreased over time. In 1981, a survey of 34 intensive care units observed that 90% of patients were routinely administered NMBAs [207], which 12 years later had fallen to 9% [208, 209]. If a causative relationship exists between NMBAs and ICU-AW, a corresponding decrease in ICU-AW should be evident. Leatherman *et al.* published a retrospective series of 96 asthmatic patients requiring invasive ventilation admitted between 1983 and 1995, which found no association between NMBA use and ICU-AW [210]. More importantly, the same authors following a change in practice to use deep sedation rather than muscle paralysis, examined the records of 74 asthmatics admitted between 1995 and 2004 and again found no difference in the

incidence of ICU-AW, (20% vs. 14%, p=0.23) [211]. These data add support to the argument for a lack of causal relationship between NMBA use and ICU-AW.

6.1.7 Glucocorticoids

Skeletal muscle atrophy secondary to glucocorticoids administration is well known in human physiology- glucocorticoids induce catabolism, inhibit anabolism, and potentially exacerbate the effects of immobilisation [212-214]. What is not well understood is the relationship between muscle atrophy, steroid dose, and timing of administration. In critical care, the indications for glucocorticoid use are contentious. Over time, indications have included ARDS [215, 216], severe sepsis [217], and secondary hypoadrenalism [218, 219], in addition to disease specific situations such as rapidly progressing glomerulonephritis and autoimmune conditions.

Leaving aside the lack of consensus on indications for use, doses recommended in these conditions vary: 200mg of hydrocortisone/day [218, 220] in sepsis to 1-2mg/kg/day of methylprednisolone [215, 216] in ARDS, as do the length of administration. The latter is key as with time spent on the ICU (and therefore immobilised), there is likely to be up-regulation of glucocorticoid receptors in the muscle [221]. This phenomenon is poorly characterised: recent animal studies have demonstrated the opposite effect [222]. In either event, the timing and length of administration is likely to have different effects at the various stages of critical illness. There is a clear lack of fundamental understanding of the role of glucocorticoids in maintaining muscle mass in sepsis; in a recent animal model, low dose dexamethasone (equivalent to 12.5mcg/kg) suppressed endotoxaemia-related muscle loss [123]. A recent meta-analysis of sepsis trials failed to show an increased incidence of neuromuscular weakness [223]. The CORTICUS trial, a multi-centre randomised control trial of hydrocortisone in septic shock yielded similarly results [217]. Whilst it is likely that the methodology of diagnosing ICU-AW was poor in these studies, an RCT with a small number of patients receiving steroids failed to detect an increase in ICU-AW using Manual Muscle Testing as a diagnostic tool [87, 205]. Both a mechanistic understanding of glucocorticoids interaction with protein homeostasis in

the setting of systemic sepsis, and high quality randomised controlled trials with a priori decisions to screen for ICU-AW are needed. The latter is hampered by the lack of objective diagnostic tools.

6.1.8 Inflammation

Sepsis and systemic Inflammation are common in critically ill patients, with sepsis reported as the third leading cause of death in the United States [224, 225]. Variations in the circulating concentrations of amino acid have been demonstrated in the early stages of sepsis, in addition to decreased MPS [226, 227]. The exact role of altered MPB in the critically ill is unclear, with conflicting data from studies [226, 228]. Endotoxin administration to healthy volunteers, which is used as a model for systemic sepsis, demonstrated a decrease in MPS with an adaptive decrease in MPB [226]. In contrast, protein turnover studies performed in 19 patients with severe burn injuries showed an 83% increase in MPB [228], although it is appreciated that patients with burn injury and extensive soft tissue loss are not necessarily representative of patients managed in general intensive care.

6.1.9 Age

Sarcopenia (muscle loss associated with ageing) has been shown to occur at a rate of approximately 0.5-2% per annum after the age of 65, although exercise training has been shown to preserve muscle mass [229, 230]. Interestingly, basal muscle protein turnover rates do not differ with advancing age [231] but a blunted synthetic response has been observed such that training produces less of an anabolic response in older subjects [232]. An increasing proportion of the critically ill patient population are elderly and at high risk of developing muscle wasting during critical illness as a consequence of this blunted MPS response. This is particularly important as frail older sarcopenic patients who start from a compromised position with a lower muscle mass have a further reduction in muscle mass. Thus, rehabilitation resistance training in this patient group may be even more important, but from previous data harder to achieve and demonstrate benefit to the muscle. The definition of ageing is complex, and will become increasingly so. Lazarus and Harridge argue that it is not chronological age, but physiological age that is important [233, 234]. In this setting, they argue that it is the effects of increasing levels of disuse that are responsible for much of the decline in

physiological function typically associated with aging- resonating well with current COPD research [235, 236]. Perhaps what is needed is a more functional definition, such as frailty. The Rockwood frailty scale is a global assessment of function, cognitive and physical reserve, that is part of geriatric practise [237]. Frailty is particularly attractive as a concept to critical care physicians, given its overlap with co-morbidity and disability: one can have co-morbid states, but not be frail [35]. As yet we have not seen the association between pre-critical ill frailty and subsequent outcome, but data are emerging which suggest a strong link [238].

6.1.10 Nutrition

Starvation is associated with loss of muscle mass, and muscle is acutely sensitive to (if not dependent upon) protein ingestion for both stimulation of MPS and the delivery of amino acids for synthesis. Very little work has been performed in the critical care setting examining the nutritional contribution to muscle mass maintenance as a primary aim: the focus has always been on survival, hampered yet again by the lack of objective tool to measure ICU-AW. Unlike the healthy state, in critically ill patients, we do not know enough about the interaction of ingested amino acids, the gastrointestinal mucosa [239] and muscle protein synthesis [240], nor if continuous feeding is appropriate [241]. Our lack of understanding of the role nutrition plays in the critically ill regarding mortality (without addressing the lesser issue of protein homeostasis) can be seen in the differences between the European and North American guidelines on initiation and composition of feeding, summarised in table 3.

	ESPEN[242, 243]	**ASPEN[244]**
Initiation of Enteral Nutrition (EN)	Within first 24 hours	24-48hours of admission, unless haemodynamic compromise present-withhold
Initiation of Parenteral Nutrition (PN)	Within 3 days (If EN not possible)	Within 7 days (If EN not possible)
Calories	Indirect Calorimetry, or 20-30kcal/kg/day	Indirect Calorimetry, or 20-30kcal/kg/day
Glucose	2g/kg/day	No target, maintain good glucose control
Lipid	0.7-1.5g/kg/day	No target
Amino Acids	1.3-1.5g/kg/day	1.2-2.0g/kg/day

Table 2: European and North American guidelines for Enteral and Parenteral Nutrition

Furthermore, determining the optimal mode of nutrition (Enteral versus Parenteral) and the use of supplementary PN remains the primary endpoint of several large randomised controlled trials [245-248], several ongoing (http://clinicaltrials.gov/: NCT00512122, NCT01369147, NCT01206166, NCT00883948, NCT00512122, NCT01142570, and CALORIES [7]).

In human models outside critical care, there are indications of interaction with risk factors such as age [249] and immobility [152, 214].

6.2 Measuring protein homeostasis

In human beings, the measurement of muscle protein synthesis (MPS) is achieved through the constant infusion of stable isotope-labelled amino acids such as [1,2-$^{13}C_2$] Leucine and measurement of its incorporation into muscle, samples of which are obtained through biopsy. The measurement of muscle protein breakdown (MPB) is less robust, relying on the measurement of the arterio-venous difference of constantly infused [D_5] Phenylalanine coupled with measurements of blood flow across a limb. In healthy young humans, the basal fractional rate of myofibrillar protein synthesis is in the region of 0.02 to 0.06 % per hour [250]. MPS is stimulated transiently by feeding (and, specifically, exclusively by essential amino acids, particularly Leucine [137, 251]) and exercise, but in healthy young adults a balance is maintained with MPB such that muscle mass is maintained [137].

6.2.1 Controversies in stable isotope methodology

Whilst both stable labelled Leucine and Phenylalanine have been widely used for fractional synthetic rate(FSR) measurements, the use of labelled Leucine has been shown to produce more consistent measurements [252]. Further, the use of double labelled Leucine allows very low enrichments (often seen in human studies) to be reliably measured by Gas Chromatography Mass Spectrophotometry [252].

6.2.2 Primed constant infusions versus flooding dose techniques

Infusions of essential amino acids (with the exceptions of Valine and Isoleucine) stimulate protein synthesis [253, 254]. Two techniques exist for measuring FSR. Primed constant infusions can be used to prevent stimulation of protein synthesis, although this may involve a longer experimental protocol (to reach a steady state in plasma). Whilst lengthy infusion times may be advantageous to the quality of measurements[252], they may not be practical in the context of clinical experiments.

The flooding dose technique involves a bolus of stable isotope, affording a much simpler protocol including a shorter time to sampling. Unfortunately flooding dose techniques involving Leucine or Phenylalanine stimulates protein synthesis [255, 256], raising concerns as regards the validity of the measurements obtained. Further, as lower doses are often used in humans, true flooding is not reliably maintained [257].

6.2.3 Whole body protein turnover techniques

Urinary 3-Methyl Histidine (3MH)

3MH is formed by methylation of Histidine in actin and myosin, crucially, when these proteins are broken down with re-use for protein synthesis [167]. 3MH is neither re-utilised nor metabolised and is excreted in the urine [258]. The proportion of 3MH that is produced by skeletal muscle is unknown, but is presumed to be high [53]. Measurements are made on a 24 hour urine collection, removing diurnal variations and integrating 24 hour profiles [258] .

Several cautionary notes must be added to the use of 3MH. Urinary 3MH may reflect myofibrillar synthesis as well as breakdown, as urinary 3MH may be produced by selective breakdown of myofibrillar protein that has been synthesised [53]. Animal studies have suggested that significant amounts of 3MH are produced by the gastrointestinal tract and other unknown sources [259, 260]. Human studies comparing urinary 3MH excretion with femoral vein 3MH efflux have found a dissociation, providing further evidence for non-skeletal muscle sources [261]. Whilst data exist contesting the contribution of the gastrointestinal tract [262, 263], no other data exist as regards the

remainder of the body. More recent work using stable isotope infusions has added to these data, and supports the view that the gastrointestinal tract and in addition, the liver, are major contributors to whole body 3MH turnover [264].

Whilst 3MH excretion has been measured from 24hour collections, the use of 3MH:Creatinine ratios has the advantage of overcoming diurnal variation and allowing for single time point analysis [258]. Correcting for urinary creatinine (a measure of muscle mass [265]) eliminates inter-individual variation. Unfortunately, infection and trauma have both been shown to increase urinary creatinine, limiting its use in the critically ill [258, 266, 267]. Furthermore, observations exist that renal failure affects the 3MH/creatinine ratio [258, 268].

In summary, urinary 3MH is an imperfect tool in the measurement of protein breakdown, given the uncertainties of the relative contribution of solid organs to whole body turnover. This is likely to be worsened in the critically ill, with renal impairment and gastrointestinal tract dysfunction further impairing its use. The use of stable isotope infusions or arterio-venous 3MH fluxes has superseded urinary 3MH as tool in the measurement of protein turnover [264, 269].

Urinary Nitrogen balance

In addition to the arguments above regarding the relative contributions of muscle mass and other solid organs to whole body protein turnover, urinary nitrogen is affected by protein intake. In conditions where protein malnutrition was prevalent, nitrogen balance was maintained until near death, by reducing nitrogen excretion. This adds further evidence to the dissociation between measures of muscle mass and nitrogen balance [270].

6.3 Protein homeostasis in the critically ill

Whilst several studies of muscle protein turnover have been carried out in the ITU, the majority have been flawed- as a result of poor standardisation of timings, or techniques used. Whole body protein turnover studies are difficult to interpret, given that the relative contributions of large organs in the

body (such as the gastrointestinal system relative to skeletal muscle) are unknown [137, 271-274]. MPB has been shown to be high in patients suffering from severe burns [228, 275, 276], but these have significant direct tissue (and muscle) damage, which is highly likely to affect measurements of MPB performed by D_5-Phenylalanine dilution. Thus, extrapolating data from burns patients to all critically ill patients cannot be done with confidence. MPS rates have been measured as an endpoint as part of Growth Hormone trials [119]. These studies suffer from methodological weaknesses, such as the lack of standardised timings in muscle biopsies, small subject sample size and the use of flooding doses of Leucine and Phenylalanine isotopes (both of which are known to stimulate MPS) [137, 277, 278]. The same technique was recently used in 8 critically ill patients, demonstrating a variable MPS rate, and increased MPB [279]. Aside from the use of flooding dose techniques, baseline Phenylalanine and methyl-Histidine levels were back-calculated, rather than directly measured. Finally, single time point biopsies were taken (range 1-18 days) in subjects, making time course comparisons impossible. The same group had found stable rates of MPS in a larger cohort, but again with variable time points, flooding dose techniques and back-calculation of baseline levels [280].

6.4 Protein homeostasis-summary

In critically ill patients, the exact imbalance in protein homeostasis remains unclear. It is likely that muscle protein synthesis is altered, given that this facilitative process is affected by several physiological insults to which these patients are known to be subjected. Sequential stable isotope infusions at standardised time points are needed to elucidate the pathophysiology, in conjunction with muscle specific (as opposed to whole body) measurements.

Intracellular regulators of muscle protein synthesis and degradation have received significant attention in animal models, and in humans. Whilst comparison of actual protein turnover and intracellular signalling pathways in humans have yet to show the concordance demonstrated in rodent models, there seems little doubt that these pathways are key regulators of muscle mass.

What remains unclear is the complex interdependent relationship between the components of the pathways. Several proteins have demonstrated non-linear activation (polyubiqutin, GSK3B, 4EBP-1 [281]) or autoregulation (mTOR [281]) and it is likely that the oft-suggested binary relationship between active/inactive (or phosphorylated/non-phosphorylated) forms is too simplistic.

7. Mechanisms governing protein homeostasis

A starting point in understanding the components (or more accurately component groups) of these pathways is to understand their roles in normal protein homeostasis. This delineates both their function, and the irreconcilable interdependence of these components: whilst the search for a final "regulator" continues, this seems less and less likely to exist. Rather more plausible will be the existence of a group of regulators that may be amenable to therapeutic targeting.

The processes of Muscle Protein Synthesis (MPS) and Muscle Protein Breakdown (MPB) are the end product of a complex series of intracellular processes. We currently lack an understanding of the relationship between elements of the pathways to each other and to the ultimate end product-protein turnover[138]. Recent work in humans has further demonstrated dissociation between signalling and processes of MPB and in MPS [282].

7.1 Intracellular drivers of anabolism

The process by which new proteins are constructed is relatively well known. Protein synthesis is an energy dependant process, with three phases, controlled in part by three groups of proteins: initiation (controlled by Eukaryotic Initiation Factors, or EIFs), elongation (Eukaryotic Elongation Factors, EEFs) and termination (Eukaryotic Release Factors, ERFs). Following termination, proteins undergo tertiary and quaternary structure development-folding. These groups of proteins are, in turn, controlled by pathway components upstream, which are in turn modulated by a variety of proteins and stimuli.

Upstream control of anabolism is mediated by several pathways, convergent on Protein Kinase B (AKT) in the main, with some independent activity via the Insulin-receptor substrate (IRS-1) [283]. Regulation of activity of these pathways is not completely understood, and though commonly

referred to as the Insulin-like Growth Factor 1/ Phosphotidylionositol-3-kinase/Protein kinase B (IGF-1/PI3K/AKT) pathway, anabolism can also occur via other signalling molecules such as Nuclear Factor Kappa Beta (NFκB) [283, 284]. Membrane receptor control of anabolism is poorly understood, but the activators of IGF-1/PI3K/AKT and NFκB pathways are believed to be key.

7.2 Intracellular drivers of catabolism

Protein breakdown is, however, less well understood. Whilst it is an essential component of homeostasis, breakdown also provides amino acids necessary for gluconeogenesis and energy production during states of energy deficit [136]. This dual nature no doubt contributes to the complex physiology of the process of MPB. Three pathways have been described which control protein breakdown in humans: the autophagy-lysosomal pathway, the cytosolic proteases and the ubiquitin proteasome pathway.

7.2.1The autophagy lysosomal pathway

Extracellular and cytosolic proteins are taken up by endocytosis and degraded within the lysosome by acid-based proteases such as cathepsins and acid hydrolases [136, 285, 286]. Current belief is that the lysosomal pathway deals with extracellular and membrane surface proteins turnover rather than cellular turnover under normal circumstances [285, 287].

7.2.2 Cytosolic proteases

Calcium activated proteolysis involves the cysteine proteases called calpains [288]. The cytosolic proteases are independent of ATP, acting by responding to a rise in intracellular calcium and therefore likely to play a role in tissue injury and necrosis [285, 288]. It remains unclear as to their role in normal skeletal muscle.

Caspases (also known as Interleukinβ converting enzyme related proteases) are part of the apoptotic pathways. They respond to DNA damage or noxious stimuli, and lead to programmed cell death[285].

7.2.3 The Ubiquitin-Proteasome Pathway (UPP)

First described in the 1970s, this ATP dependant pathway is considered to be the mechanism via which the bulk of intracellular protein is degraded [246, 285, 289, 290]. Ubiquitin activating enzymes (E1 ligases) create an active form of ubiquitin, and bind it to an ubiquitin carrier protein (E2 ligases). The ubiquitin is then transferred to the substrate by an ubiquitin protein ligase (E3 ligases, such as Muscle Ring Finger-1(Murf-1) or Atrogin-1 (also known as MAFbx)). This occurs repeatedly until a poly-ubiquitin chain is formed, which is recognised by the 26s proteasome and degraded by the 20s core.

The UPP has been demonstrated to be the final common proteolysis pathway in disease models of starvation [291], diabetes [292], acidosis [259] cancer cachexia [136], sepsis [146], disuse [291], and glucocorticoid therapy [293]. In healthy tissue blocking of other pathways leads to only a minor reduction in protein breakdown [294]. An important caveat to this is our lack of awareness of all components of the UPP, and their relationship to each other. For example the MuRF-1 component of the UPP is regularly measured [285, 290]. Murf-2 and Murf-3 have now been shown to exist, and other currently undiscovered proteins may too [295, 296]. One feature of the UPP relevant to critically ill patients that must be considered is its ATP dependant nature [295, 297]. Mitochondrial dysfunction has been demonstrated in critically ill patients, and the ATP availability of cells in such patients is unknown [139, 298]. All studies mentioned above were carried out using animal models, either *ex vivo* or *in vivo*. Whilst there are numerous issues with interpreting this in the clinical context, it is worth noting that up-regulation of the UPP has been documented in many disease states in humans, including COPD [299], sepsis [300, 301], trauma [302], statin myopathy [303], cancer cachexia [286], burns [304] and in human experimental models of immobilisation [305], and in response to exercise [306].

7.3 Pathway components of interest

Figure 5 is a simplified schematic of the pathways purported to govern the processes of MPS and MPB. These proteins have complex interdependence and effects [281], much of which remains unclear. This is likely to be a major contributory factor to the dissociation demonstrated in human studies between actual protein synthesis and breakdown rates, and measurements of these proteins[282]. Currently, no single protein in the cascades mediating anabolism or catabolism has been demonstrated to be a useful biomarker, or potential therapeutic target.

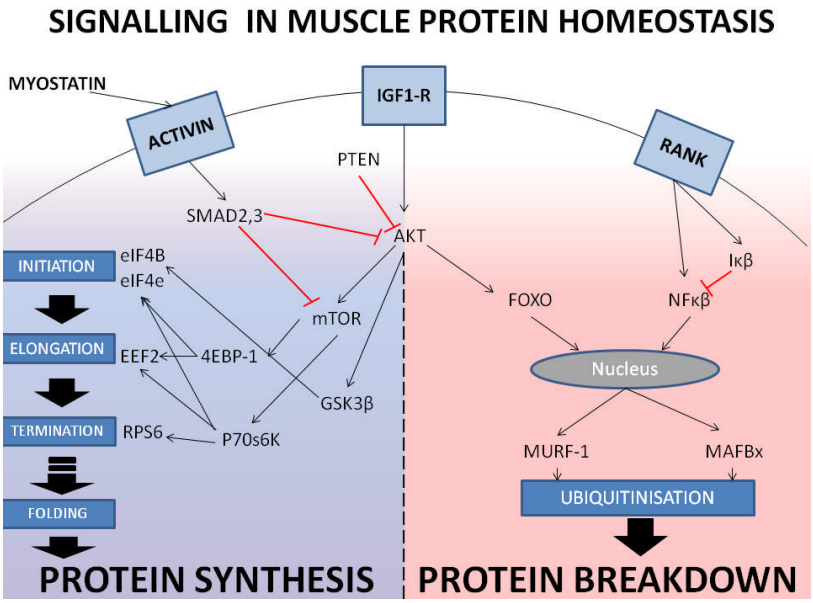


Figure 5: Anabolic and catabolic pathways involved in muscle protein homeostasis. IGF1-R= Insulin like Growth Factor-1, PTEN= Phosphatase and Tensin homolog, AKT= Protein Kinase B, FOXO= Forkhead Box Class O-1, MURF-1= Muscle Ring Finger protein 1, MAFBX= Muscle Atrophy F-Box-1, NFκβ= Nuclear Factor Kappa Beta, Iκβ= Inhibitor of Kappa Beta, RANK= Receptor Activator of Kappa Beta, mTOR=mammalian Target Of Rapamycin, 4EBP-1= Eukaryotic Initiation Factor 4E binding protein 1, P70s6K= 70-kDa S6 protein kinase GSK3B=Glycogen Synthetase Kinase 3 beta, EIf4B= Eukaryotic Initiation Factor 4-B, EIF4e=Eukaryotic Initiation Factor 4e, EEF2=Eukaryotic Elongation Factor 2, RPS6=Ribosomal Protein S6. SMAD 2,3= vertebrate homolog of Drosophila protein MAD and Caenorhabditis protein SMA 2,3.

Akt

Akt is a serine/threonine protein kinase, also known as Protein Kinase B (PKB). In humans, 3 separate Akt proteins exist (Akt-1 Akt-2, Akt-3) [281]. Akt is a regulator of mTOR and FOXO, and is in turn (partially) regulated by PI3K[281]. Precisely how the functions of Akt isoforms differ is not understood. Phosphorylation of Akt inactivates FOXO and therefore inactivates protein breakdown mechanisms. This has been demonstrated to lead to muscle hypertrophy, with upregulation of the protein synthetic pathway [307].

FOXO

The Forkhead Box O (FOXO) group of transcription factors are inactivated (phosphorylated) by Akt [290]. Active FOXO (or, more specifically, FOXO-1) has been shown to translocate to the nucleus and to upregulate mRNA expression and protein production of E3 ligases (and thus promote muscle breakdown) [308, 309]. In the setting of critically ill patients, the exact role of the FOXO group is increasingly unclear, with emerging evidence of a pleiotropic role [124].

E3 ligases

The role of the E3 ligases has been described above. It is generally agreed that they play a role in muscle protein breakdown, and have been shown to be upregulated in critical illness [310]. Much remains unknown in regards to the exact nature of this role and contribution: knockout mice are only slightly more resistant to atrophy compared to normal mice [311].

NFκβ

Nuclear Factor Kappa Beta (NFκβ) is likely to be involved in muscle atrophy in the critically ill. It has been shown to upregulate E3 ligases in an Akt independent fashion [284, 312, 313]. A parallel pathway to Insulin like Growth Factor 1 (IGF-1), NFκβ is likely to be involved in muscle atrophy in the critically ill. It is activated by members of the Tumour Necrosis Family (TNF), and up-regulation has been demonstrated in sepsis and disuse [312, 313]. A specific member of the TNF family TWEAK (TNF-related Weak Inducer of Apoptosis) has been seen to induce muscle atrophy [314]. Animal

studies have linked the NFκB pathway to ubiquitinisation, as a mode of promoting protein breakdown [284].

As demonstrated by figure 1, Akt plays a central role in "switching" from anabolism to catabolism and vice versa. Several signalling molecules have modulatory effects on Akt and were therefore examined.

IGF1R

Insulin-Like Growth Factor 1 receptor is the substrate of IGF-1 (Insulin-like Growth Factor 1), which can be activated by repeated muscle contraction, and which can block transcriptional upregulation of atrogenes [309]. However, blocking the IGF-I receptor does not impair hypertrophy of muscle in response to mechanical overload or indeed activation of the AKT pathway [315, 316].

IRS1

The Insulin receptor substrate 1 (IRS-1) is phosphorylated by the Insulin receptor, in response to insulin of IGF1R [317]. IRS-1 mediates the effects of IGF1R activity on Akt [317].

PTEN

The Phosphatase and Tensin homolog on chromosome 10 (PTEN) is a phosphatase counteracting Phosphoinositidine-3OH kinase (PI3K) activity by dephosphorylating its active products, phosphatidylinositol bisphosphate and phosphatidylinositol triphosphate (PIP2 and PIP3) [281]. PI3K is an upstream regulator of the mammalian target of rapamycin (mTOR) [281].

The intracellular signalling governing anabolism is better understood. Key elements are:

GSK3β

Glycogen Synthetase Kinase 3 Beta (GSK3β) is downstream of Akt. Upregulation of the active form is associated with hypertrophy [318]. GSK3β has regulatory control over the eukaryotic initiation factors- a key role in the process of protein synthesis [319]. However, like FOXO, GSK3β is a pleiotrophic molecule, and is involved in a range of regulatory processes [320].

mTOR

The mammalian target of rapamycin (mTOR) regulates protein synthesis by modulating its downstream targets: the Eukaryotic Initiation Factors (EIFs), Eurkaryotic Initiation Factor 4E binding protein (E4BP-1) and p70S6 kinase[281]. Whilst mTOR itself is downstream of several other regulatory proteins, it is worth noting that auto-phosphorylation can occur via an intrinsic serine/threonine pathway [321]. The exact nature, and upstream/downstream effects of such intrinsic regulation remains currently unknown, but-it is likely that mTOR expresses environment-sensitive receptors, and responds to alterations in surrounding nutrient and energy levels [281].

P70s6K

Currently, the role of the S6kinases is understood to be in upregulation of the translational apparatus of mRNA (e.g. ribosomal proteins and elongation factors) [322, 323]. In addition, the 70 kDa ribosomal protein S6 kinase (P70S6K) inactivates E4BP-1 via phosphorylation, aiding initiation of protein synthesis [281, 324].

4ebp1

The Eukaryotic Initiation Factors (EIFs), as a group, regulate ribosomal recruitment and activation. Between the EIF4 factors, mRNA is bound and unwound, facilitating binding to the 40s component of the ribosome [325]. The 4-Eukaryotic Binding Protein family (4EBPs) are repressors of the EIFs (by preventing binding of the EIFS to each other) and are inactivated by phosphorylation by mTOR [281]. Under many circumstances, initiation and ribosome binding has been suggested as the rate limiting step in protein synthesis [326].

EEF2

Eukaryotic Elongation Factor 2 (EEF2) is a translation factor, inactivated by phosphorylation, mediated by a specific kinase-EEF2 kinase. EEF2 has at least three phosphorylation sites, which mTOR acts on [281].

Myostatin and other pathways

In recent years, it has become increasingly clear that although the pathways described above (and in particular the UPP) are likely to be the final pathways of protein breakdown, *control* of these pathways is both complex and poorly understood.

Myostatin (otherwise known as GDF-8) is a member of the Transforming Growth Factor Beta (TGF-β) family, which promotes muscle atrophy and inhibits satellite cell renewal [327]. Knockout and mutation studies have demonstrated muscle hypertrophy in animal studies [328, 329], and in humans [330]. Myostatin binds to the Type IIB activin receptor, and acts via phosphorylation of Smad2 and Smad3 (mothers against decapentaplegic homolog) in turn de-phosphorylating FOXO [331], and inhibiting muscle protein synthesis by inhibiting mTOR [332, 333]. Myostatin also acts via non-Smad pathways- the MAPK[334], ERK 1/2 [335] and JNK pathways [336] have all been implicated. Whilst promising results from blockade of this pathway exist, translation into human studies has yet to occur [337]. Furthermore, Myostatin *per se* may not be a key regulator, but one of a group of proteins acting on the activin receptor [338]. Like many highly conserved proteins, Myostatin has pleiotrophic effects in metabolic modulation [332], and its exact role at this stage remains to be defined.

7.4. Exploring intracellular signalling in critically ill patients

Performing invasive physiological experiments on critically ill patients is difficult. Aside from the technical difficulties of invasive procedures (e.g. haemostasis, infection prevention) and physiological measurements (intrinsically difficult in unstable patients), the physical environment is challenging. Furthermore, communication with relatives to gain informed assent is fraught. In order to gain further insight into altered physiology, researchers have sought to extrapolate from other scenarios- either healthy humans exposed to pathological stimuli thought to affect muscle homeostasis (e.g. immobilisation or inflammation) or by constructing animal models of critical illness

[5, 339]. Human experiments are useful in the dissection of the varied insults critically ill patients are exposed to, allowing assessment of these insults as causative or not. This is limited in that 1) many purported insults are difficult ethically to recreate in healthy volunteers (e.g. prolonged neuromuscular blockade without sedation) and 2) the cumulative effect of multiple insults cannot be measured (for the same reasons).

Animal models therefore have distinct attractions. Researchers are able to perform good longitudinal interventional and observational work, with robust controls. Multiple insults and stimuli can be measured, dose-titrated and modelled. Indeed much of our mechanistic understanding of science has been derived in this fashion.

Unfortunately the clinical utility of animal work is limited, in that extrapolation to humans is limited in some fields. Muscle physiology is a prime example of this [340, 341]. Total protein turnover in adult rats is 3-4 times greater than in humans, with a approximately 2.5-fold greater protein synthetic rate [342].This is likely to be related to the differences in metabolic stability (the ability to maintain homeostasis) between mice and humans-the basal metabolic rate per gram body weight is 7 times greater in rats [339] and as a consequence there are different rates of ageing between the two[343]. Rodents used in studies are often immature and still growing, very much unlike human subjects in clinical trials. A recent review delineates the list of animal studies where very short periods of muscle unloading (5 hours) initiates a proteolytic response in animals, whereas humans regularly unload their muscles for 8 hours via sleep to no such effect [138]. Phillips *et al*. crystallize succinctly the effect these differences have conceptually-studies on rodents would indicate that protein breakdown is the driving force in muscle mass homeostasis, yet for the reasons outlined above, comparisons between humans and rodents are likely to be fundamentally flawed. Furthermore, these conclusions are in the main drawn from studies on the Ubiquitin Proteasome Pathway rather than actual measures of breakdown- a further flaw. Finally, Phillips argues that many studies were performed in the *ex-vivo* setting, where normal rat muscle display lower MPS and

higher MPB than in vivo- alterations affecting MPS would then be interpreted falsely as affecting MPB.

In summary, animal studies remain currently invaluable for mechanistic and structural understanding of muscle physiology. In respect to translation into clinically useful ideas and measures, their contribution remains unclear. If we are to clearly understand the effect of critical illness on muscle physiology (and thereby develop anti-wasting interventions), human studies are needed.

7.5 Studies in critically ill patients

Several studies have examined the alterations in anabolic and catabolic signalling in critically ill patients. *Interpretation* of these studies is difficult, as a result of significant methodological flaws. Sample sizes have been small with cross sectional rather than longitudinal data. Standardisation of biopsy time points has not occurred. Whilst a single study has suggested a homogeneity of metabolic response to critical illness [117, 118], we are currently unaware of how sex, age and presenting illness affect muscle mass loss in the critically ill. These studies lack measurements of actual alterations in muscle mass, making temporal and effect associations difficult. Critical illness is a dynamic process [217, 344-346]with secondary complications common [19-21]. Biopsies taken at a single time point are unlikely to reflect the complex metabolic adaptation that occurs in the critically ill. Longitudinal data are needed, coupled to objective measures of muscle mass and clear patient phenotyping with simultaneous measures of dynamic protein turnover. Currently this has yet to be performed.

Within these limitations, cross-sectional data have suggested increased anabolic signalling with blunted proteolysis [347]. Others have suggested increased proteolysis, but have been limited by lack of anabolic signalling measurements [279, 301, 304, 348]. Constantin *et al.* have added further levels of complexity- in 10 patients and 10 aged matched controls, they demonstrated increased E3 ligase protein concentrations and dephosphorylation (inactivation) of anabolic signalling [310]. However this was accompanied by increased mRNA levels of anabolic signalling- implying a

cellular programme of anabolic stimulus in the face of muscle loss, possibly in preparation of recovery.

7.6 Muscle wasting in the critically ill- gaps in our knowledge

Muscle wasting in critical illness is common, and has significant consequences for patients, carers and the healthcare and welfare systems, as a result of protracted functional disability. The pathogenesis of muscle wasting remains unclear, specifically with regard to altered protein homeostasis. Animal models and patients suffering from burns are not appropriate models to extrapolate from, as a result of fundamental differences in metabolism. This lack of understanding prevents the development of prophylactic measures and therapeutic interventions.

I therefore propose the following hypotheses:

H1 Loss of muscle mass occurs early and rapidly in critical illness

H2 Critical illness depresses muscle protein synthesis and increases its breakdown

H3 Protein turnover dysregulation is the result of decreased activity of the anabolic pathways, and increased activity of the catabolic pathways

H4 Clinical indicators may be used to predict H1-3

I sought to examine these hypotheses in a prospective longitudinal study of critically ill adult patients. All were intubated and ventilated, and expected to remain so for 48 hours, spend at least 7 days in intensive care, and survive. Patients were recruited on day 1 of intensive care admission, and underwent serial measurements of Rectus Femoris Cross Sectional Area, serial muscle biopsies for Fibre cross sectional area measurements and Protein/DNA ratios. Furthermore signalling molecules of components of the anabolic and catabolic signalling pathways were examined by western blotting. In a sub cohort, serial infusions of stable isotopes were performed with concurrent muscle biopsies and arteriovenous sampling to determine Leucine incorporation and Phenylalanine dilution.

Chapter 2 described the methods used and chapter 3 the characteristics of patients recruited. Chapter 4 and 5 outline the results of investigations into alterations in muscle mass and quality

respectively. Chapter 6 describes protein homeostasis as measured by stable isotope studies and chapter 7 the anabolic and catabolic signalling pathways.

METHODS

1. Study overview

As discussed in chapter 1, muscle wasting is a common consequence of critical illness in adults, causing early functional dependence and seeming likely to contribute substantially to subsequent functional limitation (figure 6) [349]. Survivors attempt to regain muscle mass and functional independence.

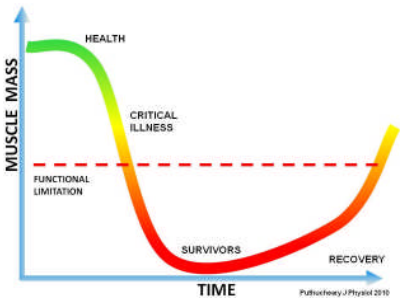


Figure 6: Schematic of acute muscle loss and recovery

However we do not know

1. its time course and extent
2. The role of altered protein homeostasis (balance of synthesis/ breakdown)
3. Its clinical drivers and associations, or
4. The nature and extent of qualitative changes within skeletal muscle

I therefore set out to address these gaps in our knowledge through a prospective study of adult general intensive care patients, amongst whom serial muscle biopsies were collected.

1.1 Time course and extent

There is no 'gold standard' for assessing muscle wasting in such subjects, amongst whom a variety of confounders may affect any one measure. Three techniques were thus applies:

- Muscle ultrasound
- Protein/DNA ratios
- Histological assessment of muscle for fibre cross sectional area

1.2 Role of altered protein homeostasis (balance of synthesis/ breakdown)

- Assessment of Leucine incorporation and Phenylalanine dilution
- Alterations in protein concentrations of signalling molecules driving protein homeostasis

1.3 Clinical drivers and associations

- The collection of detailed physiological data
- The collection of serial blood samples for appropriate study-specific biochemical analyses

1.4 Nature and extent of qualitative changes within skeletal muscle

- Histological analysis of muscle

Each of these elements will be dealt with in turn in this chapter.

2. Study Design

A prospective observational cohort study was registered at www.clinicaltrials.gov as NCT01106300. Ethical approval was given by the *Joint UCL/UCLH Committees on the Ethics of Human Research Committee A* COREC ref: 05/q0505/30. Research and Development approval was given at each hospital site, and a consortium agreement was drawn up instead of a Material Transfer Agreement, for exchange of specimens across sites.

2.1 Patient Recruitment

Patients were recruited from the Whittington Hospital NHS Trust and Kings College Hospital NHS Trust ICUs. Infra-structure was developed at St Thomas' Hospital ICU initially, to ensure adequate subject availability, although this was ultimately not needed to complete recruitment. Ethical approval for assent under section 17 of the Metal Capacity Act was given. Whilst those persons eligible to give assent included a wide group, only those considered to be next-of-kin were approached, given the highly emotive setting. Retrospective consent was sought from surviving patients, once capacity was regained.

2.2 Inclusion and exclusion criteria

All adult invasively ventilated patients admitted to the ICU who were considered likely to remain intubated for 48 hours, remain on the ICU for 7 days and to survive were considered eligible for study. As no current scoring system is able to predict this, clinical experience and acumen was relied upon (as is standard practise in such trials). To fit the inclusion criteria, ZAP (having successfully completed Intensive Care training in the United Kingdom and with senior fellow experience) and the senior clinician in charge of the patients had to concur.

Excluded were those who

1. were pregnant
2. had active disseminated malignancy
3. were unilateral/bilateral amputees
4. suffered from a primary neuromyopathy
5. were participants in an interventional trial
6. were inappropriate for clinical reasons (e.g. victims of domestic violence, admissions secondary to iatrogenesis, presence of a language barrier)

To be maintained in the study, patients had to continue to meet inclusion criteria. Thus if a patient was extubated in <48 hours, left the ICU in <7days or died <10 days (primary analysis criteria), they were thereafter excluded.

2.3 Database construction and content

A bespoke database was constructed by Montgomery Solutions (London, UK), compatible with SQL Server®2008 R2 Management Studio (Microsoft Corporation, USA). Data was collected at the bedside using a proforma, and entered into the database at a later stage. The full proforma is in appendix X but, in summary, I documented data relating to:

- Admission physiological data
- Sex
- Admission age and co-morbidities
- Six-hourly arterial blood gas analyses, heart rate, blood sugar and blood pressure
- Daily blood profiles
- Daily Sequential Organ Failure Assessment scoring
- Sequential nutritional and Insulin supplementation
- Drugs administered
- Daily fluid balance

2.4. Bedside protocol

Simultaneous serum, plasma and urine samples were taken. Muscle biopsies were performed ***EITHER*** on days 1, 3, 7 and 10 ***OR*** on days 1 and 7 with primed tracer infusions.

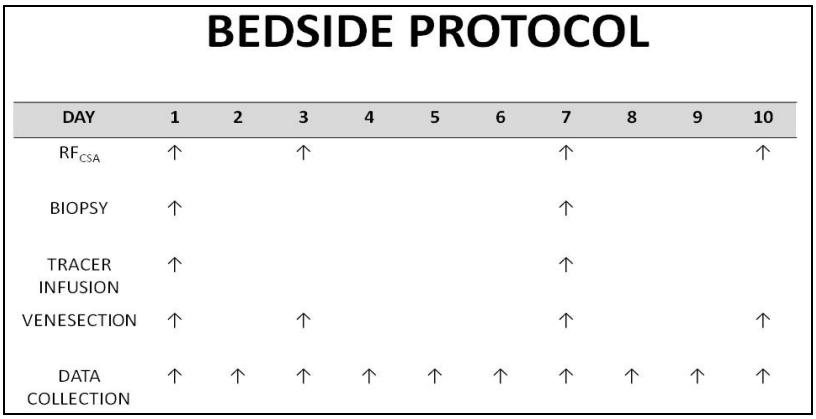


Figure 7: Bedside protocol of the MUSCLE study. RF_{CSA}= Rectus Femoris Cross Sectional Area

2.5. Tracer infusion protocol

In healthy normal subjects, the dose of [1,2-$^{13}C_2$] Leucine and D_5-Phenylalanine used for primed constant infusions are based on actual weight. However, this poses a challenge in a study such as this: the use of weighing beds and slings were not practical, given the severity of disease on day 1, whilst the impacts of fluid resuscitation on weight could not be readily determined. A pragmatic approach was thus taken, that being to use a dose based on a 90kg weight in the anticipation that only if patients were to weigh a significant amount more would the incorporation of Leucine or dilution of Phenylalanine be under detectable limits.

Thirty millilitres of D_5-Phenylalanine and 30 ml [1,2-$^{13}C_2$] Leucine were thus co-administered from a single 60ml syringe, with a prime dose of 10.2ml, and a constant infusion at 17.6ml/hr.

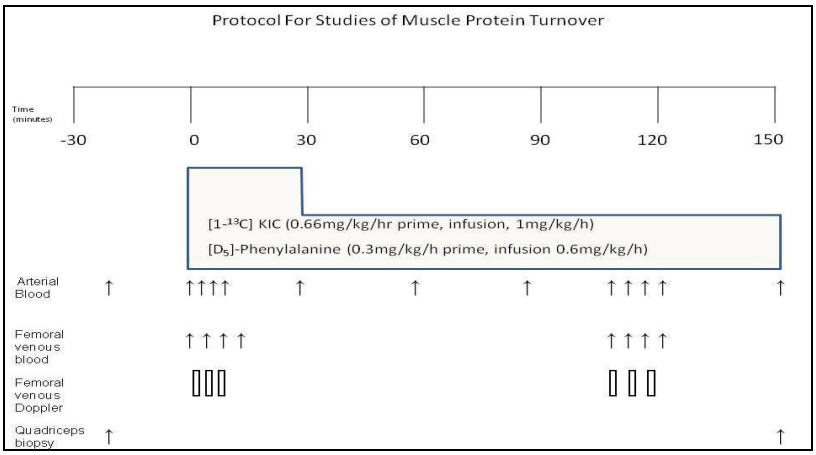


Figure 8: bedside protocol for primed constant isotope infusions

3. Muscle biopsy protocol

3.1 Biopsy technique

The Conchotome technique was used [350]. Preference for this technique over the Bergstom needle was a result of the familiarity of use within the critical care setting (by Prof Mervyn Singer [139] and Dr John Coakley [68].) Training was provided by both Prof Singer and the laboratory staff at the department of Clinical Physiology in Nottingham, under Prof Michael Rennie. The belly of the right Vastus Lateralis was located clinically, and prepared using universal sterile techniques. Xylocaine with 1% Adrenaline (1:200 000, Astra Zeneca, London, UK) was infiltrated. The skin and fascia were incised with a scalpel. Henkel-Tilley forceps (5.5mm x114m, www.surgicalholdings.co.uk) were used to obtain biopsy specimens of at least 50micrograms, which were then processed at the bedside for histological assessment, or snap frozen in liquid nitrogen and stored at -80^{o} C for Leucine incorporation or intracellular signalling pathway analysis. All equipment was disposable, bar the forceps, whicnthropomorphich were sterilised according to hospital guidelines.

3.2 Coagulation

Patients were biopsied if they were not coagulopathic, as defined by International Normalised Ratio (INR) <1.6, Activated Partial Thromboplastin Time Ratio (APTTR) <1.6 and platelet count > $40x10^9$/litre. Decisions were made in conjunction with the multi-disciplinary team caring for the patient- in the cases of disagreement regarding the safety of a biopsy, no procedure was carried out. If any coagulation abnormalities were to be corrected, muscle biopsy was timed to occur during transfusion of the required blood products.

3.3 Post biopsy care of the patient

Each biopsy was closed using steri-strips™ (3M, Nexcare, Bracknell, UK) before being dressed with an absorptive dressing, covered by a Tegaderm™ dressing (3M, Bracknell, UK). Wounds were visually inspected by ZAP and/or JR every 48 hours, or by bedside staff as clinically indicated.

4. Non invasive measurements of muscle mass

4.1 Ultrasound measurement of Rectus Femoris Cross Sectional Area

Rectus Femoris Cross Sectional Area (RF_{CSA}) was measured on days 1, 3, 7 and 10 by B-mode ultrasonography, using an 8MHz 5.6cm-linear transducer array (PLM805, Toshiba Medical Systems Ltd, Crawley, UK) at Kings College Hospital, and a 6-15MHz 4cm linear transducer array (Phillips Envisor HD 1.3 system with Pan-view software, Guildford, United Kingdom) at the Whittington Hospital. This was performed using a similar method to that of Seymour *et al.* [92]. The transducer was placed perpendicularly along the superior aspect of the thigh, two-thirds of the distance between the anterior superior iliac crest and the superior patellar border. Patients were supine with a 30^{o} upwards incline at the head, except where the clinical conditions dictated otherwise. An excess of gel was applied to minimise distortion. At Kings College Hospital, RF_{CSA} was calculated using inbuilt planimetric software (Nemio™, Toshiba Medical Systems Ltd, Crawley, UK) and, at the Whittington Hospital, using off-line using Image J (National Institute of Health, U.S.A). RF_{CSA} was taken as an average of three consecutive measurements within 10% of one another.

4.1.1 Training

Training was performed by both Dr Paul Sidhu (Consultant radiologist at Kings College Hospital) and Dr John Seymour (whose PhD had focussed on muscle ultrasound). Beginning with basic anatomy and methodology, training then progressed through bedside scanning of patients. Competence was considered attained when blinded measurements were within 10% of JS, and PS reviewed scans off line to ensure a lack of anatomical distortion (the result of poor technique).

4.1.2 Reproducibility

Two blinded operators (ZP and BC) performed RF_{CSA} scans on 1 in 3 subjects. A mean of three readings was taken. Correlation coefficients were calculated and a Bland-Altman plot generated.

4.2 Anthropomorphic measurements

Serial measurements were taken of Mid Thigh Circumference (MTC) on days 1 and 10. Measurements were taken at the same level as RFCSA, that being two-thirds of the distance between the anterior superior iliac crest and the superior aspect of the patella. Mid Arm Circumference (MAC) was measured simultaneously, at the midpoint between the olecranon process of the ulna and the acromion process of scapula. Both points were marked and covered with a waterproof transparent dressing for repeated measurements.

5. Protein/Deoxyribonucleic Acid ratio (Protein/DNA ratio)

Quantification of the ratio of protein to deoxyribonucleic acid (protein/DNA; Qubit®, Life Technologies™, Paisley, UK) was performed by an external group (qStandard, London, UK) blinded to all subject data. The technique is as follows:

5.1 Homogenisation

Approximately 1 mm^3 piece of muscle tissue was removed from a frozen biopsy sample on dry ice and placed into a 2ml tube containing a cooled 5 mm steel bead and 300µl cold lysis solution (2% SDS, 5 mM EDTA, 1% protease inhibitor cocktail). Samples were homogenised cold in a Tissuelyzer LT (Qiagen) at 50 Hz for one minute. Lysates were centrifuged at 13,000 rpm at $4^{o}C$ for 5 min to pellet debris. For each sample, a 5 µl aliquot of the clear supernatant was transferred to a 1.5 ml tube and diluted 40-fold with nuclease-free water.

5.2 Protein concentration

A working solution of Quant-it protein assay buffer (Invitrogen, Life Technologies, Paisley, UK) was prepared according to manufacturer's instructions. In order to ensure that standards contained the same concentration of SDS and EDTA as the samples, protein standards were prepared by mixing 180 µl working solution with 10 µl 20-fold diluted lysis solution and 10 µl protein standards. Sample tubes were prepared in triplicate by adding 20 µl 40x diluted lysate supernatant to 180 µl of working solution. Samples were incubated at room temperature for 15 min. The Qubit fluorimeter was calibrated with three protein standards and sample concentration was read and recorded in µg/ml.

5.3 DNA concentration

A working solution of Quant-it DNA HS assay buffer (Invitrogen, Life Technologies, Paisley, UK) was prepared according to manufacturer's instructions. In order to ensure that standards contained the same concentration of SDS and EDTA as the samples, DNA standards were prepared by mixing 180 µl working solution with 10 µl 20-fold diluted lysis solution and 10 µl DNA standards. Sample tubes were prepared in triplicate by adding 20 µl 40x diluted lysate supernatant to 180 µl of working solution. Samples were incubated at room temperature for 2 min. The fluorimeter (Qubit 2.0®Invitrogen, Life Technologies, Paisley, UK) was calibrated with two DNA standards and sample concentration was read and recorded in ng/ml. DNA concentrations were converted to µg/ml prior to calculation of the Protein:DNA ratio.

Sample acquisition is described above. Once the sample had been washed in 0.9% saline and blotted to remove adherent blood, mounting could occur [351].

6. Sample preparation

6.1 Sample orientation

For the majority of analyses of interest, transverse sections were needed. Muscle samples were thus orientated

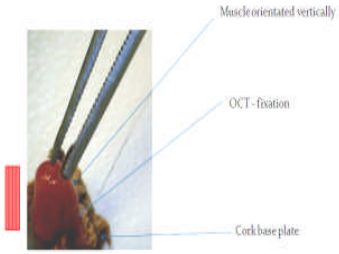


Figure 9: Mounting and orientation of biopsy specimen on cork base, with O.C.T. ® fixation

vertically, and fixed using Optimal Cutting Temperature compound (O.C.T., a proprietary polyethylene glycol mounting agent, Tissue-Tek®, USA) on a cork base (figure 9).

Occasionally, samples are composed of fibres orientated in multiple directions, and/or have a large amount of fat and connective tissue. In these settings blunt dissection using a fine pair of tweezers

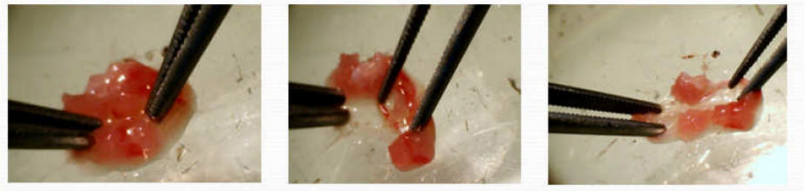


Figure 10: blunt dissection of muscle fascicles

separated the fascicles from the connective tissue (figure 10). These were then aligned on a strip of moist tissue paper with all fascicles orientated vertically. This was then rolled to form a substantive piece (figure 11).

Figure 11: fascicle orientation on wet tissue, with subsequent rolling to form a package for mounting

6.2 Sample freezing

Once mounted successfully, samples were frozen in 100% isopentane (Sigma-Aldrich, Dorset, UK), suspended in liquid nitrogen. This allows cooling by convection, and prevents ice-crystal formation (for example if cooled directly with liquid nitrogen). The sample was then wrapped in foil, taking care to prevent contact (rapid warming and refreezing will cause ice-crystal artefact), and stored at -80^{o}C.

6.3 Sample sectioning

The frozen samples were sectioned on a Cryostat (Bright Instrument Company Limited, Cambridgeshire, UK) at -20^{o}C with the blade set to cut 10 micrometer sections. Two sections were cut on to slide for each biopsy, and 30 slides cut. Cut sections on slides were then stored at -80^{o}C until staining was performed.

7. Histology staining protocols

Both dye-based and immunostaining protocols were used. Choice of protocols was based on the need to assess:

- changes in muscle mass
- alterations in muscle quality

7.1 Staining to assess changes in muscle fibre cross-sectional area ($Fibre_{CSA}$)

Two techniques were used: Adenosine Tri-phosphatase enzyme staining (ATPase) and immunostaining using Anti-Fast antibodies. Fibre cross sectional measurements was carried out using Scion image (National Institute of Health, USA, http://www.nist.gov/lispix/imlab/labs.html). Several criteria were used to judge slides as interpretable for $Fibre_{CSA}$ measurement:

Exclusion (quality control)

1. Distortive ice crystal artefact
2. <70 measureable fibres in transverse section per time point

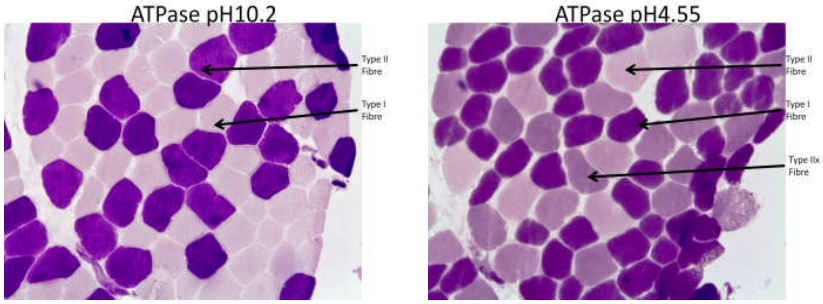
Exclusion (morphometric [351])

1. Fibre destruction
2. Fibre splitting
3. Basophilic or regenerative fibres
4. Time points measured <7 days apart to give a daily rate of loss.

7.1.1 ATPase staining

This method utilises the inherent difference between specific fibre types [351]. Pre-incubation at a specific pH inactivates myosin-ATPase enzymes in specific fibre types. Acid incubation inhibits myosin ATPase in type II fibres, while alkali incubation inhibits myosin ATPase in Type I fibres. ATP is then reintroduced which is consumed by muscle ATPase, releasing Phosphate. This Phosphate is attached to a Calcium ion ($CaPo_4$), replaced with a Cobalt ion, and then precipitated when compounded with ammonium sulphide as a black solid [7].

Analysis of ATPase stained sections allowed differentiation between Type I and Type II fibres and, in the case of the acid stain, Type IIx fibres(Figures 12 and 13). I did not attempt to differentiate between subtypes of Type II fibres.



Figures 12 and 13: Adenosine Triphosphatase (ATPase) staining. Figure 1 shows pre-treatment with alkaline buffer (pH10.2) with light type I fibres and dark type II fibres. Figure 2 shows pre-treatment with acid buffer (pH4.55) with light type II fibres, dark type I fibres and intermediate staining IIx fibres

The protocol used the following reagents:

1. *Acid buffer*: 0.2M Na acetate, pH adjusted to required with glacial acetic acid.
2. *Alkali buffer*:
    a. 3.1g Na barbital+1.17g Na acetate in 100ml dH_20 (Solution A)
    b. 3g $CaCl_2.2H_20$ in 100ml dH_20 (Solution B). Use 1.82g/100 for $.3H_20$ salt
    c. Add A to B, and adjust pH with NaOH or glacial acetic acid as required.
3. 3% $CaCl_2$
4. 1% $CoCl_2$

5. 1% NH_4 polysulphide (by colour, freshly made- exact concentration not important).

ALKALI ATPase	**ACID ATPase**
Incubate one section per pH in alkali buffer (pH 10.2 and 10.4) for 20minutes at RT	Incubate one section per pH in acid buffer (pH 4.5 and 4.4) for 5minutes at RT
Rinse solutions in distilled water once, and in alkali buffer pH9.4 once	Rinse solutions in distilled water once, and in alkali buffer pH9.4 **twice**

Incubate sections in alkali buffer pH9.4 containing ATP at 0.5-1.0mg/ml for 60 minutes at room temperature

Rinse sections in 3x changes of approx 60s each in $CaCl_2$

Transfer sections to $CoCl_2$ solution for 3 minutes

Rinse sections in 3x changes of distilled H_20

Transfer sections to NH4 polysulphide for approx 60s, expecting colour to develop within seconds

Rinse in tap water, dehydrate through alcohols, clear and mount

7.1.2 Anti-Fast antibody immunostaining

On occasion, possibly linked to the qualitative changes seen in the biopsy specimens [114], the ATPase staining did not produce adequate staining for analysis. On these occasions, immunostaining (figure 14) was carried out, using the following protocol:

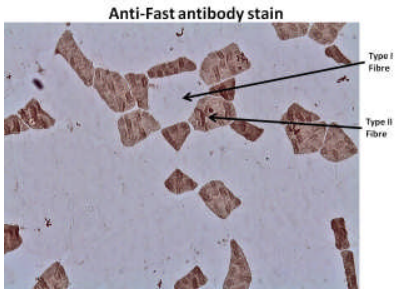


Figure 14: Anti fast antibody stain showing type II fibres. Type I fibres are visible with a slight pink tinge.

1. Wash slides with phosphate buffered solution (PBS)
2. Draw around fibres with hydrophobic pen
3. Incubate with anti-fast antibody (1:6000, dilute with PBS with 1% Bovine Albumin Serum(BSA)) for 3-4 days
4. Wash 3x with PBS, then add 1:100 biotinyalted anti mouse antibody for 1-2 days
5. Wash 3x with PBS then add Streptavidin for 1 day
6. Wash 3x with PBS then add to PBS+H2O2+DAB solution (Diaminobenzidine)
    a. 1 drop of H_2O_2 per 20ml PBS
    b. Use very small amount of DAB

7.1.3 Training and quality assurance

Scion Image (National Institute of Health, USA, www.scioncorp.com) has a bespoke training programme for area measurements. Once this was completed, measurements on 26 fibres were taken and compared in a blinded fashion against the measurements of Dr Anthea Rowlerson and a further 52 against Miss Marie Guojon-Svrzic (a research technician). Competence was considered achieved when the correlation between the two sets was >0.95.

7.2 Development of necrosis, and general qualitative assessment

A Haematoxylin and Eosin stain was used. Haematoxylin is a natural dye, whose active form is the oxidised product haematin [351]. This is

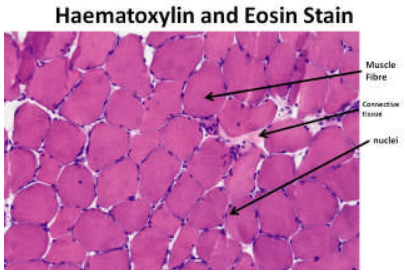


Figure 15: Haematoxylin and Eosin stain. Muscle fibres are red, with black nuclei. Connective tissue stains a paler pink

expensive, so haematoxylin is often oxidised by the addition of iodine-based solutions. It is combined with a metallic salt (termed mordants, in this case potassium or ammonium alum) to make a good nuclear stain (figure 15). Washing in tap water changes the purple nuclei dye to a blue, giving a much better contrast. The Eosin is a red counter stain. As it is dissolved in 95% ethanol, if left exposed to 95% for long it will de-colourise. This can be minimised by rapid progress through the alcohols before clearing and mounting [7].

The protocol is as follows, with these solutions:

1. Cole's haematoxylin solution (Cole 1943)
    a. Haematoxylin 1.5g
    b. Saturated aqueous potassium or ammonium alum 700ml
    c. 1% iodine in 95% alcohol 50ml
    d. Distilled H_2O 250mls

    Dissolve (a) in (d) using gentle heat. Add this to (c) then boil, and then cool quickly. Mix with (b) then filter.
2. Eosin-Phloxine mix
    a. Eosin 1% in distilled water (100mls)
    b. Phloxine 1% in dH2O 100mls
    c. Glacial acetic acid
    d. 95% alcohol 780mls

- Incubate in Haematoxylin for 5 minutes
- Rinse in tap water (and further incubate with acid- alkali if too blue, 10 seconds per check)
- Incubate for 1-3 minutes with Eosin (wash with tap water if too red)

Rinse rapidly in tap water

- 2-3 minutes in 70% 90% and 2x 5min in 100% alcohol,

- Place in xylene I and II for 5 minutes then mount.

7.2.1 Quantification of necrosis

Whilst initially examined and quantified by ZP, final reporting was carried out by Dr Rahul Phadke (Senior lecturer and honorary consultant histopathologist at the National Hospital for Neurology and Neurosurgery, Queens Square, London). Necrotic fibres were identified in HE stained section as fibres with loss of cytoplasmic eosinophilia and basophilic nuclear staining, lytic changes and infiltration by macrophages. More severe and acute necrosis was accompanied by fascial oedema and influx of neutrophils and macrophages. Regenerating fibres were identified on HE stained sections as fibres with increased cytoplasmic basophilia and large vesicular nuclei with prominent nucleoli. Regenerating fibres with or without necrotic fibres were only identified in biopsies taken on day 7 or day 10. Biopsies with sharply circumscribed groups of necrotic/regenerating fibres were excluded from the analysis, as we could not exclude that the necrosis was not related to the previous biopsy.

The biopsies contained 10-15 fascicles, yielding ca. 500 fibres on average when examined under 5 consecutive medium power fields (MPFs) which were sufficient in covering >80% of the section. The necrosis was graded semi-quantitatively; mild 0-5/5 MPFs; moderate 5-10/5 MPFs and marked >10/5MPFs and, as described above, further distinction was made between the pattern (scattered versus circumscribed confluent).

7.3 Detection of collagen

Collagen detection and quantification was attempted initially with the Weigert Van Geison stain, and then with immunostaining.

7.3.1Weigart-Van Geison stain

Van Geison's stain distinguishes between acidophilic tissues. Collagen is an "open" texture

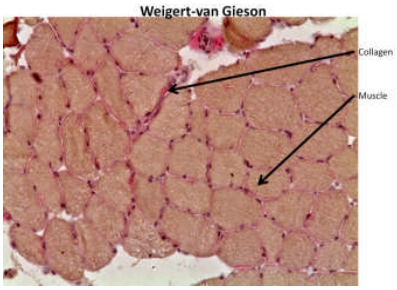


Figure 16: collagen staining (pink), muscle (yellow) and black

material, and therefore stains red. Muscle restricts access to larger dyes, only allowing the yellow stain through. Weigart's is an acid resistant nuclear stain, and is therefore used to stain nuclei (figure 16).

The protocol used the following solutions:

1. Weigart's iron solution: Mix equal volumes of (a) and (b) just before use
    a. 1% Haematoxylin in 95% ethanol
    b. 1-2% $FeCl_3$ in 1%HCL
2. Van Gieson's solution
    a. Picric Acid 100ml
    b. 1% acid fuchsin 5-15ml

Method:

1. Incubate for 1 minute in Weigart's iron solution
2. Wash in tap water- if too blue-wash in acid-alkali briefly
3. Incubate for 1 minute in van Geison. 70% alcohol can decrease staining if too red.
4. Rinse briefly, then dry and mount.

7.3.2 Collagen I antibody staining

The protocol was as follows:

1. Wash in PBS for 15 minutes
2. Draw around sections with hydrophobic pen
3. Incubate with Collagen I antibody (Collagen I antibody (Sigma-Aldrich C2456)) at 1:50, for 3 days.
4. Wash 3x with PBS, and incubate with anti-mouse (Alexa Fluor 488 goat anti-mouse (Molecular Probes A11029) 1:100 for 2 days.
5. Wash 3x with PBS and mount with DAPI.

7.4 Identification of cellular infiltrates

A CD68 stain was used for detection of macrophage infiltrates. The CD68 stain was added to a Laminin stain (outlining muscle fibres), and nuclear counterstaining performed with 4'-6-Diamidino-2-phenylindole (Molecular Probes ProLong Gold anti-fade reagent P36931, Cat. No. P36931). The following antibodies and dilutions were used, in a similar fashion to that described above:

- CD68: (Dako Monoclonal Mouse IgG1 isotype Kappa (anti-CD68) Code M0718) 1:50 dilution for 3 days. Secondary used was Alexa Fluor 488 goat anti-mouse (Molecular Probes A11029).
- Laminin: (Laminin (rabbit, cat. no. Z0097), Dako, polyclonal IG fraction) 1:100 for 3 days. Secondary used was Alexa Fluor 568 goat anti-rabbit (Molecular Probes A11036; Invitrogen A/S, Taastrup, Denmark).

8. Stable isotope incorporation and dilution analysis

8.1 Myofibrillar protein isolation

Muscle (50-70 mg) was minced using fine scissors in ice cold extraction buffer (0.02 M Tris, 0.15 M NaCl, 0.1 M EDTA, 0.1% Triton X). The homogenate was centrifuged at 10,000 rpm for 10 min, the supernatant removed, and the myofibrillar-collagen pellet re-suspended in 0.3 M NaOH. The soluble myofibrillar protein and the insoluble collagen were pelleted by centrifugation. The myofibrillar fraction was removed and precipitated with 1 M perchloric acid; the resulting pellet was washed twice with 70% ethanol and collected by centrifugation.

Myofibrillar protein was hydrolysed in a 0.05 M HCl/Dowex slurry (50W-X8-200 Sigma-Aldrich, Poole, United Kingdom) at 110°C overnight. Amino acids were purified by ion exchange chromatography on Dowex H^+ resin and eluted in 2 M NH_4OH. The amino acids were subsequently derivatized as their N-acetyl-n-propyl ester[352]. Incorporation of [1,2-$^{13}C_2$] Leucine into protein was measured by

capillary gas chromatography combustion isotope ratio mass spectrometry (Delta-plus XL; Thermo Fisher Scientific, Hemel Hempstead, United Kingdom); separation was achieved on a 25 m x 0.25 mm x 1.0 film DB 1701 capillary column (Agilent Technologies, West Lothian, United Kingdom).

8.2 Plasma amino acid and keto-acid labelling

To determine labelling (atom % excess; APE) and concentration of arterialized venous and venous Phenylalanine, 200µl of plasma was deproteinized with 100% ethanol, dried and re-suspended in 0.5 M HCl. Lipids were removed by extraction with ethyl acetate and the amino acids fraction dried then converted to their *tert*-butyldimethylsilyl derivatives by adding 70 µl Acetonitrile and 70 µl MTBSTFA and incubating at 90^{o}C for 60 min.

Venous plasma a-ketoisocaproate (KIC) was chosen as the surrogate precursor for leucyl-t-RNA labelling. Briefly, 200µl of plasma was deproteinized with 100% ethanol and dried down, then the quinoxalinol derivative of KIC was formed by incubation with 200µl H_20 and 100µl 0.15% (ortho-phenylenediamine, OPD) in 4N HCl at 90^{o}C for 60 min. The quinoxalinol was extracted into ethyl acetate, dried down, and further derivatized as the t-butyldimethylsilyl-quinoxalinol by incubating 50 µl Acetonitrile and 50 µl MTBSTFA at 90^{o}C for 30 min. Enrichment of Phenylalanine and KIC was determined by gas chromatography–mass spectrometry (Trace DSQ GC-MS Thermo Fisher Scientific, Hemel Hempstead, United Kingdom), using selected ion monitoring.

8.3 Calculations of muscle protein synthesis and breakdown

The fractional synthetic rate (FSR) for myofibrillar protein synthesis was calculated from the increase in incorporation of [1,2 $^{13}C_2$]Leucine enrichment between subsequent muscle biopsies (E_{m1} & E_{m2}) using the labelling of venous plasma a-ketoisocaproate to represent the average precursor enrichment between biopsies (Ep). The FSR was calculated by using the standard precursor-product

method as follows: FSR (%/h) = $(E_{m2} - E_{m1})/E_p$ x $1/t_2$-t_1 x 100, where t_2 and t_1 represent the time between biopsies.

Leg protein flux (i.e., breakdown) was calculated from the arterio-venous dilution of [D_5] Phenylalanine tracer using the following equation; rate of appearance of Phe, Ra = (E_A/E_V)-1 x C_A x BF, where E_A and E_V are the mean enrichments of phenylalanine at steady state in arterial and femoral venous plasma, C_A is the mean concentration in the arterial blood and BF is blood flow in ml per leg and adjusted for the haematocrit. Measurements were made over the last hour of the 150 min study period, i.e. the mean of 4 separately analyzed samples. The net AA balance was calculated as the difference in arterial and venous concentrations multiplied by the plasma flow, also the average of 4 measurements adjusted for haematocrit.

9. Intracellular signalling molecule quantification

9.1 Luminex platform

The Luminex platform (Millipore xMAP©, Billerica, MA, USA) was used for quantification of the majority of signalling molecules.

9.1.1Principle

Antibodies are attached to coded beads known as microspheres. Each microsphere is filled with a combination of fluorescent dyes, the mixture of which gives each bead a unique identifier. 100 bead identities can be labelled, each of which can be coated with a specific capture antibody. After an analyte is captured by the bead, a biotinylated detection antibody is introduced. The third step is the incubation with Streptavidin-PE conjugate, which acts as a florescent reporter molecule.

Samples are then placed in a Luminex platform machine (FLEXMAP 3D®, Merck, Darmstadt, Germany). The Luminex platform contains two lasers. The firsts excites the dyes within the beads, marking the microspheres individually. The second quantifies the bioassay, based on fluorescent Streptavidin-PE signals.

9.1.2 Arrays used

The Luminex platform assays have distinct advantages and disadvantages over conventional western blotting. Multiple proteins are detectable in a single run (up to a maximum of 11). However the buffers used for homogenisation are proprietary and separate buffers are used for total and phosphorylated isomers – therefore ensuring they cannot be quantified in a single run. This prevents direct comparison of the MFIs of two runs, hence the expression of the data as Phosphorylated: Total ratios. All antibodies and assays were Merck Millipore Milliplex® kits (Merck , Darmstadt, Germany). Assays used were:

Analyte	Total protein	Phosphorylated
AKT	TOTAL	Ser 473
GSK3B	TOTAL	Ser 9
IGF1-R	TOTAL	TYR1135/1136
IRS-1	TOTAL	Ser 312
mTOR	TOTAL	Ser 2448
P70S6k	TOTAL	Thr412
PTEN	TOTAL	Ser 380
GAPDH	TOTAL	
NFKB	TOTAL	Ser 536

Table 4: Merck Millipore Milliplex® kits used

9.1.3 Calibration

Normal human muscle was used to test median fluorescence at different concentration (10 µg/µl, 25µg/µl and 50µg/µl) and increasing numbers of beads (50, 100 and 150), to ascertain the optimum

concentrations needed and minimum numbers of beads. Details of this experiment are in the thesis appendix.

9.2 Western Blotting

Where appropriate antibodies were not available for Luminex analysis, Western Blotting was performed. First described in the late 1970s [353] protein blotting can be used to detect low amounts of proteins in complex tissue-e.g. muscle. Western blotting is a complex from of protein blotting. It involves separation of the protein mixture by gel electrophoresis, and subsequent transfer to a suitable membrane. Protein identification occurs by labelling with a specific antibody, with subsequent immuno-detection.

9.2.1 Protocol for whole muscle homogenisation and preparation

1. Add protease and phosphatase inhibitors to homogenisation buffer (or add before freezing buffer to -80°C).
2. On ice, crush muscle with approx. 0.2ml per 10mg muscle.
3. For soluble proteins, spin at 13000-14000rpm for 10 min at 4°C.
4. Transfer to new tube. Aliquot out, and save pellet separately.
5. Thaw sample at point of use, and mix with equal volume of 2X sample buffer
6. Boil for 5 min (100°C) (may need to make a hole in the top of the eppendorf)
7. Spin briefly to collect droplets.

9.2.2 Choosing Gel for Electrophoresis

(based on 37:1 acrylamide: bis acrylamide ratio)

Gel %	**Protein size**
4-5%	>250kDa
7.5%	250-120kDa
10%	**120-40kDa**
13%	40-15kDa
15%	<20kDa

Table 4: Appropriate acrylamide percentages for different sized protein detection

9.2.3 Loading and Running Gel

1. Load gels onto to gel holder, and fill inner buffer tank first, above level of the wells
2. Flush wells out with 20-50µl of running buffer
3. Check for leaks at this stage- leaks will affect running of gel
4. Load 10 µl of sample into each well, with two wells on either side for 15 µl of marker
5. Fill outer buffer tank to below well line (half full)
6. Place on ice slurry (Ice and water)
7. Run at 100V for 2hours-2hours20 min (Exact time will vary, E4Bp1 runs at 17kDa (lowest green marker, and care must be taken not to run it off!)
8. Transfer gel, on glass plate into tray of fresh transfer buffer, and agitate gently for 20min, to remove salts and allow the methanol to shrink it.
9. If protein readout of the gel is needed, stain with Coomaisey, and destain with methanol/acetic acid combination.

Current should be approx. 60mA per gel (120mA for2), dropping over the run to around 30mA/gel, caused by change in buffer ions-a slow rise in resistance. Ohms law (v=I*R), at constant voltage, a rise in resistance results in a drop in current.

9.2.4 Electrophoretic Transfer

1. Cut a nitrocellulose membrane to size of gel. In most practises, cut top left hand corner to mark orientation. Or accept that the markersx2 will be enough, combined with future labelling with pencil.
2. Soak in distilled water (add at 45 degrees to prevent bubble trapping) then equilibrate in transfer buffer for 10min)
3. If using Polyvinylidene fluoride (PVDF) membrane, activate by soaking in 100% methanol for 10 seconds, followed by distilled water for 10 min, and then transfer buffer for 10min. contrasts between the two are :

Properties	**Nitrocellulose**	**PVDF**
Physical strength	Poor	Strong
Protein binding Capacity	80-100µg/cm^2	100-300µg/cm^2
Storage ability	No	Yes

Table 5: properties of Nitrocellulose and Polyvinylidene fluoride (PVDF) membranes

4. Soak sponges in transfer buffer, and scrunch to remove bubbles.
5. Set up sandwich, rolling out bubbles with a glass tube.

BOTTOM
Black bracket
Sponge
Filter paper X2
Gel
Nitrocellulose membrane

Filter Paper X2 Red Bracket TOP

6. Place tank in colour coded orientation- the current will run protein to red hence "Run to Red". Do not overfill as prevents localisation of current through gel- may "short out".
7. Run transfer for 2.5hours at 100V on ice, starting mA200 which should rise as the system heats up. Ensure a magnetic stirrer is used.
8. Visual verification of transfer and protein loading can be accomplished using Ponceau red staining (0.5g Ponceau S, 1ml Glacial HAc, 99ml dH_2O), which can then be scanned, followed by rinsing with TBS-Tween20.
9. Block membranes for 1 hour with 1% BSA TBS-T ***OR*** use the SNAP-ID™ with 1% BSA.

9.2.5 Antibody incubations

10. Incubate with primary antibody overnight, at 4°C if necessary.
11. Wash quickly with agitation x2 with distilled water.
12. Wash 4X 5min in TBS-Tween.
13. Incubate with secondary antibody
14. Wash quickly with agitation x2 with distilled water.
15. Wash 4X 5min in TBS-Tween.

9.2.6 Chemi-luminescent detection

1. Ensure adequate levels of developing fluid and fixatives, and ensure developer is warmed up
2. Mix ECL detection reagent
    a. 500µl Solution A
    b. 12.5 µl Solution B

3. Using a plastic wallet, blot dry excess liquid and arrange membranes.
4. Apply reagent, 0.5-1ml per blot, avoiding excess.
5. Agitate for 5 min, then wipe the wallet to remove all excess liquid
6. Place wallet in film cassette with x-ray film (with right corner chopped off for orientation) and expose. If background disastrous, two stacked films may overcome this.

9.2.7 Stripping and Reprobing

1. Warm stripping buffer
2. Add 10mls per plastic box,
3. Place in water bath at 50°C for 15 minutes, agitating occasionally
4. Pour off stripping buffer in fume cupboard
5. Rinse quickly 2-3 times with agitation in distilled H_20
6. Wash a minimum of 4X 5min in TBS-T, until all traces of Beta mecaptoethanol are removed
7. Ready for blocking stage.

9.2.8 Quantification of Western blots

Blots were scanned and uploaded into Scion image (National Institute of Health, USA, www.scioncorp.com). Images were grey-scaled and densitometry calculated. Values were normalised against tubulin densitometry.

9.2.9 Antibodies used for Western blotting.

Primary antibody	Supplier	Product Code	Concentration	Monoclonal
T- EEF2	Cell signalling	#2332	1:2000	N
P- EEF2 (THr56)	Cell signalling	#2331	1:4000	N
T-AKT	Cell signalling	#4691	1:1000	C67E7
P- AKT (Ser473)	Cell signalling	#4060	1:1000	D9E
T 4e-BP1	Cell signalling	#9644	1:4000	53H11
P- 4E-BP1 (Thr37/46)	Cell signalling	#2855	1:4000	236B4
T- FOX01	Cell signalling	#2880	1:1000	C29H4
P-FOX01 (ser 256)	Cell signalling	#9461	1:1000	N
Fbx32	Abcam	Ab92281	1:5000	N
MURF1	Abcam	Ab96857	1:4000	N
Secondary antibody	**Supplier**	**Product Code**	**Concentration**	**Monoclonal**
anti goat	Abcam	Ab97110	1:5000	N
Anti rabbit	Cell Signalling	#7074	1:3000	N

Table 6: Antibodies used for Western blotting

10. RNA extraction and Real-Time quantitative PCR

Quantitative PCR was carried out by an independent group (qStandard, London, UK). Quantitative PCR was used to measure mRNA of Murf-1 and MAFBx (to corroborate findings of protein quantification) and mRNA of Myostatin (as no good antibody exists). Their methods are as follows:

RNA integrity was assessed using the Agilent Bioanalyzer. RIN was variable indicating that some RNA exhibited signs of degradation. RIN is moderately correlated with the normalisation factor (Spearman rank correlation, $R=0.561$, $P<0.001$). RNA purity and concentration were measured using a NanoDrop spectrophotometer. A260/280 ratio >1.8 was seen for all samples. The absorbance peak was at 260 nm for all samples, which is the correct peak for nucleic acids A260/230 >0.14 . The low A260/230 ratio for RNA extracted with the RNeasy kit is usually due to high levels of GITC - these do not affect the qPCR. However, the presence of PCR inhibitors that absorb at 230 nm cannot be excluded. Where the RNA concentration permitted, 250ng RNA were reverse transcribed using the Qiagen Quantitect reverse transcription in a 10uL reaction according to the manufacturer's instructions. In cases where the RNA yield did not reach 250ng, 5uL of RNA were reverse-transcribed. This RT kit includes a mandatory gDNA wipe-out step, which was extended to 4 min at 42C for these samples. RT duplicate reactions were performed for nine samples. The completed reaction was diluted 5-fold with 5ug/mL tRNA in water. Two microlitres of cDNA were amplified in a 10uL reaction using Agilent Brilliant III Ultrafast SYBR green qPCR mix with each primer at a final concentration of 500nmol/L. The no-template control reaction contained 2uL of tRNA 5ug/mL. qPCR standards (107-101 copies/reaction) for each gene were included in each run. Amplification parameters: 95^{o}C for 5 minutes followed by 40 cycles of 95^{o}C for 5 seconds, 57^{o}C for 1 second using a Rotor-Gene 6000. Melt curves were checked for product specificity (single peak) and the presence of primer dimmers. All GOI primers used were intron-spanning designs that do not amplify gDNA. Copy numbers/reaction were derived from the standard curves using the Rotor-Gene software. Four

of the five reference genes were identified as most stable using geNorm software and GOI copy numbers were normalised using the normalisation factor. The melt curves exhibited a single peak at the expected temperature and a single band of the correct size was observed on a gel was observed.

Primer name	Primer Sequence	NCBI Accession number(s)
hsa_MSTN_001 fwd	cattaccatgcctacagagtct	NM_005259
hsa_MSTN_001 rev	ctcgacgggtctcaaatatatcc	
hsa_FBXO32_001 fwd	gaagaaactctgccagtaccactt	NM_058229, NM_148177, NM_001242463
hsa_FBXO32_001 rev	atactgctctttccttgggtaacat	
hsa_TRIM63_001 fwd	gagagccattgactttgggaca	NM_032588
REFERENCE GENES		
hsa_B2M_002 fwd	ctctctctttctggcctggag	NM_004048
hsa_B2M_002 rev	acccagacacatagcaattcag	
hsa_GAPDH_001 fwd	tgcaccaccaactgcttagc	NM_002046
hsa_GAPDH_001 rev	ggcatggactgtggtcatgag	
hsa_HPRT1_002 fwd	ggaaagaatgtcttgattgtggaag	NM_000194
hsa_HPRT1_002 rev	gggtccttttcaccagcaagc	
hsa_PLA2G6_003 fwd	tccaaaatcggcagacttgtcac	NM_003560
hsa_PLA2G6_003 rev	gttctaggttgtttaggctgatcg	
hsa_PPIA_002 fwd	cgaggaaaaccgtgtactattagc	NM_021130
hsa_PPIA_002 rev	caccctgacacataaaccctg	
hsa_YWHAZ_001 fwd	acttttggtacattgtggcttca	NM_003406, NM_145690, NM_001135699, NM_001135700, NM_001135701, and NM_001135702

Table 7: Primer name, primer sequences and NCBI accession numbers of both genes studies and reference genes

11. Statistical analysis

Sample size calculations were carried out in Stata V.11 (using the ANCOVA method). Using alpha 0.05 and 50% subject dropout throughout, and 90% power for change in RF_{CSA} and 80% for protein synthesis:

11.1 Rectus Femoris Cross-Sectional Area (RF_{CSA})

Primary end point was 15% RF_{CSA} loss in 10 days[92] (pilot data in the on-line supplement and below in table 8). Thirty patients were needed. Intra-class correlation was estimated using pilot data provided for six patients.

	AGE	SEX	APACHE II	RF_{CSA} in mm^2 (SD)			
				Day 1	Day 3	Day 7	Day 10
1	40	M	20	975(54)	885 (23)	812 (18)	878 (19)
2	30	F	4	1003(25)	872 (19)	796 (3)	851 (14)
3	77	M	34	442(18)	476 (21)	431 (7)	362 (7)
4	57	M	11	589 (19)	487 (24)	481 (7)	456 (5)
5	58	M	15	492 (4)	427 (2)	384 (7)	395 (5)
6	38	M	15	608 (8)	520 (14)	501 (15)	444 (16)
	Mean (SD)			**684 (22)**	**611 (17)**	**568 (6)**[#]	**566 (11)**[#]

Table 8: Pilot data of loss of Rectus Femoris Cross Sectional Area in 6 critically ill patients. # indicated significant change from baseline (Friedman Test, Dunn's post-hoc comparison). APACHE II= Acute Physiology and Chronic Health Evaluation II score. RF_{CSA}= Rectus Femoris Cross Sectional Area. SD= Standard Deviation.

11.2 Muscle protein turnover

Using a coefficient of variance of 15% for measures of protein synthesis, and an expected difference over time of 20%, 10 patients were needed [282]. One-way analysis of variance was to be used.

11.3 Intracellular signalling

11.3.1 Individual molecules

Changes were analysed between days 1 and 7. Paired Wilcoxon Signed rank tests were performed, as the majority of samples did not pass the normality test of D'Agostino & Pearson (Graphpad Prism® 5, GraphPad Software Inc).

11.3.2 Principle component analysis (PCA) of signalling network

First, log-fold change was calculated for each pre-post pair of protein measurements, for every subject and for each protein of interest, as $\log_{10}$ (post/pre). These values were then entered into a 35 x 24 matrix (number of subjects x number of variables) for subsequent PCA. Prior to analysis, missing data were replaced using *mdCheck* and data were *z*-normalized and mean-centred, all in PLS Toolbox 6.5 (Eigenvector Research Inc., Wenatchee, US) running in Matlab 7 (Mathworks, Natick, MA, USA). PCA was then conducted using the *svd* algorithm (again in PLS toolbox). The number of principal components was selected by visual inspection of a screen plot as recommended[354]. Subsequent correlation analysis of principal components vs. other data was conducted using SPSS Statistics 18 (IBM Corporation, Armonk, NY, USA).

11.4 Multivariate and univariate analysis

Multivariate and univariate analysis were performed using statistical software (Statistical Package for the Social Sciences version 17 (SPSS, Inc, Chicago, Ill), and MedCalc version 12.3.0 (MedCalc software, Mariakerke, Belgium). Measures of central tendency for continuous variables were compared using the *t* test (parametric variables) following normality testing and the Mann-Whitney *U* test (nonparametric variables). Parametric variables were reported as mean (SD); and nonparametric variables, as medians (interquartile ranges). Statistical significance (95% confidence interval [CI]) was reported for $p < .05$ with a trend toward significance for $P < .10$. Linear regression

was performed with change in RF_{CSA} at day 7 and again at day 10 as a continuous dependent variable. Independent variables statistically significant in univariate analysis for correlation with RF_{CSA} were entered into a backwards multivariate analysis if the *p* value achieved on univariate analysis was .10 or less. Logistic regression was used to determine predictors of RF_{CSA} change at the 10% level, based on chronic obstructive pulmonary disease (COPD) rehabilitation data [355].

RESULTS: MUSCLE STUDY OVERVIEW

1. Study recruitment

Patients were recruited between August 2009 and April 2011, from the Whittington Hospital NHS Trust and Kings College Hospital NHS Trust. Sufficient patients were recruited from these sites, such that recruitment from St. Thomas' Hospital was not initiated.

1.1 STROBE flowchart

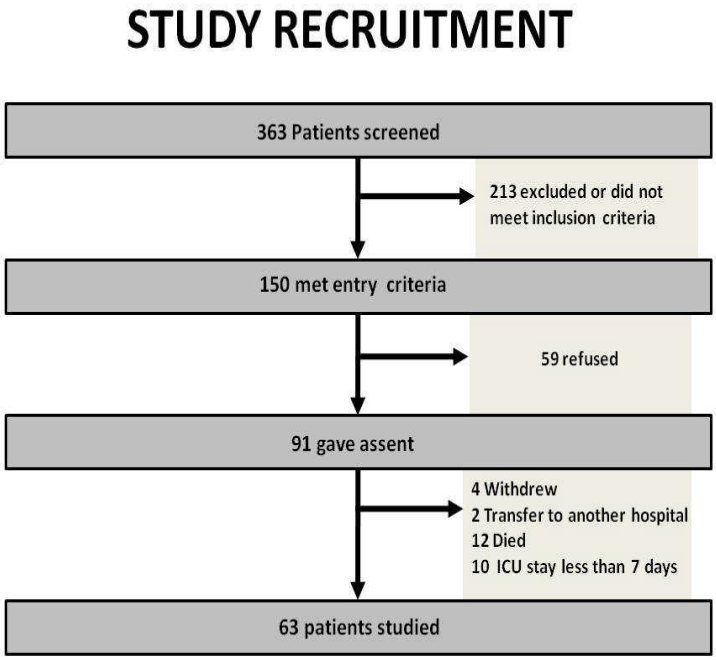


Figure 17: STROBE flowchart of study recruitment

1.2 Cohort sub-divisions

Not all patients received serial muscle biopsies. Of the 63 patients in the study, 42 patients were biopsied, and of those 35 were biopsied on days 1 and 7. Whilst all 35 patients were analysed for changes in intracellular signalling pathways, only 28 patients had paired RF_{CSA}, Protein:DNA ratios and $Fibre_{CSA}$ measured, as 7 patients samples did not meet histological quality criteria. This is schematically demonstrated in figure 18:

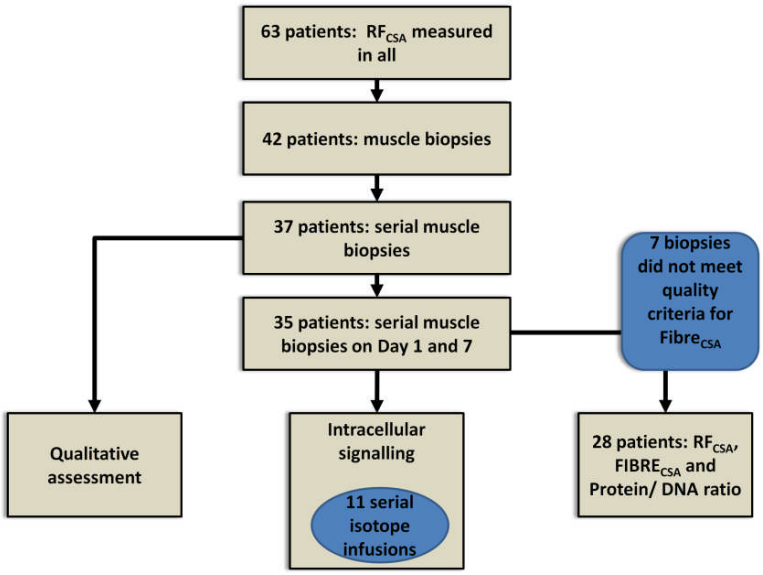


Figure 18: Cohort subdivisions of patients studied. RF_{CSA}= Rectus Femoris Cross Sectional Area, $Fibre_{CSA}$= Myofibre Cross Sectional Area.

2. Patient Characteristics

No differences were seen between sub-cohorts except more male patients were biopsied (table 8).

Characteristics	All Patients	Serial Muscle Biopsies and Ultrasound	Muscle Ultrasound alone	Stable Isotope incorporation
N	63	42	21	11
Age	54.5± 18.0	55.3± 18.7	53.1± 16.9	62.7± 18.8
Male sex - n(%)¥	37(58.7)	**30(71.4)***	7 (31.3)	**9 (81.8)***
Pre-ICU LOS-days#	1 (1-45)	1(1-6)	1 (1-45)	1(1-6)
Days ventilated#	10(2-62)	8.5(2-62)	10(4-24)	12 (2-62)
ICU LOS-days#	16 (7-80)	15.5(7-80)	17(7-73)	18(8-80)
Hospital LOS-days#	30 (11-334)	29.5(11-212)	33 (13-334)	50(17-212)
APACHE II score	23.5±6.5	23.3± 6.3	24± 7.0	27±6.2
SAPS II score	45.5±14.9	43.4±13.4	49.7±17	47±11
ICU survival- n(%)¥	61 (97)	40 (95)	21 (100)	10 (91)
Hospital survival- n(%)¥	56 (89)	37(88)	19(90)	9 (82)
Renal Replacement Therapy – n(%)	19(30.2)	13(31.0)	6(29.0)	4(36.4)
Days NMBA use#	0 (0-6)	0 (0-6)	0 (0-5)	0 (0-6)
Hydrocortisone dose(mg)$				
Day 1	69.0±150.5	83.2±169.6	40.6±100	161.6±236.4
Total by Day 10	601.4±1081.8	642.8±1143.7	518.6±967.1	1129.5±1649.3
HMG-CoA reductase inhibitor use-n(%)	11(17.4)	7(16.7)	4(19)	1(9.1)
Blood glucose (mmol/l) #	7.4(5.1-11.4)	7.3 (5.1-10.3)	7.6(5.6-11.4)	7.9 (6.1-9.5)
Cumulative insulin (iu)	198.9±282.4	177.0±295.9	242.8±254.3	245.6±469.6
Admission diagnosis-n(%)				
Sepsis	31(49.2)	19 (45.3)	12 (57.1)	6 (54.5)
Trauma	16 (25.4)	13 (31.0)	3 (14.3)	4(36.4)
Intra-cranial bleed	5(7.9)	4(9.5)	1(4.8)	0
Acute liver failure	5(8.0)	3(7.0)	2(9.5)	1(9.1)
Cardiogenic shock	6(9.5)	3(7.1)	3(14.3)	0
Co-morbidities- n(%)				
COPD	9 (14.3)	7 (16.7)	2 (9.5)	2 (18)
Ischaemic heart disease	10 (15.9)	7 (16.7)	3 (14.3)	1 (9.1)
Hypertension	13 (19.0)	8 (19.0)	5 (23.8)	1 (9.1)
Diabetes mellitus	8 (12.7)	6 (14.3)	2 (9.5)	1 (9.1)
Liver cirrhosis	6 (9.5)	4 (9.5)	2 (9.5)	1 (9.1)
Chronic pancreatitis	2 (3.2)	1 (2.4)	1 (4.7)	0
Haematological disease	4 (6.3)	2 (4.8)	2 (9.5)	0
Obesity	3 (4.8)	2 (4.8)	1 (4.7)	0
Previous CVA	1 (1.6)	1 (2.4)	0	0
Renal impairment	2 (1.6)	1 (2.4)	1(4.7)	0
Crohn's disease	1 (1.6)	0	1 (4.7)	0
Thyroid disease	3 (4.8)	1 (2.4)	2 (9.5)	0

Table 8: ICU=intensive care unit, LOS= Length of stay, APACHE II= Acute Physiology and Chronic Health Evaluation score, SAPS II= Simplified Acute Physiology Score. NMBA=neuromuscular blocking agents, COPD=Chronic Obstructive Pulmonary Disease, CVA=Cerebro-vascular accident. $=Corticosteroid dosing as hydrocortisone equivalents. Mean

values with plus-minus values indicate SD, except for # indicating median with range. *Student's T-test was used except for ¥* (Chi-squared) and # (Mann Whitney U),* indicates p<0.05 when compared to ultrasound alone group.

RESULTS: CHANGES IN MUSCLE MASS IN THE CRITICALLY ILL PATIENT

1. Muscle Ultrasound

1.1 Reproducibility of measurements

Blinded independent measurements of RF_{CSA} were made in 21 patients by ZP and BC. The correlation coefficient of measurements was 0.97 (Figure 19). A Bland-Altman plot (Figure 20) reveals bias (SD) and 95% limits of agreement to be 7 (37) mm^2 and -66.1 to +80.5 mm^2 respectively.

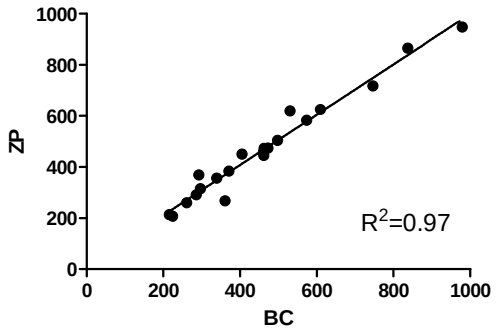


Figure 39: Inter-rater reliability of Rectus Femoris cross sectional area measurements (mm^2) made in 21 patients by Zudin Puthucheary (ZP, x-axis) and by Bronwen Connolly (BC, y-axis). Each point represents a single patient.

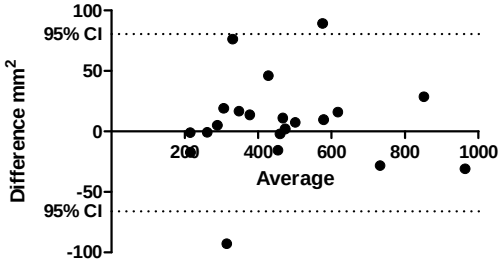


Figure 20: Bland-Altman plot demonstrating inter-rater reliability of Rectus Femoris cross sectional area (RF_{CSA}) measurement

1.2 Rectus Femoris Cross Sectional Area Measurements (RF_{CSA})

Of the 63 patients included in the analysis, one patient could not undergo RF_{CSA} assessment due to morbid obesity (BMI 67kg.m^{-2}). In the group overall, RF_{CSA} decreased significantly from days 1 to 7 (12.5 ± 12.9%; p<0.01), and continued to decrease to day 10 (17.7±12.1%; p<0.001). Using a 1-way repeated measures analysis of variance (ANOVA), the magnitude of this fall was significant at day 7 (p<0.05) and highly significant at day 10 (p<0.001) (figure 21, table 9).

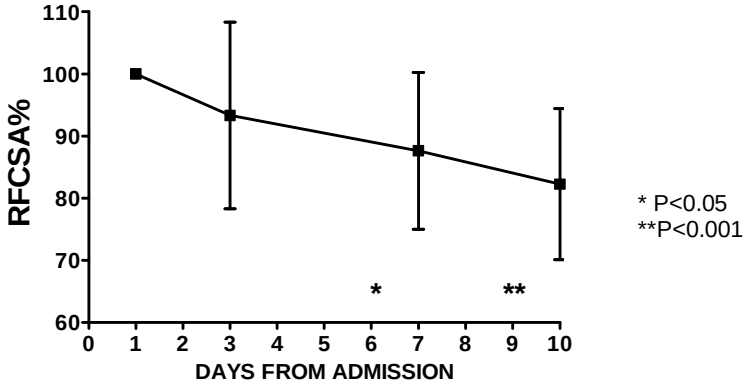


Figure 21: Loss of Rectus Femoris Cross Sectional Area (RF_{CSA}) over 10 days for all patients. *denotes significant difference from day 1, **highly significant, using repeated measures 1-way analysis of variance.

DAY	Percentage of Day 1 RF_{CSA}: Mean (SD)
1	100 (0)
3	93.3 (15)
7	87.6 (12.6)*
10	82.3 (12.1)**

Table 9: Loss of Rectus Femoris Cross Sectional Area (RF_{CSA}) over 10 days for all patients. *denotes significant difference from day 1, **highly significant, using repeated measures 1-way analysis of variance.

Figure 22 is an example of a serial ultrasound measurement in a representative patient.

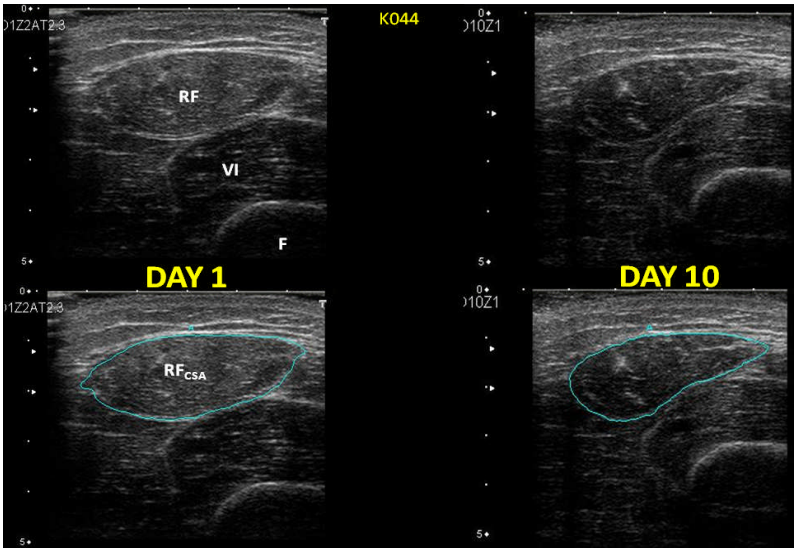


Figure 22: Ultrasound measurements of Rectus Femoris cross-sectional area (RF_{CSA}) on day 1 (left panel) and day 10 (right panel) in a representative patient. From top left, anticlockwise: RF=Rectus Femoris, VL=Vastus Lateralis, F=Femur.

2 Histological measurements of Fibre Cross Sectional Area ($Fibre_{CSA}$)

2.1 Reproducibility of measurements

A total of 23 measurements were compared against those of Dr Anthea Rowlerson (AR), and a further 52 compared against those of Miss Marie Goujon-Svrzic (MS). The correlation coefficients were 0.99 and 0.97 respectively (figure 23). Bland-Altman plots demonstrated that bias (SD) and 95% limits of agreement were 4.5 (4.5) and -4.36 to 13.4 micrometers and -5.8(5.2) and -16.1 to 4.5 micrometers respectively (figure 23).

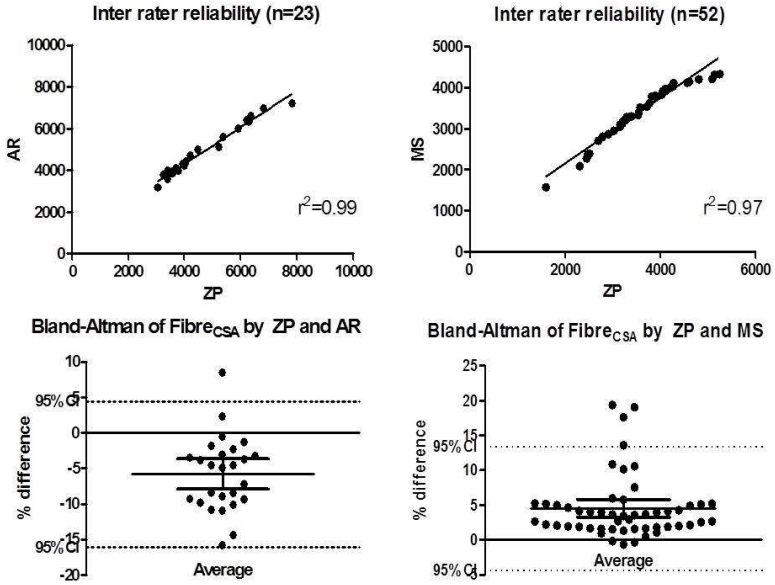


Figure 43: Correlations between ZP and AR, and ZP and MS, with corresponding Bland-Altman plots. ZP=Zudin Puthucheary, AR=Anthea Rowlerson, MS= Marie Goujon-Svrzic

2.2 Quality assurance of preparations

As stated in the study overview (chapter 3), not all of the 42 patients who were biopsied had these performed serially or had samples which passed quality control (figure 24).

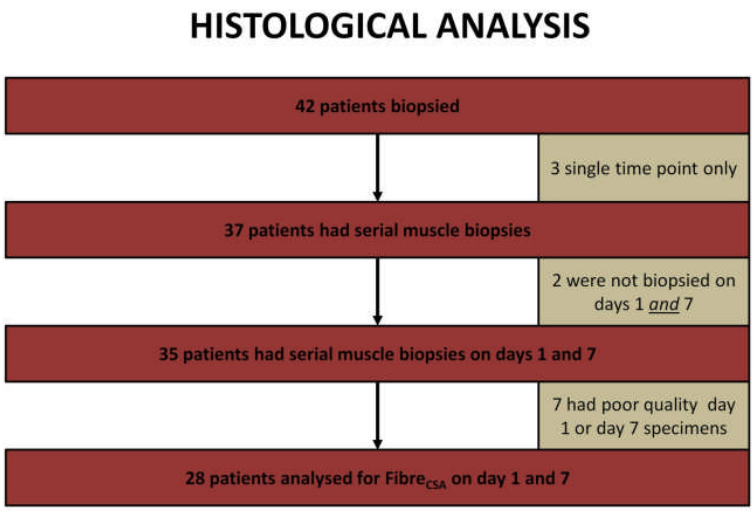


Figure 24: Flowchart of quality control of samples for morphological assessment

2.3 Change in Fibre$_{CSA}$ over 7 days

In the 28 patients assessed on day 1 and 7, Fibre$_{CSA}$ decreased by 17.5 ± 30.2% ($p<0.001$) which was not significantly different from the changes in RF$_{CSA}$ seen in the same 28 patients (10.3 ± 10.9%, $p=0.31$) (figure 25).

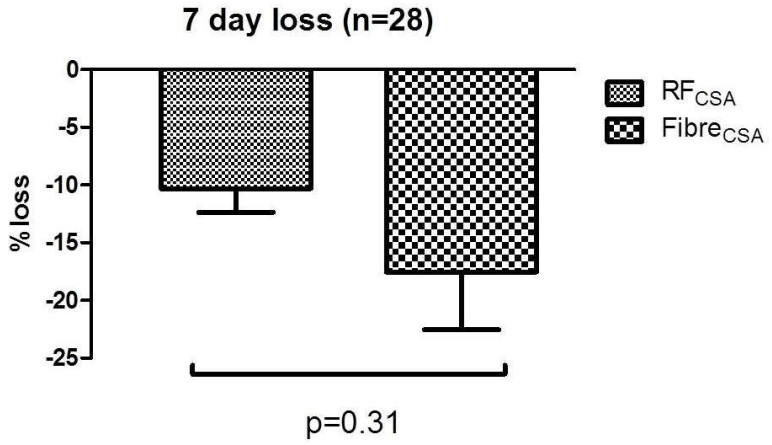


Figure 25: Comparison of loss of muscle mass by ultrasound and histological assessment. RF_{CSA}= Rectus Femoris Cross Sectional Area, $Fibre_{CSA}$= Vastus Lateralis Muscle Fibre Cross Sectional Area

3. Protein/DNA ratio

Protein/DNA measurements were carried out by an independent commercial group (qStandard, London, UK) blind to all data. In the 28 patients that had all three measurements of muscle mass, the protein/DNA ratio decreased significantly ($p < 0.001$), and to a greater degree when compared to RF_{CSA} (29.5 ± 41.5%, p=0.03) (figure 26).

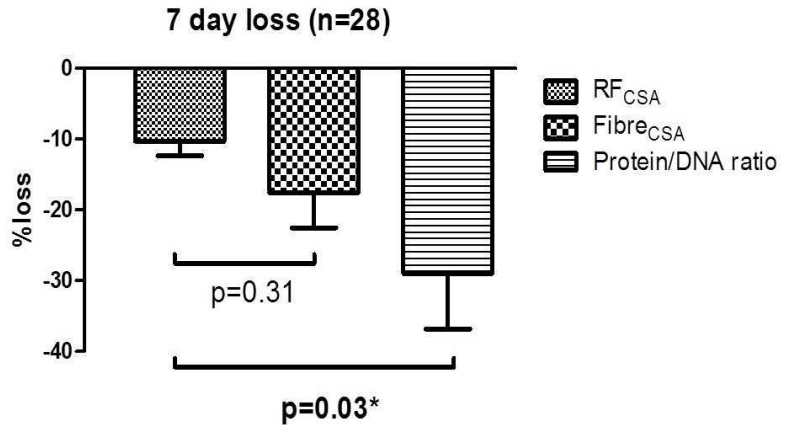


Figure 26: Comparison of loss of muscle mass by ultrasound, histological assessment and protein/DNA ratio. RF_{CSA}= Rectus Femoris Cross Sectional Area, $Fibre_{CSA}$= Vastus Lateralis Muscle Fibre Cross Sectional Area

4. Clinical correlates, patient stratification and risk factors for muscle wasting

Increasing organ failure score correlated with ΔRF_{CSA} (r^2=0.23, p<0.0001). ΔRF_{CSA} differed between patients with multiple- rather than single-organ failure (day 3 -8.7±16.3% vs. -1.8 ± 9.6% respectively, p<0.01; day 7 -15.7±11.2% vs. -3.0 ± 13.6%; p <0.0001). ΔRF_{CSA} was greater in those with ≥4 than in those with 2-3 organ failure (-20.3±12.1% vs. -13.9±10.5% respectively, p<0.01). The differential impact of organ failure became more pronounced by day 10 (Figure 27 and 28, Tables 10 and 11).

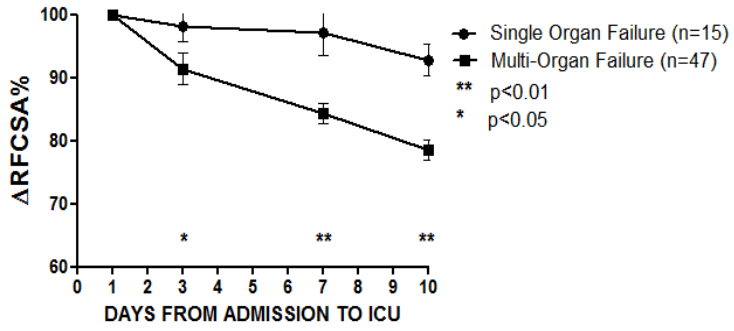


Figure 27: Sequential loss of Rectus Femoris Cross Sectional Area as a percentage (ΔRFCSA %) over 10 days for two sub-groups. SOF= Single Organ Failure, MOF= Multi-organ Failure. Using 1-way ANOVA *indicated significance, and ** high significance

DAY	Percentage of Day1 RF_{CSA}: Mean (SD)	
	SOF	**MOF**
1	100(0)	100(0)
3	98.2 (9.6)	91.3(16.3)*
7	97.0(13.6)	84.3(11.2)**
10	92.8 (9.7)	78.5(10.6)**

Table 10: Sequential loss of Rectus Femoris Cross Sectional Area as a percentage (ΔRFCSA %) over 10 days for two sub-groups. SOF= Single Organ Failure, MOF= Multi-organ Failure. Using 1-way ANOVA *indicated significance, and ** high significance

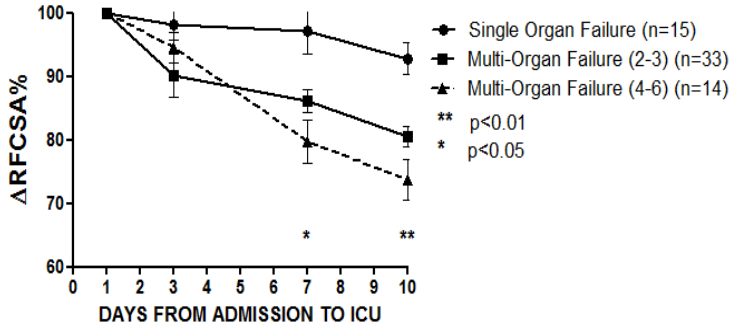


Figure 28: Sequential loss of Rectus Femoris Cross Sectional Area as a percentage (ΔRF_{CSA} %) over 10 days for three sub-groups. SOF= Single Organ Failure, MOF= Multi-organ Failure. (2-3)=2 or 3 organ failure, 4-6= 4,5 or 6 organ failure. Using 1-way ANOVA *indicated significance, and ** high significance between MOF (2-3) and MOF (4-6).

DAY	Percentage of Day1 RF_{CSA}: Mean (SD)		
	SOF	**MOF (2-3)**	**MOF (4-6)**
1	100(0)	100(0)	100(0)
3	98.2 (9.6)	90.1(18.6)	94.5(8.1)
7	97.0(13.6)	86.1(10.5)	79.7(12.1)*
10	92.8 (9.7)	80.5(9.4)	73.7(12)**

Table 31: Sequential loss of Rectus Femoris Cross Sectional Area as a percentage (ΔRF_{CSA} %) over 10 days for three sub-groups. SOF= Single Organ Failure, MOF= Multi-organ Failure. (2-3)=2 or 3 organ failure, 4-6= 4,5 or 6 organ failure. Using 1-way ANOVA *indicated significance, and ** high significance between MOF (2-3) and MOF (4-6).

In a univariate linear regression, a weak correlation exists between ΔRF_{CSA} at day 10 (r^2=0.09, p=0.02) and ICU length of stay, seen in figure 29. However on multivariate linear analysis, ΔRF_{CSA} at day 10 was not an independent predictor of ICU length of stay.

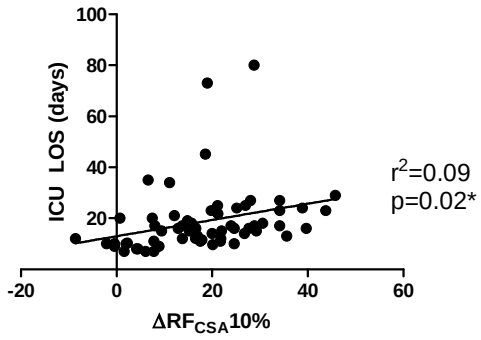


Figure 29: linear regression of change in muscle mass and intensive care length of stay. dRF$_{CSA}$10%=change in Rectus Femoris Cross Sectional Area in10 days expressed as a percentage, ICU LOS= Intensive Care Length of Stay in days

In multivariate linear analysis, ΔRF_{CSA} at day 10 was inversely correlated with serum bicarbonate, PaO_2/FiO_2 ratio and haemoglobin concentration on ICU admission (r^2=0.51, p<0.001), and positive associations with the degree of organ failure, mean CRP and total protein delivered over the study period were identified (Table 12abc).

Variable	Slope	95% CI	Intercept	R^2	p-value	Slope	Beta	p-value
Organ Failure[#]	0.516	0.273-0.763	8.475	0.226	**<0.0001**	0.266	0.264	**0.038**
Age (years)	0.110	-0.001-0.003	11.900	0.027	0.194			
C-Reactive Protein[#]	0.006	0.002-0.011	10.385	0.108	**0.009**	0.003	0.261	**0.049**
Chronic disease[&]	1.047	-5.123-7.265	17.465	0.002	0.735			
Insulin[#$]	0.510	-0.076-1.096	16.115	0.048	0.087			
Protein[#$]	0.583	0.066-1.156	14.016	0.076	**0.030**	0.801	0.459	**0.0003**
Calories[#$]	0.045	-0.007-0.098	10.735	0.047	0.088			
Male Sex	-4.627	-10.79-1.535	20.712	0.036	0.138			
Days of NMB	1.283	-0.961 - 3.528	16.940	0.02	0.26			
Corticosteroid dose-day1[@]	0.009	-0.014- 0.032	17.415	0.01	0.44			
Corticosteroid dose- total[@]	0.002	-0.001- 0.005	16.976	0.02	0.24			
Days of RRT	5.919	0.585-12.423	16.136	0.05	0.074			
HMGCoA RI[£]	-2.169	-10.204-5.882	18.336	0.01	0.59			
Median glucose[#]	-0.605	-3.292- 2.082	22.477	0.00	0.65			
DAY 1 PHYSIOLOGY								
APACHE II	0.309	-0.163-0.785	10.642	0.028	0.192			
SAPS II	0.162	-0.042-0.366	10.544	0.040	0.118			
Day 1 SOFA	-0.624	-1.642-0.393	23.912	0.024	0.225			
Temperature (Celsius)	-1.095	-3.286-1.096	58.921	0.016	0.321			
Mean Arterial Pressure (mmHg)	-0.183	-0.478 to 0.112	33.207	0.024	0.220			
Heart Rate (beats/minute)	0.147	-0.022 to 0.316	5.093	0.047	0.087			

Table 12a

DAY 1 HAEMATOLOGY AND BIOCHEMISTRY

Variable	Slope	95% CI	Intercept	R^2	p-value	Slope	Beta	p-value
Haemoglobin (g/dl)	-1.422	-2.767--0.078	32.738	0.069	**0.038**	-1.0907	-0.256	**0.053**
White Cell count (10^9 c/l)	0.207	-0.190-0.605	15.261	0.017	0.302			
Platelets (10^3 mm^3)	-0.012	-0.037-0.013	20.344	0.014	0.356			
INR	4.075	-4.731-12.881	12.319	0.014	0.358			
APTTR	5.160	-3.746-14.068	10.949	0.021	0.251			
Sodium (mmol/l)	0.193	-0.430-0.818	-9.528	0.006	0.537			
Potassium (mmol/l)	0.252	-4.819-5.325	16.826	0	0.921			
Urea (mmol/l)	0.336	-0.205-0.878	14.995	0.025	0.219			
Creatinine (µmol/l)	0.050	-0.001-0.101	12.538	0.059	0.056			
Alkaline Phosphatase (iu/l)	0.023	-0.047-0.095	16.121	0.007	0.510			
Aspartate Transaminase (iu/l)	0.003	-0.013-0.019	17.543	0.002	0.709			
Bilirubin (mg/dl)	-0.028	-0.128-0.070	18.567	0.005	0.565			
Albumin (g/l)	-0.414	-0.819-0.009	29.326	0.065	**0.045**			
Corrected Calcium (mmol/l)	-7.894	-4.305-8.412	34.836	0.015	0.333			
Phosphate (mg/dl)	2.053	4.689-27.702	15.635	0.006	0.521			
Magnesium (mg/dl)	16.196	4.689-27.702	2.341	0.116	**0.007**			

Table 12b

DAY 1 ARTERIAL BLOOD GASES

Variable	Slope	95% CI	Intercept	R^2	p-value	Slope	Beta	p-value
pH	-81.60	-122.893 to -40.314	618.643	0.206	**<0.001**			
PaO_2 (kPa)	-0.653	-1.436 to 0.128	26.822	0.044	0.100			
SaO_2 (%)	-0.033	-0.306 to 0.241	21.058	0	0.813			
$PaCO_2$(kPa)	2.809	0.258 to 5.360	2.674	0.074	**0.031**			
p/f ratio	-0.347	-0.563 to -0.131	29.261	0.147	**0.002**	-0.247	-0.385	**0.021**
Base Excess (mmol/l)	-0.964	-1.782 to -0.147	15.866	0.089	**0.018**			
Bicarbonate (mEq/L)	-1.191	-0.097-3.143	44.535	0.09	**0.02**	-0.945	-0.256	**0.030**
Lactate (mmol/l)	1.522	-0.0975 to 3.143	14.738	0.055	0.065			
Chloride (mmol/l)	0.227	-0.348 to 0.803	-7.171	0.010	0.433			
Glucose (mmol/l)	-0.401	-2.162 to 1.360	21.154	0	0.650			

Table 12c

Table 12abc: Univariate and multivariate analysis of bedside physiology versus loss of Rectus Femoris cross sectional area by day 10. Organ failure defined by SOFA scoring. All values are for day 1 of ICU admission, except # which denotes area under curve for 10 days. $ indicates total delivered normalised to Ideal Body Weight. @ Corticosteroid doses calculated in hydrocortisone equivalents. £ denotes use on admission, and continued through study period. & chronic disease defined by hospital and general practise coding for management of chronic disease. For the multivariate model any variable with a univariate p value of less than 0.10 was presented to the multivariate linear regression in backward mode with 0.10 used as a continued p value removal criterion. For the final multivariate model the constant was -20.714, R^2 was 0.503 and the model p-value <0.001. Only variables retained in the final multivariate model are presented in the right hand columns. NMB=Neuromuscular blockade, RRT= Renal Replacement Therapy, HMGCoA RI= 3-hydroxy-3-methyl-glutaryl-CoA reductase inhibitor treatment, APACHE II= Acute Physiology and Chronic Health Evaluation II score, SAPS 2= Simplified Acute Physiology Score 2, SOFA= Sequential Organ Failure Assessment, paO_2= Partial pressure of Oxygen in arterial blood, $paCO_2$= Partial pressure of carbon dioxide in arterial blood, SaO_2= Oxygen Saturation in arterial blood, FiO_2= Fraction of inspired oxygen, INR= International Normalised Ratio, APTTR= Activated Partial Thromboplastin Time Ratio.

Logistic multivariate regression analysis demonstrated age, admission bicarbonate and PaO_2/FiO_2 ratio to be associated with >10% loss in RF_{CSA} at day 10 (AUROC=0.90 95%CI 0.790-0.958 sensitivity 84%, specificity 89%; p<0.001) (Table 13ab).

Variable	OR	95%CI	P value	OR	95%CI	p-value
Organ Failure[#]	1.118	1.0373 to 1.2065	**0.004**			
Age	1.038	1.0046 to 1.0741	**0.026**	1.0483	1.005-1.106	**0.028**
C-Reactive Protein[#]	1.001	1.0004 to 1.0027	**0.01**			
Chronic disease[&]	1.571	0.5144 to 4.8009	0.428			
Insulin[#$]	1.164	0.9704 to 1.3974	0.101			
Protein[#$]	1.292	1.0016 to 1.6672	**0.049**			
Calories[#$]	1.012	1.0017 to 1.0235	**0.024**			
Male Sex	0.919	0.2993 to 2.8232	0.88			
Days of NMB	NUMBERS TOO SMALL					
Corticosteroid dose-day1[@]	1.001	0.997 to 1.005	0.70			
Corticosteroid dose- total[@]	1.000	0.999 to 1.001	0.97			
Days of RRT	1.810	0.506 to 6.473	0.36			
HMGCoA RI[£]	2.057	0.398 to 10.631	0.39			
Median glucose[#]	1.057	0.651 to 1.716	0.82			
DAY 1 PHYSIOLOGY						
APACHE II	1.042	0.9531 to 1.1394	0.366			
SAPS II	1.026	0.9852 to 1.0699	0.211			
Day 1 SOFA	0.998	0.8314 to 1.2001	0.99			
Temperature (Celsius)	0.965	0.648 to 1.438	0.862			
Mean Arterial Pressure (mmHg)	0.961	0.909 to 1.016	0.170			
Heart Rate (beats/minute)	1.033	0.998 to 1.069	0.064			
DAY 1 HAEMATOLOGY AND BIOCHEMISTRY						
Haemoglobin (g/dl)	0.888	0.6914 to 1.1409	0.353			
White Cell count (10^9 c/l)	0.986	0.919 to 1.0572	0.686			
Platelets (10^3 mm^3)	0.994	0.9894 to 0.9992	**0.022**			
INR	5.133	0.7198 to 36.6045	0.102			
APTTR	13.201	1.1755 to 148.2512	**0.037**			
Sodium (mmol/l)	1.039	0.9284 to 1.1646	0.50			
Potassium (mmol/l)	0.883	0.3599 to 2.1686	0.787			
Urea (mmol/l)	1.059	0.9453 to 1.186	0.322			
Creatinine (µmol/l)	1.005	0.994 to 1.015	0.344			
Alkaline Phosphatase	1.002	0.989-1.016	0.678			

(iu/l)

Table 13a

Variable	OR	95%CI	P value	OR	95%CI	p-value
Aspartate Transaminase (iu/l)	1.001	0.997 to 1.004	0.498			
Bilirubin (mg/dl)	0.999	0.982 to 1.017	0.979			
Albumin (g/l)	0.920	0.849 to 0.998	**0.046**			
Corrected Calcium (mmol/l)	0.452	0.025 to 8.134	0.59			
Phosphate (mg/dl)	0.786	0.258 to 2.394	0.67			
Magnesium (mg/dl)	14.413	1.095 to 189.696	**0.042**			
DAY 1 ARTERIAL BLOOD GASES						
pH	0.000	0.000 to 0.008	**0.009**			
PaO_2(kPa)	0.876	0.756 to 1.016	0.08			
SaO_2 (%)	0.738	0.529 to 1.029	0.07			
$PaCO_2$(kPa)	1.752	0.771 to 3.975	0.1803			
Base Excess (mmol/l)	0.854	0.717 to 1.017	0.078			
Bicarbonate (mEq/L)	0.807	0.651 to 1.001	0.052	0.717	0.524-0.907	**0.011**
Lactate (mmol/l)	2.013	0.968 to 4.184	0.061			
Chloride (mmol/l)	1.052	0.942 to 1.175	0.359			
Glucose (mmol/l)	1.077	0.783 to 1.479	0.647			
p/f ratio	0.919	0.872 to 0.969	**0.002**	0.880	0.819-0.947	**<0.001**

Table 13b

Table 13ab: Univariate and multivariate logistical analysis of bedside physiology versus loss of Rectus Femoris cross sectional area by day 10. Organ failure was defined by SOFA scoring. All values are for day 1 of ICU admission, except # which denotes area under curve for 10 days. $ indicates those normalised to Ideal Body Weight. @ Corticosteroid doses calculated in hydrocortisone equivalents. £ denotes use on admission, and continued through study period. & chronic disease defined by hospital and general practise coding for management of chronic disease For the multivariate model the constant was 9.883, the c statistic was 0.894 (95%CI 0.790-0.958), p<0.001 and the Hosmer and Lemeshow p-value=0.667 indicating the model was not overfitted. NMB=Neuromuscular blockade, RRT= Renal Replacement Therapy, HMGCoA RI= 3-hydroxy-3-methyl-glutaryl-CoA reductase inhibitor treatment APACHE II= Acute Physiology and Chronic Health Evaluation II score, SAPS 2= Simplified Acute Physiology Score 2, SOFA= Sequential Organ Failure Assessment, paO_2= Partial pressure of Oxygen in arterial blood, $PaCO_2$= Partial pressure of carbon dioxide in arterial blood, SaO_2= Oxygen Saturation in arterial blood, FiO_2= Fraction of inspired oxygen, INR= International Normalised Ratio, APTTR= Activated Partial Thromboplastin Time Ratio.

5. Anthropomorphic measurements

Thirty-three measurements of Mid-Arm Circumference (MAC) were made and compared to Mid-Thigh Circumference (MTC), showing a poor correlation (r^2=0.22). There was no relationship seen between change in MAC and MTC in 10 days (n=15, r^2=0.18, p=0.1, figure 29). Change in MTC at 10 days (ΔMTCd10%) and ΔRF_{CSA}d10% correlated weakly (n=45, r^2=0.11, p=0.03, figure 30). Patients

with single organ failure (SOF) lost thigh circumference, unlike those with multi-organ failure (MOF) (Table 14).

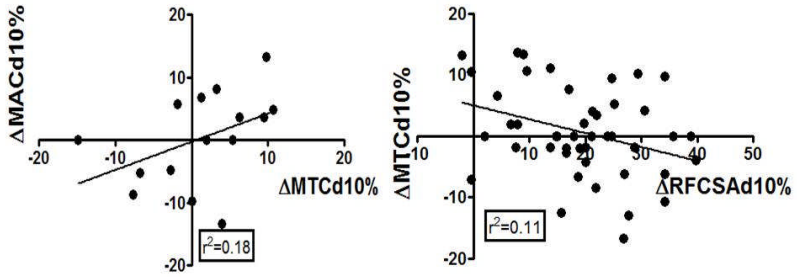


Figure 29 and 30: anthropomorphic measurements in critically ill patient and correlation with ultrasound measurements. ΔMACd10%= percentage change in mid arm circumference in 10 days. ΔMTCd10%= percentage change in mid thigh circumference in 10 days. ΔRF_{CSA}d10%= percentage change in Rectus Femoris Cross Sectional Area in 10 days.

Measurements	SOF	MOF	
ΔMTCd10% (loss)	5.5 ±6.96 (n=9)	-0.09±6.8 (n=36)	**P=0.03***

Table 14: ΔMTCd10 (loss) = loss of Mid-Thigh Circumference (MTC) in 10 days. SOF=Single Organ Failure, MOF=Multi-Organ Failure. Figures are mean values plus/minus SD, with numbers examined in brackets. Paired Student's T-Test was used between groups, * indicating significance.

6. Discussion

Skeletal muscle wasting, characterised for the first time in a longitudinal cohort by three independent measures, occurs early and rapidly in critical illness. Specifically, ultrasound-derived Rectus Femoris cross-sectional area, histologically-determined Vastus Lateralis muscle fibre cross-sectional area, and Vastus Lateralis protein/DNA ratio decreased over the first week.

Reduction in RF_{CSA} was associated with organ failure burden. In particular, patients with single-organ failure (SOF) demonstrated limited wasting whilst those with 4-organ failure showed muscle loss of over 20% by the end of the first week. Patients with multi-organ failure (MOF) experienced greater physiological derangements previously implicated in the pathogenesis of muscle wasting [226, 356-

358]. Whilst immobility [152] is associated with wasting, all our patients were effectively bed-bound and it is unlikely that mobility differences contributed significantly to organ failure-related differential muscle loss. Those in SOF lost only very little muscle mass in the first 7 days (3%) with greater loss by 10 days (7.2%). This is in keeping with bed rest studies in healthy volunteers, in which lean body mass declined by 6.3% over 10 days [359]. Importantly, muscle wasting purely due to immobilisation accelerates over time [360]. It is likely that these patients would develop significant muscle wasting if their critical illness was prolonged, though these data were not captured in this study.

These data demonstrate a direct correlation between muscle wasting and both inflammation (C-reactive protein) and acute lung injury (P_aO_2/FiO_2 ratio). Specifically, inflammation affects protein homeostasis [227] and lung-derived inflammatory mediators (e.g. tumour necrosis factor-alpha) are associated with muscle wasting in chronic lung disease [356]. Acute lung injury results in the release of systemic inflammatory cytokines, which may drive muscle wasting [10, 361-365]. In addition, metabolic acidaemia was associated with wasting, in keeping with a possible causal role [357]. The mechanism of muscle wasting associated with acidaemia is unknown, although correcting of chronic acidaemia in patients with chronic kidney disease results in an increase in lean body mass [357, 366]. Finally, low haemoglobin concentrations, often a biomarker of chronic disease [358], were also associated with muscle wasting. The small numbers of patients receiving neuromuscular blockade and the confounders of accompanying disease state and severity preclude the dissection of the impact of paralysis *per se* or its cumulative effect with corticosteroids.

Although data were generally consistent across techniques, variation in muscle loss between them may relate to differences in technique or the muscles studied. Specifically, Rectus Femoris was assessed by ultrasound and Vastus Lateralis biopsy specimens to measure fibre cross-sectional area and protein/DNA ratio. Protein/DNA ratio measured by spectrophotometry is unaffected by water content, unlike ultrasound measurement and histology [116]. $Fibre_{CSA}$ has been shown to increase in the face of fluid resuscitation in porcine models of sepsis [116]. Interestingly, endomysial oedema

was not seen in non-septic pigs. Muscle oedema may thus have contributed to an underestimation of loss of ultrasound-derived Rectus Femoris cross-sectional area. Alternative explanations could include differential muscle wasting in Rectus Femoris and Vastus Lateralis, which has not been well described [360]. Rectus Femoris is a bi-articular muscle, as opposed to Vastus Lateralis which is uni-articular. Small movements may therefore have a differential mechanical load across two joints, allowing some preservation of RF_{CSA}.

It is difficult to compare these data with those of previous studies, as few were longitudinal in nature, and none had standardised time points from point of admission [98-100, 115, 367]. Those studies that are comparable were performed over two decades ago in a vastly different clinical arena [125].

In keeping with previous studies, anthropomorphic measurements correlated poorly with direct measures of muscle mass [98, 99], and seemed to be worse in those with MOF, likely due to dependant oedema.

6.1 Clinical implications

Early and rapid muscle wasting during the first week of critical illness was more pronounced in those with multi-organ failure. These data indicate a role for using organ failure scoring to stratify critically ill patients at risk of muscle wasting. This approach may help identify those who would benefit from early mobilisation strategies [150, 368]. The sample size precludes meaningful exploration of the association of wasting with specific disease entities. However, homogeneity of muscle loss with stratification by organ failure suggests that the specific disease state may not be the most significant driver of muscle loss in the first week. Whilst these data may be relevant to all patients during the acute stages of critical illness, expanded disease-specific studies are advocated.

6.2 Summary

Muscle wasting occurs early and rapidly during the first week of critical illness, and is more pronounced in multi-organ failure.

RESULTS: CHANGES IN MUSCLE QUALITY IN THE CRITICALY ILL PATIENT

Histology specimens were stained with Haematoxylin and Eosin, and examined first by myself, and then reported in a blinded fashion by Dr Rahul Padhke, consultant histopathologist at the National Hospital for Neurology and Neurosurgery, Queen's Square, London. Samples were noted to develop necrosis between days 1 and 7, but no other myopathic changes were observed. All myopathic changes reported in the cohort overall at days 7 and 10 were already present on day 1.

1. Muscle necrosis

1.1 Quality control

Several samples exhibited acute circumscribed areas of muscle necrosis and regeneration (Figure 31). Whilst previously reported [367], this potentially was an artefact from lignocaine infiltration.

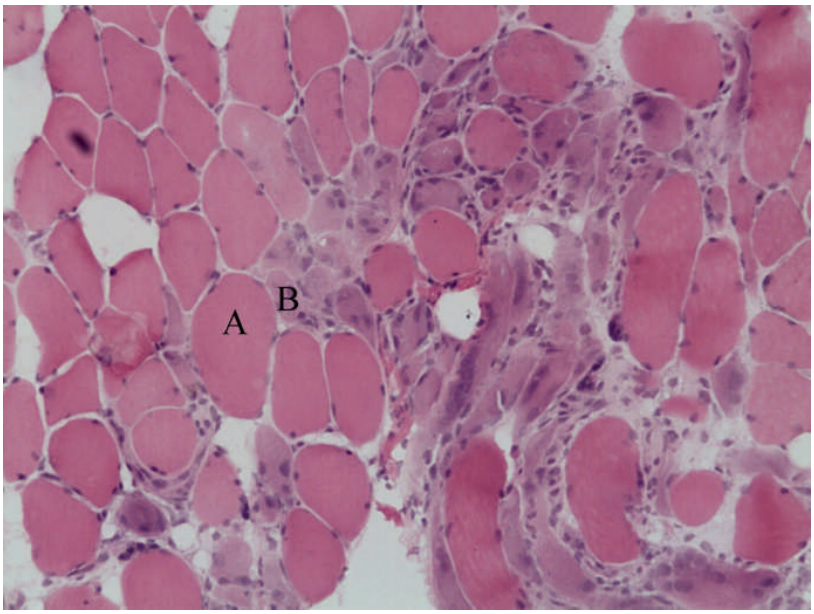


Figure 31: Area of circumscribed necrosis and regeneration. A=normal fibre, B= regenerating fibre

Thus the decision was made to report this conservatively as iatrogenic damage. Of note, the early regeneration seen is similar to that seen amongst patients undergoing blunt trauma from road traffic accidents (figure 32).

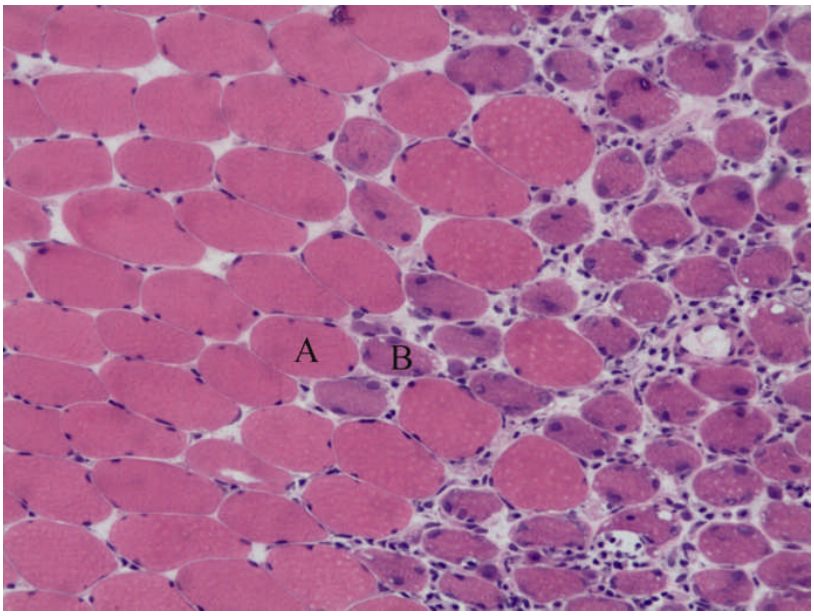


Figure 35: Area of regeneration following blunt trauma. A=normal fibre, B= regenerating fibre

1.2 Incidence of muscle necrosis

Serial muscle biopsies in 37 patients were analysed. Fourteen patients had evidence of muscle necrosis by day 7, and 20 by day 10. In 6 patients, necrosis secondary to previous biopsy could not be excluded (quality control section above) and were excluded from analysis. All necrotic samples demonstrated macrophage infiltrates. Figures 33 and 34 are representative examples of necrotic change.

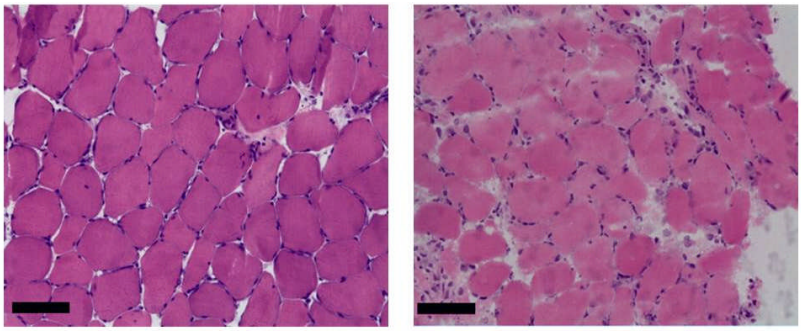


Figure 36 and 34: Representative samples showing Haematoxylin and Eosin stains of Vastus Lateralis muscle on day 1 and 7. Healthy muscle is seen on day 1(figure 33) with necrosis and a cellular infiltrate on day 7 (figure 34).

1.3 Clinical correlates with muscle necrosis

No clinical variables demonstrated adequate sensitivity/specificity in predicting necrosis, by receiver operator curve analysis (area under curve <0.7, p>0.05), Table 15.

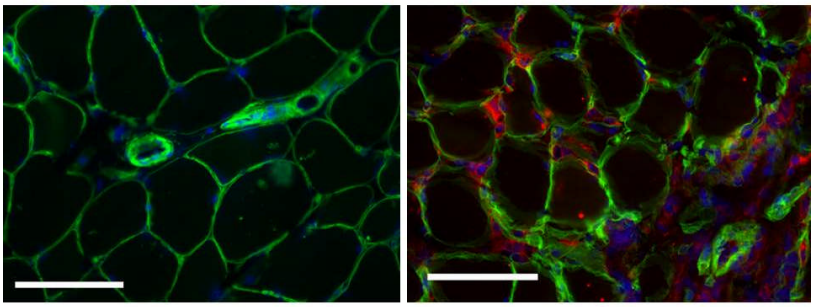
Variable	95% Confidence Interval	AUROC	Significance
$\Delta RF_{CSA}10\%$	0.3109 - 0.7391	0.53	0.81
Organ Failure[#]	0.3268 - 0.7441	0.54	0.74
Age	0.3471 - 0.7612	0.55	0.61
C-Reactive Protein[#]	0.3953 - 0.8047	0.60	0.34
Chronic Disease	0.3059 - 0.7191	0.51	0.91
Insulin[#$]	0.3009 - 0.7158	0.51	0.94
Protein[#$]	0.3533 - 0.7717	0.56	0.55
Calories[#$]	0.2943 - 0.7223	0.51	0.94
Male Sex	0.3166 - 0.7292	0.52	0.83
Days of NMB	0.3353-0.7206	0.53	0.77
Corticosteroid dose-day1[@]	0.3767-0.7498	0.56	0.51
Corticosteroid dose- total[@]	0.3837-0.7605	0.57	0.46
Days of RRT	0.3138-0.6921	0.50	0.96
HMGCoA RI[£]	0.3274-0.7049	0.52	0.87
Median glucose[#]	0.3195-0.7011	0.51	0.92
DAY 1 PHYSIOLOGY			
APACHE II score	0.2908 - 0.7134	0.50	0.98
SAPS II score	0.3888 - 0.7987	0.59	0.37
Day 1 SOFA score	0.3254 - 0.7455	0.54	0.74
Temperature (Celsius)	0.3332 - 0.7543	0.54	0.68
Mean Arterial Pressure (mmHg)	0.3207 - 0.7543	0.54	0.72
Heart Rate (beats/minute)	0.3692 - 0.7933	0.58	0.44
DAY 1 ARTERIAL BLOOD GASES			
pH	0.3110 - 0.7390	0.53	0.81
PaO_2(kPa)	0.3056 - 0.7277	0.52	0.87
$PaCO_2$(kPa)	0.3088 - 0.7245	0.52	0.87
SaO_2 (%)	0.4078 - 0.8130	0.61	0.30
paO_2/FiO_2 Ratio	0.3766 - 0.7901	0.58	0.43
Base Excess (mmol/l)	0.3045 - 0.7205	0.52	0.91
Bicarbonate (mEq/L)	0.3414 - 0.7586	0.55	0.64
Lactate (mmol/l)	0.4261 - 0.8281	0.63	0.23
Chloride (mmol/l)	0.3317 - 0.7475	0.54	0.71
Glucose (mmol/l)	0.4561 - 0.8606	0.66	0.13
DAY 1 HAEMATOLOGY AND BIOCHEMISTRY			
Haemoglobin (g/dl)	0.3015 - 0.7235	0.51	0.91
White Cell count (10^9 c/l)	0.4333 - 0.8333	0.63	0.20
Platelets (10^3 mm^3)	0.3018 - 0.7232	0.51	0.91
INR	0.2952 - 0.7131	0.50	0.97
APTTR	0.2986 - 0.7180	0.51	0.94
Sodium (mmol/l)	0.3260 - 0.7448	0.54	0.74
Potassium (mmol/l)	0.4192 - 0.8225	0.62	0.25
Urea (mmol/l)	0.3866 - 0.8009	0.59	0.37
Creatinine (µmol/l)	0.3950 - 0.8008	0.60	0.35
Alkaline Phosphatase (iu/l)	0.3246 - 0.7420	0.53	0.75
Aspartate Transaminase (iu/l)	0.4044 - 0.8206	0.61	0.29
Bilirubin (mg/dl)	0.3400 - 0.7559	0.65	0.55
Albumin (g/l)	0.3245 - 0.7422	0.53	0.75
Corrected Calcium (mmol/l)	0.3245 - 0.7422	0.54	0.68

Phosphate (mg/dl)	0.3696 - 0.7846	0.58	0.46
Magnesium (mg/dl)	0.3896 - 0.8104	0.60	0.34

Table 15: Predictors of muscle necrosis. All values are for day 1 of ICU admission, except # which denotes area under curve for 7 days. $ indicates total delivered normalised to Ideal Body Weight. @ Corticosteroid doses calculated in hydrocortisone equivalents. £ denotes use on admission, and continued through study period. & chronic disease defined by hospital and general practise coding for management of chronic disease. $\Delta RF_{CSA}10$= change in rectus femoris cross sectional area over 10 days. NMB=Neuromuscular blockade, RRT= Renal Replacement Therapy, HMGCoA RI= 3-hydroxy-3-methyl-glutaryl-CoA reductase inhibitor treatment, APACHE II= Acute Physiology and Chronic Health Evaluation II score, SAPS 2= Simplified Acute Physiology Score 2, SOFA= Sequential Organ Failure Assessment, paO_2= Partial pressure of Oxygen in arterial blood, $paCO_2$= Partial pressure of carbon dioxide in arterial blood, SaO_2= Oxygen Saturation in arterial blood, FiO_2= Fraction of inspired oxygen, INR= International Normalised Ratio, APTTR= Activated Partial Thromboplastin Time Ratio

2. Phenotyping of cellular infiltrates

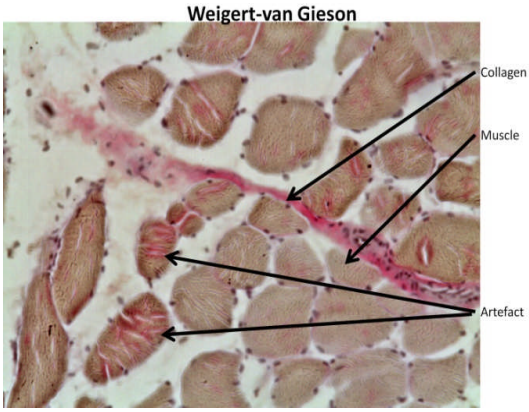
Examination of Haematoxylin and Eosin-stained biopsies suggested the cellular infiltrate seen in necrotic samples to be of macrophagic origin. This was confirmed with CD68 immunostaining (see chapter 2). Figures 35 and 36 shows the same samples as in figures 33 and 34 above, but stained with CD68 antibody (red).



Figures 35 and 36: Characterization of cellular infiltrates. Slides were stained for Laminin (green), CD68 antibody (red) and with 4',6-diamidino-2-phenylindole (DAPI, blue). Scale bars represent 100 micrometers.

3. Collagen quantification

3.1 Weigart-Van Geison stain



Whilst the Weigart-Van Geison stain (described in the methods chapter) worked well, the nature of dye infiltration and bonding made quantification difficult. Muscle fibres that were damaged stained positive for collagen at the points of damage (figure 37). Whilst in general terms one is able to separate damage as a result of ice-crystal formation at time of freezing from pathological damage, the confounding influences of damage as a result of section cutting or necrosis are difficult to account for quantitatively.

Figure 37: Weigart- van Geison stain of patient K057 on day 1. This demonstrates artefactual pink staining on muscle as a result of local muscle damage.

In summary, Weigart-Van Geison staining is not an appropriate method for quantitative analysis of collagen content in skeletal muscle in this study.

3.2 Collagen Immunostaining

Despite appropriate staining of collagen (as per methods chapter), the signal was too weak to quantify presence of collagen replacement due to the presence of artefacts (figure 38).

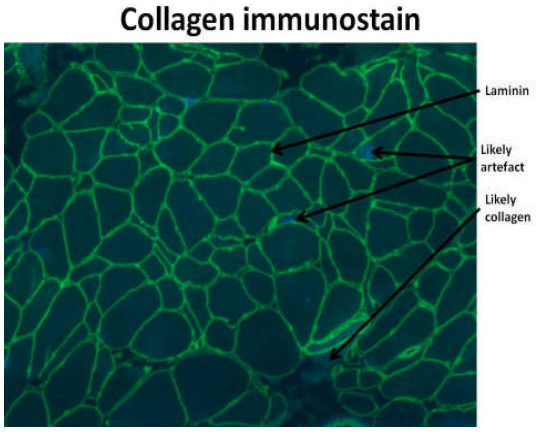


Figure 38: collagen immunostain showing high levels of artefactual staining

4. Discussion

The incidence of acute myofibre necrosis (40% of patients affected) was higher than that previously described [125], despite the exclusion of those where iatrogenic muscle damage was considered a possibility [369, 370]. A macrophage cellular infiltrate was only found in those samples with evidence of necrosis. This has not been reported prior to this study, and there is no indication as to the role of the macrophages- their presence being either a result of the necrosis or causative. The occurrence of necrosis was independent of the disease state causing admission and of the patient's age. Further, whilst chronic diseases from which our subjects suffered (COPD, liver cirrhosis, heart failure) can cause muscle fibre atrophy, necrosis or macrophagic infiltrates have not been reported [131, 371]. The presence of apoptotic cells has been reported in a few patients with chronic disease [372, 373] though conflicting reports exist [129, 374]. Nor was necrosis due to blunt trauma: its pattern (patchy and not confluent, and unassociated with haemorrhage) was not that associated with blunt injury, whilst the features observed in the trauma cases were indistinguishable from those seen in others.

These facts, together with the observed aggressive and progressive evolution in wasting and necrosis, are consistent with the existence of an early and aggressive myopathic process related to critical illness. While no clinical variables were associated with necrosis, several of the potential contributing factors were not measured. Arterial hypoxia was not associated with the development of necrosis, but this does not exclude roles for cellular dysoxia or microvascular re-distribution. While the micro-circulation can be measured using near-infra spectrometry (NIRS) [375] and has been used on peripheral muscle [376, 377], several factors precluded its use. The current protocol is time-intensive (especially in regards to the isotope infusions) and further additions would add time pressures to both the clinical staff and relatives, which would be inappropriate in the setting of the unstable critical care patient. Secondly the infra red blade depth and measurements of tissue oxygenation are significantly affected by tissue oedema [378], rendering the results unreliable in

dependant sites, such as quadriceps. Finally, tissue oxygenation measurements by NIRS is acutely affected by cardiac output [379] and peripheral vascular resistance [380] and therefore measurements within the first 24 hours of critical care admission are likely to be labile, and single time point measurements will poorly reflect tissue oxygenation over this hyper-acute period.

Pharmacotherapy is another potential candidate for the development of necrosis. Whilst glucocorticoid use has been associated with muscle necrosis [112] this was seen in small case reports, with multiple confounding factors. Animal studies have consistently failed to show a development of necrosis with glucocorticoid use [381], and recent work has suggested that low dose steroids may even be protective [123]. In non-critical care inflammatory myopathies, glucocorticoids are the first line of treatment [382] adding further weight to a non-causal relationship. Vasopressors have been implicated in animal studies [383, 384]. One major criticism of these studies is the doses used -20mml/kg boluses [383] or 5mg/kg boluses [384], which would be lethal in a human. Specific correlations between the use of vasoactive drugs and muscle necrosis were not sought in this study, due to the difficulties in rationalising the different agents used (norepinephrine, epinephrine, Dobutamine and vasopressin). It should be noted that many of the single organ failure patients in the study received vasoconstrictors for maintenance of cerebral perfusion pressure in the face of raised intracranial pressure. None of these patients developed muscle necrosis, suggesting a non-causal relationship.

Future research into the development of muscle necrosis in critical care should concentrate (at a molecular level) on the apoptotic pathways and their upstream regulators. Whilst some data exist on the associated non-ATP dependant pathways [385], these derive from small studies with single, variable timepoints. Further, an understanding of the drive to apoptose is needed, and the relative contributions of intracellular need (secondary to mitochondrial damage [139, 386]) or extracellular drive, potentially mediated by Tumour Necrosis Factor Alpha [283, 310]). Experimental models of muscle wasting and necrosis may be needed to further characterise the macrophages seen, and thus clarify their role.

Attempts to investigate the previously noted increase in connective tissue [133] were not successful. Historical data for the development of muscle fibrosis is weak [133], and it seems unlikely that the growth of new collagen or the replacement of healthy muscle with collagen would occur within the first seven days of critical illness.

4.1 Summary

Skeletal muscle necrosis with an accompanying macrophagic infiltrate is a common adjunctive finding in critically ill patients within the first 7 to 10 days.

RESULTS: PROTEIN HOMEOSTASIS

1. Limb protein turnover

1.1 Quality control-bedside experiments

Two potential points of weakness exist in the protocol- the achievement of steady state of labelled D_5Phenyalanine and the accuracy of the Doppler flow measurements: the latter may cause large variation in readings, as the least objective measurement is the radius of the femoral artery, which affects this equation for limb blood flow:

$$LBF\ (l.min^{-1}) = Mean\ Blood\ Velocity\ (cm.sec^{-1}) \times \pi \times (femoral\ artery\ radius\ (mm))^2$$

To confirm low intra-observer variation, 7 patients had their limb blood flow measured 3 times at 90 minutes and 3 times at 130 minutes from initiation of primed constant infusions on day 1 (when the patients were deemed to be at their most unstable haemodynamically). Measurements correlated well (r^2=0.92, p<0.001, figure 39) and a Bland Altman plot reveals bias (SD) and 95% limits of agreement to be -0.0001 (0.6) $l.min^{-1}$ and -0.12 to +0.12 $l.min^{-1}$ respectively, figure 40.

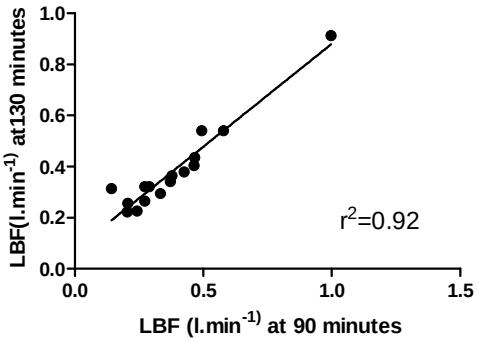


Figure 39: Correlation between limb blood flow measurements taken at the femoral artery at 90 minutes and at 130 minutes from initiation of primed constant infusion. All measurements performed by ZP. Each time point refers to an individual patient. LBF ($l.min^{-1}$) = Limb blood flow per minute

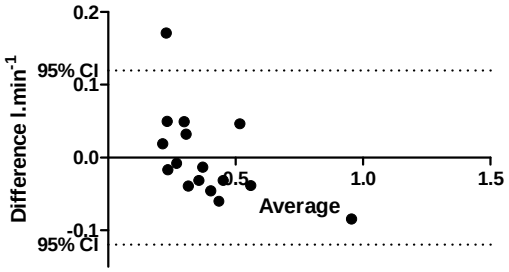


Figure 40: Bland Altman plot of limb blood flow measurements taken at the femoral artery at 90 minutes and at 130 minutes from initiation of primed constant infusion. All measurements performed by ZP. Each time point refers to an individual patient.

Interim analysis of the same samples in Prof Rennie's laboratory at University of Nottingham, confirmed the universal achievement of steady state by 90 minutes.

1.2 D_5-Phenylalanine limb protein turnover

Leg protein breakdown was elevated in comparison to leg protein synthesis from days 1 to 7 (8.5±5.7 vs. 6.6±6, (p=0.05) to 10.6±5.7 vs.9.31±4.1, (p<0.01) µmol phe/min/kg/IBW*100) resulting in a net catabolic balance, figure 41:

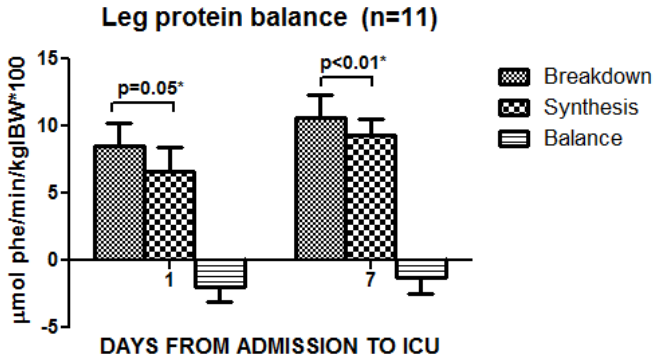


Figure 41: B: Leg protein balance on day 1 and 7, determined by D5-Phenlyalanine dilution expressed per minute/kilogram of ideal body weight*100. * denotes significant differences by Students't-test. Data are mean and standard error

2. Leucine incorporation

Muscle protein fractional synthetic rate (FSR) on day 1 was depressed to rates observed in fasted healthy controls[387](0.035 ± 0.018%/hr vs. 0.039 ± 0.011%/hr, respectively; p=0.57), before rising by day 7 (from 0.035 ± 0.018%/hr to 0.076 ± 0.066%/hr; p=0.03) to levels observed in healthy fed controls [387] (0.076 ± 0.066%/hr vs. 0.065 ±0.018%/hr; p=0.30), figure 42:

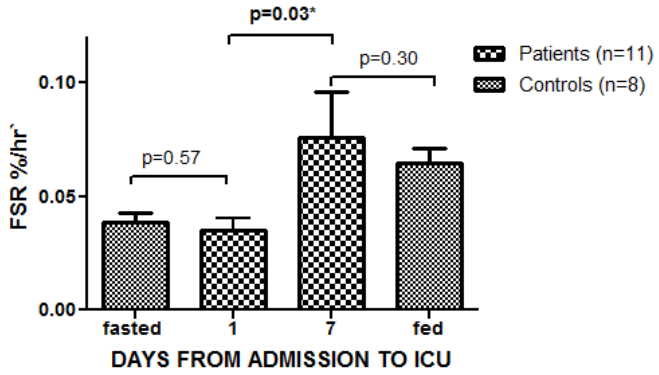


Figure 72: Fractional Synthetic Rate of muscle protein synthesis, determined by [^{1,2}-13C$_2$] Leucine incorporation in patients and controls. FSR%/hr= Fractional Synthetic Rate per hour. * denotes significant differences by Student's t-test. Data are mean and standard error.

2.1 Effect of nutrition delivery on day 1

Nasogastric feeding was successfully initiated in 9 of the 11 patients (in whom Leucine incorporation studies were performed) on day 1 and in all patients by day 7 (<200 millilitres of nasogastric aspirate every 4 hours).

Whilst 11 patients had sequential primed isotope infusions, a total of 14 were studied on day 1. Dividing them into a fed cohort (defined as initiation of nasogastric feed, with <200ml aspirated in first 4 hours or initiation of Total Parenteral Nutrition) and an unfed cohort (neither enteral nor parenteral nutrition initiated) demonstrated no difference in Fractional Synthetic Rate (p=0.47, figure 43).

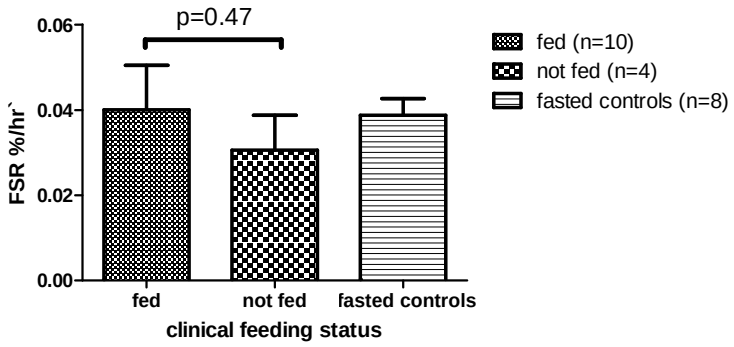


Figure 43: FSR% Hr= Fractional Synthetic Rate of muscle as a percentage per hour. p=0.47, One tailed Mann Whitney U test. Fed=initiation of NG feed, with <200ml aspirated in first 4 hours or initiation of Total Parenteral Nutrition. Not fed= neither enteral nor parenteral nutrition initiated.

Following consultation with statistical experts, univariate and multivariate analyses were not performed, owing to the low numbers.

3. Discussion

Muscle wasting (as described in chapter 4) is a consequence of both depressed muscle protein synthesis (MPS) and an elevation in muscle protein breakdown (MPB) relative to protein synthesis, resulting in a net catabolic state. MPS was depressed to levels equivalent to the healthy fasted state on day 1 but rose to rates similar to that in the healthy fed state by day 7, although the net balance remained catabolic. The trajectory of change implies an increase in MPS towards more normal values. This variation in muscle protein synthesis with time may explain the unclear results from studies performed at varying time point in critical illness [385].

It has been postulated that increased muscle protein breakdown is the major driver of muscle wasting [388, 389], whilst others have insisted that suppressed protein synthesis must be the pathophysiological process [156]. In man, MPS is the facilitative process, with MPB adapting as a result, which is the reverse of rodents [137, 138, 343]. Hence this depression in MPS is entirely in keeping with the response of protein synthesis to known pathophysiological conditions [151, 226].

That breakdown is higher relative to synthesis on day 1 is to be expected (due the MPS depression). Interestingly, whilst MPS recovers by day 7 to levels found in fed normal healthy volunteers; breakdown is still elevated relative to synthesis. This is of relevance both pathophysiologically and in the development of therapeutic interventions. These data suggest that early interventions might best be targeted to increase muscle protein synthesis, whilst later ones may utilise the normalisation of the synthetic response. Concurrently, as patients spend increasing time in the intensive care, the role of muscle protein breakdown becomes increasingly relevant: later interventions will have to counterbalance this process too.

Unfortunately, rates of limb protein breakdown seen in this study are not easily compared to those published in the literature- D_5Phenylalnine dilution rates are usually expressed per kilogram actual body weight [226] or more accurately, leg volume measured by Dual-energy X-ray absorptiometry [390]. Neither exact body weights nor leg volumes were viable possibilities in this population.

Importantly, these overall effects occurred despite the administration of nutrition. In the clinical correlates section of chapter 4, higher protein delivery in the first week was associated with greater muscle wasting. This finding is in keeping with an adverse effect of early targeted feeding [391]. In a large randomised control trial of 4640 patients, early feeding was associated with increased lengths of stay, greater number of infections and longer ventilation and renal replacement therapy requirements [391]. In a smaller single centre study, 300 patients were randomised to standard feeding versus supplemented feedings [392]. This had no effect on length of mechanical ventilation, length of hospital stay or mortality [392]. Smaller single centre studies attempting to target nutritional needs early in critical illness with supplementation have also found an associated increase length of stay [245].

One hypothesis to explain this worsening of the clinical state with apparently "normal" nutrition may be the "muscle full effect". During a continuous infusion of amino acids, MPS peaks between 30-120 minutes before dropping off to fasted rates, despite the continuing availability of amino acids [241]. This hypothesis was first put forward by Millward *et al.* [393]. Similar data have been produced by

continued amino acid availability by the oral route [394]. It is likely that the excess amino acids are then oxidised, with increased concentrations of urea being seen [241]. The muscle full effect still occurs if Leucine (which stimulates MPS) is infused as a sole amino acid [395]. A contentious physiological phenomena that may exacerbate the blunted anabolic response is "anabolic resistance" [156]. This is the inability to mount a synthetic response in the face of an anabolic challenge i.e. amino acids, which has been seen in immobility studies [152] and aging [396]. These data cannot unequivocally demonstrate anabolic resistance in the critically ill *per se*- the muscle full effect not being accounted for. However, it is plausible that these patients may demonstrate both these phenomena.

The timing and mode of nutritional support (continuous vs. intermittent) thus needs further consideration.

4. Conclusions

Muscle protein synthesis is depressed on day of critical illness, to rates associated with fasted controls. Recoveries of rates comparable to fed controls are seen by day 7. Protein breakdown is elevated throughout.

RESULTS: INTRACELLULAR DRIVERS OF PROTEIN HOMEOSTASIS

Thirty-five of the 63 patients had muscle biopsy samples taken on days 1 and 7 of ICU admission. These samples were analysed for changes in signalling molecule concentration. Image analysis was performed by both Dr Cristiana Velloso and I for Experiment 1, and repeated analyses demonstrated a good correlation (r^2=0.89). 1 in 3 samples underwent repeated analysis and a strong correlation was seen (n=40, r^2=0.97). Figure 44 (seen before in Chapter 1 as figure 5) describes the relationship between these molecules:

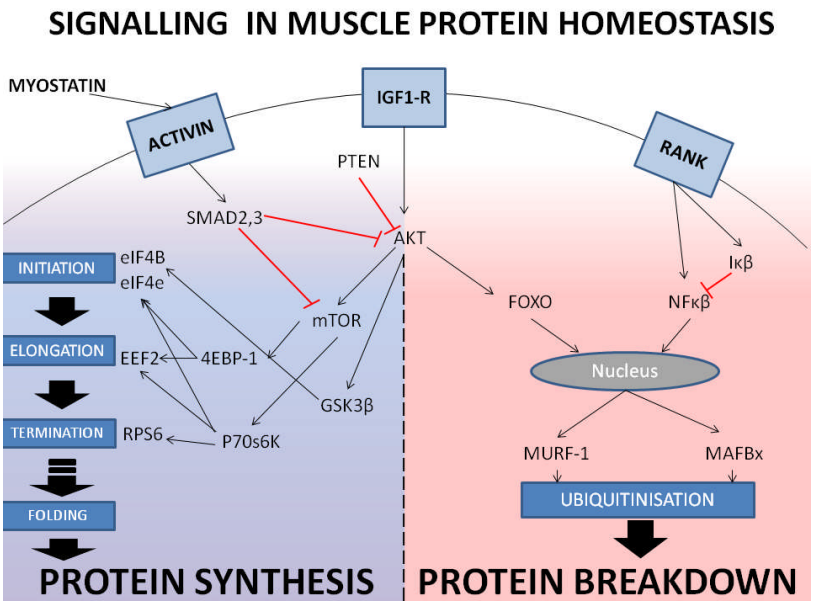


Figure 44: Anabolic and catabolic pathways involved in muscle protein homeostasis. IGF1-R= Insulin like Growth Factor-1, PTEN= Phosphatase and Tensin homolog, AKT= Protein Kinase B, FOXO= Forkhead Box Class O-1, MURF-1= Muscle Ring Finger protein 1, MAFBX= Muscle Atrophy F-Box-1, NFκβ= Nuclear Factor Kappa Beta, Iκβ= Inhibitor of Kappa Beta, RANK= Receptor Activator of Kappa Beta, mTOR=mammalian Target Of Rapamycin, 4EBP-1= Eukaryotic Initiation Factor 4E binding protein 1, P70s6K= 70-kDa S6 protein kinase GSK3B=Glycogen Synthetase Kinase 3 beta, EIf4B= Eukaryotic Initiation Factor 4-B, EIF4e=Eukaryotic Initiation Factor 4e, EEF2=Eukaryotic Elongation Factor 2, RPS6=Ribosomal Protein S6. SMAD 2,3= vertebrate homolog of Drosophila protein MAD and Caenorhabditis protein SMA 2,3.

Molecules measured are listed below (table 16):

ABBREVIATION	DEFINITION
MAFBx	Muscle Atrophy F-Box-1
MURF-1	Muscle Ring Finger protein 1
AKT	Protein Kinase B
FOXO	Forkhead Box Class O-1
NFκβ	Nuclear Factor Kappa Beta
IGF1-R	Insulin like Growth Factor 1 Receptor
IRS-1	Insulin Receptor Substrate-1
PTEN	Phosphatase and Tensin homolog
P70s6K	70kDa ribosomal protein S6 kinase
mTOR	Mammalian Target of Rapamycin
GSK3β	Glycogen Storage Kinase 3 Beta
EEF2	Eukaryotic Elongation Factor
4EBP-1	Eukaryotic Initiation Factor 4E binding protein 1
Myostatin	Myostatin

Table 16: Signalling molecules measured

Total and phosphorylated concentrations of all of these molecules were measured (except those of MURF-1 and MAFBx, where no phosphorylated forms exist). Exemplar western blots are shown in figure 45 and 46.

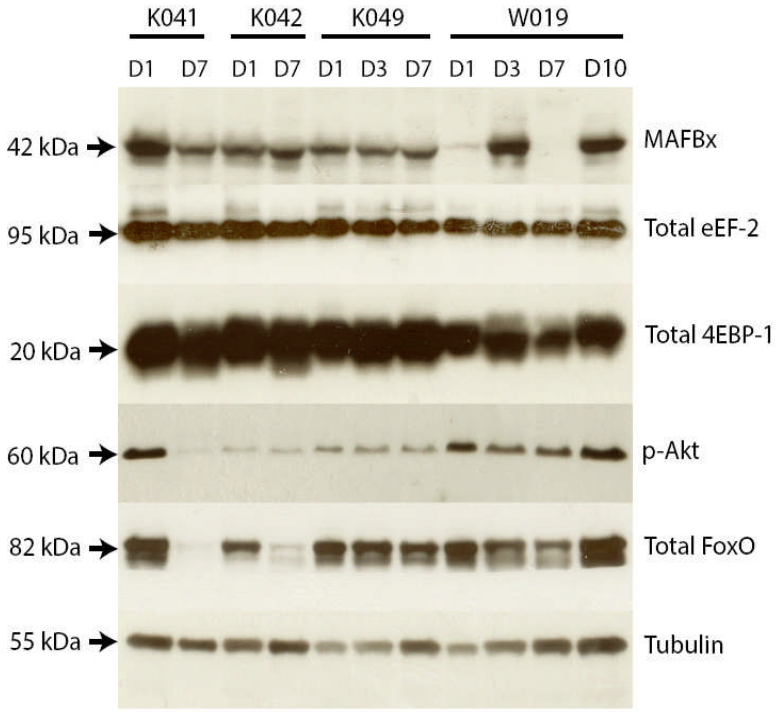


Figure 45: Western blots of signalling molecules. K and W numbers represent unique identification numbers. D1 D3 D7 D10 represent days from admission to ICU. p-AKT= phosphorylated Protein Kinase B, T-FoxO= Total Forkhead Box Class O-1, MAFBx= Muscle Atrophy F-Box-1, t-4EBP-1= Total Eukaryotic Initiation Factor 4E binding protein 1, Total eEF2= Total Eukaryotic Elongation Factor

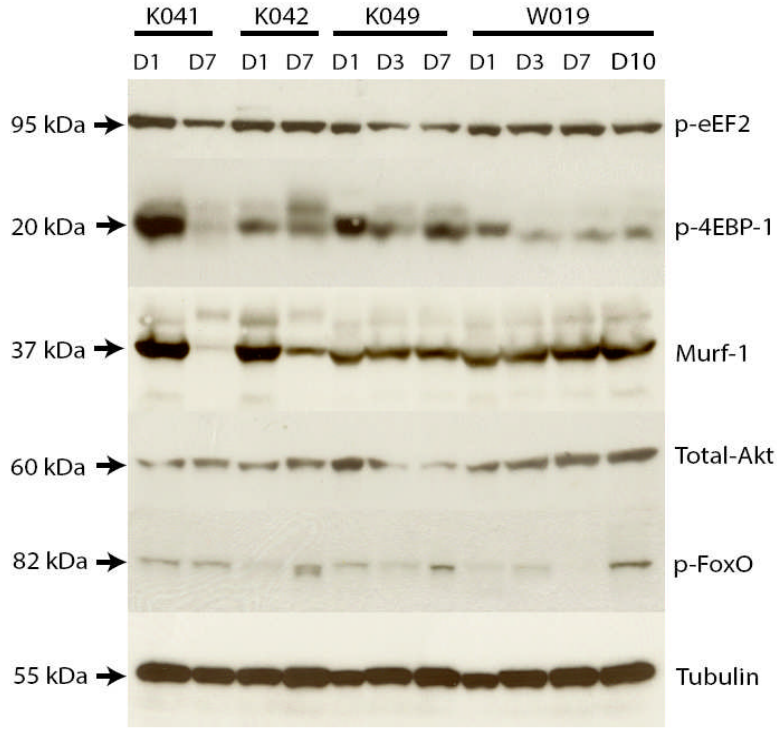


Figure 46: Western blots of signalling molecules. K and W numbers represent Unique Identification numbers. D1 D3 D7 D10 represent days from admission to ICU. T-AKT= Total Protein Kinase B, p-FoxO= phosphorylated Forkhead Box Class O-1, MURF-1= Muscle Ring Finger protein 1, p-4EBP-1= phosphorylated Eukaryotic Initiation Factor 4E binding protein 1, peEF2= phosphorylated Eukaryotic Elongation Factor

1. Components of the catabolic signalling pathway

Total AKT did not change significantly (p=0.66). P-AKT increased, but the magnitude of rise did not reach statistical significance (p=0.06). The ratio of P:T AKT did not change significantly (p=0.10). This is summarised in figure 47. Total FOXO decreased significantly between day 1 and 7 (p<0.01). P-FOXO also decreased, but the scale of change once again did not reach statistical significance (p=0.09). The ratio of Phosphorlyated:Total FOXO (P:T FOXO) did not change significantly (p=0.57),

summarised in figure 48. Murf-1 levels dropped significantly over 7 days (p=0.01). Mafbx also decreased significantly over this period (p<0.01), displayed below in figure 49. No significant changes were seen in total or phosphorylated forms of NFκβ or the P:T ratio between day 1 and 7 (p=0.1, p=0.71, p=0.63) as displayed in figure 50.

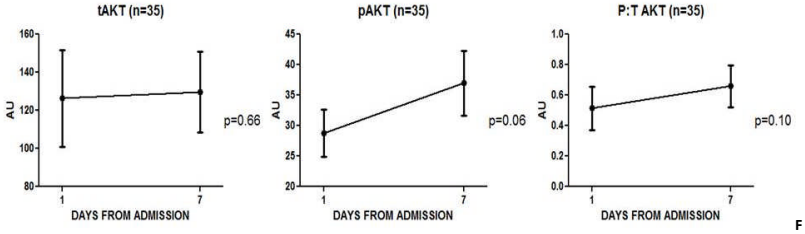


Figure 47: Graphs from left to right: Total Protein Kinase B (AKT), Phosphorylated AKT, and Phosphorylated:Total ratio. AU=arbitrary units, statistical analysis performed using Wilcoxon Signed Rank test.

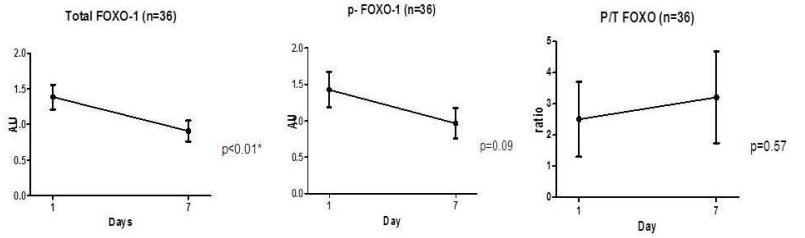


Figure 48: Graphs from left to right: Total Forkhead Group O-1 (FOXO-1), Phosphorylated FOXO-1, and Phosphorylated:Total ratio. AU=arbitrary units, statistical analysis performed using Wilcoxon Signed Rank test.

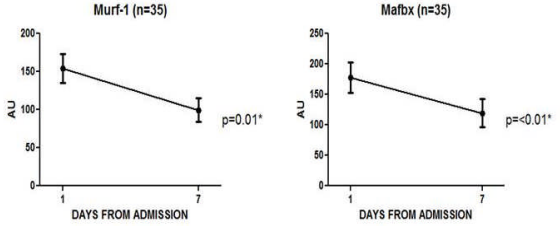


Figure 49: Graphs from left to right: Muscle Ring Finger 1 (MURF-1), Atrogin-1 (MAFBx). AU=arbitrary units, statistical analysis performed using Wilcoxon Signed Rank test.

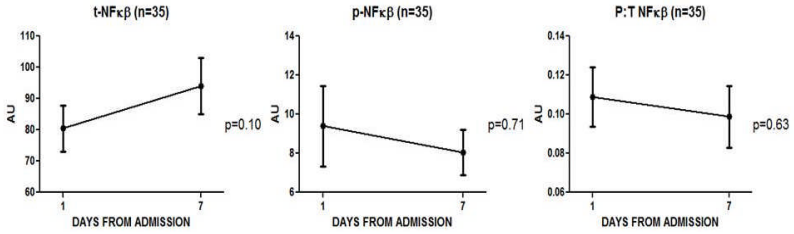


Figure 50: Graphs from left to right: Total Nuclear Factor Kappa Beta (NFκβ), Phosphorylated NFκβ, and Phosphorylated:Total ratio. AU=arbitrary units, statistical analysis performed using Wilcoxon Signed Rank test.

2. Proximal modulators of AKT

No significant changes were seen in total or phosphorylated forms of IGF1R or the P:T ratio between day 1 and 7 (p=0.76, p=0.09, p=0.31), as displayed in figure 51. No significant changes were seen in total or phosphorylated forms of IRS-1 or the P:T ratio between day 1 and 7 (p=0.87, p=0.13, p=0.89), as displayed in figure 52. Both total and phosphorylated levels of PTEN increased in a highly significant fashion (p<0.001). However a numerical increase in P:T ratio did not achieve significance (p=0.08) as demonstrated in figure 53.

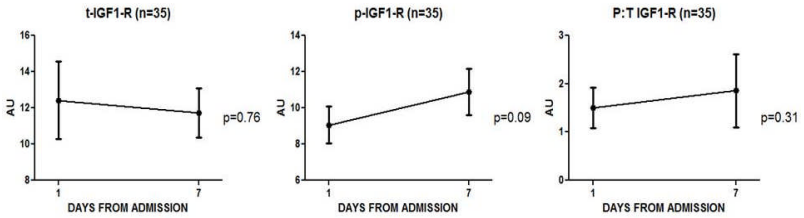


Figure 51: Graphs from left to right: total Insulin like Growth Factor 1 Receptor (IGF1-R), phosphorylated IGF1-R, and Phosphorylated:Total ratio. AU=arbitrary units. Statistical analysis performed using Wilcoxon Signed Rank test.

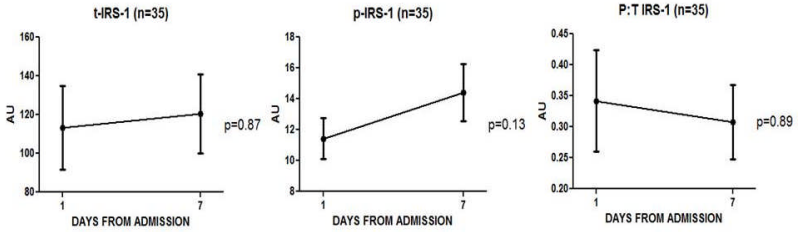


Figure 52: Graphs from left to right: total Insulin Receptor Substrate 1 (IRS-1), phosphorylated IRS-1, and Phosphorylated:Total ratio. AU=arbitrary units. Statistical analysis performed using Wilcoxon Signed Rank test.

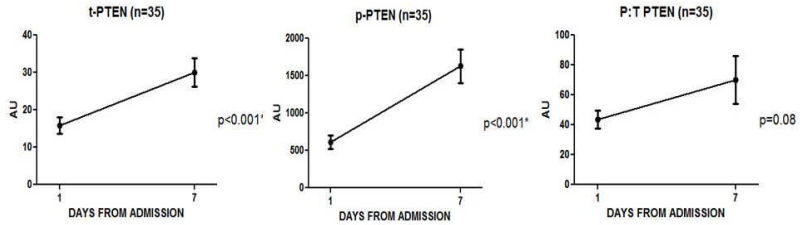


Figure 53: Graphs from left to right: Phosphatase and Tensin homolog (PTEN), phosphorylated PTEN, and Phosphorylated:Total ratio. AU=arbitrary units. Statistical analysis performed using Wilcoxon Signed Rank test.

3. Anabolic signalling components

No significant changes were seen in total or phosphorylated forms of GSK3β or the P:T ratio between day 1 and 7 (p=0.82, p=0.89, p=0.80), as displayed in figure 54. No significant changes were seen in total or phosphorylated forms of mTOR or the P:T ratio between day 1 and 7 (p=0.85, p=0.08, p=0.08), as displayed in figure 55. Whilst total p70s6K did not change significantly (p=0.94), the phosphorylated form increased significantly over 7 days (p=0.04), although the P:T ratio did not alter significantly (p=0.40, figure 56). No significant changes were seen in total or phosphorylated forms of 4eBP-1 or the P:T ratio between day 1 and 7 (p=0.16, p=0.52, p=0.78), as displayed in figure 57. No significant changes were seen in total form of EEF2 (p=0.26). Phosphorylated EEF2 dropped significantly over 7 days (p<0.01) though the P:T ratio between day 1 and 7 did not change significantly (p=0.5), as displayed in figure 58.

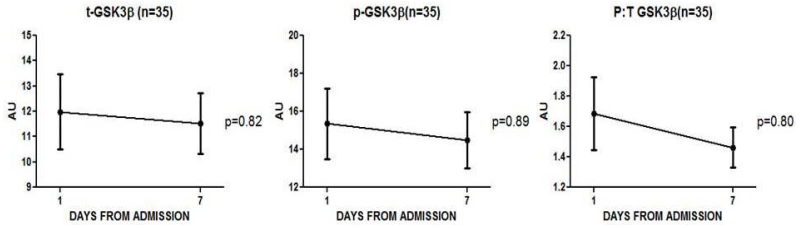


Figure 54: Graphs from left to right: Glycogen Storage Kinase 3 Beta (GSK3β), Phosphorylated GSK3β, and Phosphorylated:Total ratio. AU=arbitrary units, statistical analysis performed using Wilcoxon Signed Rank test.

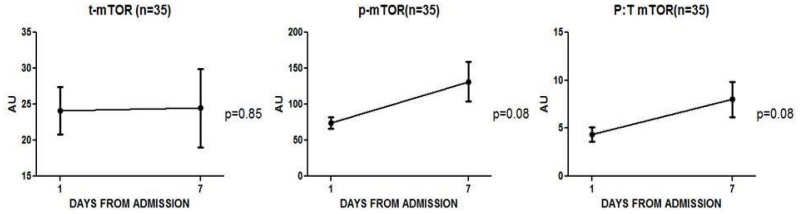


Figure 55: Graphs from left to right: mammalian Target of Rapamycin (mTOR), phosphorylated mTOR, and Phosphorylated:Total ratio. AU=arbitrary units Statistical analysis performed using Wilcoxon Signed Rank test.

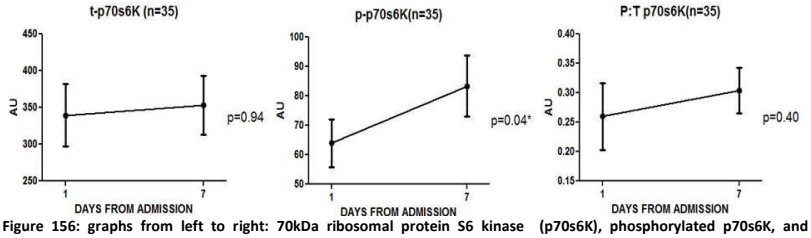


Figure 156: graphs from left to right: 70kDa ribosomal protein S6 kinase (p70s6K), phosphorylated p70s6K, and Phosphorylated:Total ratio. AU=arbitrary units, statistical analysis performed using Wilcoxon Signed Rank test.

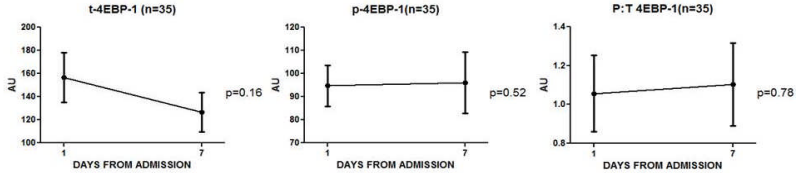


Figure 57: graphs from left to right: 4-Eukaryotic Binding Protein 1 (E4BP-1), phosphorylated E4BP-1, and Phosphorylated:Total ratio. AU=arbitrary units, statistical analysis performed using Wilcoxon Signed Rank test.

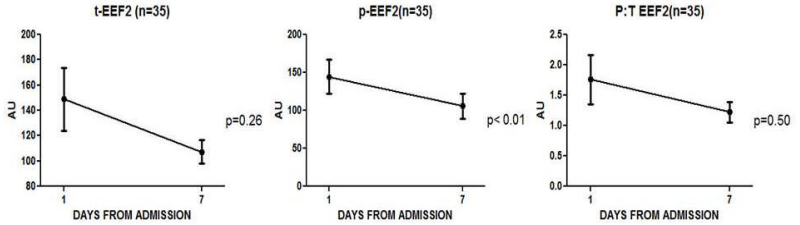


Figure 58: graphs from left to right: Eukaryotic Elongation Factor 2 (EEF2), phosphorylated EEF2, and Phosphorylated:Total ratio. AU=arbitrary units. Statistical analysis performed using Wilcoxon Signed Rank test.

4. Analyses of changes in signalling molecule protein concentrations

A lack of obvious pattern of change was noted in terms of P:T ratios, and whilst the degree of change approached statistical significance for a few proteins, none achieved this.

4.1 Separation by muscle wasting

Given the heterogeneous nature of the wasting response (and thence, potentially, in changes in related signalling pathways), data were sub-stratified according to the magnitude of muscle wasting. A cut-off of a 10% loss of RF_{CSA} was used, this being a level of muscle wasting that has been shown to have a functional impact on ambulant patients with Chronic Obstructive Pulmonary Disease [355]. Comparisons were made between those patients with muscle wasting against those without (n=23 vs. 12, respectively). Both RF_{CSA} (Table 17) and Protein:DNA ratio (table 18) were used as measures of muscle wasting, neither of which demonstrated a pattern of signalling.

Molecule	Total	Phosphorylated	P:T Ratio
P70s6K	0.25	0.60	0.09
GSK3β	0.61	0.43	0.38
mTOR	0.20	0.87	0.94
PTEN	0.85	0.96	0.57
AKT	0.90	0.17	0.81
IRS-1	0.47	0.07	0.14
IGF1-R	0.70	**<0.01***	0.09
NFκβ	0.63	0.69	0.36
MURF-1	**0.01***		
MAFBx	0.18		
4EBP-1	0.48	0.15	0.48
eEF2	0.92	0.99	0.47
FoxO	0.51	0.14	0.77

Table 16: p values for correlation between 10% loss in Rectus Femoris cross sectional area (RF_{CSA}) and change in protein concentrations of signalling molecules. IGF1-R= Insulin like Growth Factor-1, IRS-1= Insulin Receptor Substrate 1, PTEN= Phosphatase and Tensin homolog, AKT= Protein Kinase B, FoxO= Forkhead Box Class O-1, MURF-1= Muscle Ring Finger protein 1, MAFBX= Muscle Atrophy F-Box-1, NFκβ= Nuclear Factor Kappa Beta, mTOR=mammalian Target Of Rapamycin, 4EBP-1= Eukaryotic Initiation Factor 4E binding protein 1, P70s6K= 70-kDa S6 protein kinase GSK3B=Glycogen Synthetase Kinase 3 beta, eEF2=Eukaryotic Elongation Factor 2.

Molecule	Total	Phosphorylated	P:T Ratio
P70s6K	0.41	0.38	0.40
GSK3β	0.99	0.72	0.98
mTOR	0.45	0.73	0.21
PTEN	0.66	0.90	0.63
AKT	0.43	0.42	0.43
IRS-1	**0.03***	0.21	0.25
IGF1-R	0.40	0.21	0.41
NFκβ	0.38	0.33	0.40
MURF-1	0.90		
MAFBx	0.24		
4EBP-1	0.11	0.53	0.08
eEF2	0.49	0.56	0.83
FoxO	0.25	0.90	0.31

Table 17: p values for correlation between 10% loss in protein/DNA ratio and change in protein concentrations of signalling molecules. IGF1-R= Insulin like Growth Factor-1, IRS-1= Insulin Receptor Substrate 1, PTEN= Phosphatase and Tensin homolog, AKT= Protein Kinase B, FoxO= Forkhead Box Class O-1, MURF-1= Muscle Ring Finger protein 1, MAFBX= Muscle Atrophy F-Box-1, NFκβ= Nuclear Factor Kappa Beta, mTOR=mammalian Target Of Rapamycin, 4EBP-1= Eukaryotic Initiation Factor 4E binding protein 1, P70s6K= 70-kDa S6 protein kinase GSK3B=Glycogen Synthetase Kinase 3 beta, eEF2=Eukaryotic Elongation Factor 2.

4.2. Relationship between anabolic signalling and fractional synthetic rate (FSR)

As described in chapter 6, eleven patients received paired primed infusions of D_5-Phenylalanine and [1,2-$^{13}C_2$] Leucine, with biopsies on days 1 and 7. Change in Fractional Synthesis Rate (FSR) was compared to alterations in signalling patterns (Phosphorylated (P):Total (T) ratios). Whilst significant relationships were seen between FSR and the P:T ratios of AKT, IGF1-R and FOXO, examination of the correlation graphs revealed this relationship to be likely over-emphasised as a result of a few data points (table 18,figure 59).

P:T of each protein	Normality passed*	R^2	P value
P70s6K	Y	0.14	0.12
GSK3β	Log10*	0.005	0.42
mTOR	Y	0.15	0.12
PTEN	Y	0.06	0.24
AKT	Y	0.62	**<0.001**
IRS-1	Log10*	0.02	0.33
IGF1-R	Log10*	0.45	**0.01**
4EBP-1	Y	0.09	0.18
EEF2	Y	0.14	0.12
FOXO	Y	0.63	**<0.01**
NFκβ	Y	0.11	0.16

Table 18: Relationship between phosphorylated to total (P:T) ratios of protein concentration of signalling molecules and fractional synthetic rate of protein synthesis, determined by leucine incorporation. IGF1-R= Insulin-like Growth Factor-1, IRS-1= Insulin Receptor Substrate 1, PTEN= Phosphatase and Tensin homolog, AKT= Protein Kinase B, FOXO= Forkhead Box Class O-1, MURF-1= Muscle Ring Finger protein 1, MAFBX= Muscle Atrophy F-Box-1, NFκβ= Nuclear Factor Kappa Beta, mTOR=mammalian Target Of Rapamycin, 4EBP-1= Eukaryotic Initiation Factor 4E binding protein 1, P70s6K= 70-kDa S6 protein kinase GSK3B=Glycogen Synthetase Kinase 3 beta, EEF2=Eukaryotic Elongation Factor 2.

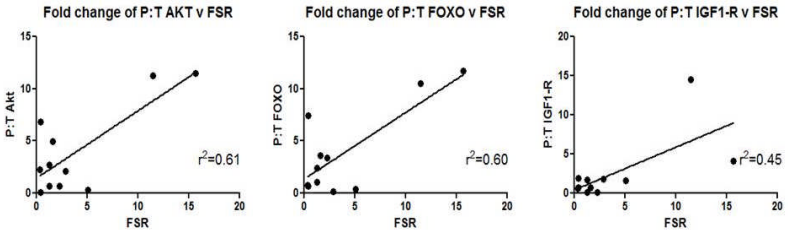


Figure 59: Fold change of phosphorylated to total protein (P:T) concentrations of protein Kinase B (AKT), Forkhead Box Class O-1 (FOXO) and Insulin-like Growth Factor1 Receptor (IGF1R) against protein Fractional Synthetic Rate.

4.5 Quantitative PCR

The decrease in E3 ligase levels seen over 7 days was mirrored by mRNA levels (figure 60). Myostatin levels remained unchanged from day 1 to day 7 (36.27±48.55 to 34.59±58.7 copy numbers/reaction, n=22; p=0.88) (figure 61).

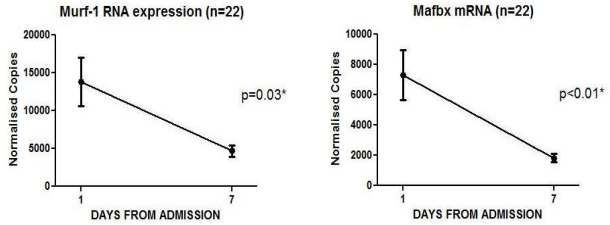


Figure 60: Expression of Muscle Ring Finger 1 (MURF-1) and Atrogin-1 (MAFbx), statistical analysis performed using Wilcoxon Signed Rank Test

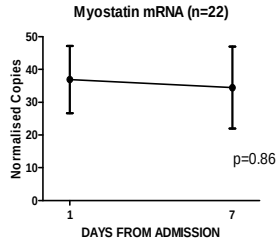


Figure 61: Myostatin messenger Ribonucleic Acid copy numbers normalised to reference genes on day 1 and 7 of critical care admission

4.4 Principle Component Analysis

Principal components analysis (PCA) of putative signalling molecules identified a pattern that correlated with both MPS and MPB. A 2-component PCA model explained 41% of the variance in the time-related log-fold changes in molecular signalling data. Principal component 2 (PC2) captured biologically-relevant relationships between signals, and 12% of the total variance in the signalling data. PC2 was tested for correlations with log-fold change in the components of protein homeostasis (defined by D_5Phenylalanine dilution data), synthesis and breakdown. Significant correlations were found between PC2 and both breakdown ($r = -.83$, $n = 9$, $p = .005$, figure 63) and synthesis ($r = -.69$, $n = 9$, $p = .041$, figure 64).

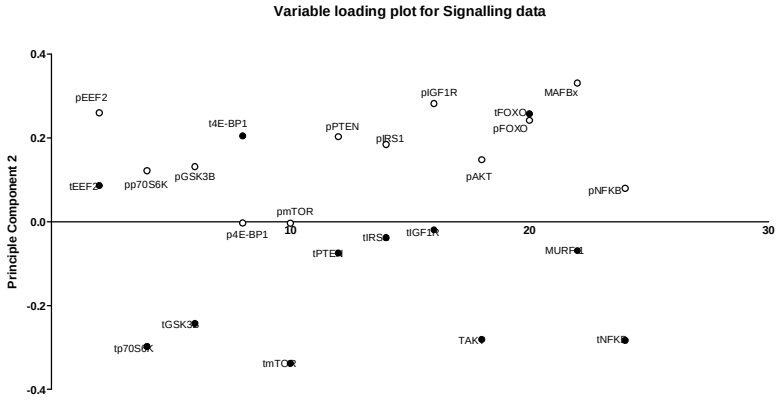


Figure 62: Loading plot of principle component 2. See text for identification of each signalling molecule.

Intracellular Signalling vs Limb Protein Breakdown

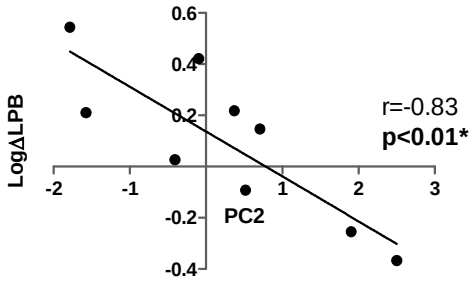


Figure 63: Correlation between Principle Component 2 and log measure of limb protein breakdown (LPB), the latter determined by D_5Phenylalanine dilution

Intracellular Signalling vs Limb Protein Synthesis

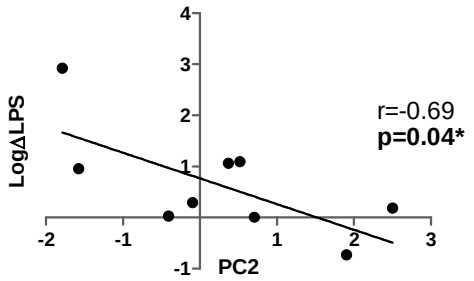


Figure 64: Correlation between Principle Component 2 and log measure of limb protein synthesis (LPS), as determined by D_5Phenylalanine dilution.

5. Discussion

Complex interactions between the different components of the anabolic and catabolic signalling pathways were identified. It would be unusual for a single molecule to represent a rate-limiting step in muscle homeostasis and, unsurprisingly, individual components of the anabolic and catabolic signalling pathways did not correlate with muscle loss or protein homeostasis. Furthermore, several of the central molecules in the signalling cascade are pleiotrophic: FOXO, for example, is involved in

carbohydrate metabolism [124]. This is in keeping with both the critical care literature- cross sectional data having variously showed increased proteolysis and both activation and inactivation of the anabolic signalling cascade. No studies have been performed longitudinally, nor have signalling molecules been analysed in a whole system fashion [279, 304, 310, 347, 348]. The heterogeneity in results is likely to be the result of the fact that intracellular signalling molecules are highly responsive to diverse (and likely heterogeneously prevalent and intense) stimuli, such as glucocorticoids [293], activity or immobilisation[305, 397], acidosis [398], sepsis [301] and starvation [291]. Whole-system principle component analysis extracted a novel pattern in the signalling data that inversely correlated with muscle protein turnover. Such analysis thus appears to have uncovered a complex molecular signal underpinning muscle protein turnover in critically ill patients.

Concentrations of anabolic signalling molecules decreased over time almost universally although, when analysed individually, none correlated with leucine incorporation. It may be that a non-linear relationship exists between muscle protein synthesis and the concentrations of active signalling molecules [323] or that the production of new molecules is decreased, perhaps secondary to a lack of intracellular ATP, the result of mitochondrial dysfunction [139, 298]. Mitochondrial dysfunction may underlie the decrease in E3 ligase concentrations seen. The significant decrease seen in both MURF-1 and MAFbx concentration was unexpected. These findings were independently confirmed by measurements of mRNA concentrations by investigators blinded to the clinical data (qStandard Group, London, UK). The Ubiquitin Proteasome Pathway is ATP dependant [295, 297] and in states where energy supply is limited [139], muscle protein breakdown may occur via ATP independent pathways such as the cytosolic proteases. Further, as described in chapter 5, muscle necrosis is a common adjuvant finding. Therefore a significant proportion of protein loss may be driven by the apoptotic pathways, lending further weight to the likelihood that the cytosolic proteases may be needed to remove necrotic tissue [285]. Individual components of the cytosolic protease families have been measured in the critically ill, but not in large numbers, or in a standardised longitudinal fashion, preventing meaningful interpretation [279]. What is needed is a systems approach,

encompassing both families of muscle protein turnover signalling rather than an examination of individual molecules.

6. Conclusions

Relationships are seen between the activity of anabolic and catabolic signalling and protein turnover when a whole system approach is used. No distinct patterns emerged from examination of individual molecules.

DISCUSSION

Skeletal muscle wasting in the critically ill has significant functional implications for patients who survive. The development of prophylactic or therapeutic interventions has been hampered by our lack of understanding of the pathophysiology driving the process of muscle wasting.

I have shown that skeletal muscle wasting, characterised for the first time in a longitudinal cohort by three independent measures, occurs early and rapidly in critical illness. Specifically, ultrasound-derived Rectus Femoris cross-sectional area, histologically-determined Vastus Lateralis muscle fibre cross-sectional area, and protein/DNA ratio, all decreased over the first week. This lower limb muscle wasting is a consequence of both depressed muscle protein synthesis and an elevation in protein breakdown relative to protein synthesis, resulting in a net catabolic state. Muscle protein synthesis was depressed to levels equivalent to the healthy fasted state on day 1 but rose to rates similar to that in the healthy fed state by day 7, although the net balance remained catabolic. Whilst the activity of no individual intracellular signalling molecule was seen to correlate with either protein synthesis or breakdown, activity of the signalling family overall as determined by PCA correlated significantly.

Muscle ultrasound significantly underestimated protein loss (as measured by the protein/DNA ratio), perhaps in part because of the presence of interstitial oedema [116]: by day 7, the median net positive fluid balance in the study was 6410mls per patient. Despite the inability to perform validation studies in the critical care population, muscle ultrasound is likely to be useful in tracking muscle wasting and gain in response to interventions.

Correlations between muscle wasting and both serum C-reactive Protein (a marker of inflammation) and PaO_2/FiO_2 ratios were identified. Further studies are warranted here, in part to determine the sites of synthesis of inflammatory markers such as Tumour Necrosis Factor Alpha (TNF-α)-specifically, possible production by injured lung rather than by muscle itself [124]. TNF-α receptor

profiling would explain the downstream effects of said cytokines. Receptor I (otherwise known as the death receptor) leads to cellular apoptosis whilst Receptor II activation leads to activation of the atrophic pathways acting via Nuclear Factor Kappa Beta [399]. This switching between atrophy and apoptosis may help to explain the pathogenesis of muscle necrosis seen in this study. Whilst the above is a description of the extrinsic apoptotic pathway, the intrinsic apoptotic pathway needs to be investigated: mitochondrial damage has been demonstrated in the critically ill previously [139, 298], and it is likely that both intrinsic and extrinsic apoptotic pathways have complex interactions with the intracellular signalling pathways investigated here. A systems biology approach will be needed to meld these, perhaps with statistical modifiers used to build in both muscle wasting and necrosis. Unfortunately we currently lack specific markers of lung inflammation. Receptor for Advanced Glycalation End Products (RAGE) has been put forward as a likely candidate [400, 401]. The relationship between lung inflammation and RAGE remains unclear as to whether RAGE is implicated in the pathogenesis of lung injury or is produced as a result.

This study has identified a macrophagic infiltrate accompanying myofibre necrosis. The CD68 stain used is a generic macrophage stain, and does not allow identification of the role of the cellular infiltrate. Whilst it is likely that this is a reactive phenomenon secondary to myofibre necrosis, inflammatory infiltrates have been described in inflammatory myopathies [402-405]. Further characterization of macrophage sub-type would be difficult in humans, as large quantities of muscle would be necessary to prepare tissue and perform flow-cytometry. Animal models of sepsis and recovery are needed.

1. Developing clinical interventions

1.1 Research Methodology

Traditionally, critical care studies have focussed on mortality as the primary outcome[406]. As previously discussed, mortality is falling from critical illness, and long term morbidity and the burden of survivorship mandates the development of new, robust, patient centred endpoints [407].

Whilst functional end points (such as activity and time to return to work) and patient-reported endpoints are desirable, these ultimately do not fulfil the function of assisting the development of primary preventative measures for acute muscle wasting. Muscle cross sectional area is strongly related to function [92, 96]. If an interventional study were to be designed using $RF_{CSA}d10$ as a primary endpoint, the non-intervention arm would see a 20% loss. Assuming a 50% improvement with said intervention, using an alpha of 0.05 and power of 80% and two time points analysed via repeated measures ANOVA, 26 patients would be needed. This is a feasible study number to perform in-depth bedside physiological studies on interventions to reduce muscle wasting. The study design described in this thesis would therefore seem to be a robust framework to assess interventions as candidates for larger randomised controlled trials.

Early mobilisation strategies have been shown to decrease ICU and hospital length of stays, and mortality [150]. No data exist regarding the effect that this has on muscle wasting (likely due to the lack of objective techniques). It seems likely that countering the anti-anabolic effects of immobilisation may ameliorate muscle wasting [152, 160]. Early, rapid muscle wasting was more pronounced in those patients with multi-organ failure. These data indicate a role for using organ failure scoring to stratify critically ill patients at risk of muscle wasting. This approach may be clinically useful in identifying those patients who would benefit from early mobilisation strategies [150, 368].

1.2 Electrical stimulation

Neuromuscular electrical stimulation (NMES) has been trialled as an alternative to resistance exercise in ambulant patients and in the critically ill, with no clear success translatable into clinical use. In my original grant application, a further hypothesis had been put forward: "*neuromuscular electrical stimulation of muscle will ameliorate muscle wasting".* This was based on data demonstrating that NMES countered muscle wasting in immobilisation models[160], and had been used with some success in severe COPD [408-410] and following total knee arthroplasty [411-413].

However NMES is an initially catabolic stimulus (as all resistance exercise is), and has been shown to cause direct muscle damage, to a greater extent than resistance exercise [414]. Therefore NMES use as an intervention was precluded for two reasons. Muscle necrosis was a common finding, and additional muscle damage would not be in the patient's best interest. Secondly, muscle protein synthesis was depressed on day 1, despite nutritional delivery (a potent stimulus of protein synthesis). The anabolic stimulus of exercise and feeding would therefore seem very unlikely to occur. The results from this study suggest that NMES used early in critical illness would likely be of little benefit to patients, with a possibility of harm. With the normalisation of the protein synthetic response seen later in the ITU stay, NMES may have a role in recovery and rehabilitation, rather than in primary prevention. Recently a small study used Computer Tomography to assess muscle mass in 8 patients undergoing unilateral NMES, starting within 72 hours of admission to critical care with septic shock [97]. Whilst they too noted a significant decrease in muscle volume (2.3% a day), no difference was seen between the legs after 7 days of NMES 60 minutes a day. This is in keeping with the presence of depressed protein synthesis, unresponsive to stimuli. A pilot study comparing NMES in long stay ICU patients (greater than 14 days) versus acute (less than 7 days) found that only the former group benefitted from NMES after 4 weeks [415]. However the gain was 4.9% muscle limb thickness using ultrasound and one could argue that such a small gain may be a sampling error. A second study using muscle limb thickness found a difference of 11 millimetres between stimulated and un-stimulated legs [416]. However there was a lack of standardisation of intervention, with those who received NMES to the left leg having no benefit, and those that received NMES to the right leg seeing a 6-7% difference in Rectus Femoris diameter [416]. There are 4 randomised controlled trials registered on www.clinicaltrials.gov investigating the use of NMES in critically ill patients.

1.3 Feeding

As discussed in chapter 6, continuous delivery of enteral and parenteral amino acids results in a blunted synthetic response, protein synthesis peaking at 90-120 minutes from ingestion [417]. The remaining amino acids are likely to remain in the circulation, before being metabolised by the liver. Continuous feeding therefore mitigates the anabolic stimulus of feeding, and bolus feeding should be considered, especially in the early stages of critical illness, when protein synthesis is depressed.

Bolus feeding is not new to intensive care, having been the subject of multiple varying sized clinical trials [418-421]. These trials have focussed on prevention of ventilator-acquired pneumonia, and the alterations in gastric pH accompanying continuous enteral feeding. However these studies demonstrate that not only are such trials feasible, but that complications such as regurgitation, delivery errors and poor absorption did not differ between groups [418]. Careful study design will be needed: the physiological effects of supplemental feed boluses in a recent trial would have been masked by the concurrent continuous feeding [392]. Furthermore, the interplay between bolus nutrition and insulin resistance secondary to critical illness is likely to have consequences. It may be that accompanying insulin/potassium boluses are needed, which will have considerable logistical and safety implications. One weakness that must be acknowledged in this study is the definition of feeding. Whilst a pragmatic clinical definition has been used, future studies need to be clear on the effect of gut integrity on nutritional absorption. Measurements of alterations in serum amino acid profiles will be needed, which was not possible in the current study design.

Bolus feeding may have a role outside the first seven days. Whilst the trajectory of change in protein synthesis implies an increase towards more normal values, the building of muscle bulk requires both resistance exercise and nutrition. The synergistic effects of these stimuli would be blunted by continuous enteral feeding. Continuing bolus feeding in recovery period may decrease the time to independent nutritional maintenance.

In summary, high quality bolus feeding studies are needed before it can be concluded that nutritional delivery in the acute period of critical illness is deleterious to muscle mass.

1.4 Pharmacological intervention

Data from this study suggests that early interventions to enhance anabolism may be required in addition to reducing catabolism in order to effectively limit or even prevent muscle wasting. However, targeting individual components of the protein homeostasis signalling system may be less effective than physiological targeting of the protein signalling network.

With this in mind, proteasome inhibitors such as bortezomib have met with limited success, likely due to the lack of substrate specificity [422]. The lack of current knowledge on the regulation of the ubiquitin proteasome pathway has hindered the development of therapeutic agents. The Hdm2 oncogene product, HDM2, is an ubiquitin protein ligase. Research has focussed on the interaction between HDM2 and the p53 tumour suppressor oncogene. Small molecules that disrupt this relationship have been the subject of Phase I studies [423]. It may be that in the development of effective chemotherapies future research may reveal an appropriate specific target for intracellular manipulation.

1.4.1 Beta blockade

Beta blockade use in sepsis was first suggested in the 1970s [424] following animal and humans studies. Whilst there has been little interest in their use until recent times [425], some evidence exists that they may have a role in preventing muscle wasting, either as an immune-modulatory agent, or in decreasing oxygen consumption.

The normal immunosuppressant properties of catecholamines are attenuated in septic shock, though the precise nature of the relationship is unclear [426]. Epinepherine does not suppress TNFα levels in septic patients [426]. Non-selective beta blockade therapy upregulated the anti-inflammatory response in animal models, but resulted in an increased mortality [427]. Selective β1 receptor blocking does decrease circulating pro inflammatory cytokines in animal models [427, 428]. In a study in severely burnt children, propranolol decreased TNF αand IL-1B concentrations without

increasing mortality, highlighting the dangers of extrapolating animal work to clinical studies. In burnt adults a decrease in IL-1B was seen with propranolol therapy [429].

Non-selective beta blockers have been used in severely burnt children [430]), and decreases were seen in both resting energy expenditure (measured using a metabolic cart) and in limb protein breakdown (measured using labelled arterio-venous phenylalanine dilution).

Beta-blocker use in patients remains highly experimental. While the basic science has begun to describe a role for beta blockade in clinical studies, further clarity is needed as to their interactions with inotrophic agents before this can occur [431].

1.4.2 Leucine

One potential complication of using stable leucine isotopes for incorporation studies described in chapter 6 is the leucine itself can stimulate muscle protein synthesis. In healthy humans, leucine supplementation in the post exercise setting stimulates muscle protein synthesis [432-436]. However long term oral leucine supplementation has not been shown to increase muscle mass or strength in healthy elderly men [437] or in elderly type II diabetics [438]. This is despite evidence of increased muscle protein synthesis [439]. None of the above models can be completely extrapolated to the critically ill, where this study has demonstrated both increased limb protein breakdown and decreased muscle protein synthesis. It may be that leucine (or indeed mixed essential amino acid) supplementation has a role in ameliorating muscle wasting, but this again will need to be in the setting of bolus feeding, to prevent a "muscle full" effect confounder.

1.4.3 Tumour Necrosis Factor alpha blockade

All clinical trials so far have not included measures of muscle mass (due to lack of available techniques). Using mortality as a primary outcome both the NORASEPT and INTERSEPT trials showed no difference in mortality or organ failure scoring [440]. However a single bolus of TNF α antibody did reduce cytokine production, but had no effect on whole body protein [441].

2. Critique of the study design

Whilst these data relate to the largest cohort of critically-ill patients to have undergone longitudinal deep phenotyping, the pragmatic nature of this study raises a methodological issue. The first day of ICU admission does not necessarily reflect the first day of critical illness. However, whilst unable to quantify physiological derangement prior to admission, the median time from hospital to ICU admission was only 24 hours. In addition 27 subjects were trauma patients, suffered intracranial events or acute coronary syndromes and were not exposed to antecedent decline.

3. Conclusion

Muscle wasting occurs early and rapidly during the first week of critical illness, and is more pronounced in multi-organ failure. Muscle wasting is associated with a decrease in muscle protein synthesis and a net catabolic state, with acute myofibre necrosis and macrophage infiltration a common adjunctive finding. These novel data provide important directions for both the clinical management of and future research into muscle wasting in critically ill patients.

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APPENDIX

Author	Year of Study	Mean Age (yr)	Diagnosis	Mean APACHE Score (SD)	Design	Frequency of ICU-AW	Steroid Use	Neuromuscular 1Blocking Agent Used	Mean Length of Use of Neuromuscular Blocking Agent (d)	Days Ventilated Prediagnosis of ICU-AW	Diagnostic Method for ICU-AW	Association with Neuromuscular Blocking Agent
Leijten *et al.* (117)	1995	<75	Heterogeneous	Not reported	Prospective observational	29/50	Not recorded	Vecuronium	Not reported	Not standardized	EP Testing	N (U)
Latronico *et al.* (70)	1996	50.2	Heterogeneous	Not reported	Prospective observational	24/24	4/24	Pancuronium or atracurium	For procedures only	Not standardized	Clinical examination	N (U)
Kesler *et al.* (98)	2009	39	Asthma	Not reported	Retrospective observational	10/74	All	Vecuronium or atracurium	2 patients	Not standardized	Clinical examination	N (U)
Leatherman *et al.* (97)	1996	38	Asthma	Not reported	Retrospective observational	20/96	All	Vecuronium or atracurium	1	Not standardized	Clinical examination	N (U)
Behbehani *et al.* (67)	1999	47	Asthma	14.3 (6.2)	Retrospective observational	9/86	All	Vecuronium or pancuronium	3.1	Not standardized	Clinical examination	Y (M)
Segredo *et al.* (66)	1992	45	Respiratory failure	Not reported	Prospective observational	7/16	None	Vecuronium	7	Not standardized	EP testing*	Y (U)
Witt *et al.* (118)	1991	43	Heterogeneous	Not reported	Prospective observational	30/43	Not recorded	Not recorded	Not reported	Not standardized	EP testing	N (M)
Douglass *et al.* (31)	1992	N/A	Asthma	Not reported	Prospective observational	9/25	All	Vecuronium	Not available	Not standardized	Clinical examination	Y (U)
Adnet *et al.* (68)	1995–1999	40	Asthma	Not reported	Retrospective observational	10/55	All	Pancuronium or vecuronium	4.2	Not standardized	Clinical examination	Y (M)
Nanas *et al.* (119)	2005–2006	55	Heterogeneous	15 (7)	Prospective observational	50/474	38/474	Not recorded	61 patients	Not standardized	Clinical examination	N (M)
De Jonghe *et al.* (5)	1999–2000	62	Heterogeneous	SAPS II 48.7	Prospective observational	23/95	26/95	Vecuronium	3.3	>7	Clinical examination	N (U, M)
Garnacho-Montero *et al.* (69)	1996–1999	62	Heterogeneous	17.5 (6.9)	Prospective observational	46/73	11/73	Vecuronium or atracurium	10/73	>10	EP testing	Y (M)
Campellone *et al.* (120)	1995–1995	53	Liver transplantation	24.4	Prospective observational	8/77	All	Pancuronium or vecuronium	Not reported	>7	Clinical examination	N (U,M)
de Letter *et al.* (121)	1994–1996	70	Heterogeneous	Not reported	Prospective observational	32/98	34/98	Vecuronium	Not reported	>15	Clinical examination	N (M)
Garnacho-Montero *et al.* (71)	1999–2002	61	Severe/sepsis	22.2	Prospective observational	34/64	Not reported	Vecuronium or atracurium	13/64	Not standardized	EP testing	N (M)
Bednarik *et al.* (122)	2000–2002	59	Multiorgan failure	SOFA 7	Prospective observational	17/61	None	Pipecuronium	Not reported	Not standardized	Clinical examination	N (M)

Definition of abbreviations: EP testing = electrophysiological testing; ICU-AW = intensive care unit–acquired weakness; M = multivariate; N = no; U = univariate; Y = yes.
*Limited to ulnar nerve stimulation.

Table 16: Observational studies investigating the relationship between Neuromuscular Blocking Agent use and Intensive Care Acquired Weakness. From Puthucheary et al. AJRCCM 2012

1. Western Blotting

First described in the late 1970s (Towbin et al 1979), protein blotting can be used to detect low amounts of proteins in complex tissue-e.g. muscle. Western blotting is a complex from of protein blotting. Briefly, it involves separation of the protein mixture by gel electrophoresis, and subsequent transfer to a suitable membrane. Protein identification occurs by labelling with a specific antibody, with subsequent immuno-detection.

1.1 Choice of Membranes

Both Polyvinylidene and Nitrocellulose membranes are commercially available. The former has a higher protein binding capacity (determined by the inner surface area of the pores Mansfield 1994) in addition to other generic advantages. In the context of this study, whilst both forms were trialled, these advantages had little real utility, and Nitrocellulose membranes were used. Protein adsorption

is the result of binding of hydrophobic amino acid side chains and the hydrophobic domains with the membrane surface.

1.2 Gel electrophoresis

Strictly speaking, complex proteins mixtures (i.e. muscle) are separated using a uni-dimensional sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins are separated by their molecular weight, using an electrical field.

The polyacrylamide concentrations can be homogenous or stepped-using a separate concentration for stacking/ separating gels. Different concentrations suit different sized proteins. 7.5% and 10% acrylamide gels were trialled; the major difference for this study was seen in the resolution of T/P-E4BP-1 (figure 65), the latter delivering similar results to that advertised by the supplier.

Figure 65: left to right. 7.5% gel with poor resolution of T4E-BP1. 10% gel with better resolution

1.3 Electophoretic transfer

A tank transfer method was used. Gels/membrane stack were immersed in a buffer reservoir, and a constant current applied. The transfer buffer provides electrical continuity between the electrodes, and provides an environment for the proteins to remain solubility of the proteins, without affecting adsorption of proteins by the membrane. Buffers undergo Joule heating during transfer-hence the need for external cooling. Tris-glycine buffer at pH8.3 was used, with methanol. pH 8.3 is higher than the isoelectric point of most proteins. Proteins that have separated on a gel are negatively charged, and migrate towards the anode.

1.3.1 Methanol in electrophoretic transfer

Methanol performs two major functions- gel stabilisation and stripping of SDS from protein molecules. Polyacrylamide is a hydrogel. In pure water, gels increase in size. This results in loss of resolution, preventable by the methanol component of the buffer. SDS is necessary for protein

resolution on gels. SDS confers a high negative charge to the protein, causing rapid movement through the membrane. Additionally, by coating the protein, the contact time with the membrane is reduced. The combination results in impaired transfer efficiency, which is resolved by the addition of methanol to strip SDS. Prior to transfer the gel is left to equilibrate in transfer buffer, to begin removal of SDS. There are certain circumstances where SDS is useful in small concentrations e.g. in blotting for extremely high Molecular weight proteins (bolt and Mahoney 1997).

1.3.2 Current and length of transfer.

100V was used for 2.5 hours. Concerns with higher currents and longer transfer revolve around increased migration of proteins through the membrane. 100V was a standard used in our laboratory and shorter transfer times were trialled (figure 66) to ensure that 2.5 hours resulted in a better transfer.

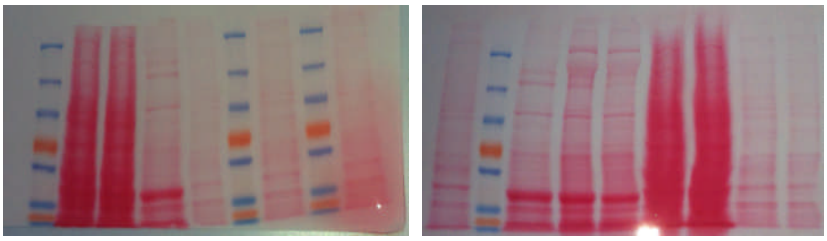


Figure 66: left to right: 90 minute transfer and 150 minute transfer, the latter showing greater protein concentration on staining with Ponceau Red.

1.4. Antibody reactions and immune detection.

1.4.1 Blocking

Prior to incubation with antibodies, unoccupied membrane sites are blocked with an inert protein (1% Bovine Serum Albumin was used) to prevent non-specific binding of antibodies. This is suspended in detergent (Tween® 20%), which has a renaturing effect on antigens-improving recognition.

1.4.2 Antibody binding

A primary antibody is bound to the protein, and a secondary to the primary. The secondary antibody is conjugated to a dye that allows localisation (figure 67). Whilst the staged process may seem cumbersome, advantages exist. Signal amplification may occur if the secondary antibody binds to more than one primary. A labelled secondary antibody can be used for many different primary antibodies, reducing the total number of antibodies needed. Antibodies are carried in 1% BSA as a vehicle, with a small amount of Tween®20 to prevent non-specific antibody aggregates.

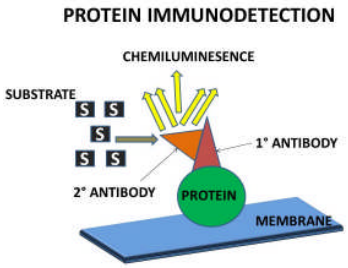


Figure 67: Schematic of protein detection using chemiluminesence. S=Substrate

1.4.3 Chemiluminescent Detection

A chemical reaction resulting in the production of light, chemiluminescense is highly sensitive. The ECL™ detection reagents are prepared by adding p-iodophenol to luminol (Hengen 1997). Detection is achieved by exposing the blot to X-ray film.

1.5 Membrane stripping and re-probing

Bound antibodies can be stripped from the membranes, allowing the bound proteins to be re-probed. A stripping buffer, made from Detergent and Beta mecaptoethanol was used. Over-stripping (removal of protein in addition) and under-stripping (insufficient removal of antibodies) can occur. Laboratory experiment 1, described below, had the stripping protocol incorporated for verification of efficacy.

2 .Recipes Used for Molecular Biology

10% separating Gel (0.75mm, 5mlper gel, 4 gels =20ml total)

-prepare on ice, and allow polymerizing for 2 hrs minimum, preferably overnight

Lower Tris pH 8.8	4000µl
40% Acrylamide	5000 µl
10% APS From Fresh Stock: 0.1g in 1ml DH20	400 µl
Temed	100 µl

4% Stacking gel (0.75mm, 5ml per gel=20ml total)

-prepare on ice

Upper Tris pH 6.8	4000 µl
40% Acrylamide	2000 µl
10% APS	400 µl
TEMED	100 µl
Bromophenol Blue	500 µl
DH20	13000 µl

Harsh stripping Buffer

200 microlitres of Beta Mercaptoethanol (fresh) to 50ml Tris/SDS

Laemmli Upper Tris- 500ml of 5X

37.85g Trizma Base	0.125M Trizma Base=1x=7.57g, 5X=37.85g
2.5g SDS	=0.5%SDS
Make up with DH20	

pH to 6.8

Laemmli Lower Tris- 500ml of 5X

113.357g Trizma Base	0.375M Trizma Base=1x=22.7g, 5X=113.357g
2.5g SDS	=0.5%SDS
Make up with DH20	
pH to 8.8	

Tris Buffered Saline (TBS) - 1litre of 10X

24.2g Trizma base	2.42g Trizma base for 1X
80g NaCl	8g for 1X
pH to 7.4	

TBS-Tween 20- 1litre of 10X

Trizma Base	24.2g
NaCl	80g
Distilled H2O	Make to 1Litre
Tween 20 (0.2%)	2ml, add at end
pH to 7.5	

Tris-Glycine SDS Running Buffer-10X

Tris base (formula weight 121.14)	0.25M=30g
Glycine (FW75.07)	1.92M=144g
10% SDS- final SDS solution of 1%	100ml or 10gSDS in 1litre
pH 8.3 before adding SDS	Some protocols say do not pH
DH2O	Make to 1 litre

Transfer buffer-2l of 1x

Tris Base	6.06g
Glycine	28.8g
Methanol	20% (200ml)
The pH is 8.3, do not adjust as may create salt and or increase ionic strength-and thus	If the protein has an isoelectric point equal to the buffer pH, transfer will not be promoted

conductivity

1% BSA- 2g in 200ml DH2O

3. Laboratory Experiment 1

Objectives:

1. To optimise Western Blotting protocol for samples from the MUSCLE study.
2. To quantify phosphorylation of AKT (p-AKT/t-AKT ratio) in samples manipulated to give varying levels of p-AKT

Human myoblasts were cultured in a variety of mediums, in order to increase p-AKT signalling incrementally. Table 1 details the mediums used:

Sample Number	Sample Type	Preparation
1	Human Muscle	Snap frozen
2	MARKER	MARKER
3	Human Myoblast	Serum free medium
4	Human Myoblast	Serum treated medium
5	Human Myoblast	Insulin treated medium
6	Human Myoblast	Insulin and Serum treated medium

Table 1: Sample preparation for Western Blotting. Sampled numbers correspond to well position on blots

Following gel electrophoresis (100V for 2 hours on ice) and transfer to nitrocellulose membrane (100V for 90 minutes on ice) visual verification of transfer was achieved by staining of the membrane with Ponceau Red staining (figure 68).

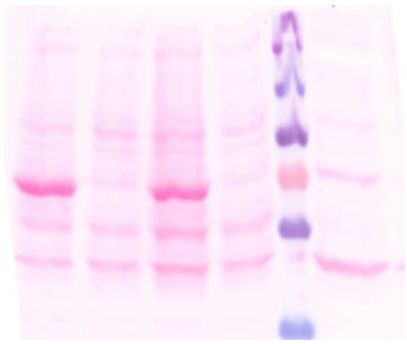


Figure 68: Nitrocellulose membrane stained with Ponceau red post transfer, visualising protein transfer.

The membrane was subsequently processed with antibodies for pAKT. Figure 69 is the resultant blot, with well numbers corresponding to table 1:

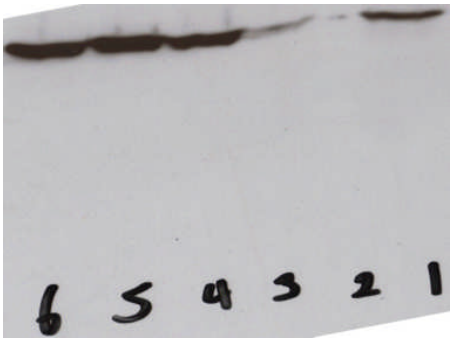


Figure 69: Western blot for pAKT. 1=Healthy human, 2=marker 3=myoblasts in serum free medium, 4=myoblasts in serum treated medium, 5= myoblasts in insulin treated medium, 6= myoblasts in insulin and serum treated medium.

The membrane was subsequently treated with an antibody stripping protocol, preparing it for reprobing with further antibodies. Prior to reprobing, the membrane was treated with secondary antibody and re-exposed to demonstrate lack of primary antibody (figure 70).

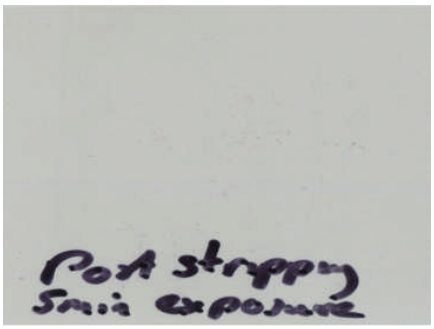


Figure 70: Western blot post stripping procedure, demonstrating lack of primary antibody.

The membrane was reprobed for tAKT, stripped and reprobed once more for and beta tubulin used as a loading control[442]. Figures 71 and 72 show this respectively.

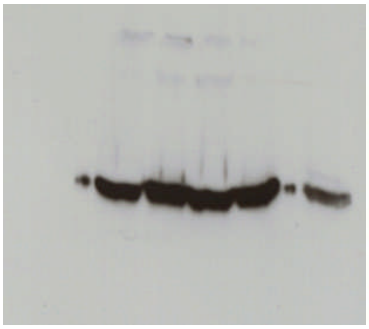


Figure 71: Western blot of same membrane, reprobed for tAKT.

Figure 72: Western blot of same membrane, reprobed for beta tubulin

The ratio of pAKT and tAKT was then plotted for these samples (Figure 73):

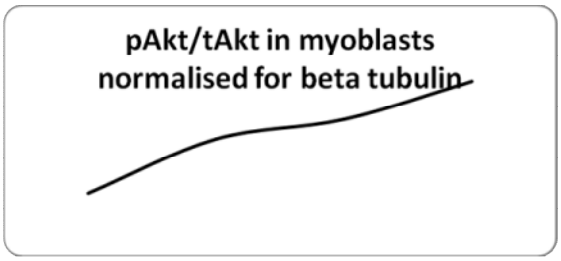


figure 73:ratio of pAKT/tAKT in the myoblast series.

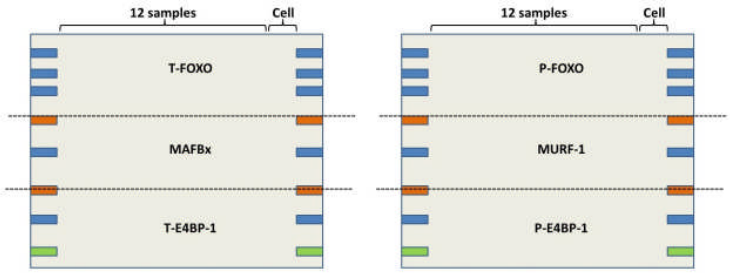
Conclusions

1. Western blotting protocol as above is viable
2. The strip and reprobe protocol is viable
3. This provides information of sufficient sensitivity as to distinguish between differing concentrations of p-AKT
4. To probe for Beta-tubulin, it would be preferable to re-strip the membrane, as the AKT/pAKT bands are close in molecular weight to that of beta tubulin.

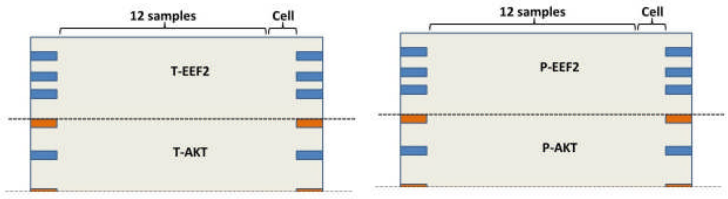
4. Algorithm for Western blotting of muscle samples

In total 127 samples were processed in this fashion. Only 48 were probed for T-AKT/PAKT- levels will be determined using the Luminex platform. T-AKT/P-AKT was measured by Western Blotting for comparison of techniques. 2x15 well gels were run concurrently, with two wells for molecular markers, and one well for an immortalised cell line. This may allow for inter-gel comparison.

Stage1: Dashed lines represent level at which the membrane was cut, to allow multiple protein detection.



Stage 2: Following exposure, membranes were striped and re-probed (Lowest segment discarded).



Stage 3: Following exposure membranes were striped and re-probed (Uppermost segment discarded).

6. Laboratory Experiment 2: Luminex Median Fluorescence

Normal Human muscle was used to test median fluorescence at different concentrations and increasing numbers of beads.

"M4" was processed at 10mcg/mcl, 25 mcg/mcl and 50mcg/mcl

T-Akt beads were used, with 2x runs at 50, 1x at 100 and 1x at 150 beads (figure 74). Coefficient of Variation of the Median Florescence Index (MFI) was also calculated (figure 75).

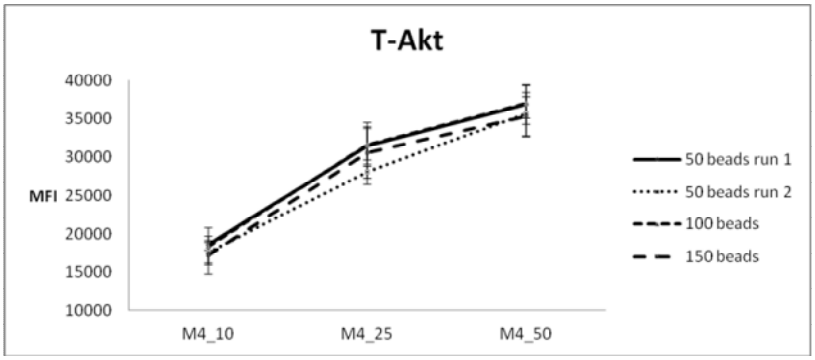


Figure 74: Median Florescense Index (MFI) for 3 concentrations of muscle preperations, run for different bead counts

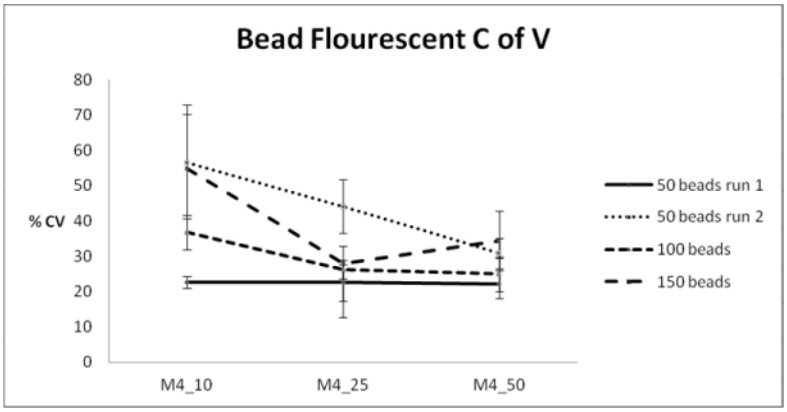


Figure 75: Coefficient of Variation of Median Florescence Index at different concentrations and bead counts

Summary:

1. The use of larger number of beads confers no added benefit.

2. 25mcg/mcl is an appropriate protein concentration.
3. Increasing number of beads does not decrease the Coefficient of Variation, which is 22% for 50 beads.