The metabolic phenotype of skeletal muscle in early critical illness

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Running Title: Skeletal muscle phenotype in early critical illness

What is the key question?
To investigate if adenosine triphosphate (ATP) bioavailability and lipid metabolism are drivers of the early and rapidly acute skeletal muscle wasting that occurs during critical illness.

What is the bottom line?
Skeletal muscle wasting in critical care is associated with impaired lipid oxidation and reduced ATP bioavailability, driven by intramuscular inflammation and altered hypoxic signaling which may account for the inconsistent outcome observed in the nutrition and exercise clinical trials.

Why read on?
In this first study to investigate the relationship between bioenergetics changes and skeletal muscle muscle wasting during early critical illness, alterations in fat metabolism and ATP abundance are associated with the severity of inflammation and altered oxygen signaling, which could be considered as targets for future therapies.

Descriptor number: (on website)
ABSTRACT

Objective: To characterise the skeletal muscle metabolic phenotype during early critical illness

Methods: Vastus Lateralis muscle biopsies and serum samples (days 1 and 7) were obtained from 63 intensive care patients (59% male 54.7 ± 18.0 years APACHE II score 23.5 ± 6.5).

Measurements and Main Results: From day 1 to 7, there was a reduction in mitochondrial beta-oxidation enzyme concentrations, mitochondrial biogenesis markers (PGC1α mRNA expression (-27.4CN (95%CI -123.9-14.3); n=23; p=0.025) and mitochondrial DNA copy number (-1859CN (IQR -5557-1325); n=35; p=0.032). Intramuscular ATP content was reduced compared to controls on day 1 (17.7mmol/kg/dw (95%CI 15.3-20.0) vs. 21.7mmol/kg/dw (95%CI 20.4-22.9); p<0.001) and decreased over 7 days (-4.8 mmol/kg dw (IQR -8.0—1.2); n=33; p=0.001). In addition, the ratio of phosphorylated:total AMP-K (the bioenergetic sensor) increased (0.52 (IQR -0.09-2.6) n=31; p<0.001). There was an increase in intramuscular phosphocholine (847.2AU (IQR 232.5-1672); n=15; p=0.022), intramuscular TNFR1 (0.66 μg IQR-0.44-3.33 n=29; p=0.041) and IL-10 (13.6ng IQR 3.4-39.0; n=29; p=0.004). Serum adiponectin (10.3μg (95%CI 6.8-13.7); p<0.001) and ghrelin (16.0ng/ml (IQR -7-100); p=0.028) increased. Network analysis revealed a close and direct relationship between bioenergetic impairment and reduction in muscle mass and between intramuscular inflammation and impaired anabolic signaling. ATP content and muscle mass were unrelated to lipids delivery.

Conclusions: Decreased mitochondrial biogenesis and dysregulated lipid oxidation contribute to compromised skeletal muscle bioenergetic status. In addition, intramuscular inflammation was associated with impaired anabolic recovery with lipid delivery observed as bioenergetically inert. Future clinical work will focus on these key areas to ameliorate acute skeletal muscle wasting.

Word count:250
MeSh: Muscle, Skeletal; Critical illness; Physiology
INTRODUCTION

Acute skeletal muscle wasting occurs early and rapidly in critical illness\(^1\) and is reported to be a major driver of longterm disability\(^2\). Indeed, muscle wasting is associated with increased length of intensive care unit (ICU) stay\(^1\), hospital stay\(^1\) and mortality\(^3\). It is underpinned by a fall in muscle protein synthesis\(^1\), a process which, in healthy individuals, is responsive to exercise and amino acid loading\(^4\). However, clinical trials of enhanced nutritional support\(^5\)-\(^8\) and early exercise training\(^9\)-\(^12\) in acute critical illness have yielded inconsistent results in terms of amelioration of muscle wasting and improvement in physical function.

Muscle protein synthesis is ATP dependent\(^13\)\(^-\)^14. The observed reduction in early critical illness\(^15\)\(^\)\(^-\)^16 may be the result of decreased mitochondrial number or function, or both\(^15\)\(^\)\(^-\)^16. These defects of metabolism may be the result of decreased substrate utilisation including glucose, a well-recognised complication of critical illness. Furthermore, the metabolic anomalies observed could be the consequence of the decreased utilisation of other energy substrates, such as fatty acids. This may, in part, account for the failure to show benefit in the previous nutrition and exercise clinical trials\(^5\)-\(^8\)\(^\)\(^17\)\(^\)\(^-\)^9\(^\)\(^-\)^12.

Systemic hypoxia and inflammation are part of the phenotype of critical illness\(^18\)-\(^20\) and both can impair mitochondrial function\(^16\) and muscle protein synthesis\(^21\)\(^\)\(^-\)^22. These interactions are complex\(^23\) and, as yet, incompletely elucidated with limited data available reporting the relationship between loss in muscle mass and skeletal muscle hypoxia and inflammation.

Whilst mitochondrial dysfunction has reported\(^15\)\(^\)\(^-\)^16\(^\)\(^\)\(^-\)^24, these data have not investigated the relationship with skeletal muscle wasting and muscle protein
turnover. We investigated the relationships between loss in muscle mass in early critical illness and skeletal muscle inflammatory, hypoxic and protein homeostatic signaling together with the bioenergetic status.

**METHODS**

Patients were those enrolled in the previously-described MUSCLE study (NCT01106300, [www.clinicaltrials.gov](http://www.clinicaltrials.gov))\(^1\). Ethical approval was obtained from University College London Ethics Committee A. At enrolment, written assent was obtained from the next-of-kin with retrospective patient consent obtained when full mental capacity was regained.

Patients were recruited within 24 hours of admission to a university hospital and a community hospital intensive care unit (ICU). All were anticipated to be invasively ventilated for >48 hours and to spend >7 days in, and survive, ICU. Patients were excluded if these criteria were not met and if they were pregnant or a lower limb amputee, or suffering primary neuromuscular disease or disseminated cancer.

Vastus Lateralis biopsies (days 1 and 7) were performed and snap-frozen in under 10 seconds. Serum samples (days 1, 3, 7 and 10) and physiological data were collected as described\(^1\).

**Mitochondrial components**

Electron transport chain component protein concentrations were determined, as were those of key enzymes of fatty acid transport (Carnitine Palmitoyltransferase-1), beta-oxidation (Medium Chain Acyl-CoA Dehydrogenase) and oxidation of unsaturated fatty acids (2,4-dienoyl-CoA reductase 1) using muscle homogenates (Luminex technology: Flexmap3d, Merck Millipore). The peroxisomal beta-oxidation
enzyme Multifunctional Enzyme-2 was quantified as was the electron transport system electron carrier Electron Transferring Flavoprotein (ETF).

Bioenergetic quantification

Intramuscular adenosine triphosphate (ATP), free creatine (Cr) and phosphocreatine (PCr) levels were fluorometrically-measured\textsuperscript{25}. These were compared against unpublished data from 31 healthy subjects comprising of both those with and without stable chronic disease, as per our patient cohort (Table S7). Intracellular energy sensor Adenosine Monophosphate-activated Protein Kinase (AMP-K) protein concentration was measured (Western Blotting).

Muscle metabotyping

Thawed muscle tissues underwent extraction and the organic phase used for ultra performance liquid chromatography and tandem mass spectrometry (UPLC-MS) optimised for lipid profiling as previously described\textsuperscript{26}.

Mitochondrial biogenesis

Mitochondrial DNA copy number and messenger RNA (mRNA) expression of members of the peroxisome proliferator-activated receptor gamma co-activator 1 family (PGC1\textalpha, PG1\textbeta and PPRC-1, central regulators of mitochondrial metabolism and biogenesis\textsuperscript{27}) were independently quantified (Quantitative Polymerase Chain Reaction: qStandard, London UK) and reported as normalized copy number (CN). Expression of Dynamin-related protein 1 (DRP-1; a marker of mitochondrial fission\textsuperscript{28}) was determined.
Adipokine and ghrelin response

Enzyme-linked Immunosorbent Assay (ELISA, Merck Millipore) of serum ghrelin and adipokinin concentrations was performed.

Intramuscular hypoxia and inflammation

We measured intramuscular concentrations of Hypoxia-Inducible Factor 1-alpha (HIF1α, the primary hypoxia-induced transcription factor) (Luminex technology; Flexmap3d, Merck Millipore) and pro/anti-inflammatory cytokines (high sensitivity Evidence Investigator chip array, Randox, Co Antrim).

Statistical analysis

Data were assessed for normality (D’Agostino and Pearson omnibus tests), and analyzed using Student’s t-test, Pearson’s coefficient, Mann-Whitney U test and Wilcoxon’s signed Rank Tests as appropriate. Parametric data are reported as mean( confidence intervals) and non-parametric as median (Interquartile range).

Metabotyping: Two forms of data reduction (principal components analysis and partial least squares discriminant analysis) were used for visualisation of untargeted profiling data. Principal component analysis (LCMS data) visualised inherent clustering and identified outliers. Partial least squares discriminant analysis was performed to maximise class differences between day 1 and day 7 samples while minimising variability unrelated to class. These were used to identify metabolites whose concentration was different between day 1 and day 3 and thus determine
metabolites of importance. These were then presented as raw data to confirm the changes identified in the multivariate methods.

**Network Analysis:** Each variable was represented as a node, and edges weighted by pairwise pearson correlation coefficient (threshold r > 0.4). Temporal changes (change from day 1 to day 7) in concentrations of active moieties of the anabolic/catabolic signaling pathways, physiological and nutritional data and muscle mass were included\(^1\). Clinical variables include were those that achieved statistical significance in our previously published multi-variate analysis\(^1\). MCLclust\(^{29}\) was implemented in Cytoscape (www.cytoscape.com)\(^{30}\) to reveal complex relationships otherwise hidden. As in all network analyses, multiple comparison were not corrected for as p values were not determined\(^{31}\).

(See Online Supplement for further methodological details).

**RESULTS**

The characteristics of the cohort have been previously described\(^1\). Of the 91 patients recruited, 63 fulfilled criteria for longitudinal analysis. Serial Vastus Lateralis biopsy samples were available from 33 patients and serum for 59, and metabotyping performed on a sub-cohort of 15 (figure S1). The characteristics of the whole cohort, patients biopsied and those metabotyped did not differ (all p>0.05), except that the proportion of males was higher amongst those metabotyped (80.0%, v 58.7%; p=0.047) (Table 1).
<table>
<thead>
<tr>
<th>Characteristics</th>
<th>All Patients</th>
<th>Intramuscular bioenergetic data</th>
<th>Lipidomics</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>63</td>
<td>33</td>
<td>15</td>
</tr>
<tr>
<td>Age</td>
<td>54.5 (50.0-59.1)</td>
<td>54.6 (37.5-62.0)</td>
<td>57.4 (49.2-65.7)</td>
</tr>
<tr>
<td>Male sex - n(%)</td>
<td>37 (58.7)</td>
<td>24 (72.7)</td>
<td>12 (80.0)*</td>
</tr>
<tr>
<td>Pre-ICU LOS-days#</td>
<td>1 (1-45)</td>
<td>1(1-36)</td>
<td>1 (1-6)</td>
</tr>
<tr>
<td>Days ventilated#</td>
<td>10 (2-62)</td>
<td>8 (2-62)</td>
<td>7 (2-23)</td>
</tr>
<tr>
<td>ICU LOS-days#</td>
<td>16 (6-80)</td>
<td>16 (7-80)</td>
<td>16 (6-20)</td>
</tr>
<tr>
<td>Hospital LOS-days#</td>
<td>30 (10-334)</td>
<td>30 (11-212)</td>
<td>38 (10-141)</td>
</tr>
<tr>
<td>APACHE II score</td>
<td>23.5 (21.9-25.2)</td>
<td>22.6 (20.1-25.0)</td>
<td>23.3 (19.3-27.3)</td>
</tr>
<tr>
<td>SAPS II score</td>
<td>45.5 (41.8-49.3)</td>
<td>44.2 (39.4-49.0)</td>
<td>44 (38.1-50.0)</td>
</tr>
<tr>
<td>ICU survival- n(%)</td>
<td>61 (97)</td>
<td>31 (94)</td>
<td>14 (93)</td>
</tr>
<tr>
<td>Hospital survival- n(%)</td>
<td>56 (89)</td>
<td>28 (84.8)</td>
<td>13 (87)</td>
</tr>
<tr>
<td>Renal Replacement Therapy – n(%)</td>
<td>19 (30.2)</td>
<td>10 (30.3)</td>
<td>4 (28.6)</td>
</tr>
<tr>
<td>Days NMBA use#</td>
<td>0 (0-6)</td>
<td>0 (0-6)</td>
<td>0 (0-2)</td>
</tr>
<tr>
<td>Hydrocortisone dose(mg)$#</td>
<td>0 (0-800)</td>
<td>0 (0-800)</td>
<td>0 (0-800)</td>
</tr>
<tr>
<td>Day 1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total by Day 10</td>
<td>0 (0-4533)</td>
<td>0 (0-4533)</td>
<td>266.5(0-4533)</td>
</tr>
<tr>
<td>HMG-CoA reductase inhibitor use-n(%)</td>
<td>11 (17.4)</td>
<td>5 (15.2)</td>
<td>2 (14.3)</td>
</tr>
<tr>
<td>Blood glucose (mmol/l) #</td>
<td>7.4 (5.1-11.4)</td>
<td>7.5 (7.1-7.9)</td>
<td>7.5 (6.9-8.1)</td>
</tr>
<tr>
<td>Cumulative insulin (iu)#</td>
<td>93 (0-1704.0)</td>
<td>103.9 (0-30.5)</td>
<td>32.9 (0-11.6)</td>
</tr>
<tr>
<td>Protein delivered (g/kg/ibw)</td>
<td>6.7 (5.3-8.2)</td>
<td>5.4 (4.5-6.2)</td>
<td>5.3 (3.8-6.7)</td>
</tr>
<tr>
<td>Calories delivered (kcal/kg/ibw)</td>
<td>158.4 (143.9-172.9)</td>
<td>148.4 (126.0-170.8)</td>
<td>159.5(122.0-197.0)</td>
</tr>
<tr>
<td>Fat delivered (g/kg/ibw)#</td>
<td>4.87(4.1-5.6)</td>
<td>4.55(3.7-5.4)</td>
<td>5.95 (3.3-8.6)</td>
</tr>
<tr>
<td>Cumulative Propofol (mg)</td>
<td>8684 (6320-11048)</td>
<td>10825 (6619-15030)</td>
<td>7590 (2264-12916)</td>
</tr>
<tr>
<td>Admission RFCSA (mm²)</td>
<td>500.6 (448.5-552.8)</td>
<td>511.6 (435.8-587.4)</td>
<td>551.8 (441.9-661.7)</td>
</tr>
</tbody>
</table>
**Mitochondrial components**

No change was observed in individual mitochondrial respiratory complex protein concentrations between day 1 and 7 (all p>0.050, figure S2, Table S4). *Mitochondrial* beta-oxidation enzyme concentrations decreased (all p<0.050, figure S3, Table S5), whilst concentrations of Multifunctional Enzyme-2 (*extra-mitochondrial* beta-oxidation) were unchanged (p=0.666).

**Bioenergetic data**
Intramuscular ATP content on day 1 in critically ill patients was lower than that of healthy subjects (17.7 mmol/kg dry weight (dw) (95% CI 15.3-20.0) vs. 21.7 mmol/kg dw (95% CI 20.4-22.9); p=0.005) (figure 1A). Day 1 ATP content was lower in critically ill patients with chronic disease than in those without (13.61 mmol/kg dw (95% CI 9.9-17.4) vs. 21.26 mmol/kg dw (95% CI 19.0-23.6); p=0.001, figure 1A). Correspondingly, ATP content on day 1 was similar in healthy individuals and critically ill patients without chronic diseases (22.17 mmol/kg dw (95% CI 20.7-23.7) vs. (21.26 mmol/kg dw (95% CI 18.9-23.6); p=0.383). However critically ill patients with pre-existing chronic disease had lower ATP content on day 1 than ambulant COPD controls (13.6 mmol/kg dw (95% CI 9.9-17.4) vs. 21.1 mmol/kg dw (95% CI 19.0-23.2); p<0.001).

ATP content declined from day 1 to day 7 in the whole cohort (-4.8 mmol/kg dw (IQR -8.0—1.2); n=33; p=0.001). The difference between those with and without pre-existing chronic diseases persisted at day 7 (10.43 mmol/kg dw (95% CI 7.3-13.5) vs. 16.47 mmol/kg dw (95% CI 14.2-18.8) respectively; p=0.002), although ATP content change over 7 days was no different between those with and without pre-existing chronic diseases (-3.18% (95%CI -8.1- 1.7) vs. -4.79% (95%CI-6.8--2.8); p=0.503). The ratio of phosphorylated to total AMP-K rose from day 1 to day 7 in the critically ill cohort (0.88 (IQR 0.62-2.52) vs. 1.57 (IQR 0.68-3.99) n=31; p<0.001, figure 1B).

Phosphocreatine concentration was also lower in patients than in healthy subjects on day 1 (41.7 mmol/kg dw (95% CI 32.6-50.4) vs. 72.7 mmol/kg dw (95% CI 69.0-76.4); p<0.001) and declined over the following 7 days (-14.7 mmol/kg dw (95% CI -23.3—6.1); n=33; p=0.039; figure 1C). Total creatine content was similar to that found in healthy subjects at day 1 (116.9 mmol/kg dw (95% CI 105.7-128.0) vs.126.0 mmol/kg dw (95% CI 117.4-134.6); p=0.141, figure 1D).
dw (95%CI 120.7-131.2); p=0.142; figure 1D) but declined over 7 days (-25.39 mmol/kg dw (IQR -42.4-1.44); n=33; p<0.048). Phosphocreatine and total creatine content were independent of chronic disease status.

Metabotyping

A 2-component cross-validated model had an $R^2_Y$ of 0.72 and $Q^2$ of 0.41 and AUROC of 0.95 for predicting day of muscle sampling. Following correction for multiple comparisons, a decrease in intramuscular triglyceride (-1366AU (95%CI-2605--127); n=15; p=0.048) and an increase in intramuscular phosphocholine ((side chains 19:0, 22:0); 847.2AU (IQR 232.5-1672); n=15; p=0.022) over 7 days retained statistical significance (Figure 2).

Mitochondrial biogenesis

PGC1α mRNA expression decreased (-27.4CN (95%CI -123.9-14.3); n=23; p=0.025) between day 1 and 7 as did that of the marker of mitochondrial fission DRP-1 (-42.7CN. (95%CI -77.2—8.2); n=23; p=0.018, figure S4, Table S6). Mitochondrial DNA copy number also decreased over the first 7 days (-1859CN (IQR -5557-1325); n=35; p=0.032). No change was observed in PGC1β or PPRC1 expression over 7 days (both p>0.05).

Adipokine and ghrelin response

Serum adiponectin concentration increased from day 1 to day 7 (10.3μg (95%CI 6.8-13.7); n=59; p<0.001), as did those of ghrelin (16.0ng/ml (IQR -7-100); n= 59; p=0.028, table S8). Both increases were sustained at day 10. Resistin concentration
was unchanged (-0.75μg (95%CI-1.6—0.1); n=59; p=0.918), as was leptin concentration (-0.01; n=59; p=0.113). In the obese subgroup (n = 16, body mass index ≥ 30 kg.m⁻²), leptin levels decreased over 10 days (26.6ng/ml (95%CI 22.7-30.5) to 19.2ng/ml (95%CI13.6-24.8); n=16; p=0.048).

**Hypoxic and inflammatory signaling**

Intramuscular HIF1α protein concentration increased from day 1 to 7 (-12.0AU (IQR 2.3-47.8); n=33; p<0.001; Figure 3C). Change in HIF1α was unrelated to changes in the partial pressure of oxygen in arterial blood, or to the saturation of arterial haemoglobin with oxygen (both r²<0.1, p>0.10; table S9).

Intramuscular tumor necrosis factor receptor 1 (TNFR1) concentration increased from day 1 to day 7 (0.66 μg (IQR-0.44-3.33 ); n=29; p=0.041; figure 3A), as did intramuscular interleukin (IL)-10 concentration (13.6ng (IQR 3.4-39.0); n=29; p=0.004; figure 3B). Intramuscular concentrations of IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-8, tumor necrosis factor alpha, TNFR2, Interferon gamma, monocyte chemoattractant protein 1, and endothelial growth Factor did not change over 7 days (n=29; p>0.05; Table 2, figure 3ABD).
### Table: Cytokine Levels

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Day 1</th>
<th>Day 7</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-α</td>
<td>11.2 (0.6-32.0)</td>
<td>0.6 (0.6-24.0)</td>
<td>0.375</td>
</tr>
<tr>
<td>TNFR1#</td>
<td>0.34 (0.0-1.5)</td>
<td>1.1 (0.0-3.5)</td>
<td><strong>0.042</strong>*</td>
</tr>
<tr>
<td>TNFR2#</td>
<td>0.01 (0.01-1.1)</td>
<td>1.4 (0.01-2.7)</td>
<td>0.083</td>
</tr>
<tr>
<td>IL-1α</td>
<td>6.8 (5.2-9.8)</td>
<td>7.6 (6.4-10.2)</td>
<td>0.715</td>
</tr>
<tr>
<td>HIF1-α$^*$</td>
<td>14.0 (9.8-22.5)</td>
<td>26.0 (21.0-69.8)</td>
<td>&lt;<strong>0.001</strong>*</td>
</tr>
<tr>
<td>IL-1β</td>
<td>28.4 (21.6-44.0)</td>
<td>30.8 (27.2-37.2)</td>
<td>0.229</td>
</tr>
<tr>
<td>IL-2</td>
<td>51.2 (0.9-66.0)</td>
<td>48.8 (0.9-56.8)</td>
<td>0.294</td>
</tr>
<tr>
<td>IL-4</td>
<td>150.0 (88.6-370.0)</td>
<td>242.0 (152.2-719.4)</td>
<td>0.206</td>
</tr>
<tr>
<td>IL-6</td>
<td>19.2 (6.8-59.8)</td>
<td>37.2 (12.2-84.2)</td>
<td>0.495</td>
</tr>
<tr>
<td>IL-8</td>
<td>21.6 (7.4-58.2)</td>
<td>52.8 (10.6-177.0)</td>
<td>0.100</td>
</tr>
<tr>
<td>IL-10</td>
<td>11.2 (0.37-41.8)</td>
<td>24.8 (14.8-298.4)</td>
<td><strong>0.005</strong>*</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>6.8 (0.4-8.8)</td>
<td>8.4 (3.0-9.2)</td>
<td>0.353</td>
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<tr>
<td>MCP-1</td>
<td>84.8 (18.1-122.2)</td>
<td>116.0 (88.4-267.2)</td>
<td>0.168</td>
</tr>
<tr>
<td>EGF</td>
<td>22.8 (2.0-40.6)</td>
<td>21.2 (1.0-29.6)</td>
<td>0.301</td>
</tr>
</tbody>
</table>

**Integration of data: network analysis**

While simple linear regression analyses did not provide useful insight (Tables S8,12,-14), nine data clusters were delineated, comprising 91 different interactions between longitudinal data from clinical, protein homeostatic, inflammatory, mitochondrial, bioenergetic and nutritional data (Figure 4). Of these, 79 (87%) interactions have been observed previously in human tissue, and the remainder in animal tissue (Table S11), further validating of our approach. The network revealed the close relationship between bioenergetic impairment and reductions in both Rectus Femoris Cross Sectional Area and Protein/DNA ratio (Figure 4A), and between intramuscular inflammation and impaired anabolic signaling (Figure 4B). Bioenergetic impairment and fall in muscle mass appeared unrelated to the delivery
of phospholipids, or of saturated, monounsaturated or polyunsaturated fatty acids, delivered (Figure 4C).

Further results are available in the online supplement.

**DISCUSSION**

In this study of patients during early critical illness, we report novel data detailing the relationships between skeletal muscle wasting and the inflammatory, hypoxic and protein homeostatic signaling together with the bioenergetic status of the muscle. A network analysis has been used to describe these relationships in the context of clinical physiological data. Three important novel findings were observed: i) Decreased ATP, creatine and phosphocreatine availability are closely and directly related to acute skeletal muscle wasting (protein/DNA ratio and Rectus Femoris Cross Sectional Area). ii) Activation of hypoxic and inflammatory signaling are closely and directly related to impairment of anabolic signaling pathways. iii) Changes in intramuscular ATP content and skeletal muscle mass are unrelated to the quantity of lipids delivered.

*Change in bioenergetic state during early critical illness*

Decreases in PGC1α, markers of mitochondrial fission (DRP-1) and mitochondrial DNA copy number reflect reduced mitochondrial biogenesis. Mitochondrial complex concentrations did not change over the first week of critical illness, although mitochondrial beta-oxidation enzyme concentrations fell. Although functional mitochondrial measurements could not be performed, decreases in beta-oxidation
and oxidative phosphorylation explain the finding that myocellular ATP content was lower in patients than in healthy controls on day 1, and decreased further over the first week. The measured decrease in muscle ATP, along with phosphocreatine and creatine content was accompanied by an increase in AMP-K, implying a compromised bioenergetic state and potentially decreasing anabolism. Assimilating previously published data on muscle mass and protein homeostasis regulation\(^1\), network analysis confirmed the relationship between impaired bioenergetic status, bioenergetic sensing signal and loss in muscle mass.

Neither muscle ATP content nor muscle mass were related to the quantity of fatty acids delivered as part of nutrition or sedative use. This was in keeping with both the measurements of lipid oxidation performed, and the increase in myocellular phosphocholine in a separate analysis. These data suggest that current critical care nutritional management is unable to prevent development of an impaired bioenergetics status, which may be causal in regard to acute muscle wasting.

**Metabolo-inflammatory response during early critical illness**

The complex relationship between inflammation, anabolism and metabolism was demonstrated in the network analysis and may impact on both skeletal muscle mass and quality. Intramuscular inflammatory mediators clustered with anabolic signaling proteins, suggesting a deleterious effect of inflammation. Previous work by Constantin et al\(^32\) demonstrated anabolic signaling transcription upregulation with no corresponding increase in protein expression. Inflammatory signalling likely inhibits this translation of programmed anabolic restoration\(^32\) and therefore muscle mass maintenance and has been demonstrated in animal models\(^22,33\).
Myonecrosis with an associated macrophagic infiltrate has also been reported. Elevated intramuscular IL-10 as seen in these data may activate macrophages and induce phagocytosis of myofibre debris, and represent another potential mechanism of switching in macrophage phenotype. This may counteract the detrimental effects of the observed lipid accumulation, which may affect switching from the M1 (pro-inflammatory) to M2 (anti-inflammatory) macrophage phenotype, impeding autophagy and recovery from myonecrosis. In addition, IL-10 demonstrates a suppressive effect on the Th1 cytokine response in injured muscle i.e. decreasing the inflammatory effects of M1 associated effectors, such as Il-6 and TNFα. The final macrophage phenotype remains unclear but is likely to be the result of a balance of these factors, which may be altered by interventions that either a) decrease inflammation or b) decrease lipid accumulation.

The rise in HIF1α was unrelated to the presence of systemic hypoxia, although the presence of cellular dysoxia cannot be excluded. Measuring the totality of the balance between tissue oxygen supply and demand in muscle beds is not technically possible in the clinical context. Nor is it easy to separate the extent to which metabolic change is dependent or consequent to altered oxygen or substrate delivery. However HIF activity can be stimulated via pro-inflammatory cytokines, circulating lactate or insulin resistance. Thus the HIF1α signaling in muscle during critical illness appears to mirror the complex relationship between hypoxic signaling and inflammation seen in solid tumours. Regardless, this increase in HIF1α concentration may reflect alterations in glucose metabolism such as the Pasteur effect, metabolic reprogramming to allow GLUT-4 membrane translocation or
attempts to stimulate glycolysis\textsuperscript{43}. Additonal examination of the complexity of glucose metabolism was beyond the scope of this manuscript.

The rise in adiponectin could represent a homeostatic response to lipid accumulation and/or insulin resistance\textsuperscript{44}, and increasing ghrelin concentrations may reflect the compromised bioenergetics state in skeletal muscle. However, adiponectin and ghrelin also play a role in the regulation of inflammation\textsuperscript{45,46}, and cluster analysis suggests a close relationship with the inflammatory response.

These data suggest that intramuscular inflammation is likely to prevent anabolic restoration, impeding the effects of exercise or amino acid supplementation on muscle protein synthesis. HIF1 $\alpha$ upregulation is associated with this inflammation and may modulate the metabolic response.

\textit{Clinical relevance}

These data are the first to suggest that the lipid component of enteral and parenteral nutrition may be, in part, bioenergetically inert in the context of critical illness. This may be of substantial clinical importance, given that lipids contribute 29-43\%\textsuperscript{47,48} of the energy content in enteral, and 50\% of parenteral, formulae\textsuperscript{49}. These findings thus offer a plausible explanation for the apparent conflict between data from observational studies demonstrating a clinical benefit from increased nutrition over the entire ICU stay\textsuperscript{50} and the lack of benefit observed with early targeted nutrition\textsuperscript{5-7}. In early critical illness, ATP turnover may not respond to higher calorific feed content and therefore differ little between intervention and usual care groups in clinical trials, particularly when parenteral nutrition is used\textsuperscript{5,6,17}.

Likewise, a failure of ATP turnover may account for a limited response to (and thus the observed limited benefit from) exercise interventions\textsuperscript{9,10,12} with concomitant
hypoxic and/or inflammatory stimuli additionally impairing muscle protein synthesis resulting in an attenuated response to intervention. However, as these adverse stimuli decrease with treatment and time, nutrition delivered may augment recovery.

Altering the composition of feed delivered in critical illness to exclude the lipid component may, in part, address the observed bioenergetic impairment and is an area for further research.

**Future work**

We consider that future clinical studies should be directed towards addressing these three core important novel findings. However, targeting a single molecule or other aspect of altered biology is unlikely to be successful. Interventions will likely need to target the whole, or multiple parts of the network (i.e. the molecular signature), in the same fashion perhaps as asthma or breast cancer.

From these data, we propose potential research to be targeted at:  

i) Skeletal muscle wasting as a consequence of a reduction in ATP, creatine and phosphocreatine availability e.g. non-invasive diagnostic techniques to measure mitochondrial function; development of treatments to ameliorate mitochondrial dysfunction and/or skeletal muscle energy content including the use of supplemental creatine.  

ii) Skeletal muscle anabolism which is negatively affected by hypoxia and inflammation e.g. targeted anti-inflammatory treatments, which may be pharmacologic, nutritional and exercise prescriptions; confirmation of timing for nutritional and exercise delivery to aid anabolic restoration.  

iii) Lipid delivery which has limited impact of skeletal muscle energy status e.g. the use of alternative non-fat fuel sources and removal of fatty acid supplementation.
In addition, the low bioenergetic baseline of patients with pre-existing chronic diseases highlights the need for tailored interventions, including nutrition\textsuperscript{50}, and stratification in trials\textsuperscript{54}.

Limitations

We did not directly measure mitochondrial function in snap-frozen samples, due to limitations in the validity of this approach\textsuperscript{55, 56}. However, our focus was on the downstream effects of previously described alterations in mitochondrial function\textsuperscript{15, 16, 24} and both the fall in ATP and increases in phospholipids are in keeping with the static measurements performed. Tissue was rapidly acquired (under 10 seconds) and snap-frozen allowing further confidence in these data\textsuperscript{57}. Limited tissue availability precluded more granular metabophenotyping, although sufficient to test the hypothesis. The consistent and marked declines in muscle ATP and the muscle creatine pool may be contributed to by muscle tissue necrosis that has been previously observed\textsuperscript{1}. Total ghrelin was measured rather than active components such as acyl-ghrelin\textsuperscript{58} and may not reflect true activity. The network analysis allowed revelation of patterns of activity that would otherwise have been hidden by traditional analysis\textsuperscript{31} of a relatively small (albeit deeply phenotyped) cohort, which nevertheless represents the largest longitudinal serial muscle biopsy cohort in acute critical illness. Ideally these findings would be replicated in a second independent sample. Given the difficulties in performing serial muscle biopsies in the critically ill, this was considered beyond the scope of the study. Our data may not be generalisable to all critically ill patients, as this cohort consisted of patients of high acuity (APACHE II score of 23.5 (95\%CI21.9-25.2) who spent a minimum of 7 days in critical care. A validation cohort of lesser acuity may offer differing results. The
patient cohort is heterogeneous in many regards, as is typical of the critically ill. However we, and others, have demonstrated that similar of patterns exist in critically ill patients with respect to acute muscle wasting in terms of muscle mass\textsuperscript{1,59} and function\textsuperscript{54,59,60}, molecular biology\textsuperscript{1} and in this current application, metabolism, despite heterogeneity in presenting disease. Moreover, the statistical approach used would reveal such heterogeneity if it existed. These data strongly support the presence of metabolic homogeneity. Of clinical importance, the mechanisms identified were present across a mixed ICU population. It is, of course, possible that the balance of such elements may vary amongst individual patients (e.g. with differing levels of inflammation, hypoxaemia, corticosteroid and vasopressor agent exposure).

Only 5.3\% of data entered into the network analysis was missing, which is within acceptable missing data threshold\textsuperscript{61}. The convergence of data from separate laboratories and techniques also increases confidence in our conclusions. The first day of ICU admission does not necessarily reflect the first day of critical illness. Whilst unable to quantify physiological derangement prior to admission, the median time from hospital to ICU admission was only 24 hours. In addition 16/34 patients suffered major trauma or an intracranial bleed and were not exposed to antecedent decline. Due to the observational nature of our study, causal links in terms of mechanism cannot be made. While longitudinal studies remain the focus of this work, we acknowledge the need for future work accounting for alterations in inflammation and hypoxia signaling relative to healthy controls.

All control muscle ATP, phosphocreatine and free creatine concentrations represent unpublished, archived, historical data that were generated prior to the analysis
performed on muscle from critically ill patients. However, all measurements were
standardized as they were performed in the same laboratory, using the same
techniques by the same research group. Further control muscle ATP, creatine and
phosphocreatine contents are noted to be within normal range\textsuperscript{25}.

Conclusions

These data have shown that there is a relationship between an impaired
bioenergetic status and acute muscle wasting during early critical illness. This
indeed may explain the reduced clinical effectiveness of the currently employed
nutritional supplementation and exercise therapy interventions used in the ICU.
Dysregulated lipid oxidation and decreased mitochondrial biogenesis contribute to
this compromised skeletal muscle bioenergetic status. In addition, intramuscular
inflammation was associated with impaired anabolic recovery with lipid delivery
during early critical illness observed as bioenergetically inert. Future clinical work will
need to focus on these key areas to develop strategies to ameliorate acute acute
skeletal muscle wasting during early critical illness.

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REFERENCES


FIGURES LEGENDS

FIGURE 1: Bioenergetic Data A) Skeletal muscle Adenosine TriPhosphatase in control subjects and on day 1 and 7 of critical illness. B) ratio of Phosphorylated to Total Adenosine MonoPhosphate Kinase on day 1 and 7 of critical illness. C) Phosphocreatine and D) Total Creatine data in control subjects and on day 1 and 7 of critical illness. Clear symbols represent patients with stable pre-existing chronic disease, whilst filled symbols represent patients without pre-existing chronic disease. Red lines represent mean and 95% Confidence Intervals. *represents p<0.05 for comparisons between time points (horizontal parentheses) and comparisons between those with and without pre-existing chronic diseases (vertical parentheses).

FIGURE 2AB: Ultra Performance liquid chromatography mass spectrometry data for muscle samples on day 1 and day 7 of critical illness. A) Phosphocholine and B) Triglyceride. *represents p<0.05.

FIGURE 3ABCD: Change in intramuscular cytokine concentration between day 1 and day 7 of critical illness. A) TNFα= Tumour Necrosis Factor alpha; TNFR1= Tumour Necrosis Factor Receptor 1; TNFR2= Tumour Necrosis Factor Receptor 2; B) il-4=Interleukin 4, il-10=Interleukin-10; C) HIF1α= Hypoxia Inducible Factor 1 alpha; D) il-1α= Interleukin 1 alpha; il-1β= Interleukin 1 beta; il-6 interleukin 6; IFN- γ= Interferon gamma; EGF=Epithelial Growth Factor. * indicates p<0.05.

FIGURE 4ABC: Multi-dimensional network analysis of components of bioenergetics status, hypoxia and inflammatory signaling, protein homeostasis signaling molecules, measures of muscle mass, clinical physiology data and lipid and carbohydrate nutrition delivered. Data are change over 7 days except for nutritional data (total delivered over 7 days) and organ failure (cumulative). Lines represent r-values, green being positive and red negative, with greater thickness representing higher values. Panels represent Markov Clusters: (A) Measures of muscle mass and energetic data: TCR=Total Creatine; PCR=Phosphocreatine; P/D=Protein to DNA ratio; ATP= Adenosine TriPhosphate; DRP-1= Dynamin Related Protein 1; RFCSA= Rectus Femoris Cross Sectional Area; AMPK= Adenosine Monophosphate Kinase; IL= interleukin; (B) Protein homeostasis and inflammation: AKT= Protein Kinase B; mTOR= mammalian target of rapamycin; P70S6K= 70-kDa S6 protein kinase; FOXO= Forkhead Box Class O-1; E4BP1= Eukaryotic Initiation Factor 4E binding protein 1; HIF1α= Hypoxia Inducible Factor 1 alpha; TNFα= Tumour Necrosis Factor alpha; TNFR1= Tumour Necrosis Factor Receptor 1; TNFR2= Tumour Necrosis Factor Receptor 2; IFN- γ= Interferon gamma; EGF=Epithelial Growth Factor; MCP-1= Macrophage Chemotactic Protein-1; (C) Nutritional lipid delivery: P.Lipid= Phospholipids; MUFA= Monounsaturated Fatty Acids; PUFA= Polyunsaturated Fatty Acids; SFA= Saturated Fatty Acids. CRP= C-Reactive Protein; IGF1-R= Insulin like Growth Factor-1; OF= Organ Failure; MXN= Mitochondrial copy number; MCT=Medium Chain Triglycerides; PS=Polysaccharides; ADP=Adenosine
DiPhosphate; HCO₃⁻= Serum Bicarbonate; CPT= Carnitine Palmitoyltransferase-1; MCAD= Medium Chain Acyl-CoA Dehydrogenase; ETF=electron Transferring Flavoprotein; DECR1=2,4-dienoyl-CoA reductase 1.

Table 1: Baseline characteristics of patients. 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= Cerebrovascular accident. $= Corticosteroid dosing as hydrocortisone equivalents. iu= international units, ibw= ideal body weight, g=grams; kg=kilograms RFCSA= Admission Rectus Femoris Cross Sectional Area. Data are mean values (95% Confidence Intervals), except for # indicating median with range. Student’s T-test was used except for ¥(Chi-squared) and # (Mann Whitney U). a indicates p<0.05 when compared to whole cohort.

Table 2: Intramuscular cytokine concentrations on day 1 and 7 of critical illness (n=29). Data are median(IQR). p values are for two tailed Student t-test for parametric data and Wilcoxon rank test for non-parametric data. TNFα= Tumor Necrosis Factor alpha. TNFR= Tumour Necrosis Factor Receptor. VEGF= Vascular Endothelial Growth Factor. IFNγ =Interferon Gamma. MCP-1= Monocyte Chemoattractant Protein-1. EGF= Endothelial Growth Factor. Units are nanogram/litre except for #=microgram/litre and $= Median Florescence Index.