**Charles University in Prague**
Third Faculty of Medicine

Doctoral thesis

# The Intensive Care Unit-Acquired Weakness: the role of mitochondrial dysfunction in its pathogenesis

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# DECLARATION

I herewith declare that this study was done independently, results are original, genuine and my own. This doctoral thesis is based on experiments performed in the Laboratory of Metabolism and Bioenergetics on the Third Faculty of Medicine, Charles University, during my Ph.D. studies.

I have prepared this doctoral thesis individually and all cited work is referred to as appropriate. I did not use any sources figures or resources than other the ones stated in the bibliography, be they printed sources or sources off the internet.

Furthermore, I declare that – to the best of my knowledge – this work has never been submitted by me or by anyone else at this or any other university and has not been used to obtain the same or any other academic degree.

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# CONFLICT OF INTEREST STATEMENT

I have no competing financial relationships with any organization that might have an interest in the submitted work in the previous five years and no other relationships or activities that could appear to have influenced the submitted work to declare.

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# CONTENTS

# ABBREVIATIONS and ACRONYMS

| | |
|---|---|
| AA | Antimycin A |
| Acetyl-CoA | Acetyl Coenzyme A |
| ACTH | Adrenocorticotropic Hormone |
| ADP | Adenosine Diphosphate |
| ALS | Amyotrophic Lateral Sclerosis |
| ARDS | Acute Respiratory Distress Syndrome |
| Asc | Ascorbic acid |
| ATP | Adenosine Triphosphate |
| ATPase | Adenosine Triphosphatase |
| BIOPS | Biopsy Preservation Solution |
| BSA | Bovine Serum Albumin |
| CAC | Citric Acid Cycle |
| CCR | Coupling Control Ratio |
| CIM | Critical Illness Myopathy |
| CIP | Critical Illness Polyneuropathy |
| CIPNM | Critical Illness Polyneuromyopathy |
| CMAP | Compound Muscle Action Potential |
| COX | Cyclooxygenase |
| CS | Citrate Synthase |
| CSs | Corticosteroids |
| CV | Coefficient of Variability |
| cyt c | cytochrome c |
| DAPI | 4',6-diamidino-2-phenylindole |
| DMEM | Dulbeco´s Modified Eagle Medium |
| DMS | Direct Muscle Stimulation |
| ECAR | Extracellular Acidification Rate |
| EDTA | Ethylene Diamine Tetraacetic Acid |
| EGTA | Ethylene Glycol Tetraacetic Acid |
| EGF | Epidermal Growth Factor |
| EMG | Electromyography |
| ETC | Electron Transport Chain |
| FAD | Flavin Adenine Dinucleotide |
| $FADH_2$ | Flavin Adenine Dinucleotide (hydroquinone form) |
| FAO | Fatty Acid Oxidation |
| $F_1F_0$ATPase | ATP synthase |
| FBS | Foetal Bovine Serum |
| FCCP | carbonyl cyanide-4-[trifluoromethoxy]phenylhydrazone |
| FFA | Free Fatty Acid |
| FGF | Fibroblast Growth Factor |
| GPDH | Glyceraldehyde-3-Phosphate Dehydrogenase |
| GH | Growth Hormone |
| GLUT-4 | Glucose Transporter 4 |
| HOM | Homogenates |

| | |
|---|---|
| HRR | High-Resolution Respirometry |
| ICU | Intensive Care Unit |
| ICUAW | Intensive Care Unit-Acquired Weakness |
| IGF | Insulin-like Growth Factor |
| IFN-γ | Interferon gamma |
| IL-1 | Interleukin 1 |
| INR | International Normalized Ratio |
| iNOS | inducible Nitric Oxide Synthase |
| KHB | Krebs and Henseleit Bicarbonate medium |
| KCN | Potassium Cyanide |
| LA | Linolaeic Acid |
| MAP | Mean Arterial Pressure |
| MAS | Mitochondrial Assay Solution |
| MES | 2-(*N*-morpholino)ethanesulfonic acid |
| MOF | Multi-Organ Failure |
| MODS | Multiple Organ Dysfunction Syndrome |
| M-PER | Mammalian Protein Extraction Reagent |
| MRC | Medical Research Council |
| MTS | (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) |
| $NAD^+$ | Nikotinamidadenindinukleotid (oxidized form) |
| NADH | Nikotinamidadenindinukleotid (reduced form) |
| NEFA | Non-Esterified Fatty Acids |
| NMBAs | Neuromuscular Blocking Agents |
| NO | Nitric Oxide |
| OA | Oleic Acid |
| OCR | Oxygen Consumption Rate |
| OXPHOS | Oxidative Phosphorylation |
| PA | Palmitic Acid |
| PBS | Phosphate Buffer Saline |
| rhGH | recombinant human Growth Hormone |
| ROS | Reactive Oxygen Species |
| Rot | Rotenone |
| RyR1 | Ryanodine Receptor 1 |
| SDH | Succinate Dehydrogenase |
| SERCA | Sarco/Endoplasmic Reticulum $Ca^{2+}$ ATPase |
| SIRS | Systemic Inflammatory Response Syndrome |
| SNAP | Sensory Nerve Action Potential |
| SR | Sarcoplasmic Reticulum |
| SSC | Surviving Sepsis Campaign |
| TMPD | Tetramethyl-p-Phenylenediamine |
| TNF-α | Tumor Necrosis Factor alpha |
| UP | Ubiquitin-proteasome |
| UCP | Uncoupling Protein |
| XF | Extracellular Flux |

# SUMMARY

**BACKGROUND:** ICU-acquired weakness impairs functional outcome in survivors of critical illness. Therefore, deepening our understanding of its pathogenesis is an important goal as muscle-specific therapeutic targets are urgently needed. Systemic inflammation and sepsis are the main risk factors of ICUAW, and these syndromes are associated with mitochondrial dysfunction. The aim of our study was to collect reliable information on the mitochondrial function of human skeletal muscle in the protracted phase of critical illness. Additionally, we explored mitochondrial respiratory parameters following experimentally induced changes in the availability or composition of selected nutrients (fatty acids and glutamine).

**MATERIALS and METHODS:** Vastus lateralis muscle biopsy samples from patients with ICU-acquired weakness and age-matched healthy controls were obtained. In human skeletal muscle tissue homogenates mitochondrial functional indices were assessed by high-resolution respirometry, individual functional capacities of respiratory complexes were measured by spectrophotometry and correlated with concentrations of electron transport chain key subunits measured by western blot. Additionally, using human myoblasts and myotubes we studied the influence of extracellular environment manipulations by extracellular flux analysis.

**RESULTS:** The ability of aerobic ATP synthesis was reduced to ~54 % in ICU patients ($p<0.01$), in correlation with the depletion of complexes III (~38 % of controls, $p = 0.02$) and IV (~26 % of controls, $p<0.01$) and without signs of mitochondrial uncoupling. When mitochondrial functional indices were adjusted to citrate synthase activity, the activities of complexes II and III were increased in ICU patients 3-fold ($p<0.01$) respectively 2-fold ($p<0.01$). In myotubes form ICU patients the mitochondrial density was 69% of healthy controls ($p=0.051$). Fatty acid oxidation (FAO) capacity in these patients was 157% of FAO capacity in controls ($p=0.015$). Moreover, exposure of ICU myotubes to FFA significantly ($p=0.009$) increased maximum respiratory chain capacity. Of note, glutamine concentrations, consistent with moderate clinical hypoglutaminemia (300µM), bring about an optimal condition of myoblast proliferation and for efficiency of aerobic phosphorylation in an in vitro model of human skeletal muscle.

**CONCLUSION:** We first adopted high resolution respirometry to homogenates of human skeletal muscle and validated this method against isolated mitochondria and we adopted the protocol of extracellular flux analysis for the use in human myotubes. To our knowledge, this is the first study to demonstrate mitochondrial dysfunction in the skeletal muscle of patients with protracted critical illness. Importantly, functional mitochondria are depleted, but remaining mitochondria have a relative increase of fatty acid oxidation capacity and a long-term exposure to free fatty acids of these myotubes in turn leads to an increase in the capacity of the respiratory chain. Further studies are needed to evaluate whether similar changes are achievable by nutritional manipulations in vivo and whether improved mitochondrial function would translate to improved functional outcomes in ICU survivors.

# SOUHRN

**ÚVOD:** Svalová slabost kriticky nemocných významně a dlouhodobě zhoršuje výkonnost a kvalitu života pacientů, kteří překonají závažné onemocnění nebo trauma. Protože specifická léčba dosud neexistuje, je výzkum její patogeneze velmi naléhavý. Hlavní rizikové faktory – sepse a syndrom systémové zánětlivé reakce - jsou sdruženy s mitochondriální dysfunkcí, proto bylo cílem naší studie získat detailní informace o funkci mitochondrií lidského kosterního svalu v protrahované fázi kritického onemocnění. Dále jsme studovali vliv složení experimentálního prostředí (mastné kyseliny, glutamin) na mitochondriální bioenergetiku.

**MATERIÁL a METODY:** Získali jsme vzorky kosterního svalu kriticky nemocných a kontrolní skupiny pacientů (biopsie m. vastus lateralis). Na homogenátu lidského kosterního svalu jsme pomocí vysokoúčinné respirometrie analyzovali mitochondriální respirační aktivitu, spektrofotometricky jsme měřili individuální funkční kapacitu jednotlivých komplexů dýchacího řetězce a technikou western blot jsme stanovovali koncentrace jednotlivých podjednotek dýchacího řetězce. Na kulturách lidských myoblastů a myotub jsme analyzovali mitochondriální respirační parametry pomocí extracelulárního fluxu a studovali jsme vliv složení extracelulárního prostředí na bioenergetiku.

**VÝSLEDKY:** Aerobní produkce ATP je u kriticky nemocných snížená na ~54% hodnot zjištěných v homogenátu kosterního svalu kontrolní skupiny ($p<0.01$). Současně jsme pozorovali depleci komplexů respiračního řetězce III (~38%, $p = 0.02$) a IV (~26%, $p<0.01$), oproti očekávání jsme nezjistily odpřažení vnitřní mitochondriální membrány. Překvapivým zjištěním je významně vyšší aktivita komplexů II a III u kriticky nemocných (3-krát ($p<0.01$) a 2-krát ($p<0.01$)). In vitro experimenty na buněčných kulturách přinesly obdobné výsledky – zjistili jsme depleci funkčních mitochondrií u myotub kriticky nemocných (69% hodnot kontrolní skupiny, ($p=0.051$)) a celkově vyšší kapacitu oxidovat mastné kyseliny (157% hodnot kontrolní skupiny, ($p=0.015$)). Dlouhodobá expozice těchto myotub mastným kyselinám navíc signifikantně ($p=0.009$) zvyšuje maximální respirační kapacitu jejich dýchacího řetězce. Mírná hypoglutaminémie (300µM) je podle našich výsledků optimální pro účinnou oxidativní fosforylaci a proliferaci lidských myoblastů i myotub.

**ZÁVĚR:** Jako první jsme adaptovali a publikovali dvě nové metodiky - vysokoúčinnou respirometrii na homogenátu lidského kosterního svalu a analýzu extracelulárního fluxu lidských myotub. Toto je první studie vůbec, která prokázala mitochondriální dysfunkci kosterního svalu pacientů v protrahované fázi kritického onemocnění. Snížení aerobní fosforylace o ~50% je způsobeno deplecí funkčních mitochondrií, u zbývajících mitochondrií dochází k relativnímu zvýšení kapacity oxidovat mastné kyseliny. Navíc mají myotuby kriticky nemocných po expozici mastným kyselinám vyšší maximální respirační kapacitu dýchacího řetězce. Věříme, že naše výsledky přispějí k pochopení patogeneze svalové slabosti kriticky nemocných a že porozumění procesům sdruženým se změnami dostupnosti konkrétních živin může sloužit jako základ pro vývoj tolik potřebné specifické léčby ICUAW.

# 1 INTRODUCTION

Intensive care unit-acquired weakness (ICUAW) is a common complication of critical illness, particularly in patients receiving mechanical ventilation and/or suffering from conditions leading to systemic inflammatory response syndrome (SIRS). Moreover, ICUAW is now considered a manifestation of the multiple-organ failure syndrome at the level of peripheral nerve and muscle. The weakness and disability have been attributed to the nerve and muscle dysfunction acquired during or exacerbated by critical illness [1-3]. It begins within hours of an intensive care unit (ICU) admission [4], can dominate the long-term course and has a strong impact on the quality of life after the patient´s hospital discharge. Despite increasing knowledge regarding ICUAW, no specific therapy exists and identifying risk factors and developing preventive measures are therefore important goals.

Interestingly, the link between mitochondrial dysfunction and specific diseases has recently been highlighted in many reviews concerning cardiovascular diseases [5-6], neurological, degenerative and psychological disorders [7-9], ophthalmic diseases [10] or diabetes [11]. Moreover, increasing attention has started to be paid to the role of mitochondrial function and dysfunction in the development of cytopathic hypoxia in the course of multiorgan dysfunction syndrome (MODS) related to sepsis and SIRS [12]. Additionally, experimental studies on both the cellular and the organ level suggest a beneficial role for the administration of mitochondria-targeted therapies [12-13].

The work presented in this thesis concerns the various approaches to studying mitochondrial function in human skeletal muscle tissue. In the introductory chapter, I will review the current literature pertaining to the ICUAW and its pathophysiology. The experimental part aimed to assess the mitochondrial function in skeletal muscle of patients with ICUAW in protracted phase of critical illness. Mitochondrial bioenergetic indices were assessed by extracellular flux analysis in human skeletal muscle cells and by high-resolution respirometry in muscle tissue homogenates (a novel technique we have established and validated recently in our laboratory).

# 2 ICUAW and MITOCHONDRIAL DYSFUNCTION

## 2.1 ICUAW - Background

### 2.1.1 Definition and classification

ICUAW is defined as weakness developing in a critically ill patient without an identifiable cause other than nonspecific inflammation. It remains essentially a clinical diagnosis made at the bedside, when the patient is difficult to wean from mechanical ventilation or develops unexpected problems with rehabilitation and mobilization [14]. Given the substantial body of literature on neuromuscular disease in critical illness, previously two subgroups have been defined by electrophysiologic testing and histopathologic evaluation:

***(a) Critical Illness Polyneuropathy (CIP)*** is a distal sensory-motor axonopathy, where electrophysiologic testing usually shows decreased compound muscle action potential (CMAP) and sensory nerve action potential (SNAP) as well, but normal nerve conduction velocity. Abnormalities may be detected in 48 hours after ICU-admission [15]. In severe axonal disease a spontaneous muscle activity with fibrillation potentials can be detected.

***(b) Critical Illness Myopathy (CIM)*** is an acute primary myopathy. Electrophysiologic differentiation between CIP and CIM is possible only in the conscious and cooperative patient. In unconscious or uncooperative patients, the use of a direct muscle stimulation method is possible and shows reduced/absent action potentials reflecting loss of muscle electrical excitability in CIM. Finally, muscle biopsy is considered a gold standard for the assessment of the muscle-involvement in the disease process.

According to light-microscopic findings three subtypes of acute myopathy of intensive care have been described: (a) diffuse non-necrotizing “cachectic” myopathy, (b) thick-filament myopathy and c) acute necrotizing myopathy. The reported findings include muscle fiber atrophy, occasional fiber necrosis, regeneration and decreased or absent reactivity in staining for myofibrillar adenosine triphosphatase activity (ATPase), corresponding to a selective loss of myosin filaments, which is practically pathognomonic for CIM [16].

CIP and CIM frequently occur concomitantly and thus the descriptive term Critical Illness Polyneuromyopathy (CIPNM) has been used in the expert literature too. Though it is tempting to categorize weakness after recovery from critical illness as either myopathy or neuropathy, there is evidence of overlap between these pathophysiological processes [17-18]. Functional denervation resulting from nerve injury in CIP may provide the link between CIP and CIM. According to another hypothesis, both CIP and CIM represent different manifestation of a single pathophysiological mechanism triggered by sepsis or SIRS [19]. Moreover, given the limited number of advanced neuromuscular testing feasible or practicable bedside in critically

ill, the terms critical illness neuropathy (CIP) and myopathy (CIM) were in literature as well as in daily ICU-routine replaced by an overarching term for neuromuscular disease in critically ill - ICUAW.

### 2.1.2 Epidemiology

Investigation of patients with prolonged sepsis and severe muscle weakness recognized during the convalescence phase of the critical illness began in the 1980s [15]. The term ICUAW first appeared in literature in 1991 [20], although other terms like critical illness myopathy (CIM) or critical illness polyneuropathy (CIP) had already been used before [21].

There is no question that ICUAW is prevalent, but its occurrence varies substantially depending on the patient case-mix, diagnostic method used, and the timing of examination.

Clinically significant ICUAW has been found in 25% of patients who received mechanical ventilation for at least 7 days [22], electrophysiological testing in a prospective study of similar patients cohort receiving mechanical ventilation for > 7 days has resulted in reports of CIP incidence reaching 50% [23]. Several groups have documented that CIM is at least as frequent as CIP [24] and the prevalence of CIP and CIM combination may be up 96%, when the comprehensive diagnostic algorithm is used (including routine electrophysiological testing complemented with muscle biopsy and/or direct muscle stimulation (DMS) [25-26].

### 2.1.3 Clinical assessment

ICUAW clinically presents as flaccid, diffuse and usually symmetric weakness involving all extremities, with the reduction in or the absence of deep tendon reflexes [21,27]. Although cranial nerves are relatively spared and a facial grimace is usually preserved, these areas can still be affected [28]. Most commonly, patients with ICUAW have undergone unsuccessful attempts of weaning from mechanical ventilation; forced vital capacity and negative inspiratory force are typically low and profound weakness is present despite a complete return of sensorium [29]. An early clue that may be noted by care-providers is that painful stimulation (such as pressure on the nail bed) leads to a limited or absent limb response, whilst facial grimace is appropriate.

The clinical approach to ICUAW is based on the identification of a generalized weakness after other possible causes of generalized weakness have been excluded. Physical examination and the measurement of muscle power in the critically ill are dependent on the cooperation of the patient, which can be confounded by delirium, encephalopathy and other ICU-related factors (sedation, intubation, edema). A systematic approach to the investigation for neuromuscular weakness in ICU has been suggested by Maramattom and colleagues [30]. It includes the careful review of the premorbid functional status and medical history, laboratory

tests (creatinekinase (CK), electrolytes, arterial blood gasses), magnetic resonance imaging of brain/spine and electromyography (EMG). The presence or absence of a neuromuscular condition existing before an admission to the ICU must be clarified.

Whenever possible, a daily manual muscle testing should be performed accomplished with the Medical Research Council (MRC) score that includes formal testing of three muscle groups in each extremity on scale from one to five (see Table 1). This scoring system has an excellent interobserver reliability and can document the extent of weakness and its changes over time (assuming intact cognition) [31]. However, the examined patient must be awake and cooperative with all extremities capable of movement against a resistance. Despite its limitations, this scoring system has been implemented in several prospective studies in the management of critically ill, actually the MRC sum-score < 48 was used as the limit for defining ICUAW [22,25,29].

**Table 1: Medical Research Council scale (adapted from** [31]**)**

Score for each movement:

| | |
|---|---|
| 0 | no visible contraction |
| 1 | visible muscle contraction, but no limb movement |
| 2 | active movement, but not against gravity |
| 3 | active movement against gravity |
| 4 | active movement against gravity and resistance |
| 5 | active movement against full resistance |

Functions assessed => upper extremity: wrist flexion, forearm flexion, shoulder abduction
=> lower extremity: ankle dorsiflexion, knee extension, hip flexion

**Medical research council score** (0-60 points)
Maximum score = 60 points (maximum of 15 points per one extremity)
Minimum score = 0 points (quadriplegia)

Additionally, magnetic nerve stimulation is a useful experimental research tool that has been used to assess a skeletal muscle function independently on cooperation and cognition of the patients [32].

### 2.1.4 Risk factors

In the absence of an experimental model of ICUAW to explore mechanisms of injury, current information is inferred largely from prospective cohort studies using multivariate analyses to assess independent risk factors.

**(*a) Systemic inflammation*** has been repeatedly identified in prospective studies as a crucial risk factor [20,33,34] and the role of multi-organ failure (MOF) in the pathophysiology

of ICUAW has in the last decade been confirmed [35]. Also De Jonghe et al [25] demonstrated that prolonged MOF is strongly associated with the development of ICUAW.

***(b) The role of corticosteroids (CSs) and neuromuscular blocking agents (NMBAs)*** have been extensively studied over the past three decades in animal models. Administration of CSs can lead to selective muscle atrophy, particularly of fast-twitch fibres [36]. However, the thick filament myopathy (similar to CIM) can be produced only by combination of CSs and denervation injury [37]. The "two-hit" hypothesis has been proposed to explain the serious forms of CIM. To conclude, the overall data are not consistent. Some prospective studies did not identify CSs as an independent risk factor for CIPNM [38-40], although three other prospective trials did [3,35,41] and one study exists, that identify CSs to be an independent protective factor for the occurrence of CIPNM [42].

The association of neuromuscular weakness with the prolonged use of NMBAs has long been recognised and it is the main reason for a decline in a routine NMBAs use in the critically ill. The association between NMBAs use and ICUAW development remains unclear, but two clinical situations are described, where the NMBAs play an important role. Firstly, the prolonged neuromuscular blockade arising from persistent NMBAs effect occurring with agents that accumulate (or their metabolites accumulate) in the setting of renal or liver failure. Secondly, patients treated for severe asthma with ventilatory failure often undergo treatment with high-dose CSs in combination with NMBAs and can develop severe myopathy [42].

Finally, CIP or CIM has been documented in patients receiving none of these agents, so it is clear they play not the essential role in the pathophysiology of ICUAW. In human trial [43], 48 hour of continuous infusion of non-depolarizing neuromuscular blocking agents did not result in an increase of the incidence of ICUAW.

***(c) Immobility*** per se cannot create ICUAW, but repeated daily passive mobilization has prevented muscle atrophy on serial muscle biopsies in patients receiving mechanical ventilation and NMBAs [44]. Strategies to mobilize critically ill patients and implementation of protocolized sedation holds into routine care in ICUs resulted in a shorter duration of mechanical ventilation [45].

***(d) Hyperglycaemia*** was identified as an independent risk factor [20] for ICUAW long ago and confirmed as a deleterious metabolic factor on the neuromuscular system in the critically ill [40,46]. These two randomised trials suggested the application of tight glycaemic control as a promising preventive intervention. On the other hand, the NICE-SUGAR trial [47] demonstrated that intensive glucose control (to target 4.5-6.0mmol/L) resulted in a significantly increased 90-day mortality when compared to conventional glucose control (to

target ≤ 10.0mmol/L) and tight glycaemic control is currently not recommended in critically ill adults in ICU.

## 2.2 ICUAW - Pathophysiology

The pathophysiology of ICUAW is complex and is considered to represent an integral part of a process leading to MODS. Therefore, shared cellular, metabolic and microcirculatory pathophysiological mechanism/s very likely play the crucial role in the development of ICUAW. Nonetheless, no such a common mechanism has been identified so far.

The key features of ICUAW are a reduction in force-generating capacity and loss of muscle mass secondary to neuropathy, the disruption of myofilament function, an altered function of sarcoplasmic reticulum, electrical inexcitability and bioenergetic failure. A contraction of skeletal muscle involves a cascade of sub-cellular and cellular events. Naturally, when an impairment of any of the steps in this cascade occurs, it can result in muscle weakness.

In spite of being considered two independent entities in the past, myopathy and polyneuropathy in the critically ill very likely represent a continuum of changes over time in response to critical illness as there are many common aspects in their pathophysiology.

### 2.2.1 Skeletal muscle wasting in critical illness

A catabolic state with progressive skeletal muscle wasting occurs rapidly in the critically ill, particularly in patients with sepsis [48]. Diaphragm wasting has been described in the mechanically ventilated brain-dead organ donors within several hours as a marked atrophy of myofibres [4].

On account of the overall catabolic state, the rate of protein synthesis does not match the rate of protein degradation and the balance is upset. Preferential loss of myosin is a typical feature of CIM with disruption to the actin/myosin ratio [49] and destruction of typical myofilament organization in the skeletal muscle tissue. Protein degradation occurs through a couple of pathways, including calcium-dependent calpains [50] and lysosomal cathepsins. However, the predominant mechanism of proteolysis in ICUAW represents the ubiquitin-proteasome (UP) pathway, which is significantly activated as a response to inflammation and inactivity during critical illness [51]. The UP system is activated in animal models of muscle atrophy [52-53] and the ubiquitin gene expression is increased by pro-inflammatory mediators like tumor necrosis factor alpha (TNF-$\alpha$), interferon gamma (IFN-$\gamma$) and interleukin-1 (IL-1) [54-55]. Interestingly, denervation and sepsis both increase the steroid sensitivity in muscle due to up-regulation of cytosolic glucocorticoid receptors [56].

In animal models of ICUAW, the increased proteolysis dominates over decreased muscle protein synthesis. On the contrary, the anabolic resistance is a prominent process underlying the acute muscle mass loss in critically ill humans [57] coupled to a decreased protein synthesis in response to immobility and sepsis [58]. Increased protein turnover is mediated by decreased local and systemic levels of insulin-like growth factors (IGF) [59]. Additionally, protein anabolism is impaired by TNF-α through insulin resistance and increase in glucagon levels [60]. In contrast to animal models, the data in humans are more or less inconsistent and studies in the critically ill patients regarding the diminished protein synthesis and enhanced proteolysis are necessary.

### 2.2.2 Reduction in force-generating capacity during critical illness

As many critically ill become weak prior to any evidence of muscle wasting and this weakness may occur within hours of ICU admission [4], the underlying mechanism is likely to be independent of a plain muscle wasting. See Figure 1.

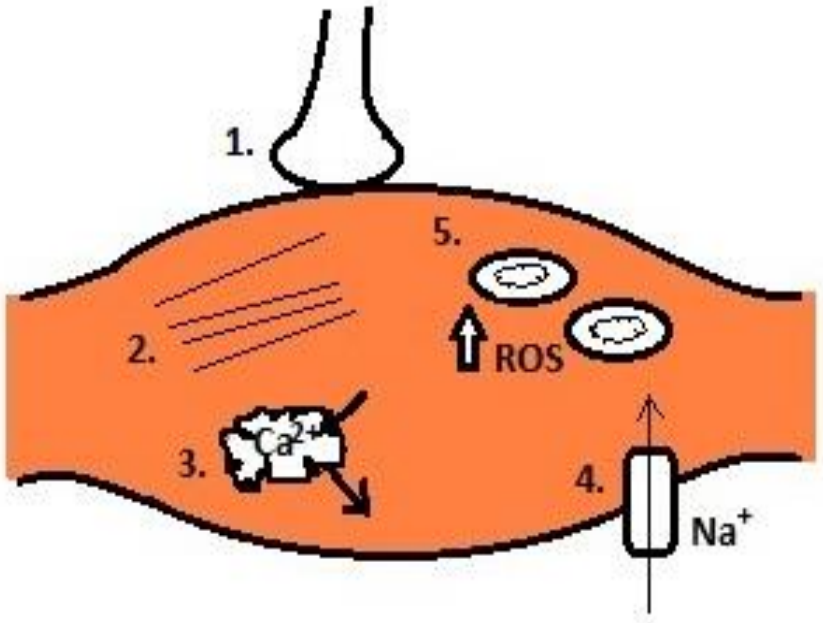


**Figure 1:** Potential mechanisms reducing the force-generating muscle capacity in ICUAW [adapted from [176]. 1) neuropathy; 2) disruption of myofibrillar organisation; 3) dysfunction of sarcoplasmic reticulum; 4) electrical inexcitability; 5) bioenergetic failure. For abbreviations see text.

#### 2.2.2.1 Muscle (and nerve) inexcitability

According to electrophysiological studies, abnormal electromyogram is a predictor of ICUAW and poor outcome in the intensive care patients [61]. Additionally, both muscle and nerve are relatively inexcitable in septic patients [62-63], although nerve histology is often normal [64] suggesting rather functional pathology. Incidentally, electrocardiographic abnormalities have been reported in septic patients, which returned to normal after recovery [65].

Historically, the popular animal model of a steroid- and denervation-induced myopathy had been studied. At first, denervation of the rat muscle fibre leads to a reduction of its resting membrane potential and to elevated expression of embryonic sodium channel isoform. The exposition of the denervated fibre to high-dose steroids results in its inability to generate action potentials, which was reversed by re-inervation [66-68]. Theoretically, functional denervation resulting from nerve injury may provide the link between polyneuropathy and myopathy.

In a rat model of ICUAW a significant reduction in sodium channel availability and overall current reduction are seen. Moreover, the available voltage dependent sodium channels become inactive in 99%, because of a shift of resting membrane potential to more negative threshold [69]. Not all fibres are affected to the same extent what may determinate the severity of muscle weakness [64].

Reduced muscle excitability is apparent in critically ill septic patients [70], and *in vitro* studies showed changes in sodium channel functions in humans [71]. I*n vivo* studies in humans in this field are lacking, and the role of the sodium channel malfunction in the pathogenesis of human CIM remains unclear. Voltage-dependent sodium channels do participate also in the regulation of neuronal excitability in human [19] and impairment of their function may underlie the ICUAW in a rodent model [72].

A significant correlation exists between renal insufficiency, elevated endoneurial potassium levels and membrane depolarization. Loss of the endoneurial membrane potential related to endoneurial hyperkalemia and/or hypoxia suggests that in patients with impaired renal function, hyperkalemia contributes to the development of polyneuropathy and patients with renal co-morbidity may have a worse outcome [73].

#### 2.2.2.2 Impaired excitation-contraction coupling

The tight regulation of $Ca^{2+}$ subcellular localization is crucial for the normal muscle contractile function as well as for the appropriate regulation of glycolytic and oxidative metabolism. Intracellular calcium levels are controlled by sarcoplasmic reticulum (SR) ryanodine receptor calcium release channel (RyR1) and the SR $Ca^{2+}$-ATPase channels (SERCAs). Changes in the excitation-contraction coupling at the level of sarcoplasmic reticulum (SR) $Ca^{2+}$ release may be involved in CIM. Long-lasting elevation of sympathetic nerve activity in critically ill causes damage to SERCAs and promotes leakage of RyR1, resulting in excitation-contraction uncoupling in a rodent sepsis model [74-75]. Interestingly, serum from ICUAW patients can reduce a force generation in permeabilised muscle fibres by blocking calcium release and have a deleterious effect on muscle membrane excitability *in vitro* [76].

#### 2.2.2.3 Neuropathy

Neuropathy associated with critical illness is a distal axonal sensory-motor polyneuropathy and affects both the locomotive and respiratory muscles [28]. In a human study, which involved the peripheral nerve, in biopsies taken early (median 15d of sepsis) most sensory nerves appeared normal, despite having reduced SNAPs [38]. Even in patients who died early after ICU admission, the autopsy demonstrated a normal nerve microscopic appearance [49]. However, late biopsies (median 56d) showed axonal neuropathy [77].

Most of the trials have focused on an “acute” neuropathy that occurs typically as a failure to wean critically ill patients off the ventilator. The nerve histology is preserved, while electrophysiological abnormalities occur and are usually reversible. But there is a subgroup of patients, who do not restore a normal function even after 2 years [78].

There is an agreement that the axonal dysfunction can be explained by a membrane depolarization defect, occurring early in the course of illness. In the absence of ultrastructural changes, impairment of the blood-nerve barrier is the first crucial step towards endoneurial oedema, hypoperfusion, hypoxemia and energy depletion. This results in a passive uptake of glucose and deficient scavenging of reactive oxygen species (ROS) [79].

In 2005, Bolton and colleagues hypothesized that microvascular alteration in the endoneurial capillaries may be the key event in the development of CIP [80]. This hypothesis was supported by the observation of an increased expression of E-selectin in endothelial cells (in peri- and endoneurial vessels) in the critically ill with ICUAW, implying an increase in the microvascular permeability occurring in the peripheral nerves [81].

Toxic factors may directly penetrate the endoneurial membrane. It has been confirmed that the severity of neuropathy in the critically ill correlates with the duration of ICU stay and serum glucose levels [82], which is of fundamental importance for clinical praxis. The passive uptake of glucose with increased generation and deficient scavenging of reactive oxygen species (ROS) in neurons [83] contribute to bioenergetic failure (see below). Additionally, the toxic oxidative effect of parenterally administered lipids (nutrition overload) can worsen the neural microvasculature damage and impair an axonal protein transport [84]. Early administration of parenteral nutrition to the critically ill has been shown to be harmful in the largest clinical trial published so far [85], as was the aggressive glutamine supplementation [86]. Our group was the first to provide evidence that glutamine can cause mitochondrial uncoupling and impair ATP generation in human skeletal muscle [87].

Microvascular injury can be caused by hypoxemia per se, or by circulating mediators released during sepsis or SIRS (e.g. serotonin, histamin or TNFα [20,88]). Moreover, in vitro experiments with sera from critically ill patients with CIP suggested that low molecular weight

neurotoxic factor may play a role in the ICUAW development, but its existence has not been confirmed and this hypothesis remains controversial [83,89]. Magnetic resonance imaging has been recently introduced as a new perspective research tool in the *in vivo* studies of blood-nerve barrier, offering a novel approach in this research field [90].

#### 2.2.2.4 Bioenergetic failure

Given the importance of impaired tissue perfusion in sepsis and/or SIRS, intensivists had focused on developing better ways to monitor and provide blood flow and $O_2$ delivery to tissue in the last decades. Finally, the initial Surviving Sepsis Campaign (SSC) guidelines were published in 2004 and its implementation in daily ICU practise has brought a convincing 15.9% absolute reduction in 28-day mortality rate in patients with severe sepsis or septic shock in randomised, controlled, multicentre study [91].

Initial Resuscitation in Surviving Sepsis Campaign 2013 is protocolized, quantitative resuscitation of patients with sepsis-induced tissue hypoperfusion (defined in this document as hypoperfusion persisting after initial fluid challenge or blood lactate concentration ≥ 4mmol/L) [92]. See Table 2.

**Table 2: Goals during the first 6 hours of resuscitation**

| | |
|---|---|
| a) | central venous pressure 8–12 mm Hg |
| b) | mean arterial pressure (MAP) ≥ 65 mm Hg |
| c) | urine output ≥ 0.5 mL/kg/hr |
| d) | central venous or mixed venous oxygen saturation 70% or 65% respectively |

However, efforts to improve systemic $O_2$ delivery later in the course of sepsis were ineffective [93] and failed to prevent multiorgan dysfunction. Finally, the hypothesis emerged that the energy metabolism in cells is deranged not because the $O_2$ delivery is impaired, but perhaps, even more importantly, because of the inability of cells to utilize available $O_2$ [94]. In the current literature, the combination of tissue hypoxia, insulin resistance and increased oxidative stress is termed bioenergetic failure [95].

A reduction in total mitochondrial activity was observed in patients with severe sepsis [96]. Additionally, increased mitochondrial biogenesis and antioxidant responses (with a trend toward depletion of respiratory chain complexes I and IV), have been reported early in the course of critical illness in quadriceps muscle of survivors as compared with non-survivors [97].

Mitochondrial dysfunction has been associated with MOF, increased severity of illness and mortality in those with septic shock and the critically ill [98-99]. Naturally, the loss of normal mitochondrial function results in an inability of cells to maintain their vital energy demands,

which in turn accelerates muscle fatigability and weakness [100-102]. Ubiquitin-proteasome activation contributes to the ATP-consumption in critical illness and excess nitric oxide (NO) production caused by induction of inducible nitric oxide synthase (iNOS) by proinflammatory cytokines inhibits mitochondrial respiratory chain complexes I and IV and reduce ATP concentration during SIRS [103]. Additionally, the opening of mitochondrial transition pores and subsequent release of cytochrom C act as a proapoptotic signal [104].

## 2.3 ICUAW - recovery and therapeutic implications

Improved mortality, but failed functional outcomes in survivors of protracted critical illness represent a major challenge of contemporary intensive care. Understanding the post-ICU morbidity experienced by these survivors has become increasingly important. In particular, neuromuscular abnormalities during critical illness are common, with a median prevalence of 57% [105]. In both patients with chronic critical illness and survivors of severe critical illness, neuromuscular weakness may be substantial and persistent [106], resulting in important decrements in physical function and quality of life for years after discharge [1,107]. In the past, routine features of general care provided in the ICU included the liberal use of sedation and immobilization of the patient, which were thought to be necessary for facilitating interventions to normalize physiological function by artificial means.

Recently, there has been a paradigm shift away from this approach towards a more conservative treatment philosophy for patients in the ICU [108-110]. This paradigm shift is consistent with the observation that long-term physical problems in survivors of critical illness, particularly those with respiratory failure, may result from the protracted ICU stay and period of immobilization during which the patient is receiving organ support that is essential for survival [110-111]. In line with this, the daily interruption of sedation policy has been widely adopted and proven to be beneficial [112] and early mobilization culture is spreading quickly across ICUs [113-115]. Indeed, these strategies, together with early physical therapy [116-121] are the only safe [122-126] and effective interventions in the prevention of long-term neuromuscular disability in survivors of intensive care.

In view of the fact that the survival rates among patients in the ICU increase, muscle-specific therapeutic targets are urgently needed. A critically ill patient may lose up to 10% of lean body mass (LBM) every week, as has been demonstrated in a study using an ultrasound technique to measure muscle thickness [127-128]. An overall loss of 25% LBM is associated with difficulties in weaning patient of mechanical ventilation, increased incidence of ventilator associated with pneumonia and delayed rehabilitation [78]. Thus, the loss of muscle mass in the critically ill significantly influences both the short- and the long-term outcomes of intensive care.

Many patients have marked functional improvement during the first 3-6 months reaching a plateau up to 1 year after critical illness [129]. Finally, neuromuscular malfunction and neuropsychological maladjustment become the greatest burdens that survivors of critical illness have to face [110,130]. In a 5-year follow-up in ARDS survivors, muscle weakness and functional impairment were frequently observed at 1 year, with a reduction in 6-min walk test to 66% of predicted value, while pulmonary functions were almost normal [1]. Interestingly, in a subgroup of ARDS survivors who underwent comprehensive neurological evaluation and electrodiagnostic testing 6 to 24 months after ICU discharge, no electrophysiological changes attributable to ICUAW were observed, while all muscle biopsies taken were structurally abnormal [77].

Unfortunately, specific treatments preventing muscle loss or enhancing muscle recovery are still to be found. At present, therapeutic interventions to attenuate ICUAW are limited to moderate glucose control [47] and early mobilization [116,118]; using stimulation of muscle contraction with neuromuscular electrical stimulation (NMES) is being investigated, as several studies have recently documented a reduction in muscle deterioration in ICU [131].

## 2.4 ICUAW – experimental approach to skeletal muscle metabolism research

However, despite proven benefit, early exercise programmes and optimum nutrition alone cannot prevent muscle deterioration. Protracted critical illness (in contrast to the acute phase) is a state of generalized anterior pituitary suppression (including low growth hormone secretion with a loss of pulsatility) and insulin resistance, whilst plasma cortisol remains elevated by non-ACTH stimulation [132]. A major candidate for anabolic support - growth hormone (GH) - was unfortunately disqualified when shown to double mortality in the critically ill [133]. Duska et al. [134] conducted a small controlled trial of low doses of GH (as frequent i.v. pulses) under strictly controlled conditions (prevention of hyperglycaemia and hypoglutaminemia). This treatment resulted in a significant effect on the maintenance of muscle mass. Pulsatile GH treatment created a unique metabolic environment with concomitant elevation of both insulin and Non-Esterified Fatty Acids (NEFA).

Skeletal muscle accounts for ~40% of adult human body weight and has a remarkable ability to regenerate. Responding to injury, new mitochondria are manufactured and myofibrillar proteins are resynthesized. As skeletal muscle stem cells, satellite cells play the key role in this highly orchestrated process. They are located on the muscle fibre under the basal lamina of muscle fibre as quiescent cells, which become activated after injury. When they leave

the fibre, they proliferate and either maintain the stem cell population or undergo myogenesis to form new fibres and reconstitute a functional contractile apparatus [135]

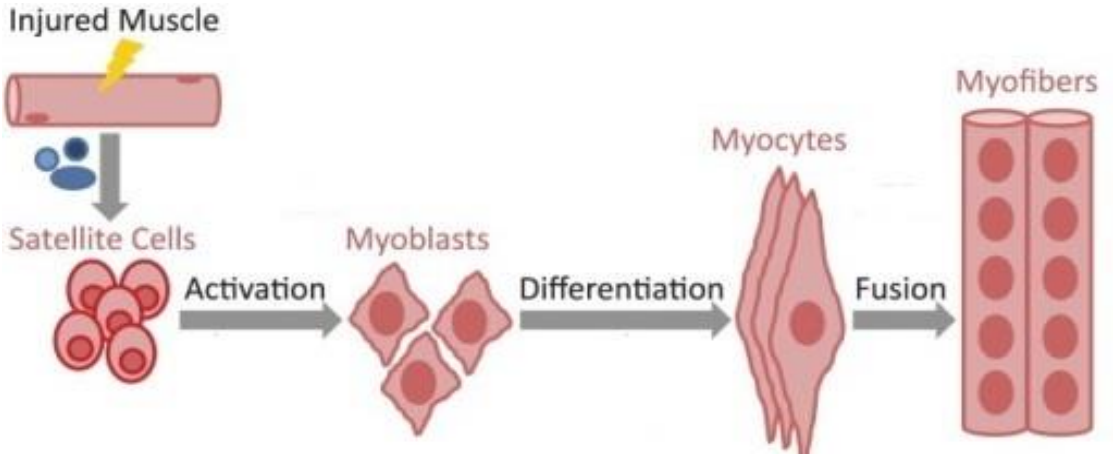


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**Figure 2:** Satellite cells remain quiescent under normal conditions. After muscle damage, satellite cells become activated and re-enter the cell cycle to produce muscle progenitor cells that regenerate new muscle fibres. They also self-renew to replenish the stem-cell population. (Adapted from (136)).

After experimental detachment from this environment, satellite cells are referred to as myoblasts. They fuse with muscle fibres and thus enrich the fibre with a new intracellular content (mitochondria, nucleus, contractile proteins etc.). The recovery of at least mitochondrial count and function in skeletal muscle seems to be crucial for the survival of sepsis with multi-organ dysfunction [97] and it may be hypothesized that the satellite cell function is essential also for regaining muscle mass and force-generating capacity during the recovery from critical illness.

Human myoblasts can be separated from muscle biopsy specimen and cultured in vitro [137]. Depending on the composition of the media they grow in, myoblasts cease division and undergo terminal differentiation into myotubes. Simultaneously, they change the phenotypic features - such as decreasing the number of GLUT-1 receptors and increasing the number of GLUT-4 receptors, thus building normal muscle reactivity to insulin [137]. Moreover, in patients with diabetes [138-140], obesity and other diseases [141-142] myotubes have been shown to retain metabolic phenotypic characteristics of the muscle tissue they have been derived from.

Previous metabolic studies on human myoblasts were limited to the detection of (phosphorylated) proteins and the analysis of intracellular concentration of energy-rich compounds [99,143]. Functional in vitro metabolic studies of muscle fibres separated from the biopsy [144], isolated mitochondria [144] or myoblasts using traditional methods (i.e. Clark electrode, radionuclide-labelled substrates) are extremely time-consuming when intended to test more than a few experimental manipulations.

Multi-well, plate-based assay (XF24 extracellular flux analyser, Seahorse Bioscience) has recently been introduced to address the need for higher throughput metabolic measurement [145]. This instrument simultaneously measures the oxygen consumption rate and extracellular acidification rate (a measure of lactate production) under a range of experimental conditions, which can be changed during the experiments by using up to 4 additives automatically added into a well. This method was successfully used in studying various cell lines [146-147]. We intend to extend its applicability to the population of human myoblasts isolated from muscle biopsies and cultured in vitro.

# 3 AIMS and OBJECTIVES

The overall aim of the study was to collect valid and reliable information on the mitochondrial function of human skeletal muscle tissue in the critically ill. Within this broad theme, the research had following objectives:

- to introduce the XF24 method on the culture of human myoblasts and myotubes in vitro;
- to determine the difference in ATP turnover rate (aerobic/anaerobic) between cultured myoblasts collected from healthy controls vs. the critically ill; to examine whether the ATP turnover rate can be influenced by substrate availability and concentration (glucose vs free fatty acids), eventually by insulin and/or growth hormone stimulation;
- in the event that the difference is detected, to perform secondary studies using enzyme inhibitors (eg. of complexes 1, 2/3 and 4 or glycolysis) in order to localize the defect;
- to understand the interaction between extracellular glutamine concentration and mitochondrial function in human skeletal muscle cells with respect to the efficiency of aerobic phosphorylation and proliferation rate in cultured myoblasts;
- to explore whether mitochondrial dysfunction persists until protracted phase of critical illness.

## HYPOTHESIS

Severe and protracted ICU-acquired weakness is associated with impaired mitochondrial function in an ex vivo model of human skeletal muscle. The ATP turnover rate under basal conditions (0mM palmitate, 6mM glucose) in myoblasts collected from weak patients is lower than in control subjects. Glucose utilization is lower at baseline and cannot be increased by insulin, but rather it depends on the glucose concentration in the environment. ATP turnover in myoblasts from weak patients can be increased by NEFA supply.

# 4 MATERIALS and METHODS

## 4.1 Patient characteristics

### 4.1.1 Control cohort of patients

For all studies, we used control muscle biopsy samples obtained from metabolically healthy patients undergoing elective hip replacement surgery that had been indicated for degenerative disease at the Department of Orthopaedic Surgery of Kralovske Vinohrady University Hospital in Prague. We excluded patients with serious mobility impairment who used compensatory aids or wheelchairs with the exception of walking sticks. Next we excluded patients with neuromuscular disorders in their medical history and patients with diabetes mellitus or with another diagnosed endocrinopathy. Patients with an appropriate substitution of subclinical hypothyroidism were enrolled.

### 4.1.2 Patients with protracted critical illness

Consecutive adults admitted to 22-ventilated bed general ICU and 10-bed medical ICU of Kralovske Vinohrady University Hospital in Prague who were receiving mechanical ventilation for more than 2 weeks were screened for eligibility. Out of the 22 eligible ICU-patients approached, only 8 consented for muscle biopsy. The detailed characteristic of study subjects are given in Table 3.

***(a) inclusion criteria:***

- age > 18 years
- invasive mechanical ventilation for > or = 2 weeks
- MRC score <48 points [148] of muscle weakness
- any surgical or other invasive procedure requiring anaesthesia is necessary

***(b) exclusion criteria:***

- pre-existing neurological disease (myasthenia gravis, Guillain-Barré, ALS...)
- severe coagulopathy (Platelets <50 G/L, INR>1.5 or heparin therapy within therapeutic range) precluding muscle biopsy
- steroids in higher than substitutional doses
- pregnancy

All patients provided a written prospective informed consent. In patients who were unable to sign the form due to muscle weakness, the consent procedure was witnessed and assented by the next of kin. The University Hospital Ethical Review Board reviewed both the protocol and the consent form and approved the study.

**Table 3: Study subject characteristics**

| Subject | Diagnosis | Sex | Age | APACHE II | Biopsy Day | MRC Score | LOS-ICU | Survived |
|---|---|---|---|---|---|---|---|---|
| 1 | Septic shock, bronchopneumonia | M | 70 | 22 | 15 | 20 | 34 | N |
| 2 | Aspiration pneumonia | M | 80 | 15 | 29 | 23 | 71 | Y |
| 3 | Sepsis | M | 60 | 31 | 40 | 25 | 92 | N |
| 4 | Cardiogenic shock | F | 65 | 27 | 41 | 4 | 45 | Y |
| 5 | CHF + CAP | F | 68 | 10 | 27 | 8 | 30 | Y |
| 6 | Chest trauma + HAP | M | 62 | 14 | 17 | 18 | 48 | Y |
| 7 | CABG, GI bleed | M | 68 | 23 | 25 | 16 | 43 | Y |
| 8 | CAP | F | 60 | 15 | 30 | 23 | 35 | Y |
| Mean±SD | | - | 67±7 | 20±7 | 28±9 | 17±8 | 50±21 | - |

*Note: CABG = coronary artery bypass grafting, CAP= community-acquired pneumonia, CHF = congestive heart failure, HAP=hospital-acquired pneumonia, MRC= Medical Research Council score of muscle power, LOS ICU = length of stay in intensive care. Survival means survival to discharge from hospital.*

## 4.2 Muscle biopsy - skeletal muscle tissue homogenate

Muscle biopsies were obtained from the vastus lateralis muscle approximately 10 cm above the knee by an open surgical technique (~500-1000 mg) as soon as the muscle was exposed during surgery or by 5mm Bergstrom needle [149] in critically ill. The sample was collected into 5 mL of ice-cold Biopsy Preservation Solution (BIOPS) with CaK2EGTA (2.77mM), K2EGTA (7.23mM), Na2ATP (5.77mM), MgCl2 (6.56mM), taurine (20mM), Na2Phosphocreatine (15mM), imidazole (20mM), dithiothreitol (0.5mM) and MES (2-(*N*-morpholino) ethanesulfonic acid, 50mM) adjusted to pH 7.1 at 0°C [150-151], stored on ice and processed for muscle homogenate immediately after the transport to laboratory (within 10 minutes).

All steps of the homogenate preparation were performed on ice. Connective tissue, fat and blood vessels were gently removed; the skeletal muscle fibres were dried by gauze and weighed on a calibrated scale (=wet weight, Ww). After the addition of K media (1 ml/100mg of muscle Ww, to obtain 10% homogenate), muscle fibres were homogenised by 4 – 6 strokes in a Elvhjem-Potter teflon/glass homogeniser. The entire procedure took approximately 10 minutes. Thereafter, the homogenate was filtered through a cheesecloth.

### 4.2.1 High-resolution respirometry on skeletal muscle homogenate

High-resolution respirometry uses the polarographic measurement of oxygen consumption by a Clark electrode and has been recently adapted to tissue homogenates [152-153]. We performed the skeletal muscle tissue homogenate respirometry assay at 30°C without preoxygenation with 0.2 ml of 10% homogenate and 1.9 ml of K media (contains KCl (80mM), Tris HCl (10mM), KH2PO4 (5mM), MgCl2 (3mM), EDTA (1mM), BSA (0.5mg/ml), adjusted to pH 7,4 at 30°C) in the respirometer Oxygraph 2K (Oroboros Instruments, Innsbruck, Austria). Two assays were performed in parallel [154] in 2 chambers of the respirometer by the serial addition of substrates and inhibitors using a Hamilton pipet.

Oxygen concentration and flux were simultaneously recorded and analysed by Dat lab software. Oxygen solubility factor used for calibration was 0.93 for K medium and 0.87 for MAS, respectively [155]. The average initial O2 concentration was 210 - 220 nmol/ml. Reagents were added into the closed chamber by using Hamilton syringes.

***a) Analysis of global mitochondrial functional indices*** was performed in sequential steps (the final concentrations in the respirometry chamber are given in brackets):

1) malate (2,5mM) + glutamate (15mM)
2) ADP (1mM)
3) cyt c (10µM)
4) succinate (10mM)
5) oligomycin (1µM)
6) FCCP (0.7µM),
7) AA (4µM)

The non-mitochondrial respiration was the $O_2$ consumption measured after the addition of AA and subtracted from the other values. The capacity of oxidative phosphorylation (OXPHOS, or 3p respiration) was the $O_2$ consumption rate when substrates for both Complexes I (malate, glutamate) and II (succinate), abundant ADP and cyt c were present. The respiratory chain capacity (state 3u) was measured after uncoupling with FCCP. The ATP synthesis rate was defined as the decrease in oxygen consumption after the addition of oligomycin when the substrates for Complex I and II were present. Additionally, the addition of cytochrome c allows for the testing perseverance of the outer mitochondrial membrane integrity during homogenisation, with the values <20% considered as acceptable [156]. In our subjects, it was 13±6% in ICU and 11±8% in control patients.

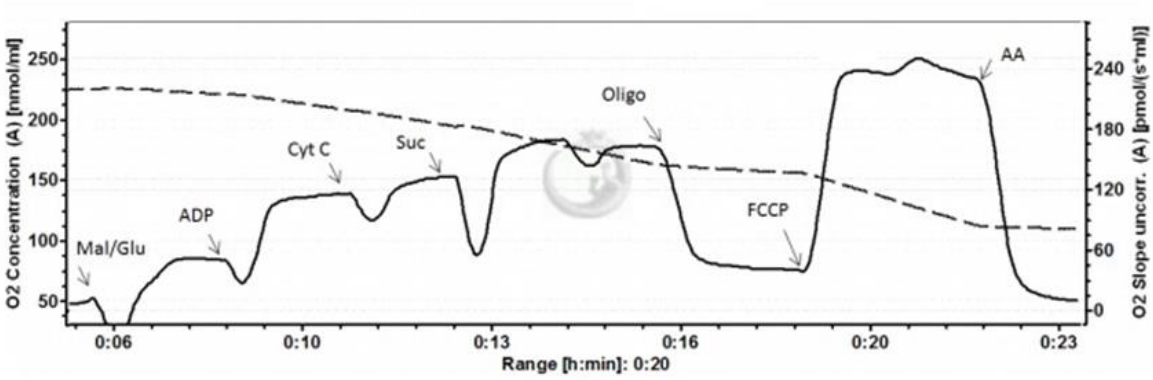


**Figure 3:** An example of high-resolution respirometry assay in a homogenate of skeletal muscle. The solid line is an oxygen consumption rate; the dashed line is the oxygen concentration. Note: Mal/Glu= malate/glutamate, suc=succinate, oligo=oligomycin, FCCP=uncoupler, AA= antimycin A [157].

***b) Functional analysis of individual respiratory complexes*** was performed using the sequential addition of:

1) malate (2.5mM) and glutamate (15mM)
2) ADP (1mM)
3) cyt c (10µM)
4) rotenone (3µM)
5) succinate (10mM)
6) malonate (5mM)
7) glycerol-3-phosphate (5mM)
8) AA (4µM),
9) ascorbic acid (10mM) and tetramethyl-p-phenylenediamine (TMPD, 200µM)
10) KCN (1mM)

Complex I activity was calculated as the decrease in oxygen consumption after its inhibitor rotenone; Complex II activity as a decrease after addition of malonate. Complex III activity was determined as an increase of oxygen consumption after the addition of glycerol-3-phosphate after both Complexes I and II had been inhibited by rotenone and malonate, respectively. Complex IV activity was measured as the increase of oxygen consumption after the addition of Complex IV substrates ascorbate/TMPD after Complex III had been inhibited by AA. See figure 4.

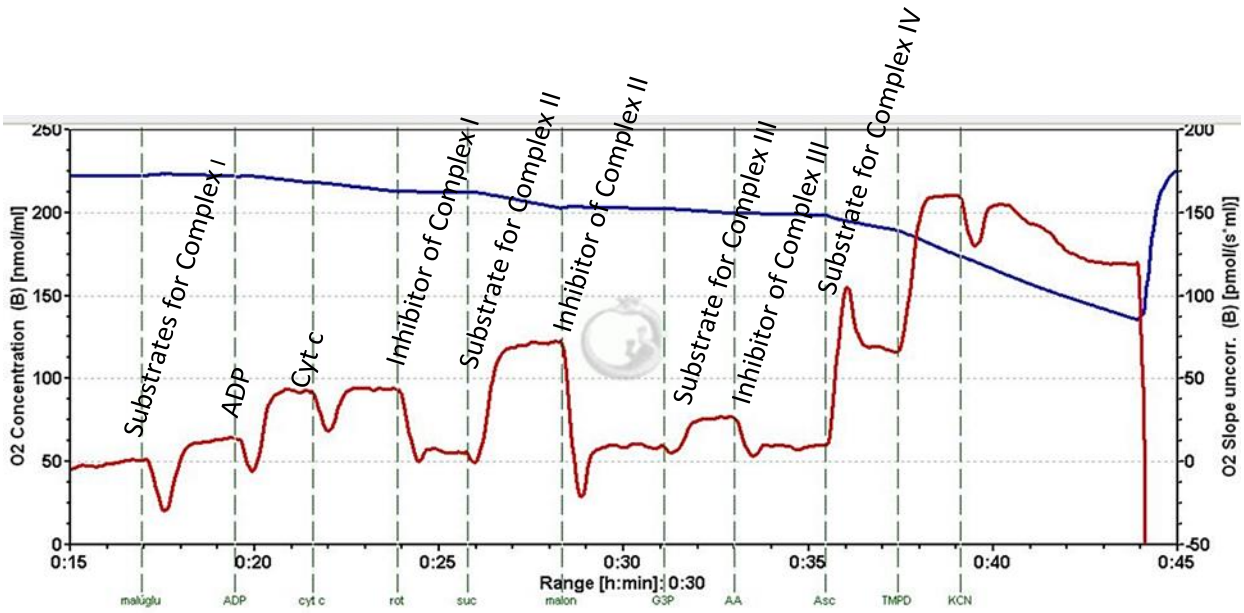


**Figure 4:** High-resolution respirometry of individual complexes in mitochondrial electron transport chain, human muscle homogenate, subj. ICUAW6 (19/12/2013). For abbreviations in detail see text.

Finally, 150 ul of M-PER (Thermo Scientific, USA) was added to 150 ul of homogenate with 5 µl of Protease inhibitor cocktail. Lysates from all samples were frozen to -80°C for later analysis of CS activity (using commercial kit from Sigma) and protein content assay (determined by Bradford assay).

### 4.2.2 Spectrophotometric analysis

Spectrophotometric analysis of individual activities of respiratory complexes has been described in detail elsewhere [158]. In brief, the frozen sample was thawed and homogenized and then exposed to three further cycles of rapid freezing thawing. Complex I assay was performed in an assay mixture composed of 25 mM potassium phosphate, 3.5 g/l BSA, 2 mM EDTA, 60 µM dichlorophenollindophenol (DCIP), 70 µM decylubiquinone, 1 µM antimycin A and 0.2 mM reduced nicotinamide adenine dinucleotide (NADH), pH 7.8. Changes in absorbance were followed at 600 nm. Rotenone sensitive activity was calculated by subtracting the activity of wells with 10 µM rotenone. Complex II activity was measured in an assay mixture containing 80 mM potassium phosphate, 1 g/l BSA, 2 mM EDTA, 10 mM succinate, 80 µM DCIP, 50 µM decylubiquinone, 1 µM antimycin A and 3 µM rotenone, pH 7.8. Changes in the absorbance were followed at 600 nm. Malonate sensitive activity was calculated by subtracting the activity of wells with 20 mM malonate. Complex III activity was measured in an assay mixture containing 50 µM ferricytochrome c, 25 mM potassium phosphate, 4 mM sodium azide, 0.1 mM EDTA, 0.025 % Tween20 and 50 µM decylubiquinol, pH 7.4. Changes in absorbance were followed at 550 nm. The antimycin A sensitive activity was calculated by subtracting the activity of wells with 10 µM antimycin A. Complex IV activity was measured in an assay buffer containing 30 mM potassium phosphate and 25 µM of freshly prepared ferrocytochrome c, pH 7.4. Changes in

absorbance were followed at 550 nm. The absorbance of samples oxidized with 10 µl of 0.5 M potassium hexacyanoferrate (III) was subtracted from all measurements, and then the natural logarithm absorbance was plotted against time and compared to the untreated control. The citrate synthase activity was measured using a commercial kit from Sigma, as per the manufacturer's instructions.

### 4.2.3 Western blots

We performed western blotting to determine the relative content of mitochondrial proteins in skeletal muscle homogenate. Samples containing 6 µg of proteins were mixed with the sample buffer and denatured by heating at 45°C for 15 min. SDS-PAGE and Western blotting were performed as described previously [159]. Briefly, proteins were separated on 12% polyacrylamide gels at 120 V and then blotted onto a 0.2µm nitrocellulose membrane (Protran BA83, Schleicher-Schuell, Dassel, Germany) for 3h at 0.25 A. The membranes were blocked in 5% weight/volume BSA in Tris-buffered saline for 30 min at room temperature. The washed membranes were probed with primary antibody cocktail Anti-human Total OxPhos Complex Kit at 4°C overnight (dilution 1:175, □ 458199, Life Technologies), containing primary antibodies against Complex II (29kDa), Complex III (core 2; 48 kDa), Complex IV (COX II subunit, 22kDa) and F1FOATPase (F1α; 45kDa) subunits.

After washing, the membranes were incubated for 2h at room temperature with mouse horseradish peroxidase-conjugated secondary antibody (dilution 1:6600; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Protein bands were visualized with an enhanced chemiluminescence detection system (Thermo Fisher Scientific, Rockford, IL, USA) using Carestream Gel Logic 4000 PRO Imaging System (Carestream Health, New Haven, CT, USA). To demonstrate equal loading, the membrane was stripped and re-probed with an anti-GAPDH antibody (dilution 1:1000, ab9485, Abcam, Cambridge, UK). Densitometry was performed using the Carestream v5.2 program (Carestream Health). Data were normalized to Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and referenced to an internal standard (a control patient sample was present on every immunoblot).

## 4.3 Muscle biopsy - human skeletal muscle cell culture

Muscle biopsies were obtained as described previously, but in contrast to the last procedure they were transported in 5 ml of ice-cold Dulbeco´s Modified Eagle Medium (DMEM) with glucose (5.56mM) and pyruvate (1mM) supplemented with glutamine (final 0.5mM), penicillin-streptomycin solution (100µg/ml) and fungizone (250µg/ml) and processed immediately for satellite cell isolation.

### 4.3.1 Culturing of human myoblasts and myotubes

100-150mg of the muscle biopsy collected in cold DMEM was processed for satellite cell isolation [160] with a few modifications [87]. In brief, skeletal muscle tissue was minced and washed using a Hank´s balanced salt solution 2–3 times and cells were subsequently subjected to a 30minutes digestion in 0.25% Trypsin/0.68% collagenase solution in a shaking water bath at 37°C. Foetal bovine serum (FBS) was added to stop the action of trypsin (due to high content of protease inhibitors e.g.α1-antitrypsin a α2-makroglobulin) and cells were collected by centrifugation at 350*g*. To remove fibroblasts, cells were subsequently pre-plated in an uncoated Petri dish for 60 minutes at 37°C with 5% $CO_2$ [161-162] and then transferred to gelatin-coated flasks (T-25, Nunc® EasYFlasks™, Nunclon® Δ Surface). Cells were grown in basal growth medium containing DMEM with low glucose (LG, 5.56mM) and pyruvate (1mM), supplemented with 15% FBS, penicillin-streptomycin solution (100µg/ml), fungizone (0.05µg/ml), dexamethasone (0,4µg/ml), fetuin (50 ng/ml), insulin (10µg/ml) and human growth factors (final EGF 10ng/ml; FGF 1ng/ml) with glutamine (0,5mM), respectively. The plates were cultured at 37°C in 5%CO2. The media were changed every 2 or 3 days until cells reached ~80%–90% confluence.

On day 0, cells were treated with trypsin to liberate from the bottom of Petri dish, counted, seeded in a 24-well gelatin-coated Seahorse plate (Seahorse bioscience, USA) and grown to ~ 85-90% confluence overnight. On day 1, either the experiment was conducted on myoblasts or the growth medium was switched to a differentiation medium DMEM with high glucose (HG, 25mM), pyruvate (1mM), 2% horse serum, penicillin-streptomycin solution (100µg/ml), insulin (10µg/ml), and glutamine (0,5mM). The medium was then exchanged every 2-3 days and cells were differentiated for 7 days prior to experimentation.

In an additional study, we examined the role of glutamine concentration on proliferation rate and energy metabolism in human skeletal muscle cells *(*overview of study design see in Figure 5). Human myoblasts (n=9) were cultured in basal grown medium supplemented from day 0 with glutamine. Cells were divided into six groups according to the final glutamine concentration in the medium. Six different glutamine concentrations were studied - 0, 100, 200, 300, 500 or 5000µM, respectively. When preparing fresh media for all but the 5000µM glutamine groups, we also considered glutamine content in FBS and spontaneous glutamine breakdown with a half-time of 3 days at 37°C [163].

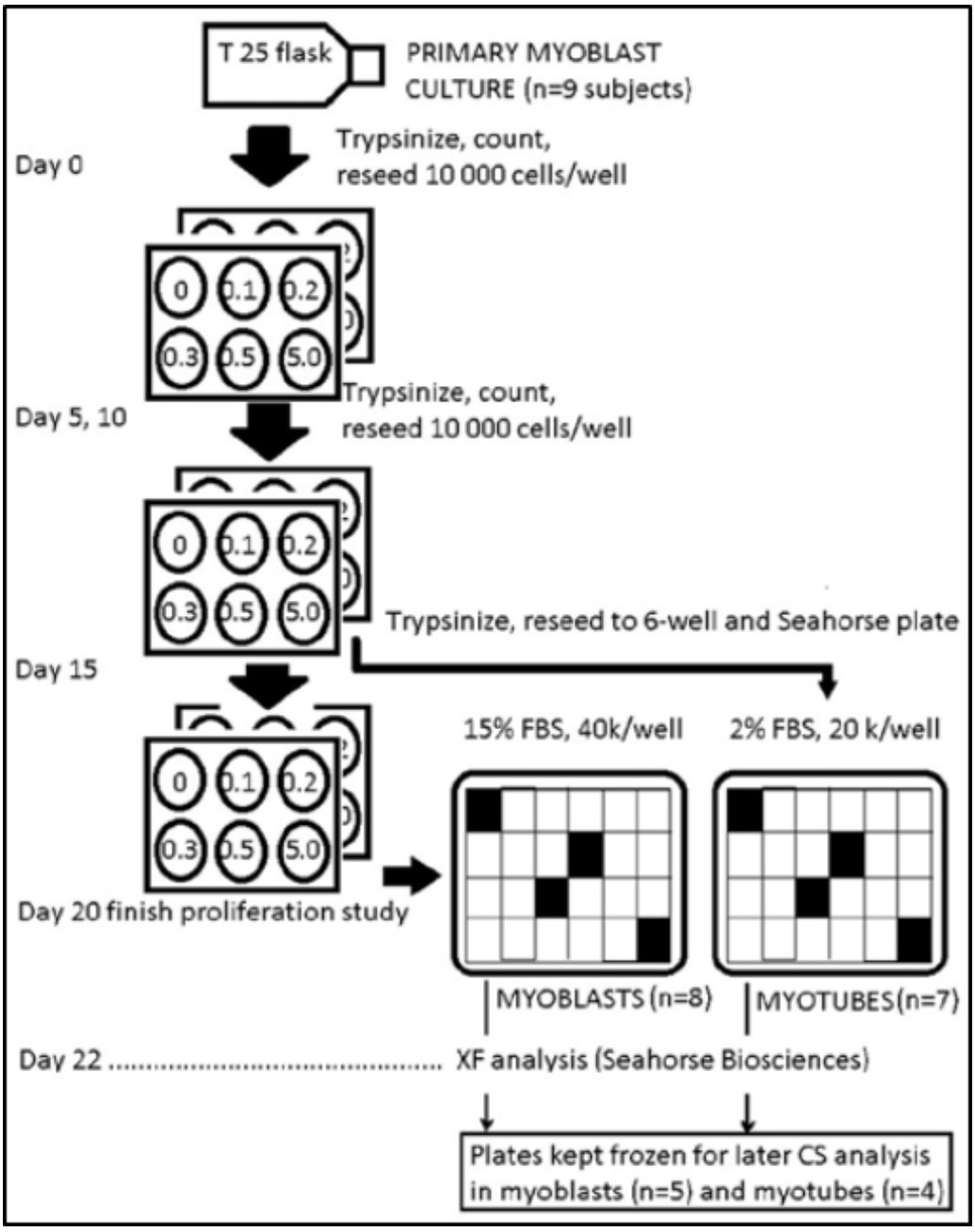


**Figure 5:** Schematic representation of the study design. CS: citrate synthase; FBS: fetal bovine serum, [87].

Cells were treated with trypsin, manually counted, and reseeded on days 5, 10, 15 and 20, and the proliferation curves were then compiled. Next, a subset of myoblasts was reseeded 20000/well on day 15 into a 24-well gelatin-coated plate (Seahorse Biosciences, Billerica, MA) and differentiated 7 days prior to experimentation. On day 20, a second subset of myoblasts was seeded into another Seahorse plate. In both Seahorse plates, the cells were continually exposed, in tri- or tetraplicates, to six different glutamine concentrations.

On day 21, the bioenergetic profile measurement was performed on both myoblasts (n=8) and myotubes (n=7). The seahorse plates were kept frozen for later analysis of protein content and citrate synthase activity.

### 4.3.2 Indirect immunofluorescence and cell viability

*Desmin immunofluorescence* staining was used to verify the purity of the cell line [141]. In brief, cells were grown to ~80% confluence on coverslips (on bottom of Petri dish) and fixed with 4% paraformaldehyde/PBS (Phosphate Buffer Saline). The coverslips were then washed thoroughly four times for 5 min in PBS (on the orbital shaker, speed 80 rpm) and permeabilized with 0,5ml of 0,1 % saponin/PBS for 10 min and washed again three times in PBS. The washed coverslips were then probed with primary antibody anti-desmin AB907 (by Millipore, dilution 1:50, 1:100, 1:200) and incubated for one hour in a covered humid chamber. After washing with PBS/0,05% Tween, the coverslips were incubated for 60min at room temperature with goat anti-rabbit IgG secondary antibody Alexa Fluor® 488 (dilution 1:200; Abcam(UK), ab150077). Finally, the rinsed coverslips were mounted on glass slides using Prolong GOLD with DAPI (4´,6´-diamidino-2-phenylindole) and sealed with nail polish. The slides were stored in a dark and cold place until confocal microscopy was performed, using Leica software.

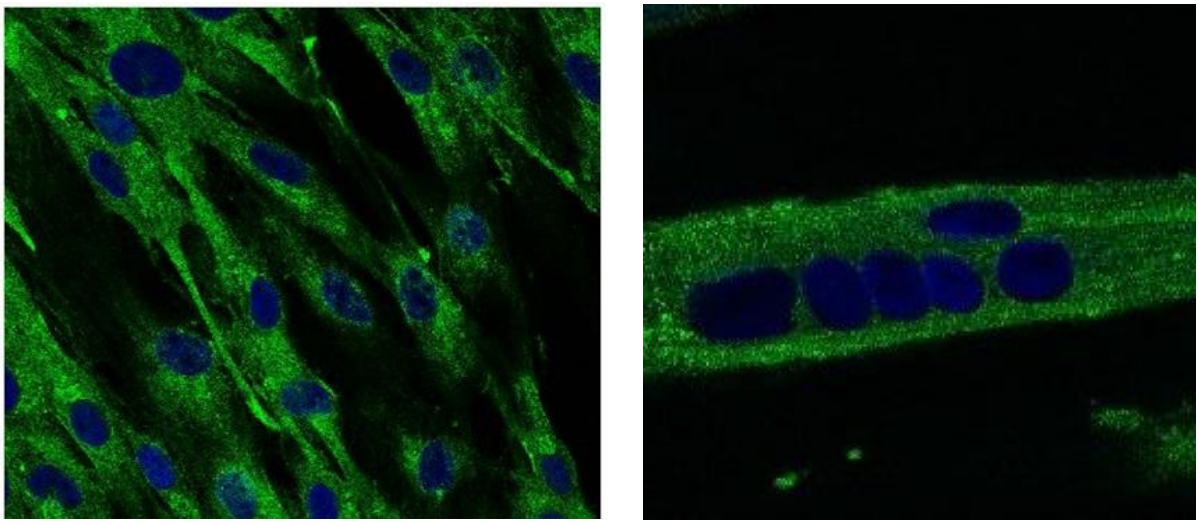

**Figure 6:** Primary human myoblasts and differentiated myotube stained with muscle filament specific antibody anti-desmin AB907 (by Millipore); confocal microscopy

*Cell viability* was measured using the CellTiter96 MTS assay (Promega, Madison, WI) as previously described [164]. Viable cells reduce the tetrazolinum dye, MTS [3-(4,5-dimethylthiazol-2-yl)-5-)3-carboxymethoxyphenyl)-2-(4-silfophenyl)-2H-tetrazolium] to purple formazan. The absorbance at 490 nm is then used as a measure of cell viability. We performed MTS on myoblasts from primary culture at baseline and then as required, e.g. on myoblasts from primary culture after 20 days of exposure to different glutamine levels.

### 4.3.3 Extracellular Flux (XF) measurement

Recently, a multi-well plate-based assay platform has been developed [146]. The XF Extracellular Analyser uses fluorescent sensors to measure real-time extracellular fluxes of

oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) from cells in custom microplates. The sensors reside on a biocartridge that fits over the cell culture plate. To detect a significant change in oxygen or proton concentration the biocartridge is lowered to approximately 200µm above the cell layer (attached to the bottom of the well) and creates a temporary 7µl microchamber with restricted diffusion. It takes 1.5 - 4min to measure the depletion of oxygen or decrease in pH. Then the fluorescent probes are lifted to a raised position (5mm above cells) within about 1ml of buffer to reequilibrate the media [145,165]. The measurement is performed at baseline (phase 1) and can be repeated every few minutes. Injector ports surrounding the fluorescent sensors over each well can automatically deliver any combination of up to 4 substrates or inhibitors. The dynamics of OCR then allows the assessment of the indices of the mitochondrial function. ECAR relates to a lactate production [165] and is used as a measure of the rate of anaerobic glycolysis.

#### 4.3.3.1 Principle of bioenergetic profile experiment

In each subject (in a 24-well plate), we performed a stress test assay – a standard bioenergetic profile experiment to assess the global mitochondrial functional indices. The basal oxygen consumption (OCR) and extracellular acidification (ECAR) rates are measured to establish baseline rates (phase 1). The cells are then metabolically perturbed through the addition of three defined compounds: an ATPase inhibitor oligomycin (1µM, phase 2), an inner membrane uncoupler FCCP (1µM, carbonyl cyanide-4-[trifluoromethoxy]phenylhydrazone, phase 3) and a complex III inhibitor antimycin A (4 µM, phase 4). Oxygen consumption after the exposure to antimycin A is considered non-mitochondrial. After this has been subtracted, OCR after oligomycin reflects proton leak through inner mitochondrial membrane and OCR after FCCP maximum respiratory chain activity. A decrease of OCR after oligomycin is a measure of ATPase activity (see Figure 7). A decrease of OCR after oligomycin is a measure of ATPase activity (see Figure 7). To reflect possible differences of cell number or mitochondrial content, we normalized basal OCR to the activity of citrate synthase (CS).

Basal OCR was calculated as OCR Phase1 – OCR Phase4. The absolute cell number is irrelevant since the same population of cells is compared. Therefore, we expressed most parameters of energy metabolism as a percentage of baseline value. ATP turnover was calculated as 100*(OCR Phase1–OCR Phase2)/Basal OCR [%]. Leakage through the inner mitochondrial membrane was calculated as 100*(OCR Phase2– OCR Phase4)/Basal OCR [%]. By analogy, respiratory chain capacity (or maximum respiration) was calculated as 100*(OCRPhase3–OCRPhase4)/Basal OCR.

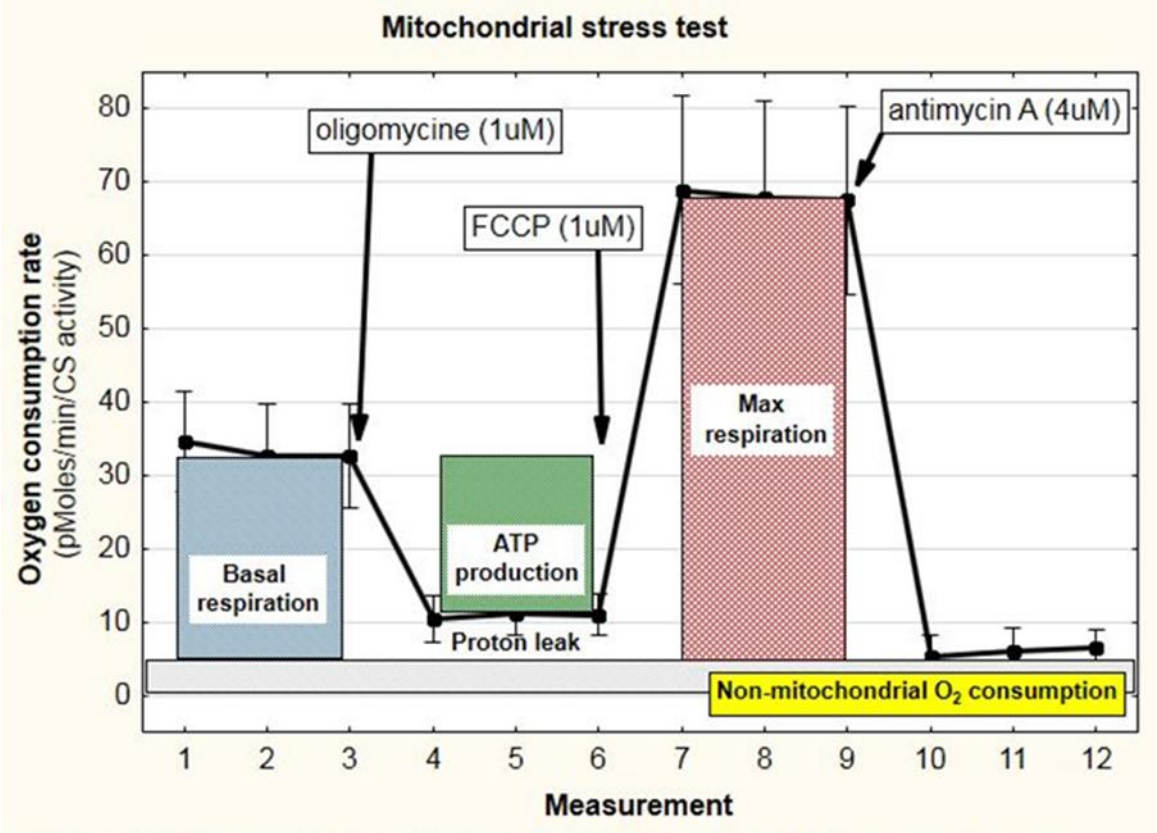


**Figure 7:** Principle of extracellular flux analysis of mitochondrial metabolism. See text for details. Bioenergetic profile experiment in ICUAW myotubes, pooled data from all subjects were used to construct this graph (means, 95% conf. int.) Note: FCCP = carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone; ATP = adenosine 5'-triphosphate;

#### 4.3.3.2 Fatty acid oxidation assay

*Fatty acid oxidation assay (FAO)* performed using the XF Analyser (Seahorse Bioscience), detects changes in oxidative respiration, as measured by the OCR resulting from the exogenous addition of fatty acids to muscle cells in culture. In contrast to the conventional radiometric assay (quantifying, radioactive end-products ($^{14}CO_2$,$^{3}HOH$) of the oxidation reaction), the XF measurement enables detection of the rate of oxygen consumption in nearly real-time [145].

FAO assay was performed on myotubes using the sequential addition of palmitate-BSA (stepwise addition to final concentration of 120 and 240µM), an uncoupler FCCP (1µM) and FAO inhibitor etomoxir (100µM). FAO rate was calculated as the decrease in OCR after the addition of etomoxir. To reflect possible differences of cell number or mitochondrial content, we normalized the basal OCR to the activity of citrate synthase (CS) and protein content.

In our final XF protocol, we performed three measurements simultaneously in the Seahorse 24-well plate (each in two columns): stress test assay (columns 1+2), FAO assay (columns 3+4) and function of Complexes I and IV on permeabilized myotubes (columns 5+6). Myoblasts from ICUAW patients (n=7) and from controls (n=7) were seeded on a 24-well gelatin-coated Seahorse plate at 20 000 cells/well and differentiated 7 days prior to experimentation.

**Table 4: Layout and sequence of individual substrates and inhibitors over the 24-well plate**

| Columns / Ports | XF assay medium | XF assay medium | KHB medium | KHB medium | MAS + mal/glu/suc | MAS + mal/glu/suc |
|---|---|---|---|---|---|---|
| port A | oligo | Oligo | PA | PA | FCCP | FCCP |
| port B | FCCP | FCCP | PA | PA | rot | rot |
| port C | AA | AA | FCCP | FCCP | Asc/TMPD | Asc/TMPD |
| port D | (medium) | (medium) | etomoxir | etomoxir | KCN | KCN |

Working concentrations of substrates and inhibitors in the Seahorse-well is given in brackets:

- oligo (1µM)
- FCCP in XF medium, KHB medium or MAS medium (1µM)
- AA (4µM)
- palmitic acid (120µM)
- etomoxir (100µM)
- rotenone (3,3µM)
- ascorbate 10mM + TMPD (0,2mM)
- KCN (3,3mM)

#### 4.3.3.3 Manipulating extracellular environment

In a subgroup ICUAW (n=4) and controls (n=4), the myoblasts were differentiated into myotubes as described before for 7 days and on day 8 the differentiation medium was exchanged for six various exposition media in tri-to tetraplicates. See Supplemetary material for details.

**Table 5: Exposition to FFA – characteristics of experimental groups**

| group | medium | Fatty acids | Hormones |
|---|---|---|---|
| 1 | medium A | - | - |
| 2 | medium B | - | - |
| 3 | medium A | PA:OA:LA = 1:1:3 - total 250uM | - |
| 4 | medium A | PA:OA:LA = 2:1:2 - total 250uM | - |
| 5 | medium A | PA:OA:LA = 2:1:2 - total 600 uM | - |
| 6 | medium A | PA:OA:LA = 2:1:2 - total 600 uM | GH/IGF-I (100 ng/ml + 500 ng/ml) |

Note: medium A (with glucose as a carbohydrate source, without FFA ), medium B (medium A with galactose as a carbohydrate source, without FFA), medium C (medium A supplemented with PA:OA:LA in 1:1:3, 250µM total concentration), medium D (medium A supplemented with PA:OA:LA 2:1:2, 250µM in total) and medium E (medium A supplemented with PA:OA:LA 2:1:2, 600 µM in total), medium F (medium E with GH 10nM + IGF-1 100nM)

The media supplemented with fatty acids were freshly prepared once a week and kept under argon atmosphere. The exposition lasted from day 8 to day 13. On day 13, a stress test was performed. The assay medium and concentrations of uncoupler and inhibitors were identical to those used in experiment on myotubes before the exposition to fatty acids.

#### 4.3.3.4 Fatty acid stock solution preparation

Palmitic (PA), oleic (OA) and linolaeic (LA) acids were used for exposition tests. Fatty acids were prepared as described elsewhere [166]. In brief, fatty acids were dissolved in 1mM NaOH at 70°C and ligated to 15% BSA. All solutions were clear after this process, and then filtered and stored at -20°C (OA and LA were stored under argon atmosphere). The concentration of fatty acids was calculated using a commercial kit (NEFA Randox). Sodium palmitate for fatty acid oxidation assay was prepared as described in the protocol from Seahorse [167].

#### 4.3.3.5 Protein content and CS activity

Immediately after the XF flux measurement, each well in a plate was washed with a cold phosphate-buffered saline (PBS) and filled with 150µl M-PER Mammalian Protein Extraction Reagent (Thermo Scientific, Waltham, MA) with 2µl Protease inhibitor cocktail. Then the plate was stored at -80°C. On the day of the assay, plates were thawed and the cellular lysate was transferred into Eppendorf tubes and centrifuged at 15,000 *g* for 15 minutes at 4°C. The supernatant was collected for protein determination and CS activity assay.

For protein determination, we used the Bradford reagent according to the manufacturer's instructions. CS activity, which was used as a measure of mitochondrial content in a sample [168], was assayed using a CS assay kit (Sigma-Aldrich) with at least 8µg of whole-cell extract protein, 0.3mM acetylCoA, and 0.1mM 5.5'-dithiobis-(2-nitro-benzoic acid). The reaction was started by adding 5mM oxaloacetate to the mixture. The CS activity of cells is stable 2–3 hours during storage on ice [169]. Enzyme activity was monitored by recording the changes in absorbance at 412 nm over the course of 1.5 minutes at 30°C.

## 4.4 Statistics

All calculations were performed in Statistica version 8 (StatSoft, Inc, Tulsa, OK). The normality of all data was tested by visual checks of the raw data histograms and by a Kolmogorov-Smirnov test ($p>0.2$). Where appropriate, we used two-tailed Student´s-test for comparisons in the case of normal distribution; for multiple comparisons, a factorial analysis of variance (one-way ANOVA with Tukey post-hoc test) was used. Differences at $P<0.05$ were considered significant. The data are presented as means ± standard deviation.

# 5 RESULTS

## 5.1 Optimisation of methodology

### 5.1.1 High-Resolution Respirometry on human skeletal muscle homogenate

We performed a series of preliminary experiments aiming to develop a protocol for the use of high-resolution respirometry (HRR) on homogenates of human skeletal muscle samples obtained by needle biopsy. HRR had been adapted to tissue homogenates [152] including those obtained from human skeletal muscle needle biopsy samples [153] and validated against well-permeabilized muscle fibres. We modified existing original protocols [152-153] and validated it against the standard technique of respirometry in isolated mitochondria. Next, we excluded the unintended influence of muscle biopsy technique (open surgery vs. needle biopsy).

#### a) protocol optimisation

**i)** ***optimal amount of homogenate:*** in order to find the optimal amount of homogenate in a chamber, we dissolved 600, 400, 200, 100 or 50µl of 10% homogenate, respectively, in K medium to final the volume of 2.1 ml and tested oxygen fluxes after the stepwise addition of glutamate/malate, followed by ADP, cyt c, succinate, oligomycine and AA. There were no significant differences in outer membrane damage (Cyt c 14.2±1.2; 11.2±6.4; 13.2±4.7 and 8.4±4.6% for 100, 200, 400 and 600µL, respectively, p=0.70, ANOVA), complex I preservation (CI/ETS 0.72±0.04; 0.82±0.09; 0.82±0.10 and 0.87±0.09, p=0.31 ANOVA) or other mitochondrial function indices among different amounts of the homogenate. However, the use of 600 or 400µl led to the rapid oxygen exhaustion, whilst with 50µl the oxygen fluxes were too low. Therefore, we decided to use 200µl of 10% homogenate for the rest of experiments (Figure 8).

**ii)** ***substrates and inhibitors concentration:*** titration of ADP and oligomycin was performed by adding 0,25mM increments and of FCCP in 0,1µM increments (up to 10µM). In order to avoid the limitations of oxygen flux through low oxygen concentration, the minimum oxygen concentration in the chamber was determined, below which the oxygen flux becomes oxygen-dependent. After the addition of malate/glutamate/succinate and ADP to the muscle homogenate, simultaneously the $O_2$ concentration and $O_2$ flux in time were recorded and the $O_2$ concentration at which the oxygen flux is reduced to 50% (Km), to 90% (C90) and to 95% (C95) were calculated. These were 11.8; 78 and 98nmol/ml (Figure 9). Thus, in all later experiments, we maintained the $O_2$ concentration above 90nmol/ml.

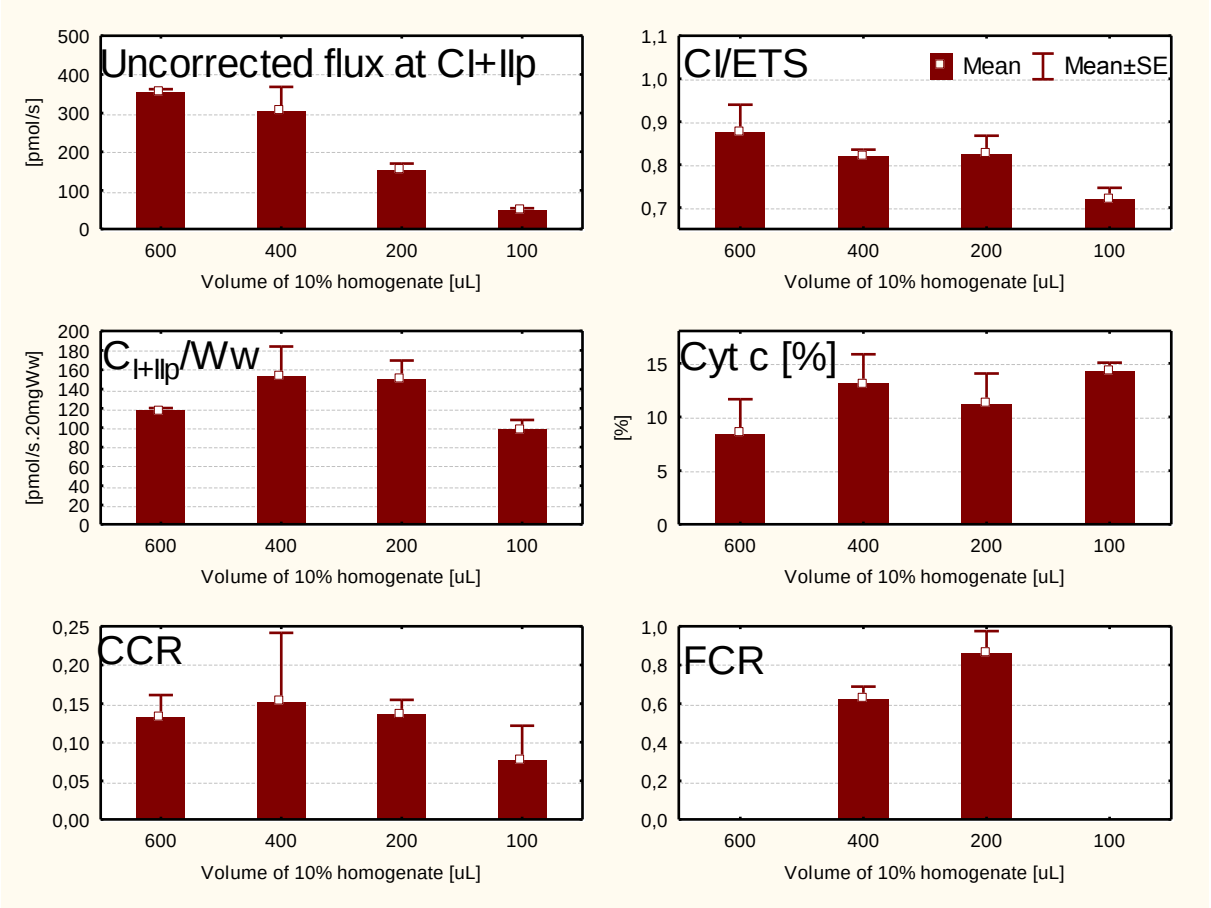


**Figure 8:** Respirometry parametrers obtained with different amounts of 10% homogenate in the chamber. Note: CI+IIp = respiration after the addition of substrates for complexes I and II, ADP and cyt c. CI+IIp/Ww is corrected to 20 mg of muscle sample in a chamber. Cyt c [%] = increase in oxygen flux after addition of Cyt c, CCR=coupling control ratio (leak/CI+IIp), FCR=flux control ratio (CI+IIp/ETS) [157].

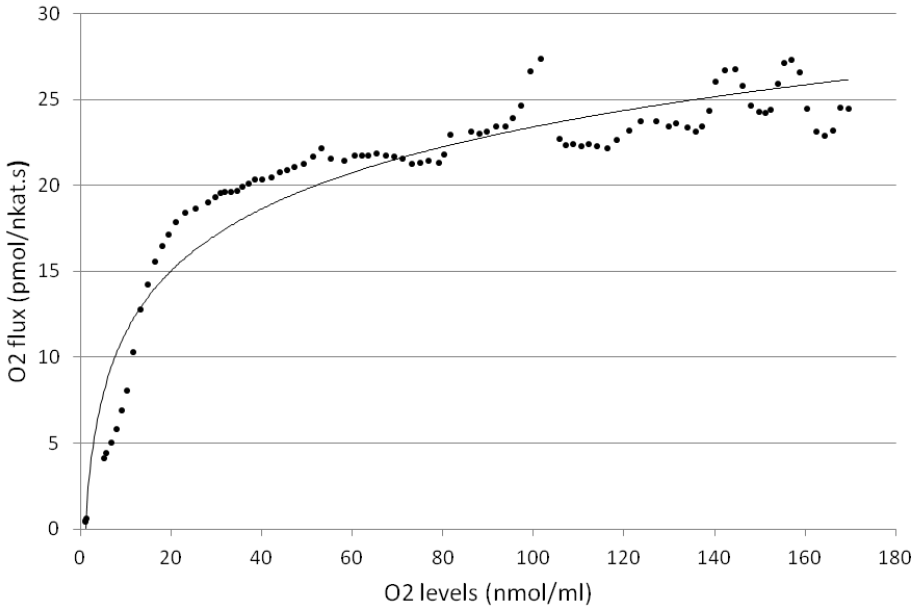


**Figure 9:** The dependency of oxygen flux on oxygen concentration after the addition of substrates for complexes I and II, ADP and Cyt c [157].

In preliminary experiments, the addition of pyruvate (10mM) to the glutamate/malate mixture caused either no change or decrease in OCR, so we decided to use standard substrates (malate/glutamate/succinate). FCCP yielded maximal flux without detectable inhibition between 0.6-0.7µM with a decrease of ORC above 1µM (Figure 10). Similarly, we observed no additional increase of oxygen flux above 1mM of ADP and no additional decrease of oxygen flux above 1µM of oligomycin. ADP titration demonstrated a stability of ADP-stimulated oxygen fluxes for up to 40 min. These concentrations were used in the final protocol.

In the final respirometry protocol, 200µl of 10% homogenate (containing 20mg of wet muscle weight) in a final volume of 2,1ml and serial addition of malate/glutamate (2.5/15mM), ADP (1mM), cyt c (20µM), succinate (10µM), oligomycine (1µM), FCCP (0.6µM), and AA (4µM) were used.

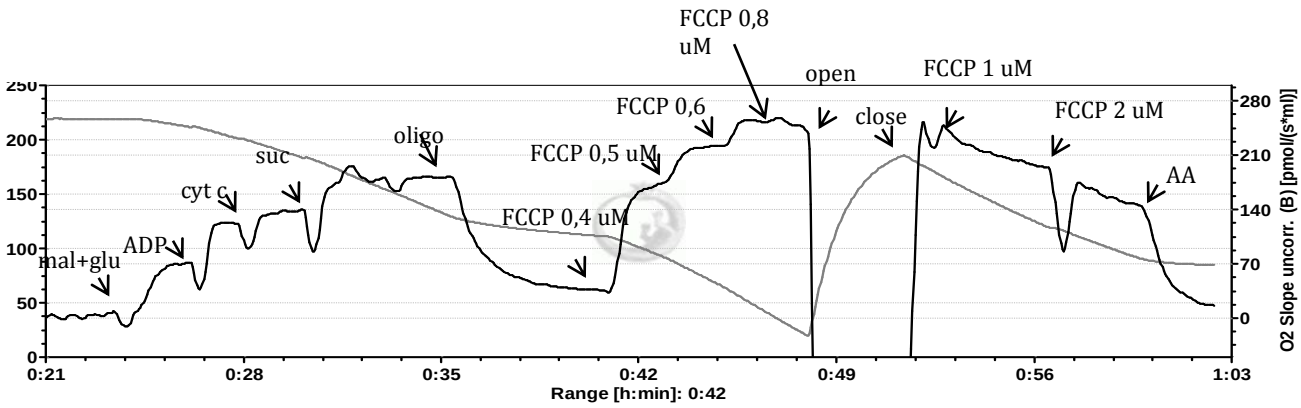


**Figure 10:** FCCP titration experiment. Note no additional increase above 0.6 µM and an inhibition with more than 1 µM [157].

**iii)** ***open surgery vs needle biopsy technique:*** to compare these biopsy techniques, two muscle biopsies were simultaneously obtained by the surgeon from the same subject (n=6). One sample was taken using the open technique with a scalpel or scissors and a second sample from the same muscle by using 5mm Bergstrom needle attached to a suction catheter. Further processing of both samples was identical and the respirometry measurements were run in parallel; each chamber containing one sample of homogenate.

We did not discover any significant differences in mitochondrial functional indices (p=0.41 – 0.99 t-test) between needle and open biopsy techniques in human skeletal muscle homogenates. Outer mitochondrial membrane was mildly disrupted in both (increments after the addition of cyt c were 13.0±6.0 vs. 12.7±6.0%, p=0.93 in needle and open biopsies, respectively).

## b) protocol validation against the standard technique

**i)** ***validation against HRR on isolated mitochondria:*** to assess reliability and reproducibility of our results, an open biopsy sample from 5 subjects were divided into two parts (n=5) and both methods were immediately performed in parallel and in duplicate. When homogenates (HOM), which had to wait for mitochondria to be prepared, were directly compared with mitochondria (MIT) isolated from the same subjects (n=5), they clearly showed signs of mitochondrial damage. Homogenate was prepared as described above and stored on ice until the mitochondria were ready. The mitochondria were prepared as previously described [170], which took 60-90 min. However, when the same experiment was repeated (n=6) on freshly prepared homogenate (HOM1), these signs were absent.

The main damage in HOM is of the outer mitochondrial membrane (cyt c 38±12 vs 12±6 vs 10±9% in HOM vs. HOM1 vs. MIT, p<0.001 ANOVA), but there is also an apparent leak through the inner mitochondrial membrane (CCR 0.40±0.10 vs. 0.12±0.06 vs. 0.24±0.11, p=0.001, ANOVA), as well as damage to complex I (CI/ETS 0.57±0.15 vs. 0.70±0.06 vs. 0.76±0.08, p=0.011, ANOVA). For other mitochondrial respiratory indices see Table 6. Of note, the coefficient of variation for OXPHOS was quite low in HOM and HOM 1 (5.2±5.1 and. 4.6±7.0%) as compared to MIT (12.4±7.6%, p=0.11, ANOVA). As the only difference between HOM and HOM1 measurement technique was the delay between the homogenate preparation and the respirometry (in case of HOM), we further explored the influence of homogenate storage on ice on respirometry results.

**Table 6: Mitochondrial functional indices in homogenate and isolated mitochondria**

| | HOM1 | HOM | MIT | P value (ANOVA) |
|---|---|---|---|---|
| N | 6 | 5 | | |
| **CI+IIp*** [pmol/s] | 128 ± 44 | 65 ± 36# | 102 ± 47# | 0.061 |
| **ETS*** [pmol/s] | 165 ± 61 | 108 ± 60 | 125 ± 92 | 0.410 |
| ETS/CS [pmol.ml/nkat.s] | 3515 ± 1534 | 4072 ± 2013 | 3301 ± 1944 | 0.357 |
| **LEAK*** [pmol/s] | 21 ± 14 | 42 ± 23 | 28 ± 21 | 0.201 |
| CI/CS [pmol.ml/nkat.s] | 2045 ± 902 | 1841 ± 1119 | 2737 ± 1235 | 0.357 |

Mitochondrial functional indices in fresh homogenate (HOM1) with homogenates (HOM) and isolated mitochondria (MIT) obtained from the same subjects and measured in parallel. Values with asterisks represent unadjusted oxygen fluxes in mitochondria from 20mg of muscle wet weight; others were normalized to citrate synthase (CS) activity. Paired samples (HOM-MIT) were also tested by paired t-test and # means significant difference (at p<0.05). Note: CI+IIp = respiration after addition of substrates for complexes I and II, ADP and cyt c. ETS=electron transfer system capacity measures after addition of substrates for complexes I and II, cyt c and an uncoupler [157].

**ii)** ***influence of measurement´s delay:*** comparing measurements immediately after the homogenate was prepared and after 120-180 min of storage on ice (n=7) revealed a trend in the outer and inner mitochondrial membrane damage, impairment of complex I with a decrease in OXPHOS capacity (Figure 11)

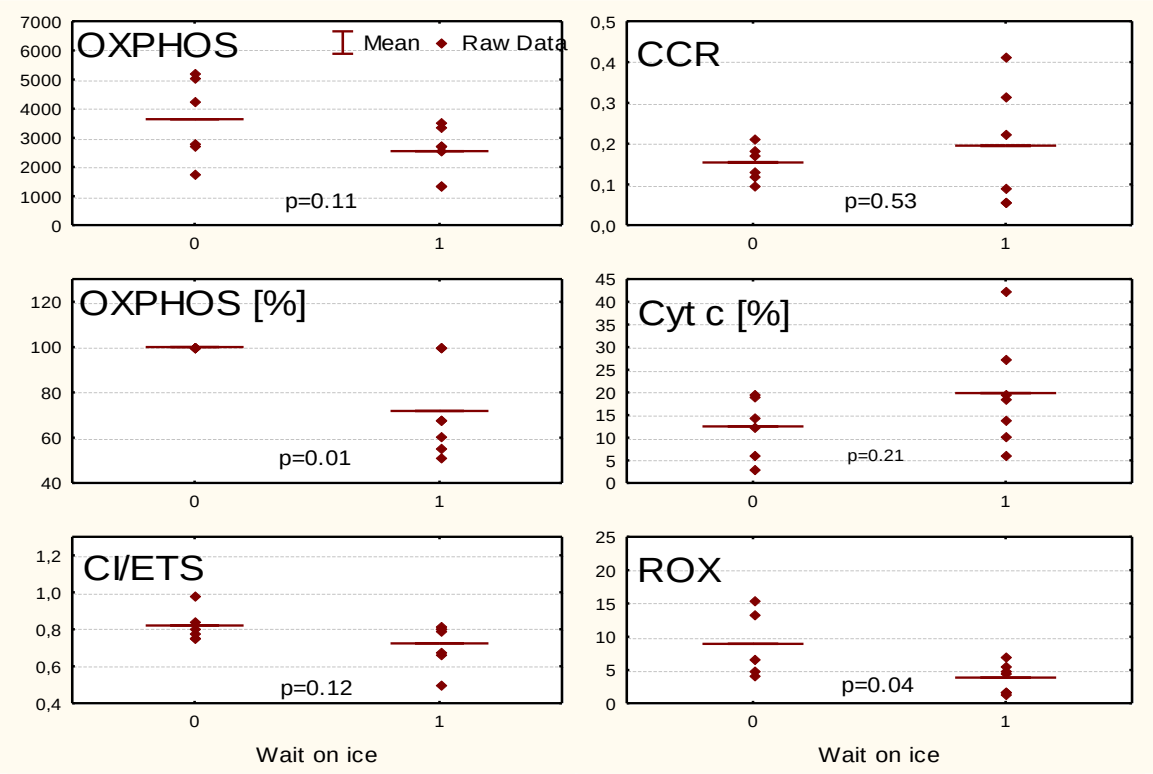


**Figure 11:** Mitochondrial functional indices in fresh homogenates (left) and after 120-180 min of storage (right). Diamonds represent individual datas, horizontal lines are means. Note: OXPHOS [pmol.ml/nkat.s]= oxidative phosphorylation capacity normalized to citrate synthase activity, CCR=coupling control ratio (leak/CI+IIs), OXPHOS [%]= relative change of OXPHOS when value of fresh homogenate is 100%,Cyt c [%]= increase in oxygen flux after the addition of Cyt c FCR=flux control ratio, CI/ETS= proportion of electron flux via complex I to electron transfer system capacity, ROX [pmol/s]= unadjusted non-mitochondrial oxygen consumption [157].

### 5.1.2 XF Flux Analysis Protocol for human skeletal muscle cells

In a series of preliminary experiments, for our research purpose we developed a novel protocol for the use of extracellular flux (XF) analysis in cultured human myoblasts by modifying existing protocols for C2C12 myoblasts [171] and human myotubes [172].

**a)** ***Seeding density:*** first, myoblasts were seeded at a density of 20000 cells per well, but a maximum OCR reached only the median of 76 or 65 pmol/min after 1 or 2µM FCCP, respectively, while OCR for the same number of C2C12 cells was approximately 10 times higher. Next, we doubled and almost tripled the number of cells that were seeded per well (40000 and 75000) and finally we achieved a maximum OCR/ well above 100 pmol/min, while $pO_2$ remained above 5kPa throughout the experiment.

**b)** ***Optimum concentration of all compounds added****: w*e compared the effects of 0.76, 1.0, and 1.5µM oligomycine, achieving maximum inhibition of ATPase with 1.0µM; similarly, we compared the uncoupling effects of 0.5, 1, and 2µM FCCP, achieving a maximum OCR with 1µM. Finally, we performed an experiment with sequential addition of 4µm AA and 0,6M KCN (complex IV inhibitor) to verify the ability of AA to inhibit the respiratory chain and no additional decrease in OCR was seen after the addition of cyanide. In our final protocol, a baseline OCR was measured in 3 cycles: 3 minutes mixing, 2 minutes waiting and 3 minutes measuring. Then these 3 cycles were repeated after adding oligomycin (1µM), FCCP (1µM), and AA (4µM)

According to the final protocol we used in the stress test assay in human myoblasts, cells were plated into 20 wells of a XF24-well gelatin-coated plate (Seahorse Bioscience, North Billerica, MA, USA) at a density of 40000/well in a basal growth medium and incubated at 37°C overnight. After 24 hours, we exchanged the medium with a XF Assay Medium (pH 7.4, $HCO_3$– free, Seahorse Biosciences) with 4mM glucose and 1mM pyruvate and incubated myoblasts for 60min at 37°C in a $CO_2$ free atmosphere. The baseline OCR was measured in 3 cycles: 3 minutes mixing, 2 minutes waiting and 3 minutes measuring. Then these 3 cycles were repeated after adding oligomycin (1µM), FCCP (1µM), and AA (4µM).

**c)** ***XF Flux Analysis of Human Myotubes****:* on day 0, myoblasts were trypsinized and seeded into a 24-well gelatin-coated Seahorse plate at a density of 20 000cells/well in a basal growth medium and grown to ~80-90% confluence. Then we exchanged the medium with a differentiation medium (DMEM) with glucose (25mM), pyruvate (1mM), 2% horse serum, penicillin-streptomycin solution (100µg/ml), insulin (10µg/mL) and glutamine (0,5mM). The medium was exchanged every 2 days for the next 6 days. On day 7 the XF assay was performed

## 5.2 Glutamine effect on mitochondrial function in skeletal muscle cells

### 5.2.1 Fastest cell proliferation was observed in 0,3mM of glutamine

Myoblast proliferation rate was maximal and exponential at 0.3mM of glutamine. Cells across a range of glutamine concentrations showed exponential proliferation, which was fastest in 300µM (Figure 12). Mean numbers (geometric means of all 4 culture intervals) of myoblasts formed from 1 ancestor cell during a 5-day cycle of culture were 10 (0µM glutamine), 15 (100µM), 18 (200µM), 25 (300µM), 24 (500µM) and 23 (5000µM). Interestingly, we observed that the proliferation rate slowed down after 10 days regardless of the glutamine concentration. However, cell viability as per the MTS test was not compromised.

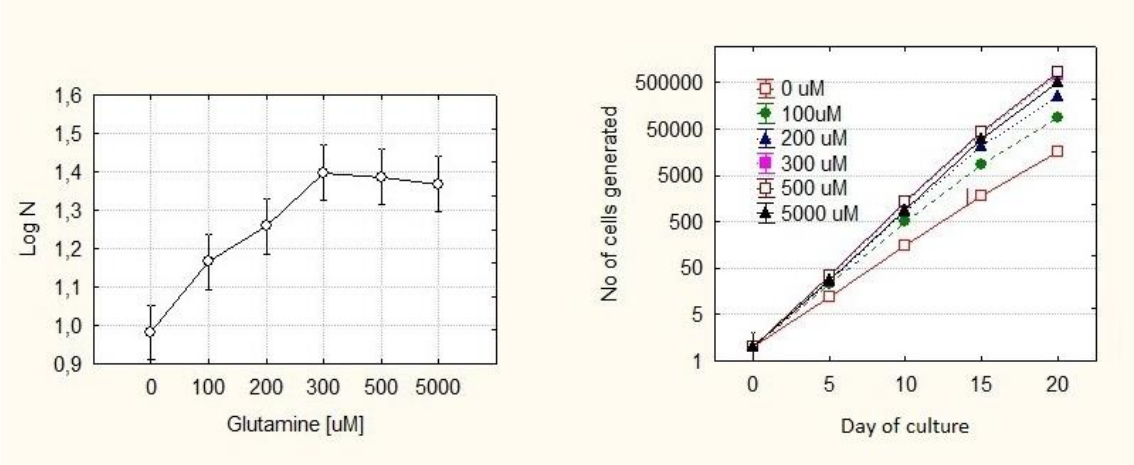


**Figure 12:** Left: Influence of glutamine concentration on the cell proliferation as per factorial ANOVA. Right: Proliferation rate of myoblasts in the rate of the relation to glutamine concentration in the media. The mean number of cells formed from a single cell at Day 0 [87].

### 5.2.2 Glutamine deprivation tends to decrease protein content

Although 20000 cells/well were seeded prior to the differentiation comparing to 40000 cells/well plated for experimentation in myoblasts, the protein content was higher in the wells containing myotubes. In general, glutamine deprivation tended to decrease the total protein content per well by up to ~50% (see 13A) in comparison to cells cultured in 5000µM glutamine. Moreover, we observed a significant correlation between the protein content in a well and glutamine in the media (Spearman $R$ = 0.46 for myotubes and $R$ = 0.38 for myoblasts; $P$ < .05 for both), but this trend was not seen for CS activity, which remained unchanged across glutamine concentrations (see 13B), and the differences in CS activity between myoblasts and myotubes approximately correspond with the number of cells seeded per well. Additionally, a measure of relative mitochondrial density - CS per protein ratio - tended to increase with the severe glutamine deprivation (see 13C) as well as OCR/ECAR ratio (see 13D), reflecting the

relative contributions of aerobic and anaerobic metabolism on ATP generation. For details see Fig. 13.

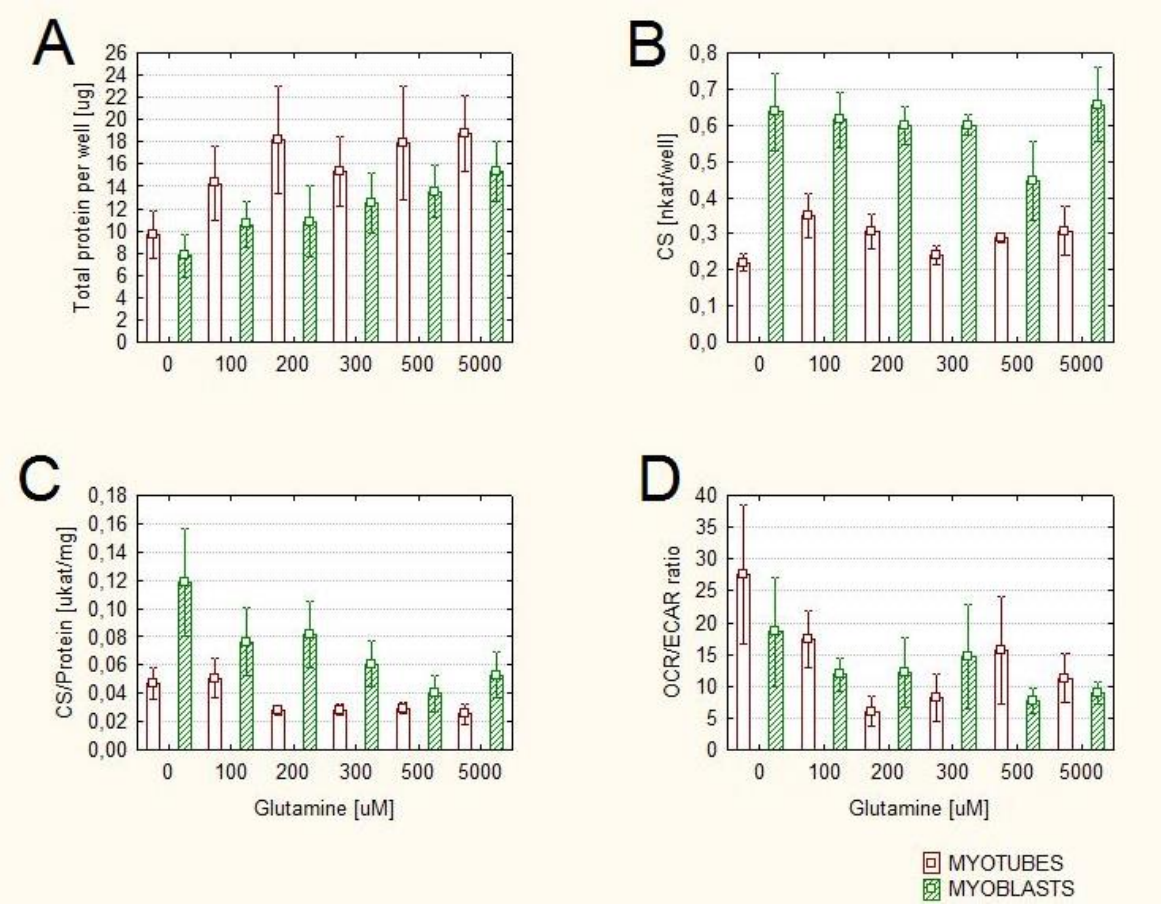


**Figure 13:** (A) Total protein content per well. (B) Citrate synthase (CS) activity per well. (C) CS per protein ratio. (D) Oxygen consumption rate (OCR) per extracellular acidification rate (ECAR) ratio. Means, vertical bars represent 95% confidence intervals [87].

### 5.2.3 Glutamine does not influence global indices of mitochondrial function except for leak through the inner mitochondrial membrane

OCR at baseline was unaffected by glutamine concentration in both myoblasts and myotubes. After the normalization to mitochondrial content (expressed as CS activity), the basal respiration does not differ either between myoblasts and myotubes ($P$=0.53) nor across glutamine concentrations ($P$=0.87).

We also did not observe any difference in anaerobic glycolysis in a range of glutamine concentrations in both cell lines. After blocking the ATP synthase with oligomycine, the anaerobic glycolysis increased to ~110 – 170% above the baseline, but this response was not affected by glutamine concentration, nor was the extra-mitochondrial oxygen consumption.

The ATP synthesis rate normalized to mitochondrial content (CS activity) was neither different between the cell lines ($P$ = 0.16) nor across the glutamine concentration ($P$ = 0.63; see

Figure 14C). The spare respiratory chain capacity (maximum respiration) is expressed as OCR after the artificial uncoupling of the inner mitochondrial membrane. If normalized to mitochondrial content (Figure 14B), the maximum respiration tends to be non-significantly higher in the myoblasts compared with the myotubes ($P$ = 0.13), and it is not influenced by glutamine ($P$ =0.94). Similarly, when related to the basal respiration, the maximum respiration is 200%–300% and not different between cell lines or among glutamine concentration.

But, the leak of protons through the inner mitochondrial membrane normalized to CS activity (Figure 14D) was significantly higher in myotubes compared with myoblasts ($P$ < 0.001) and was influenced by glutamine concentrations ($P$ = 0.041). Myoblasts had a significantly lower leak in comparison with myotubes ($P$ = 0.019), which only increased with high glutamine concentrations (from ~10% to ~30%).

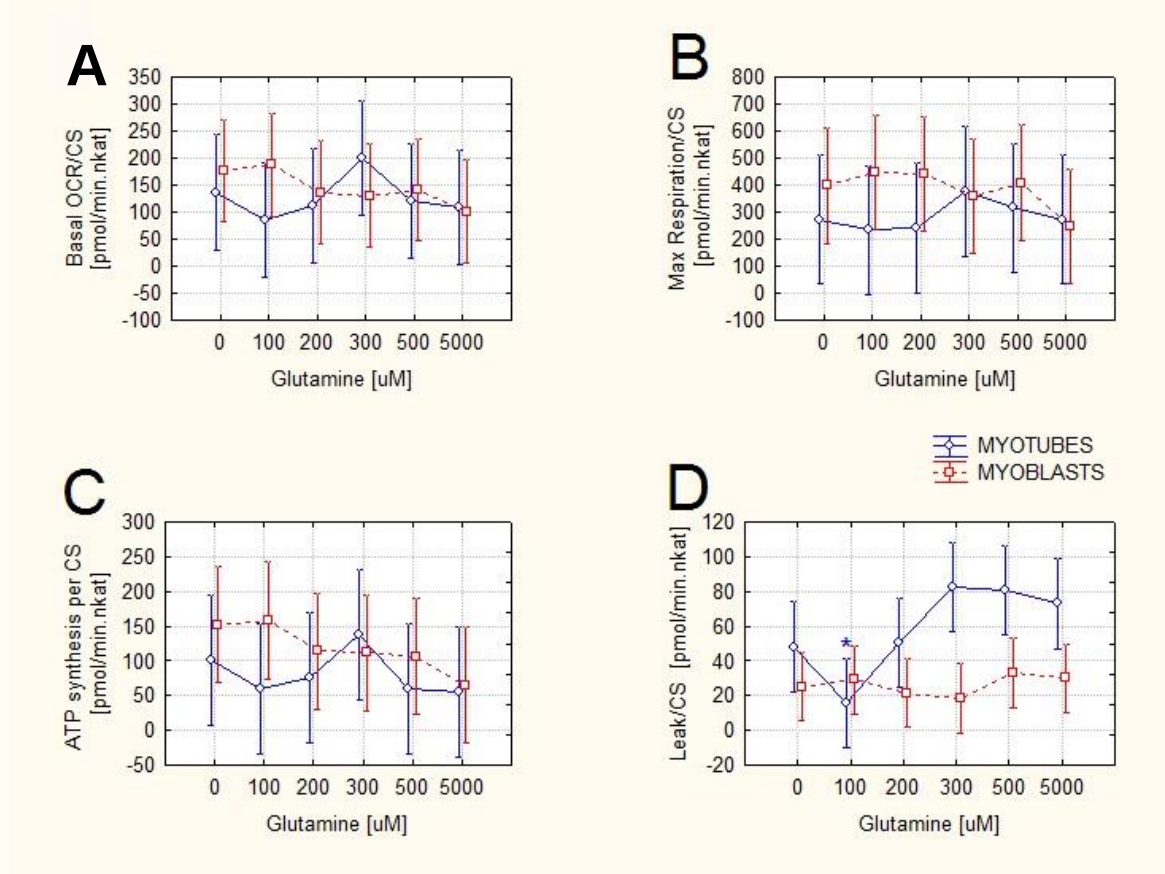


**Figure 14:** (A) Basal oxygen consumption rate. (B) Maximal respiration. (C) Adenosine triphosphate (ATP) synthesis rate. (D) Proton leak through the inner mitochondrial membrane. All values are expressed as the amount of oxygen used after the adjustment to mitochondrial content in a well expressed as citrate synthase (CS) activity (pmol/min$^{-1}$/nkat$^{-1}$). Means, vertical bars represent 95% confidence intervals. OCR, oxygen consumption rate [87].

### 5.2.4 At both extremes of glutamine concentrations we observed decreased efficiency of the respiratory chain below 70%

In myotubes, the proton leak through the inner mitochondrial membrane was lowest with 0.2-0.3mM of glutamine, whilst the lower or higher concentrations caused mitochondrial uncoupling (p=0.04) and tended to decrease ATP production (p=0.05). Similarly, the respiratory chain spare capacity was maximal at 0.2mM and decreased in both extremes of glutamine concentrations (p=0.03).

**Table 7: Energy metabolism in myotubes (medians)**

| Glutamine concentration [mM] | **0** | **0,1** | **0,2** | **0,3** | **0,5** | **5** | p (Kruskal-Wallis) |
|---|---|---|---|---|---|---|---|
| Myoblast doubling time [days] | 1,68 | 1,39 | 1,32 | 1,14 | 1,15 | 1,16 | **N/A** |
| OCR at baseline [pmol/min] | 54 (46-57) | 71 (21-100) | 44 (25-47) | 52 (45-102) | 65 (54-72) | 87 (71-103) | **0,293** |
| Leak [% OCR at baseline] | 24 (11-50) | 22 (17-26) | **8** (0-17) | 17 (13-36) | 36 (32-40) | 49 (39-51) | 0,041* |
| ATP turnover [% OCR at baseline] | 76 (50-98) | 78 (74-83) | 92 (63-100) | 83 (74-87) | 65 (62-72) | 51 (49-61) | **0,053** |
| Resp. capacity [% baseline] | 176 (132-196) | 315 (164-338) | **317** (276-520) | 202 (187-207) | 255 (183-345) | 159 (150-174) | 0,031* |

When expressed as a percentage of basal OCR, myotubes cultured at 0.2–0.3mM glutamine used 80%–90% of basal OCR for ATP synthesis and only 10%–20% of energy was dissipated as heat. At both extremes of glutamine concentration, we observed an increased mitochondrial uncoupling to ∼30%–40%, which decreased the efficiency of the respiratory chain below 70% (see Figure 22). In myoblasts, we saw similar trends which did not reach statistical significance.

To conclude, we observed the highly oxidative nature of human skeletal muscle cells and the increased leak with higher glutamine concentrations, particularly in myotubes. ATP wasted by inner mitochondrial leak was not compensated by increased anaerobic glycolysis.

## 5.3 Mitochondrial function in skeletal muscle of patients with ICUAW

### 5.3.1 Global indices of mitochondrial function (Protocol 1)

In the skeletal muscle of patients with protracted critical illness (ICUAW) compared to control subjects (Control), we demonstrated a reduction in citrate synthase (CS) activity per muscle wet weight (median 0.25 (IQR 0.16–0.28) vs 0.34 (IQR 0.28–0.43) nkat/mg Ww, p=0.03). In keeping with this, the capacity of OXPHOS and of the respiratory chain were significantly reduced in ICU patients (approximately 54 % and 52 % of that in controls, p<0.01 and p = 0.03) when expressed per muscle wet weight. Of note, there was no difference in the degree of uncoupling of inner mitochondrial membrane between ICU patients and Controls. After the adjustment to mitochondrial density (CS activity), the differences in mitochondrial functional indices disappeared (Table 8).

**Table 8: global mitochondrial functional indices measured by HRR in homogenates**

| Parametr | | Per muscle wet weight [pmol/s.mg Ww] | | | Per CS activity [pmol.nkat$^{-1}$.s$^{-1}$]. | | |
|---|---|---|---|---|---|---|---|
| | | ICUAW (n=7) | Control (n=8) | P | ICUAW (n=7) | Control (n=8) | p |
| OXPHOS (3p) | | 7,6 (5.0-8.8)* | 13.9 (11.3-17.9) | **<0.01** | 31 (28-36)* | 37 (32-74) | 0.15 |
| RC capacity (3u) | | 8.6 (6.7-10.5) | 16.4 (13.0-20.6) | **0.03** | 41 (37-44) | 42 (37-98) | 0.46 |
| Non-mito OCR | | 0.8 (0.6-1.5) | 0.8 (0.6-1.3) | 0.91 | 4 (3-5) | 2 (1-4) | 0.16 |
| $F_1F_o$ATPase activity | Absolute | 6.1 (4.8-7.6)* | 12.6 (9.2-13.0) | **<0.01** | 26 (26-30)* | 33 (29-49) | 0.46 |
| | % OXPHOS | 81 (77-83)* | 84 (80-89) | 0.36 | 81 (77-83)* | 84 (80-89) | 0.36 |
| Proton leak | Absolute | 1.3 (1.0-1.4)* | 2.2 (1.3-3.6) | 0.10 | 8 (5-9)* | 7 (4-11) | 0.95 |
| | % OXPHOS | 19 (17-23)* | 16 (11-20) | 0.36 | 19 (17-23)* | 16 (11-20) | 0.36 |

**Table 8:** Data presented as median (interquartile range), *p* value as per Mann-Whitney *U* test. *N=5 for ICU patients. *GPDH* glycerol-3-phosphate dehydrogenase, *Non-mito OCR* non-mitochondrial oxygen consumption rate, *OXPHOS* oxidative phosphorylation, *RC* respiratory chain.

OXPHOS normalized to muscle wet weight was correlated with the activity of CS ($r^2$ = 0.53, p = 0.01), the content of COX II subunit of respiratory complex IV ($r^2$ = 0.39, p = 0.03) and there was a trend towards a correlation to core 2 subunit of complex III ($r^2$ = 0.29, p = 0.06), but no relations at all were seen to the concentrations of complex II ($r^2$ = 0.04, p = 0.50) or F1α subunit of F1FOATPase ($r^2$ = 0.02, p = 0.67).

### 5.3.2 Respiratory chain complexes - analysis of function (HRR - protocol 2)

Using a sequential addition of substrates and specific inhibitors of the respiratory chain complexes, it is possible to determine maximum electron fluxes through individual complexes of electron transport chain. In order to account for the variation of mitochondrial content in samples, the oxygen consumption rates were adjusted to the activity of CS (Figure 16, upper row).

Functional capacities of Complex I and IV were not different between ICU and control group (Table 9). Surprisingly, capacity of Complex II was in ICU group ~300% of the capacity in controls (p<0.01). The capacity of complex III/GPDH was also significantly (p<0.01) higher in ICU patients as compared to controls. Very similar results were obtained when the individual activity of respiratory complexes was measured by spectrophotometry (Fig. 15).

**Table 9: high-resolution respirometry in homogenates.**

| Parametr | Per muscle wet weight [pmol/s.mg Ww] | | | Per CS activity [pmol.nkat$^{-1}$.s$^{-1}$]. | | |
|---|---|---|---|---|---|---|
| | ICUAW (n=7) | Control (n=8) | P | ICUAW (n=7) | Control (n=8) | p |
| Complex I | 4.8 (4.0-6.1) | 6.7 (5.5-8.6) | 0,19 | 23 (22-35) | 23 (18-26) | 0.35 |
| Complex II | 4.6 (2.9-6.5) | 1.5 (0.8-3.8) | 0.06 | 23 (20-28) | 8 (3-14) | **<0,01** |
| Complex III/GPDH | 1.5 (1.1-1.9) | 0.8 (0.4-1.3) | 0.12 | 7.4 (6.0-9.3) | 1.8 (1.2-3.9) | **<0,01** |
| Complex IV | 15.5 (13.0-19.5) | 19.7 (15.3-27.5) | 0.30 | 88 (69-99) | 49 (40-113) | 0,12 |

**Table 9:** Data presented as median (interquartile range), p value as per Mann–Whitney U test.

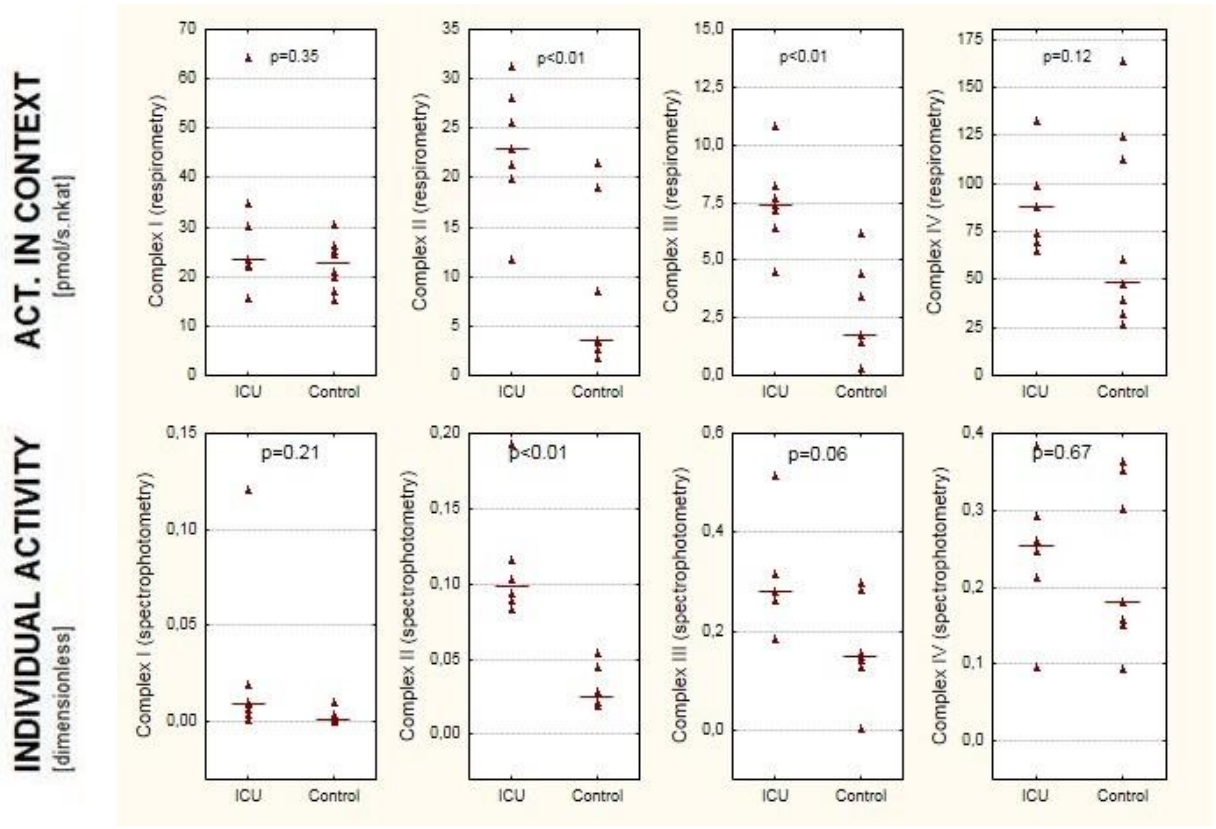


**Figure 15:** Activity of individual respiratory complexes adjusted to mitochondrial content (citrate synthase activity) measured by two independent methods. Upper row complex activity in cytosolic context determined by high-resolution respirometry in skeletal muscle homogenates. Lower row spectrophotometric analysis of the activity of individual respiratory complexes. Lines represent medians [173].

### 5.3.3 Relative content of mitochondrial proteins

Next, we measured the content of individual respiratory complexes to clarify the relationship between their individual functional capacity and relative content, due to the fact that we had observed an overall ~50% reduced capacity to synthetize ATP by aerobic phosphorylation (OXPHOS) and a simultaneously significant increase in functional capacities of Complexes II and III in the respiratory electron transport chain in ICUAW compared to Controls.

Surprisingly, in ICU patients compared to controls, there was a significant reduction of Core 2 subunit of Complex III (median content in ICU patients was approximately 38 % of that in controls, $p = 0.02$) and COX2 subunit of complex IV (approximately 26 %, $p < 0.01$). No differences were detected in the subunits of F1FOATPase (approximately 109 %, $p = 0.89$) or Complex II (approximately 90 %, $p=0.99$). We were unable to determine the content of subunits of Complex I (the signals were bellow detection limits in both ICU and control patients). See Figure 16.

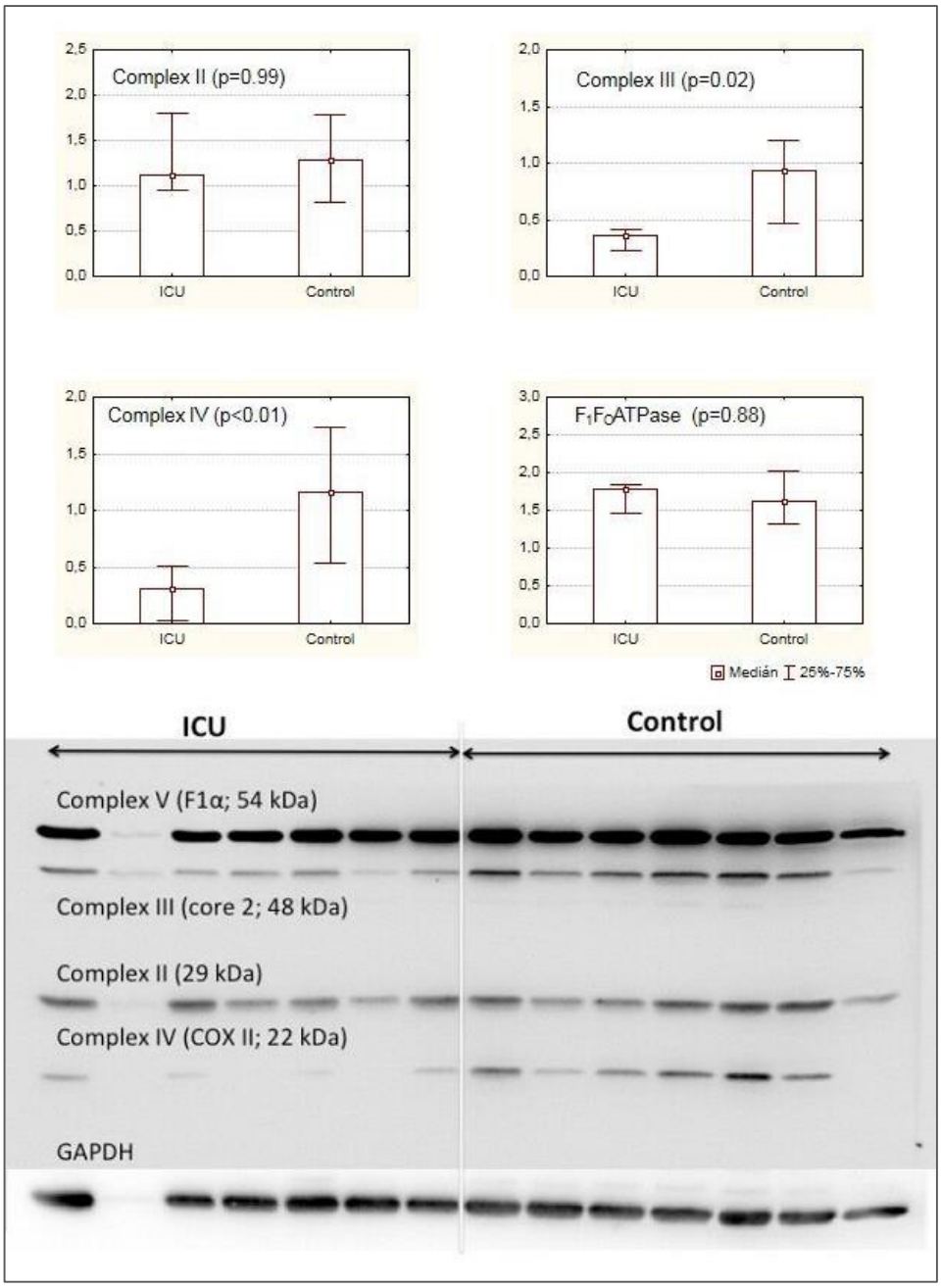


**Figure 16:** Concentration of functional subunits of respiratory complexes in arbitrary units and an example of immunoblot memrane. Data are presented as medians, vertical bars represent interquartile ranges [173].

Last we asked whether there is a relationship between the capacity of OXPHOS (or state 3p as determined in Protocol 1) and the specific functional capacity of individual respiratory complexes (as determined in Protocol 2). Complex I ($r^2$ = 0.33, p = 0.04), and even more strongly Complex IV ($r^2$ = 0.65, p <0.01) correlated to OXPHOS, whilst complexes II and III/GPDH did not ($r^2$ = 0.08, p = 0.36 and $r^2$ = 0.12, p = 0.28, respectively). See Figure 17.

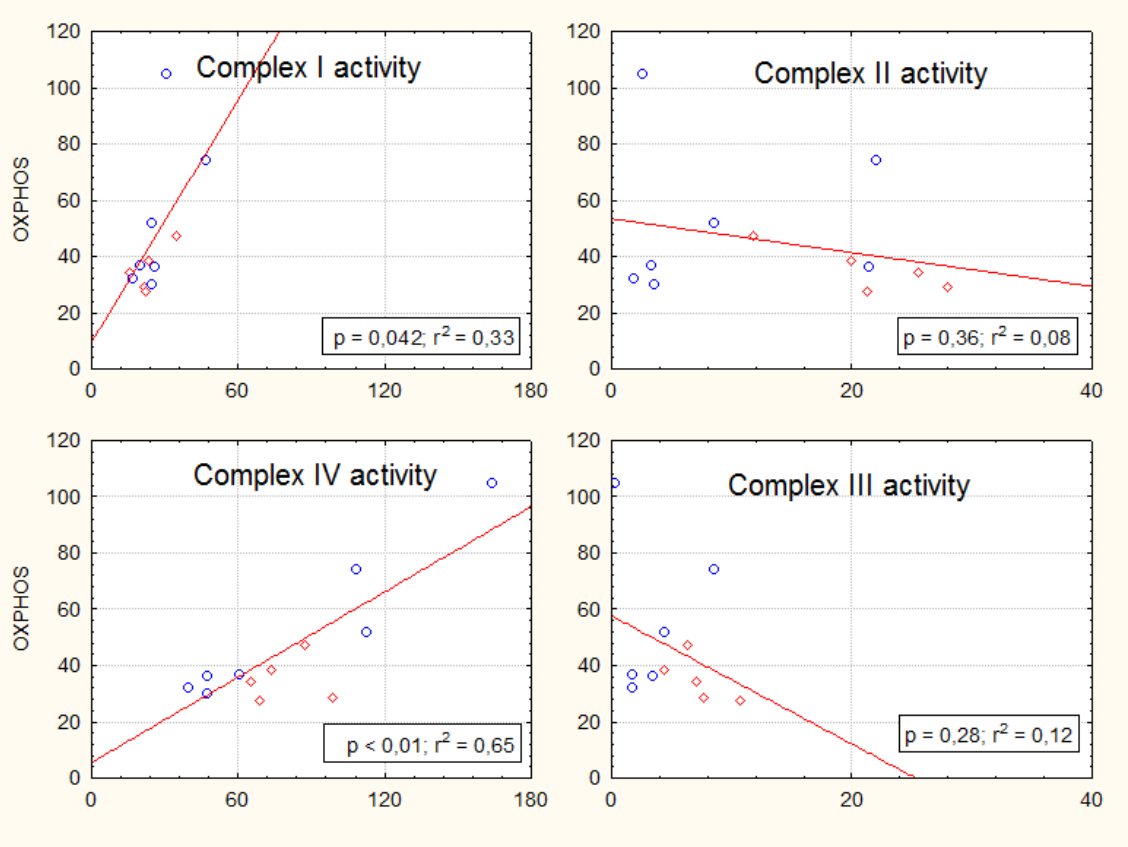


**Figure 17:** Correlations between functional capacities of respiratory complexes (x axis) and OXPHOS (oxidative phosphorylation, or state 3p, y axis). All data were adjusted to citrate synthase activity and are expressed in [pmol/s.nkat]. Note: Red circles = ICU patients, blue circles=Control.

### 5.3.4 In vitro model of skeletal muscle – cultured myotubes

In the myotubes from ICU patients, there was a trend to a reduction of mitochondrial density expressed as CS activity per well (4.10 [3.34-4.83] vs. 5.89 [5.45-8.03] nmol/ml/min; p= 0.051). After the adjustment to the mitochondrial content (i.e. the activity of citrate synthase), global mitochondrial indices were not different between ICU patients and controls. Of note, there was no sign of increased mitochondrial uncoupling in ICU patients. See table 10.

**Table 10: Mitochondrial functional indices in human skeletal myotubes as per XF analysis.**

| Parameter | ICU | Controls | p |
|---|---|---|---|
| ATP synthesis rate [% baseline OCR] | 77.1 [72.5-79.2] | 81.1 [66.9-84.1] | 0.89 |
| Proton leak [% baseline OCR] | 22.9 [20.8-27.5] | 18.8 [15.9-33.1] | 0,89 |
| Respiratory chain capacity [pmol/$s^{-1}$.$nkat^{-1}$] | 56 [38-83] | 31 [30-36] | 0,36 |
| Anaerobic glycolysis/protein content [pmol/$s^{-1}$. $\mu g^{-1}$] | 0.46 [0.24-0.57] | 0.36 [0.21-0.41] | 0.39 |
| Fatty acid oxidation/CS [pmol/s.nkat] | 18.6 [14.2-26.9] | 11.8 [14.2-26.9] | **0.015** |

Data are presented as medians (interquartile range), p value as per Mann–Whitney U test

In ICUAW patients, the fatty acid oxidation adjusted to mitochondrial density was significantly higher compared to controls (Fig.18)

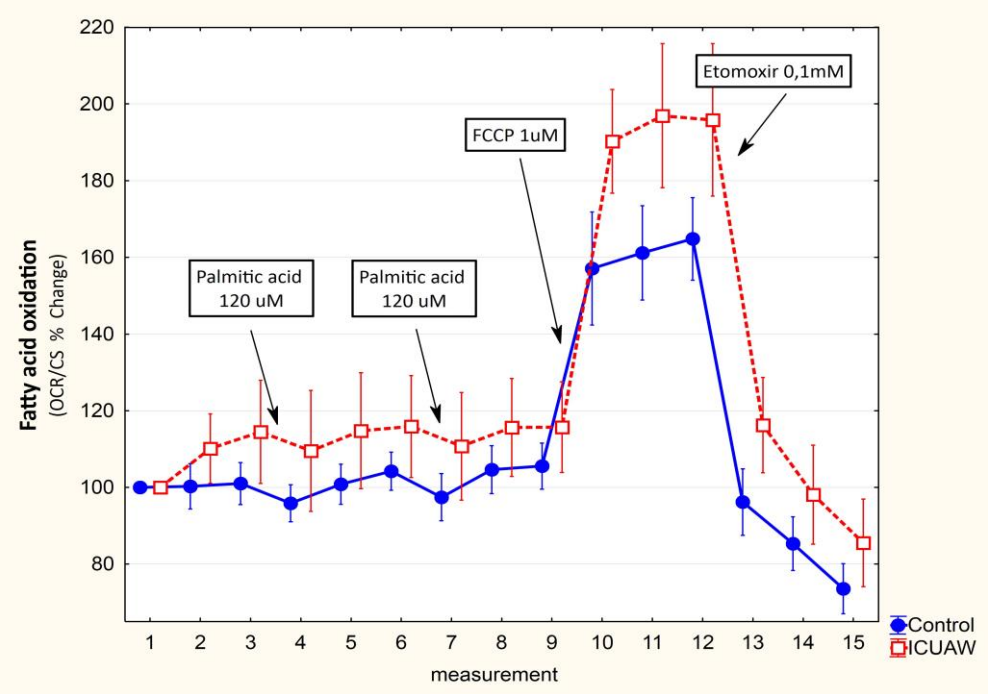


**Figure 18:** Fatty acid oxidation in myotubes from ICUAW patients compared to controls. Data are presented as % change in OCR (mean and 95% confidence intervals) adjusted to mitochondrial density; measurement 1 (baseline) = 100%; Note: CS = citratesynthase; FCCP = carbonyl cyanide–4-[trifluoromethoxy] phenylhydrazone; OCR = oxygen consumption rate;

### 5.3.5 Exposure to free fatty acids

As a follow-up to the previous experiments, we exposed the human myotubes to six media with a different concentration and composition of FFA (details in methods) to simulate the metabolic situation in the protracted critical illness. Six days of exposure to increased FFA concentration resulted in a corresponding increase of respiratory chain capacity in ICUAW myotubes (Figure 19), whilst we were not able to detect similar effect on the myotubes from healthy controls. Mitochondrial density (measured as the activity of citrate synthase) remained lower in ICUAW myotubes and was not influenced by FFA exposure. Even though there is some signal of increased mitochondrial uncoupling in ICUAW myotubes and that exposure to FFA may decrease it, these trends were not significant. There were also no differences in anaerobic glycolysis (measured as ECAR) between ICUAW and control myotubes (data not shown). We did not detect any effects attributable to the presence of GH and IGF-I in growth or differentiation media (data not shown).

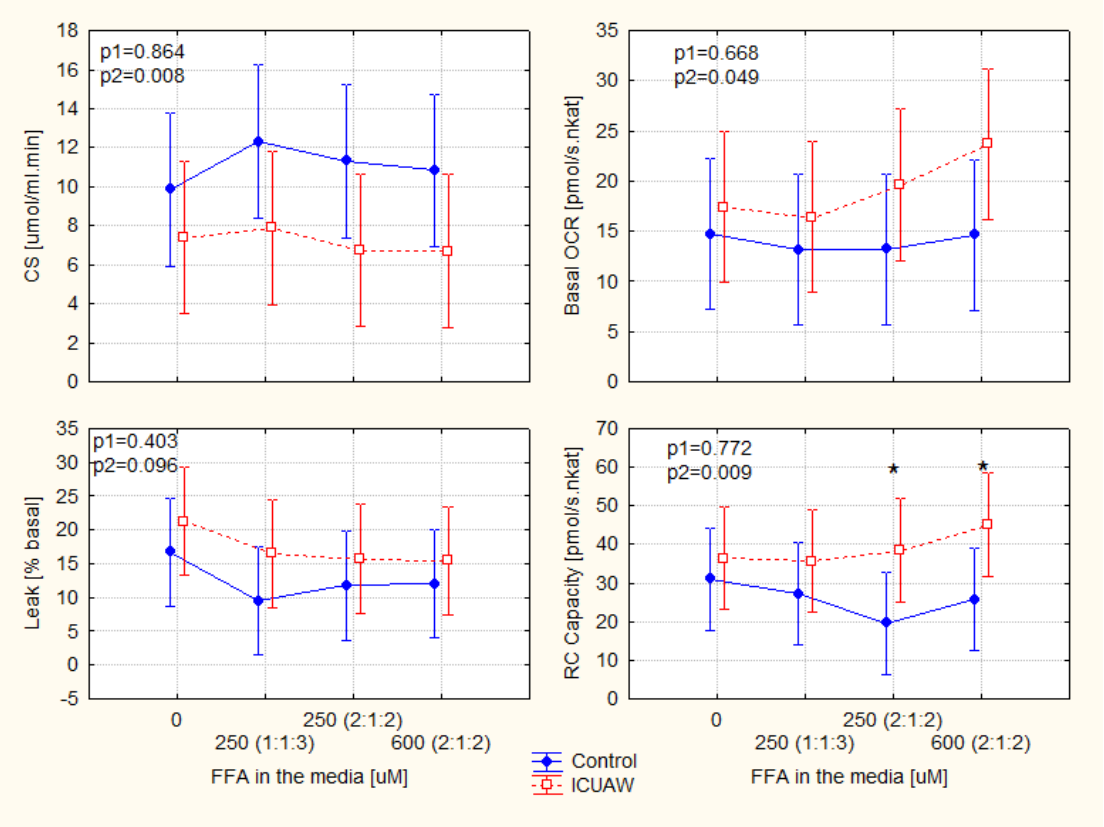


**Figure 19:** Effect of myotubes exposure to different free fatty acid (FFA) concentrations. Data presented as mean and 95% confidence intervals. Note: p1=effect of environment; p2=control vs. ICUAW (2-way ANOVA), * = p<0.05 between ICUAW and Control as per Fisher LSD post hoc test; CS=citrate synthase; RC=respiratory chain; OCR=oxygen consumption rate; 1:1:3 or 2:1:2 refer to ratio of palmitic:oleic:linoleic acids.

# 6 DISCUSSION

ICUAW is an increasingly recognised clinical consequence of critical illness, associated with significant morbidity and mortality [22,25,174]. While many authors focused on the understanding of its aetiology and on the role of risk factors in its pathogenesis in the acute phase, the protracted phase of critical illness has only recently attracted attention. Our study therefore aimed to explore whether mitochondrial dysfunction persists until the protracted phase of critical illness and to assess various mitochondrial functional indices (i.e. the capacity of oxidative phosphorylation, of respiratory chain and of individual respiratory complexes, the degree of uncoupling of inner mitochondrial membrane) in human skeletal muscle tissue in the critically ill with respect to substrate availability (glucose, FFA, glutamine), insulin and/or growth hormone stimulation.

Much of the evidence for the pathways involved in the pathogenesis of ICUAW is inferred from in vitro and animal models of muscle wasting. Since the insult of ICUAW is multifactorial, the evidence from clinical studies is sometimes contradictory. What is more, many observations demonstrate the problem in translating laboratory - based experiments into clinical studies, e.g. some human studies of immobilisation have shown greater decrease in protein synthesis compared to the increase in breakdown [175], which is the converse of that observed in rodent models [57]. In fact, there is currently a lack of human ICUAW studies [176].

Although the patients in our study had been recruited from 22-general ICU and 10-bed medical ICU of University Hospital Kralovske Vinohrady in Prague and all adults receiving mechanical ventilation for more than 2 weeks had been screened for eligibility, only 8 out of 22 eligible ICU-patients approached consented for muscle biopsy. The limited number of study subjects is the main weakness of our study which increases the risk of type II error (i.e. not being able to detect differences, which do exist). Additionally, we did not study patients with a protracted critical illness without ICUAW, and hence we are unable to distinguish which alterations are attributable to critical illness and which to ICUAW. In light of this, our results should be interpreted with caution. However, as discussed in detail below, our study is the first to demonstrate mitochondrial dysfunction in the skeletal muscle of patients with protracted critical illness and ICU-acquired weakness.

Prior to the proper study, we were aware of the need to modify the established techniques of studying mitochondrial bioenergetics, as none of available experimental protocols and tools appropriately complied with our intentions. Two novel systems that enable specific measurement of mitochondrial bioenergetics with high accuracy have been recently

introduced: high-resolution respirometry (O2k, Oroboros Instruments, Austria) and Seahorse XF system (the sensitive high-throughput Seahorse XF Extracellular Flux Analyzer, Seahorse Bioscience Inc.). The advantage of these methods is that they are not destructive to cells and tissues and can be performed in real time. Both are able to analyse the mitochondrial respiratory function in the classic in vitro experiments on isolated mitochondria as well as in cultured cells and tissues, but neither had been adapted to study human skeletal muscle cells and tissue respectively.

Firstly, we developed a protocol for the assessment of mitochondrial function using high-resolution respirometry in homogenates of human skeletal muscle. This method has previously been established only for measuring cellular bioenergetics using either isolated mitochondria, permeabilised muscle fibres or intact cultured cells. Comparing our novel technique with the standard technique of respirometry in isolated mitochondria, we have shown that it is possible to enjoy its advantages – this technique is simpler to perform and is more robust, and additionally, we can study mitochondria in cytosolic context. But certain limitations should be kept in mind.

When developing the protocol, we adopted some aspects of methodology from protocols designed for homogenates of non-muscle samples [152, 177,178] or skeletal muscle samples of animals [151]. The only existing study on human skeletal muscle homogenates [153] identified the main challenge of the technique, i.e. the damage of the very fine structure of Complex I and to outer mitochondrial membrane. Whilst Larsen and colleagues [153] focused on the technique of homogenisation, we paid attention to other details. In order to avoid adverse effects of hyperoxia on homogenate, we did not use preoxygenation but instead carefully titrated the amount of muscle, which enabled us to perform measurements at optimal oxygen fluxes without limitation by oxygen concentration. The extent to which the eventual drop of oxygen concentration limits oxygen flux is much higher in permeabilised muscle fibres (Km $\sim$ 40 nmol/ml) in comparison to isolated mitochondria (Km < 1 nmol/ml [154]), where Km is $O_2$ concentration at which the oxygen flux is reduced to 50% of the maximal rate. We determined Km of muscle homogenates to be $\sim$ 12nmol/ml. Despite using $\sim$10 times more muscle tissue in a chamber compared to Larsen [153], we were able to run all experiments above the safe limit of 90nmol/ml $O_2$, in most cases without the need of chamber opening. Titration of substrates and uncouplers in separate experiments allowed for the omission of stepwise additions of ADP and FCCP in the final protocol, which further shortened the measurement time to < 30min.

By these measures, we achieved a protocol in which outer mitochondrial membrane functional damage measured as an increase of oxygen flux after the addition of cytochrome c

was reduced to a range of 10-15%. This value is lower than the value obtained in Larsen's study (>20% [153]) and comparable to the isolated mitochondria (~10%). Damage to Complex I was also reduced, as CI/ETS ratio in our protocol was ~ 0.70 in homogenate in comparison with ~ 0.76 in isolated mitochondria and ~ 0.4 in Larsen's homogenates. In line with this, the phosphorylation capacity adjusted to CS activity (OXPHOS) is in homogenates ~ 90% of the capacity of isolated mitochondria. The mitochondrial coupling tended to be even better in homogenates in comparison with the isolated mitochondria (CCR 0.12±0.06 vs. 0.25±0.11, p=0.052), but this is because the uncoupling in our mitochondria was higher than reported by other authors [179]. The reproducibility measured as CV of duplicate measurement of OXPHOS was better in homogenates (~5%) compared to the isolated mitochondria (~12%), in keeping with Larsen's results [153].

We cannot specify which change (or combination of changes) was responsible for these improvements, but our results clearly demonstrate that homogenisation-to-respirometry time was crucial for the quality of results. The results described above are only obtained if the respirometry measurement is performed immediately after the homogenisation of the sample. Whilst human skeletal muscle sample is stable on ice for up to 24 hours [180], homogenate is not. Only after 120-180 min, we confirmed signs of inner and outer membrane decomposition and damage to Complex I resulting in a decrease in OXPHOS and increased decoupling. These changes occur in a very variable and unpredictable way, so the use of freshly prepared homogenates is of upmost importance. Moreover, our data highlights the importance of reporting homogenisation-measurement times in studies using this technique.

High-resolution respirometry has also been applied on isolated non-human [181-182] and human [153] permeabilised skeletal muscle fibres. This method offers excellent preservation of intracellular mitochondrial architecture, but is technically challenging (takes 1-2 hours [182]) and the results may be more prone to inter-operator variability. In our study, we did not compare homogenates with isolated muscle fibres, but data from Larsen et al [153] demonstrated a coefficient of variability CV ~11%. The use of hyperoxygenation is unavoidable with permeabilised fibres and puts the sample at risk of oxygen toxicity. The use of homogenates is simpler (preparation time ~10 min) and more robust (CV ~5%), but in spite of the preservation of cytosolic context, intracellular mitochondrial architecture is indeed compromised.

When compared to isolated mitochondria, our technique is simpler to perform and more robust. Homogenisation causes only minor damage to outer mitochondrial membrane and respiratory complexes and the results are identical regardless of whether the sample was

obtained by needle or by an open surgical technique. We have shown that in order to get reliable results, respirometry must be performed immediately after the homogenisation of the sample as the degradation of mitochondrial function occurs quickly.

Next, we modified the standard protocol for measuring mitochondrial functional indices and fatty acid oxidation using the technique of extracellular flux analysis (XF, Seahorse Bioscience) in C2C12 myoblasts and myotubes with the aim of studying human skeletal muscle cells. We established this technique in our laboratory, while we were studying the influence of glutamine on myoblasts proliferation rate and its effect on energy metabolism of human skeletal muscle cells. Why glutamine?

The long-term goal of our research is to contribute to the understanding of the ICUAW-pathophysiology and how the skeletal muscle metabolism is altered during the protracted phase of critical illness. In this broad area, we are specifically interested in the role that mitochondrial dysfunction and insulin resistance play in this complex process.

During the past years we have been studying the influence of glutamine supplementation on skeletal muscle metabolism [183] and the efficacy and metabolic effects of growth hormone substitution given as intravenous pulses in protracted critical illness [134,184].

Recombinant growth hormone (rhGH) became widely used in the ICU during the period between 1975-1995 as an experimental therapy for critically ill patients who had been receiving prolonged intensive care in order to prevent complications of catabolism. And its effect on nitrogen balance was ostensibly positive. Finally, two large randomized trials in 1999 revealed that the administration of rhGH (with doses 10-20 times higher than those used for replacement therapy) increases mortality in critically ill patients associated with infectious complications and development of MOF [133].

In 2008, we revisited the rhGH supplementation in prolonged critical illness and demonstrated its safety and nitrogen conservation effect together with increased serum levels of insulin-like growth factor-1 and insulin-like growth factor-1 binding protein in selected chronically ill patients receiving adequate nutrition support. However, this therapeutic approach worsens the insulin sensitivity and current guidelines continue to recommend against the use of growth hormone in critical illness [185].

Several decades ago, hyperglycaemia was identified as an independent risk factor for ICUAW and confirmed as a deleterious metabolic factor on the neuromuscular system in the critically ill [40,46]. Consequently, the application of strict glycaemic control has been implemented in the clinical praxis as a promising preventive intervention. Therefore, in this project we aimed to study mitochondrial bioenergetics with respect to insulin resistance and

optimal substrate (glucose, FFA) availability in skeletal muscle tissue. Unfortunately, results from a large, international, randomized trial NICE-SUGAR were published in 2009 and it has been confirmed that an intensive glucose control (to target 4.5-6.0 mmol/L) results in significantly increased 90-day mortality when compared to conventional glucose control (to target ≤ 10.0 mmol/L) and tight glycemic control is currently not recommended in critically ill adults in ICU [47]. In other words, safe glucose plasma concentration has been already identified in critically ill.

In line with this, glutamine attracted our attention again and our group was the first to provide evidence that glutamine can cause mitochondrial uncoupling and impair ATP generation in human skeletal muscle [87]. This generates the hypothesis that glutamine effect on mitochondrial metabolism may have contributed to the disappointing clinical outcomes that were seen with aggressive glutamine supplementation [86,186].

Rapidly dividing cells have been shown to be dependent on glutamine as a substrate for de novo nucleotide synthesis [187] and energy metabolism [187-189]. Additionally, Oudemans-van Straaten et al [190] reported that low levels of plasma glutamine (<420µmol/l) have been associated with increased mortality in the critically ill. Therefore, it has been hypothesized that glutamine is essential in critical illness due to the rapid depletion and patients would benefit from supplementation. But in 2013 Heyland et al published results of international randomized blinded trial and it has been shown that the aggressive glutamine supplementation in critically ill patients with MOF was harmful, i.e. associated with an increase in mortality [86]. The mechanism in which glutamine may cause harm remained unknown.

During metabolic stress, a major interorgan flux of glutamine occurs. Glutamine released from muscle has to subserve the increased demands of immune active and gut mucosa cells needing it for proliferation, differentiation and specific functions [191]. Increased release from muscle can be initially associated with normal plasma glutamine levels, but the low plasma glutamine concentration in protracted critical illness has been considered to be a sign of glutamine depletion [190] with the resulting debilitation of the immune system and the gut [192].

In vitro, a close relationship between the glutamine concentration and the proliferation of rapidly dividing cells is seen. This has been demonstrated for lymphocytes [193], enterocytes [194] and type II pneumocytes [195]. In accordance with these findings, a similar dependency has been shown for human myoblasts in our study. Surprisingly, supra-physiological concentrations of glutamine (5000 µM), recommended by many protocols for in vitro cell cultures [188, 196-199] including human myoblasts [172], do not bring any additional benefit in terms of myoblast proliferation. Furthermore, it may even be the case that

concentration as low as 300µM glutamine, which is close to the concentration in patients with protracted critical illness in the absence of glutamine supplementation [134] are optimal. Significant limitations of myoblast proliferation were observed at concentrations ≤200µM glutamine, which is well below the range seen in wasting diseases [200] or in acute critical illness [134,201,202]. Although it is difficult to extrapolate in vitro data to whole-body physiology, our results suggest that restoring extracellular glutamine concentration to normal levels may not improve the conditions for muscle regeneration. Of note, even cells grown in glutamine-free media remained viable and did proliferate. See Figure 20.

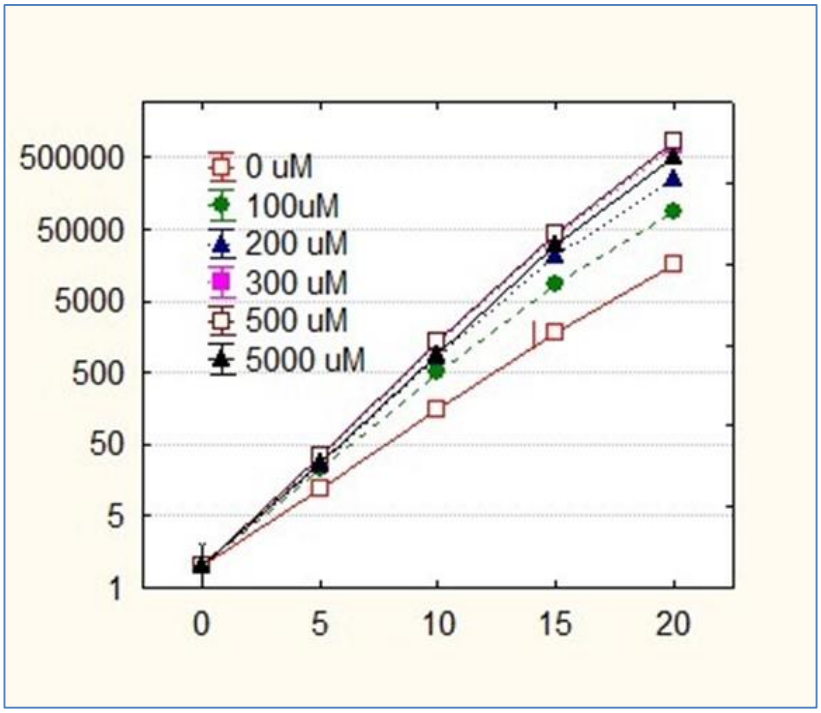


**Figure 20:** Proliferation rate of human myoblasts in relation to glutamine (Gln) concentration in the media. Mean number of cells formed from a single cell at day 0. Note log scale; confidence interval bars are not displayed for clarity [87].

A trend to a decrease in protein content per well without a change in CS activity in wells seeded with glutamine-deprived cells may reflect a lower number of cells (with more mitochondria), diminished total protein content in the same number of cells, or a combination of both. Nicklin et al [203] demonstrated, in various mammalian cell lines, that glutamine is an upstream activator of mTOR, a key regulator of protein synthesis [204]. That could represent a molecular mechanism as to how glutamine deprivation can alter intracellular protein content.

Myoblasts and myotubes cultured in a standard “laboratory” concentration of glutamine (5000µM) were largely reliant on aerobic phosphorylation, having an OCR/ECAR

ratio ~10, which is comparable to feline cardiomyocytes [205] or C2C12 myoblasts [206]. Much lower OCR/ECAR (typically between 1 and 5) ratios are reported for most cancer cell lines [207]. We observed a very small contribution of anaerobic phosphorylation to overall ATP turnover and a trend to a further increase of the OCR/ECAR ratio in glutamine-deprived cells which may reflect the fact that pyruvate—instead of being converted to lactate—is turned into oxaloacetate. This anaplerotic reaction can replenish intermediates of the citric acid cycle when there is not enough glutamine to form 2-oxoglutarate via glutamate. However, we failed to demonstrate the inhibitory effect of glutamine deprivation on anaerobic glycolysis either at baseline or after inhibition of aerobic ATP synthesis (Fig. 21)

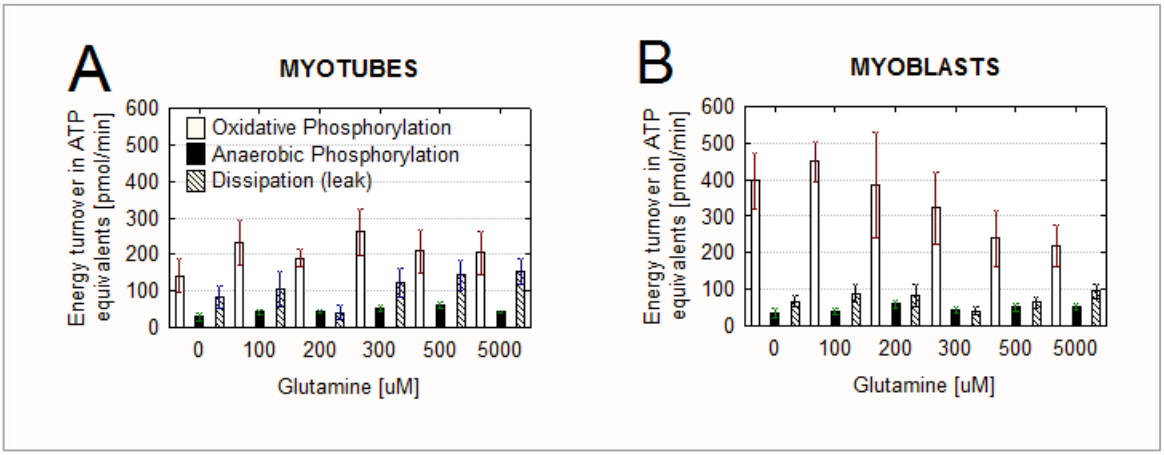


**Figure 21:** Energy profile of primary human skeletal muscle cells, see text for explanation (87)

When looking at the effect of glutamine on the energy metabolism of myotubes and myoblasts, the most interesting finding is the uncoupling effect of glutamine. In both myoblasts and myotubes cultured at 200–300µM glutamine, we observed a highly coupled respiration with ~ 90% of basal OCR being used to drive ATP synthesis. Supra-physiological concentrations of glutamine (5000 µM) decreased the efficiency of the respiratory chain to ~ 60–75% in both cell lines. In myotubes but not myoblasts, we saw a similar degree of uncoupling with extremely low glutamine (0µM). See Figure 22.

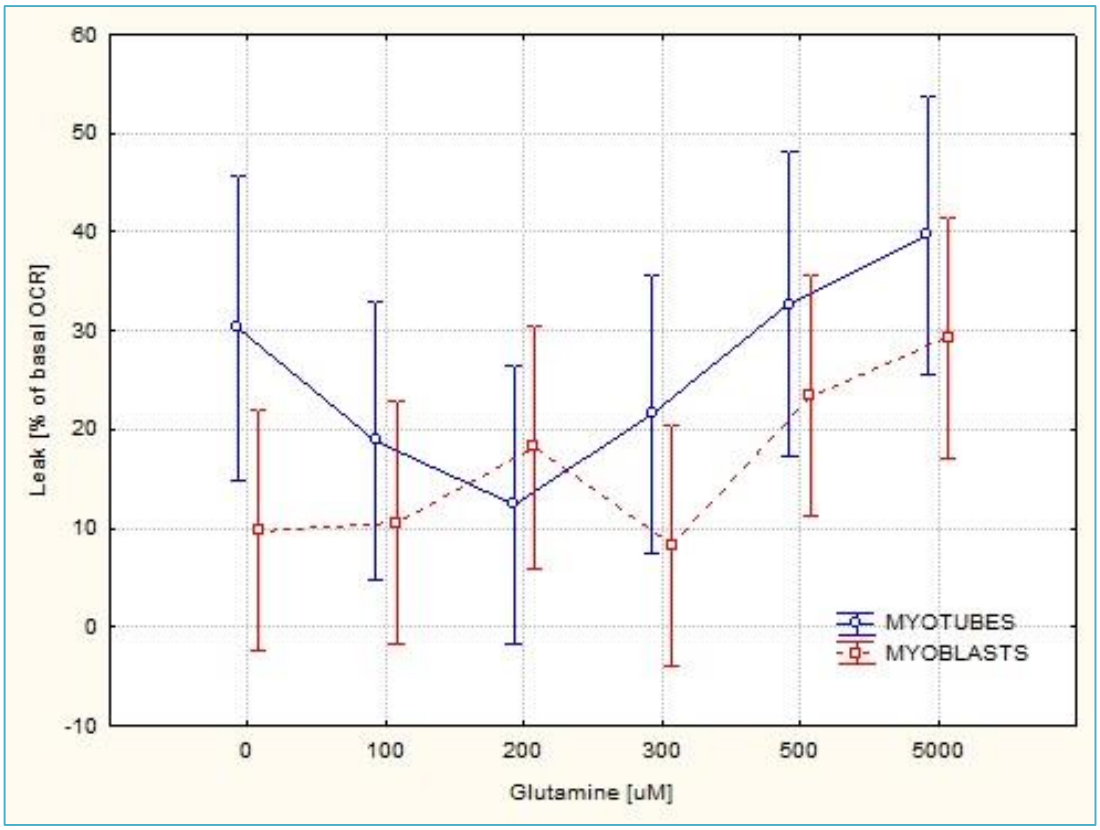


**Figure 22:** Proton leakage through the inner mitochondrial membrane expressed as a percentage of the basal oxygen consumption rate. Means, vertical bars represent 95% confidence intervals. OCR, oxygen consumption rate [87].

Mitochondrial uncoupling can result either from inner mitochondrial membrane damage or from the controlled leak of protons through the family of uncoupling proteins (UCPs). Of these, UCP-2 and UCP-3 are found in skeletal muscle [208]. In cultured macrophages, colonocytes, and pancreatic β-cells, glutamine has been shown to increase the translation of UCP-2 with the maximum induction seen at 0.4–1.0mM [209]. It has been proposed [210] that UCP-3 and UCP-2 may also serve as uniporters of pyruvate from — and glutamine into — mitochondria, respectively.

In this context, the need to transport abundant glutamine into mitochondria to join the citric acid cycle (via glutamate and 2-oxoglutarate) may have induced UCPs and caused the uncoupling seen with higher glutamine concentrations. Skeletal muscle is the main organ for glucose disposal in vivo, and glutamine-induced uncoupling should then increase substrate metabolism and energy expenditure. In line with this, glutamine supplementation in critically ill patients has repeatedly been shown to increase insulin-mediated glucose disposal [183, 211,212] and fat oxidation [184]. Iwashita et al [213] observed increased energy expenditure with glutamine in healthy volunteers. In this context, with glutamine supplementation and

uncoupling, glucose and fat can be increasingly oxidized in skeletal muscle to energize futile cycles and heat generation.

However, in our study, we have not demonstrated any significant increase in basal OCR with glutamine-induced uncoupling, and it seems that increased leak was rather at the expense of aerobic phosphorylation (albeit its changes were not significant). This would suggest a rather uncontrolled leak resulting from glutamine-induced mitochondrial damage.

Possible mitochondrial toxicity of glutamine was first noted by Groening et al [214]. When investigating the protective effect of glutamine on the activity of cytochrome c oxidase in septic rat myocardium, they found a trend towards decreased oxygen extraction and diminished cyclooxygenase (COX) activity in sham-operated (non-septic) hearts, in spite of higher COX levels with 0.75g/kg glutamine compared with placebo. The authors mention the possibility of glutamine intracellular breakdown to glutamate and ammonia, which resulted in oxidative damage of mitochondria [214].

It should be noted that muscle biopsies were taken from participants with hip arthropathy, which may have influenced muscle physiology. Additionally, the culture media with different glutamine concentrations were not isonitrogenous, and we cannot rule out the possibility that the observed effects are due to different nitrogen content rather than glutamine itself. Moreover, the methods used do not permit a deeper insight into the observed changes of energy metabolism. Further studies should explore the effects of glutamine on intracellular ATP concentration, inner mitochondrial membrane potential, and expression of UCPs, as well as measure mitochondrial DNA oxidative damage in human non-tumor cell lines. If “mitochondrial toxicity” of glutamine is confirmed, this would shed new light onto recent data from large randomized controlled trials on glutamine supplementation.

Glutamine seems to be beneficial in chronic wasting diseases but harmful in acute critical illness with multiorgan failure [86]. Our data generate the hypothesis that the reason for the discrepancy of clinical outcomes with glutamine may lie in mitochondria: bioenergetic failure is a feature of critical illness, where altered and swollen mitochondria are not able to meet extreme ATP demands of tissues, leading to multiorgan failure and death. The hypoglutaminaemia of critical illness may represent an adaptive phenomenon [215] by decreasing the circulating mitochondrial toxin below what we consider to be safe levels. This may be protective of mitochondria at the expense of other glutamine-dependent processes. After the initial insult and following mitochondrial damage and repair, the beneficial effects of higher levels of glutamine may prevail, since these other glutamine-dependent processes may become more important in the subsequent rehabilitation phase. During the recovery phase

after mitochondria have been repaired and replenished in survivors [97] and also in chronic illnesses, the beneficial effect of glutamine may prevail.

Once the methodology has been selected and customized, we continued studying mitochondrial function in skeletal muscle cells and our study is the first to demonstrate mitochondrial dysfunction in skeletal muscle of patients with protracted critical illness and ICU-acquired weakness. Biopsies were performed in ICU patients who had been ventilated for ~28 days and who had suffered from muscle weakness. We selected this cohort of patients with muscle dysfunction in order to maximize the chances to observe any alteration of bioenergetics in a non-respiratory muscle, which seems to be less affected even in acute phase of critical illness when compared to the diaphragm [216,217] or intercostal muscles [143]. Of note, we found it very difficult to consent patients for the biopsy in this non-therapeutic study, thus deriving data from a small group of highly selected subjects.

This is the first study to demonstrate mitochondrial dysfunction in skeletal muscle of patients with protracted critical illness. We have shown a ~50% reduction in the ability to synthetize ATP by aerobic phosphorylation per mg of muscle wet weight (OXPHOS/Ww). Pertaining to the relation between the capacity of OXPHOS and concentrations of individual respiratory complexes, we have discovered that OXPHOS and depletion of Complex IV are strongly correlated. Complex III was also depleted, unlike Complexes II and V. When OXPHOS was adjusted to citrate synthase activity (OXPHOS/CS), the differences between the ICU patients and the control subjects disappeared and OXPHOS/Ww strongly correlated with citrate synthase activity.

The obvious interpretation of these results is that mitochondria are depleted in ICU patients, whilst complexes II and V are relatively abundant in remaining functional mitochondria. A similar disproportionality of the concentrations of respiratory complexes has been described in skeletal muscle during aging [218] and oxidative stress [219]. Even though citrate synthase activity is widely used as a marker of mitochondrial content [99,216,220,221] it may become a subject of oxidative damage [222] and therefore it may not reliably reflect the mitochondrial density. Because we have not used an alternative method of measuring mitochondrial content (e.g. electron microscopy), we cannot state whether the depletion of Complexes III and IV occurred in isolation or occurred as part of mitochondrial depletion.

Our group initially hypothesized mitochondrial failure due to the dysfunction in ETC complexes in accordance with the findings reported by Carré et al. [97] during the trend toward the depletion of Complexes I and IV in the critically ill.

It is the concentration of the depleted Complex IV that was limiting for the mitochondrial function, in keeping with data from Levy [223], who demonstrated the relation

of Complex IV dysfunction to bioenergetics failure in acute sepsis. Contrary to our hypothesis, we have not found any signs of increased mitochondrial uncoupling (leakage of protons through inner mitochondrial membrane).

Next we have asked whether the depletion of functional mitochondria is the only abnormality or if there is also a change in the relative functional capacities of individual respiratory complexes. At first, we performed a respirometry protocol in which we used specific substrates and inhibitors of individual complexes. If expressed per muscle wet weight (Table 9), we noticed a trend towards the increase in functional capacity of respiratory Complexes II and III, whilst that of Complexes I and IV tended to be non-significantly reduced to approximately 70 % of values seen in control subjects, and correlated with OXPHOS. After the adjustment for citrate synthase activity, Complexes II and III were increased significantly (threefold and twofold respectively, $p < 0.01$) and Complexes I and IV were not different (Fig. 16).

High-resolution respirometry measures the changes in oxygen consumption in fresh intact tissue homogenates after the addition of respiratory substrates and inhibitors [152]. The sample contains intact mitochondria in a cytosolic context and it is believed that this approach better reflects physiological alterations occurring in vivo [224]. When using this method to measure the functional capacity of individual complexes, one must bear in mind that the rate-limiting step can in theory appear downstream of the complex that is being analyzed. Complexes III and IV are under physiological conditions able to accommodate the flux of electrons from both Complexes I and II and it is therefore unlikely that they become rate-limiting when fed by electrons from either Complex I or II in isolation. For testing Complex III, we used glycerol-3-phosphate as a substrate whilst Complexes I and II had been blocked. By doing so, we avoided the risk of downstream limitation (i.e., at Complex IV), but on the other hand, the rate-limiting step may be at the level of GPDH, which is functionally a part of the glycerol phosphate shuttle rather than the respiratory chain.

Next, with these limitations of respirometry in mind, we repeated the measurements of individual complex activities using classical spectrophotometry as most authors do [99,143,216]. It is a well-established method that assesses the activities of respiratory complexes by using artificial complex-specific substrates after the organelle structure has been destroyed by repeated freezing and thawing. This means that the measured activity of each complex is independent of the functionality of other complexes.

Despite different methodology, both techniques gave very similar results (Figure 16): we found no differences in the functionality of Complex I, which is known to be depleted in the acute phase of critical illness [99], nor in the capacity of Complex IV, which thus seems to be

depleted but not dysfunctional in protracted critical illness. The most surprising result was the increased functional capacity of Complexes II and III/GPDH in the critically ill as compared to control subjects.

Complex II (succinate dehydrogenase) normally drives electrons from succinate oxidation to fumarate in the citric acid cycle (CAC) via flavin adenine dinucleotide (FAD) to the respiratory chain. CAC itself is heavily dependent on the reoxidation of NADH by Complex I as it produces three molecules of NADH per one molecule of FADH2. The eventual increase in NADH/NAD+ ratio inhibits CAC. Similarly, aerobic glycolysis produces 2NADH/molecule of glucose during the conversion to pyruvate and a further 2NADH by converting pyruvate to acetyl-CoA, which is oxidised in CAC. However, during the oxidation of fatty acid and carbon skeletons of branched chain amino acids, reduced coenzymes FADH2 and NADH are produced in a 1:1 ratio. Of all catabolic pathways, fatty acid oxidation is thus least dependent on the functionality of Complex I. In the acute phase of critical illness, Complex I seems to be predominantly impaired [99] and upregulation of complex II at a later stage can be a compensatory response or an attempt to bypass dysfunctional complex I.

Insulin resistance is a well-known feature of critical illness [183,225] and it has been shown that GLUT-4 dependent transport is dysfunctional in patients with ICUAW [226] and pyruvate dehydrogenase is inhibited [227]. Skeletal muscle in protracted critical illness may consequently suffer from the starvation of carbohydrate-derived substrate for CAC. On the contrary, free fatty acids are elevated in the critically ill [184,225] and intracellular lipid droplets accumulate early in diaphragmatic and biceps muscle in brain-dead donors [216]. Branched-chain amino acids (BCAA) derived from muscle protein degradation are deaminated in skeletal muscle and their carbons are oxidized in a similar way to fatty acid oxidation. Relative up-regulation of Complex II in the context of mitochondrial dysfunction may thus represent an adaptive response to insulin resistance [228] and preferential oxidation of lipids and BCAA over carbohydrates.

Glycerol-3-phosphate can be formed from glycerol derived from lipolysis [229] and according to Mracek et al [230], it requires that respiratory complexes distal to Complex I be converted to glyceraldehyde-3-phosphate, a glycolytic intermediate. The upregulation of Complex III/GPDH seen in our ICU patients may reflect the increase in intracellular lipid turnover in the skeletal muscle of these patients. See Figure 23.

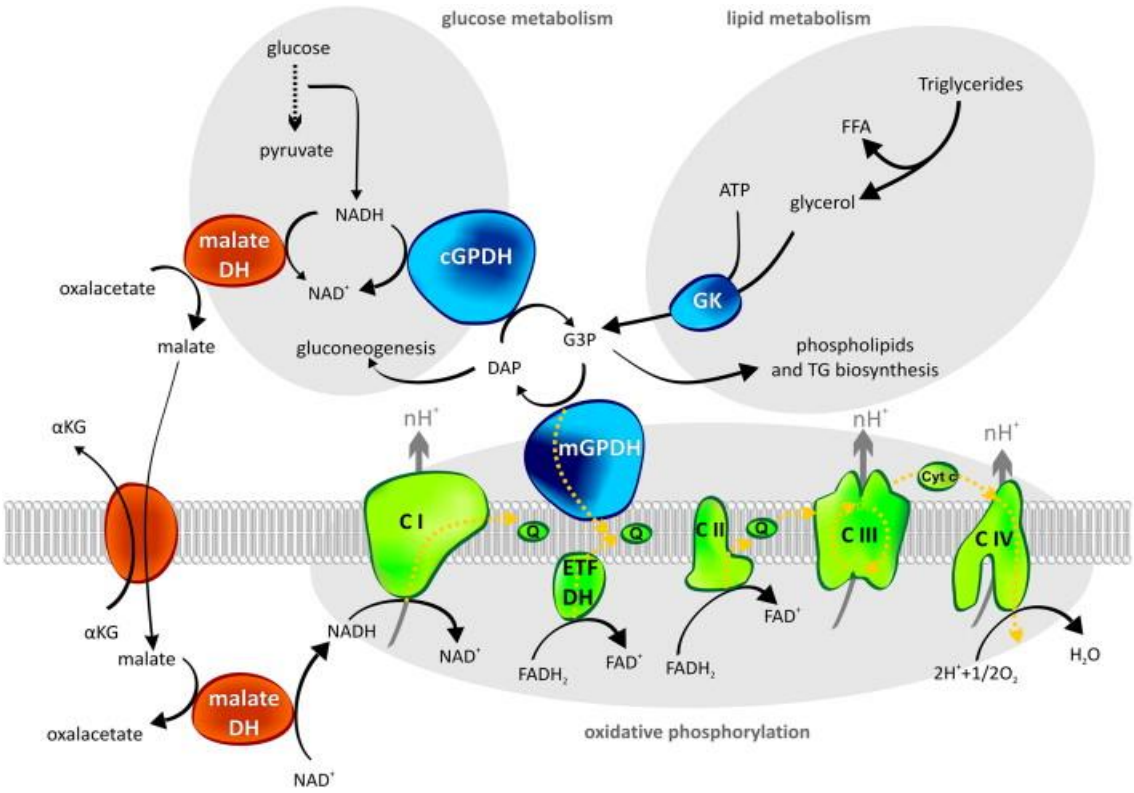


**Figure 23:** mGPDH and its placement in intermediary metabolism. Enzymes constituting GP-shuttle are depicted in blue as well as glycerol kinase (GK) which phosphorylates glycerol coming from lipolysis. Part of the malate/aspartate-shuttle as an alternative pathway of reducing equivalents transport is depicted in orange. Complexes of the mitochondrial respiratory chain are in green with yellow arrows indicating the flow of electrons through the OXPHOS. Q — Coenzyme Q, CI — complex I, CII — complex II, CIII — complex III, CIV — complex IV, ETF DH — ETF:Q dehydrogenase, FFA — free fatty acids, G3P — glycerol-3-phosphate, DAP — dihydroxyacetone phosphate [230].

However, the lack of correlation between OXPHOS and both functional capacities and relative abundance of Complexes II and III/GPDH suggests that they may perform other functions, which are not directly related to aerobic ATP production. It has recently been shown that cells accumulate succinate during hypoxia in [231-233] or inflammation [234]. When oxygenation is restored, rapid reoxidation of succinate produces electron flux, which downstream complexes are unable to absorb, and which is redirected backwards to Complex I, generating excessive amounts of reactive oxygen species [235,236]. Relative redundancy of the activity of Complexes II and III over Complex I observed by us in protracted illness could be an adaptation against cell damage when intracellular succinate levels are fluctuating.

Last but not least, we studied the influence of substrate manipulation on the skeletal muscle bioenergetics and function. In previous study we demonstrated for the first time depletion and functional changes of mitochondria in homogenates of skeletal muscle obtained from critically ill [173]. Because we found a 3-fold increase in functional capacity of complex II

in patients with ICUAW as compared to healthy controls and because complex II is fed mainly by electrons from fatty acid oxidation (FAO), in this follow up study we asked, whether the capacity to oxidise fatty acids is also increased in patients with ICUAW.

FAO is a complex process, requiring the activation of FA in the cytosol, highly regulated transport to mitochondrial matrix and finally coordinated action of four FAO enzymes with citric acid cycle and respiratory chain. Any process of artificial membrane disruption or mitochondrial isolation may disrupt these complex processes and we decided therefore to use intact cells to study it. Muscle biopsy sample contains satellite cells, which can be isolated, cultured in vitro and differentiated to myotubes. In patients with diabetes [139,140], obesity and other diseases [141,142] myotubes have been shown to retain metabolic phenotypic characteristics of the muscle they have derived from. To study mitochondrial functional indices and fatty acid oxidation, we used a well-established technique of extracellular flux analysis.

In myotubes from patients with ICUAW, there was a trend towards a reduction of mitochondrial density (expressed as CS activity per well) to 69% of values seen in healthy controls ($p=0.051$), despite the same number of cells seeded into each well. This corresponds with the finding of similar mitochondrial depletion found in skeletal muscle homogenates of patients with ICUAW [173] and suggests the perseveration of metabolic features of the donor muscle in the derived myotubes. In line with the results obtained by studying homogenates, after the adjustment to mitochondrial content, there is no difference in global mitochondrial functional indices between patients with ICUAW and healthy controls, nor are there signs of increased mitochondrial uncoupling.

However, the most interesting finding of our study is that FAO capacity in myotubes derived from the patient with ICUAW was 157% of that in myotubes from healthy controls ($p=0.015$) and that 6 days of exposure to free fatty acids was able to significantly increase the respiratory chain capacity in ICUAW myotubes, but not in myotubes derived from healthy controls. The effect is most apparent for the mixture of palmitic:oleic:linoleic acids in 2:1:2 ratio ($p=0.049$ and $p=0.038$ for 250 resp. 600 uM; Fisher LSD test), which is naturally occurring during activation of endogenous lipolysis [237], as opposed to 1:1:3 ratio seen with an infusion of standard Intralipid solution [238].

This finding may represent a functional adaptation of remaining mitochondria in mitochondria-depleted skeletal muscle to insulin resistance [239]. Transport of glucose into muscle fibre is a GLUT-4-dependent process and, in patients with ICUAW, it has been demonstrated that insulin fails to activate this process [226]. In turn, intracellular glucose starvation forces muscle to use an alternative energy substrate. Free fatty acids are elevated in plasma of critically ill [184,225] and may serve as an alternative energy source. The fact that

the increased availability of fatty acid can increase respiratory chain capacity in ICUAW, but not in control myotubes (which are able to use glucose without limitation), is in support of this explanation. It should also be noted that the oxidation of fatty acids, unlike other nutrients, feeds 50% of electrons to the respiratory chain via Complex II and bypasses Complex I. Complex I has been shown to be inhibited in acute critical illness in both rodent models [100] and in humans [97,99]. In turn, increased fatty acid oxidation may be an adaptation to long-term dysfunction of Complex I which might have been present during the acute phase of a critical illness [99].

We did not perform biopsies in patients with protracted critical illness without ICUAW, and hence we are unable to distinguish which metabolic alterations are attributable to critical illness and which to ICUAW. On the other hand, the good correlation of results obtained by extracellular flux analysis in myotubes with those obtained by high-resolution respirometry in homogenates [173] confirms the feasibility of this methodology in the critically ill.

Taken together, we have shown in an *in vitro* model of skeletal muscle of patients with ICUAW there is a trend towards a 55% reduction of mitochondrial content. After the adjustment to mitochondrial depletion, there is no difference in the mitochondrial functional indices, but rather a strong trend towards an increase of fatty acid oxidation capacity in myotubes obtained from patients with ICUAW. Long-term exposure to free fatty acids of these myotubes, in contrast to myotubes from healthy subjects, leads to an increase of the capacity of the respiratory chain. Further studies are needed to evaluate whether similar changes are achievable by nutritional manipulations in vivo and whether improved mitochondrial function would translate to improved functional outcomes in ICU survivors.

# 7 CONCLUSION

The main results of our study may be summarized into the following points:

- Our study is the first to demonstrate the mitochondrial dysfunction in skeletal muscle of patients with protracted critical illness. We have found a significant depletion of Complexes III and IV that may be the cause of a corresponding significant ~50% reduction of the ability to synthetize ATP by aerobic phosphorylation in the skeletal muscle of these patients. Contrary to our hypothesis, we did not find any signs of increased mitochondrial uncoupling.
- When taking mitochondrial depletion into account, there is no difference in global mitochondrial functional indices, but a significant increase in functional activity of respiratory chain complexes downstream to Complex I.
- In an in vitro model of skeletal muscle of patients with ICUAW, there is a similar trend towards a reduction of mitochondrial content (non-significant, p=0.051) and no difference in mitochondrial functional indices in comparison to the skeletal muscle tissue homogenates.
- We discovered a significant increment of capacity to oxidise fatty acids in ICUAW myotubes in comparison to the controls (157% of controls, p=0.015). In the context of a significantly increased functional capacity of Complexes II and III in ICUAW muscle homogenates, this finding may represent a functional adaptation of remaining mitochondria in mitochondria-depleted skeletal muscle to insulin resistance. Further studies are needed to explore these hypotheses.
- On the contrary, long-term exposure to free fatty acids of ICUAW myotubes, in contrast to myotubes from control subjects, leads to an increase of the respiratory chain capacity. We did not detect any changes caused by exposure of myotubes to hrGH or IGF-1.
- Hypoglutaminaemia in a range seen in critically ill patients (0.2-0.3mM) brings about optimal conditions for the proliferation of human myoblasts and for the ATP production in an *in vitro* model of human skeletal muscle. Glutamine concentrations above and below this range cause mitochondrial uncoupling and decrease respiratory chain spare capacity.
- We have developed and published protocol for the assessment of mitochondrial function using high-resolution respirometry of human skeletal muscle tissue homogenates. Additionally, we have modified the established protocol for the use of extracellular flux analysis in C2C12 myoblasts to study cultured human myoblasts and myotubes.

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# 9 ANNEXES

**I. Publications with IF that formed the basis of the Thesis[1]**

**JIROUTKOVÁ, K.**, KRAJČOVÁ, A., ŽIAK, J., FRIC, M., GOJDA, J., DŽUPA, V., KALOUS, M., TŮMOVÁ, J., TRNKA, J. and DUŠKA, F. Mitochondrial function in an in vitro model of skeletal muscle of patients with protracted critical illness and ICU-acquired weakness. *J Parenter Enteral Nutr.* Forthcoming 2016. ISSN 0148-607. **IF 3.151 (Supplement 1)**

**JIROUTKOVÁ, K.,** KRAJČOVÁ, A., ŽIAK, J., FRIC, M., WALDAUF, P., DŽUPA, V., NĚMCOVÁ-FüRSTOVÁ, V., KOVÁŘ, J., ELKALAF, M., TRNKA, J., DUŠKA, F. Mitochondrial function in skeletal muscle of patients with protracted critical illness and ICU-acquired weakness. *Crit Care.* 2015, 19(Dec), 448. ISSN 1364-8535. **IF 4.476 (Supplement 2)**

ZIAK, J., KRAJCOVA, A., **JIROUTKOVA, K.**, NEMCOVA, V., DZUPA, V., DUSKA, F. Assessing the function of mitochondria in cytosolic context in human skeletal muscle: adopting high resolution respirometry to homogenate of needle biopsy tissue samples. *Mitochondrion*. 2015, 21(March), 106-12. ISSN 1567-7249. **IF 3.52 (Supplement 3)**

KRAJCOVA, A., ZIAK, J., **JIROUTKOVA, K.,** PATKOVA, J., ELKALAF, M., DZUPA, V., TRNKA, J. and DUSKA, F. Normalizing glutamine concentration causes mitochondrial uncoupling in an in vitro model of human skeletal muscle. *J Parenter Enteral Nutr.* 2015, Feb; 39(2):180-9. ISSN 0148-6071. **IF 3.14 (Supplement 4)**

**II. Publication without IF related to the topic of the Thesis[2]**

**JIROUTKOVÁ, K.,** DUŠKA, F. Svalová slabost kriticky nemocných. *Anesteziologie a intenzivní medicína*. 2011, 22(3),163-8. ISSN 1214-2158. **(Supplement 5)**

**III. Abstracts on the topics of the doctoral Thesis**

**JIROUTKOVA, K.** Profylaxe svalové slabosti v akutní fázi kritického stavu. X. Kongres CSIM. Brno, CZ. 25.05.2016-27.05.2016. Zorg. Česká Společnost Intenzivní Medicíny.

**JIROUTKOVA, K.** Svalová slabost kriticky nemocných a role mitochondriální dysfunkce. VIII. Kongres CSIM. Ostrava, CZ. 12.-14.06.2014. Zorg. Česká Společnost Intenzivní Medicíny

**JIROUTKOVA, K**., ZIAK, J., KRAJCOVA, A., FRIC, M., DZUPA, V. and DUSKA F. The role of mitochondrial dysfunction in the patophysiology of ICU-acquired weakness. Jiroutkova et al. *27th ESICM Annual Congress.* Barcelona, SP. 28.09.2014-01.10.2014. Zorg. European Society of Intensive Care Medicine. *Intensive Care Medicine Experimental.* 2014, 2(Suppl 1), P29, no.abstr.0520. ISSN: 2197-425X.

**JIROUTKOVA, K.**, ZIAK, J., KRAJCOVA, A., DZUPA, V., FRIC, M. and DUSKA, F.: Role of mitochondrial dysfunction in the pathophysiology of ICU-acquired weakness. XXX. MEZINÁRODNÍ KONGRES SPOLEČNOSTI KLINICKÉ VÝŽIVY A INTENZIVNÍ METABOLICKÉ PÉČE, Hradec Králové, CZ. 07.03.2014. *Nutrition.* 2014, 30(10), 1222-24.

[1] Full text of the publications inserted, [2] title page of the publication inserted only.

KRAJCOVA, A., ZIAK, J., **JIROUTKOVA, K.**, DZUPA, V., FRIC, M., DUSKA, F. Normalizing glutamine concentration uncouples respiratory chain from ATP synthesis in an in vitro model of human skeletal muscle. A potential molecular mechanism for the harm caused by aggresive glutamine supplementation? *26th ESICM Annual Congress.* Paris, FR. 05.10.203-09.10.2012. Zorg. European Society of Intensive Care Medicine. *Intensive care medicine.* 2013, 39(suppl. 2), 241, no.abstr.0109. ISSN 0342-4642.

ZIAK, J.,KRAJCOVA, A., **JIROUTKOVA, K.,** PATKOVA, J., ELKALAF, M., DZUPA, V., FRIC, V., TRNKA, J. and DUSKA, F.: Normalizing glutamine concentration causes mitochondrial uncoupling in an in vitro model of human skeletal muscle. MITOCHONDRIAL DISEASE: TRANSLATING BIOLOGY INTO NEW TREATMENTS, CAMBRIDGE, UK. 02.10.2013-04.10.2013.

## IV. Publications not related to the topic of the Thesis

**a) with IF**

SERCLOVA, Z., DYTRYCH, P., MARVAN, J., **NOVA, K**., HANKEOVA, Z., RYSKA, O., SLEGROVA, Z., BURESOVA, L., TRAVNIKOVA, L., ANTOS, F. Fast-track in open intestinal surgery: Prospective randomized study (Clinical Trials Gov Identifier no. NCT00123456). *Clinical nutrition.* 2009, 28(6), 618-624. ISSN 0261-5614. **IF 3.274**

**b) without IF**

SERCLOVA, Z., DYTRYCH, P., MARVAN, J., **NOVA, K.,** HANKEOVA, Z., RYSKA, O., SLEGROVA, Z., BURESOVA, L., TRAVNIKOVA, L., ANTOS, F. Tolerance akcelerované pooperační rehabilitace po střevních resekčních výkonech. *Rozhledy v chirurgii.* 2009, 88(4), 178-184**.** ISSN 0035-9395.

**JIROUTKOVA, K.,** SVEHLA, J. Anestézie a pooperační analgezie u malých chirurgických výkonů. *Postgraduální medicína.* 2007, 9(7), 737-739. ISSN 1212-4184.

## V. Abstracts not related to the topic of the Thesis

SERCLOVA, Z., ANTOS, F., **NOVA, K.,** MARVAN, J., DYTRYCH, P. Akcelerovaná rehabilitace po střevních operacích. *9.gastroenterologické dny.* Karlovy Vary, CZ, 16.11.2006-18.11.2006. *Česká a slovenská gastroenterologie a hepatologie.* 2007, 61(suppl. 1), 23-4. ISSN 1213-323X.

SERCLOVA, Z., **NOVA, K**., MARVAN, J., DYTRYCH, P., ANTOS, F. Accelerared postoperativa rehabilitation including early oral feeding. *28th ESPEN Congress.* Istanbul, TR. 19.10.2006-22.10.2006. Zorg. European Society for Clinical Nutrition and Metabolism. In: *28th ESPEN Congress. Abstract Book.* B.m.: Logos, 2006, nestr.

**NOVA, K.,** SERCLOVA, Z., MARVAN, J., DYTRYCH, P., ANTOS, F. Anestezie a analgetická péče u střevních operací – Fast Track. *13. kongres ČSARIM a 12. Minářovy dny.* Plzeň, CZ. 13.06.2006-15.09.2006. Zorg. Česká spol. anesteziologie, resuscitace a intenzivní medicíny.

SERCLOVA, Z., **NOVA, K.,** MARVAN, J., DYTRYCH, P., ANTOS, F. First experience with PCA and fast track in patients after intestinal surgeries. *2nd International Forum on Pain.* Guadalajara, MX. 25.06.2006-28.05.2006.

SERCLOVA, Z., MARVAN, J., DYTRYCH, P., ANTOS, F., **NOVA, K**. PCA and fast track in patiens after intestinal surgeries. *11th Central European Congress of Proctology.* Graz, AT, 11.05.2006-13.05.2006. *Proctologia.* 2006, 7(suppl. 1), 25. ISSN 1640-5382.

# Supplement 1

**JIROUTKOVÁ, K.,** KRAJČOVÁ, A., ŽIAK, J., FRIC, M., GOJDA, J., DŽUPA, V., KALOUS, M., TŮMOVÁ, J., TRNKA, J. and DUŠKA, F.

**Mitochondrial function in an in vitro model of skeletal muscle of patients with protracted critical illness and ICU-acquired weakness.**

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# Mitochondrial function in an in vitro model of skeletal muscle of patients with protracted critical illness and ICU-acquired weakness



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| Keywords: | ICU-acquired weakness, human skeletal muscle, mitochondrial function, fatty acid oxidation, extracellular flux analysis, protracted critical illness |
| Abstract: | Background: Functional mitochondria in skeletal muscle of patients with protracted critical illness and intensive care unit-acquired weakness are depleted, but remaining mitochondria have increased functional capacities of respiratory complexes II and III (1). This can be an adaptation to relative abundancy of fatty acid over glucose caused by insulin resistance. We hypothesized that the capacity of muscle mitochondria to oxidize fatty acid is increased in protracted critical illness.<br>Materials and methods: We assessed fatty acid oxidation (FAO) and mitochondrial functional indices in vitro by using extracellular flux analysis (Seahorse Biosciences) in cultured myotubes obtained by isolating and |

# Mitochondrial function in an *in vitro* model of skeletal muscle of patients with protracted critical illness and ICU-acquired weakness

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**Summary:**

**Background:** Functional mitochondria in skeletal muscle of patients with protracted critical illness and intensive care unit-acquired weakness are depleted, but remaining mitochondria have increased functional capacities of respiratory complexes II and III (1). This can be an adaptation to relative abundancy of fatty acid over glucose caused by insulin resistance. We hypothesized that the capacity of muscle mitochondria to oxidize fatty acid is increased in protracted critical illness.

**Materials and methods:** We assessed fatty acid oxidation (FAO) and mitochondrial functional indices *in vitro* by using extracellular flux analysis (Seahorse Biosciences) in cultured myotubes obtained by isolating and culturing satellite cells from vastus lateralis muscle biopsy samples from patients with ICU-acquired weakness (n=6) and age-matched healthy controls (n=7). Bioenergetic measurements were performed at baseline and after 6 days of exposure to free fatty acids (FFA).

**Results:** Mitochondrial density in myotubes from ICU patients was 69% of healthy controls (p=0.051). After adjustment to mitochondrial content, there were no differences in ATP synthesis, or the capacity and coupling of the respiratory chain. FAO capacity in ICU patients was 157% of FAO capacity in controls (p=0.015). In myotubes of ICU patients, unlike healthy controls, the exposure to FFA significantly (p=0.009) increased maximum respiratory chain capacity.

**Conclusion:** In an in vitro model of skeletal muscle of patients with protracted critical illness we have shown signs of adaptation to increased fatty acid oxidation. Even in the presence of glucose and insulin, elevation of FFA in the extracellular environment increased maximal capacity of the respiratory chain.

**Keywords:** human skeletal muscle, mitochondrial function, fatty acid oxidation, extracellular flux analysis, protracted critical illness, ICU-acquired weakness

**Clinical Relevancy Statement**

Muscle weakness is the main contributor to failed functional outcome in ICU survivors (2), (3), (4) and evidence is accumulating that bioenergetic failure plays a key role in the development in ICUAW. We have previously demonstrated in ICU patients a ~50% reduction of the ability of skeletal muscle to syntethise ATP in mitochondria and relative increase in functional capacities of complexes II and III, which predominantly

use electrons from fatty acid oxidation (FAO). In this study (in an in vitro model of skeletal muscle) we confirmed that the ability of skeletal muscle mitochondria to oxidise fatty acids is significantly increased (157%, p=0.015) in ICU patients. Furthermore, we found that the exposure to elevated fatty acid concentrations increased spare capacity of respiratory chain in myotubes of ICUAW patients, but not in myotubes obtained from healthy volunteers. This phenomenon may represent an important functional adaptation of skeletal muscle to insulin resistance and limited intracellular availability of carbohydrate-derived substrates during critical illness (2). In turn, intracellular lipolysis achieved by active exercise or nutritional strategies leading to elevation of extracellular free fatty acid may improve the ability of skeletal muscle to meet increased ATP needs during exercise and improve functional outcomes. These hypotheses need indeed to be verified by further studies.

**Introduction**

Weakness in protracted phase of critical illness is an important clinical consequence associated with significant morbidity and protracted immobility resulting in a reduction of the quality of life in critical care survivors (6) (3). Intensive care unit-acquired weakness (ICUAW) has been attributed to muscle and nerve dysfunction acquired or exacerbated during critical illness, its epidemiology has been described and many risk factors identified (7) (8), but our understanding of its pathogenesis remains incomplete. Moreover, majority of the evidence explaining various pathophysiological mechanisms of ICUAW is inferred from animal experiments (9) (10) (11). The loss of normal mitochondrial function, i.e. inability to maintain cellular energy status and exacerbation of free radical production plays an important role in the development of ICUAW (12) (13) (14).

By using high-resolution respirometry on homogenates of skeletal muscle biopsy, our group has recently demonstrated (1) mitochondrial depletion with ~50% reduction of aerobic phosphorylation capacity in patients with ICUAW compared to metabolically healthy controls. In the remaining functional mitochondria we noticed a significant increase of functional capacity of complex II and glycerol-3-phosphatedehydrogenase/complex III. Whilst oxidation of carbohydrate-derived substrates feeds electrons to respiratory chain predominantly via complex I, fatty acid oxidation (FAO) is more reliant on downstream complexes. This generates a

hypothesis that upregulation of complexes II and III in patients with ICUAW may represent a functional adaptation to insulin resistance of critical illness (5), i.e. preferential oxidation of fatty acids when carbohydrate-derivate substrate availability is limited.

In order to elucidate this hypothesis, we used cultured myotubes derived from myoblasts isolated from biopsy samples of critically ill patients. Apart from studying primary cell culture, we also looked at changes induced by exposure of these cells to environments with different composition and concentrations of free fatty acids, which resembled changes achievable in patients by nutritional manipulations. (15).

**Methods**

Note: Unless stated otherwise, all chemicals were obtained from Sigma-Aldrich. For full detailed methods description and list of media and buffers used see the Supplementary material.

*Overview of study design (see Figure 1):* Muscle biopsies of vastus lateralis were obtained from 6 patients with protracted critical illness and from 7 metabolically healthy controls undergoing hip replacement surgery. A part of the sample (100-150mg) was processed for satellite cell isolation and culture, human primary muscle cells were differentiated to myotubes for seven days and then metabolic studies were performed by extracellular flux analysis (XF-24, Seahorse Biosciences). In addition, in a subgroup of 4 ICUAW and 4 controls we exposed myotubes for six days (in tetraplicates) to four types of media containing different fatty acid concentrations and repeated metabolic studies after exposure. XF24-well plates were kept at -80°C for later analysis of protein content and citrate synthase activity. All participants gave informed consent; in patients unable to sign the form, their next of kin witnessed the consent procedure. Our experimental protocol was approved by the Kralovske Vinohrady University Hospital Ethical Review Board.

*(Figure 1)*

*Study subjects and muscle biopsy:* Biopsies were obtained from six patients with protracted critical illness in general ICU of Kralovske Vinohrady University Hospital in Prague (proportion male/female 3/3, age 65.5 ± 3.9, body mass index (BMI) 27.5 ± 2.2)

and from seven metabolically healthy controls undergoing elective hip replacement surgery at the Department of Orthopaedic Surgery in the same hospital (proportion male/female 4/3; age 73.7 ± 6.9 years, BMI 26.8 ± 5.3). Consecutive adults admitted to general ICU who were receiving mechanical ventilation for more than 2 weeks were screened for eligibility. Patients were included if they scored <48 points of MRC score (16) of muscle weakness (scale 0-60 point where 0 means most severe weakness and 60 is normal muscle power). Patients with pre-existing neurological disease, with severe coagulopathy (platelets <50 G/L or INR>1.5) precluding muscle biopsy and patients receiving steroids in higher than substitution doses were excluded. In the period from May 2012 to September 2014 we approached 24 patients. Eight consented to muscle biopsy and myoblast isolation and culture was successful in six subjects.

Characteristics of study subjects are given in Table 1. Further details about the clinical course of their critical illness preceding the biopsy are in Supplementary material (Table S1)

(Table 1)

Muscle biopsies were taken by 5mm Bergstrom needle (17) from the vastus lateralis muscle. In order to minimize patient´s discomfort, biopsies were taken under brief general anesthesia, which was required for a routine clinical procedure unrelated to this study (e.g., changing a central line). The sample was transported in 5 ml of cold Dulbeco´s Modified Eagle Medium (DMEM) with glucose (5.56mM) and pyruvate (1mM) supplemented with glutamine (final 0.5mM), penicillin-streptomycin solution (100µg/ml) and fungizone (250µg/ml) and processed immediately.

*Cultures of human myoblasts and myotubes:* 100-150mg of the muscle biopsy collected in cold DMEM was processed for satellite cell isolation (18) with a few modifications as we described before (19). In brief, skeletal muscle tissue was minced and washed by Hank´s balanced salt solution 2–3 times and cells were subsequently dissociated by incubation in 0.25% trypsin/0.68% collagenase solution in a shaking water bath at 37°C for 30 minutes. Foetal bovine serum (FBS) was added to stop the action of trypsin and cells were collected by centrifugation at 350 *g*. To remove fibroblasts, cells were subsequently pre-plated in an uncoated Petri dish for 60 minutes at 37°C with 5% $CO_2$ and then transferred to gelatin-coated flasks (T-25). Cells were grown in DMEM with low glucose (LG, 5.56 m) and pyruvate (1mM), supplemented with 15% FBS, penicillin-streptomycin solution (100µg/mL), fungizone

(0.05µg/mL), dexamethasone (0,4µg/mL), fetuin (50ng/mL), insulin(10µg/mL) and human growth factors (EGF 10ng/mL; FGF 1ng/mL) with 500µM glutamine, respectively. Desmin immunofluorescence staining was used to verify purity of the cells (20), which were ~ 90% desmin positive (see Supplementary Figure S1)

Cells were cultured until ~90% confluence, then passaged, plated 30 000 cells per well in a XF24-well plate (Seahorse bioscience, USA) and grown to ~90-95% confluence overnight. At day 1, growth medium was changed to a differentiation medium DMEM with high glucose (HG, 25 mM), pyruvate (1mM), 2% horse serum, penicillin-streptomycin solution (100µg/mL), insulin (10µg/mL), and 500µM glutamine. Medium was exchanged every 2-3 days and cells were differentiated for 7 days prior to the experiment.

*Fatty acid stock solution preparation:* Palmitic (PA), oleic (OA) and linolaeic (LA) acids were used for exposition tests. Fatty acids were prepared as described elsewhere (21), (22). Briefly, fatty acids were dissolved in 1mM NaOH at 70°C and ligated to 15% BSA. All solutions were clear after this process, filtered and stored at -20°C (OA and LA were stored under Argon atmosphere). Concentration of fatty acids was calculated using commercial kit (NEFA Randox).

*Extracellular Flux (XF) Analysis:* The principle of the method is described elsewhere (23). In brief: oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) is measured in a thin microlayer above the cells attached to the bottom of a well. The measurement is performed at baseline (phase 1) and after the addition of up to 4 substrates and inhibitors. The dynamics of OCR then allows assessing indices of mitochondrial function. ECAR corresponds with a lactate production and reflects the rate of anaerobic glycolysis. In a 24-well plate, in each subject we performed in parallel standard assessment of global mitochondrial function (stress test) and fatty acid oxidation (FAO) assay in hexa- to heptaplicates. Stress test consisted of a sequence of an ATPase inhibitor oligomycin (1µM), an inner membrane uncoupler FCCP (1µM, carbonyl cyanide-4-[trifluoromethoxy]phenylhydrazone) and complex III inhibitor antimycin A (4 µM). Oxygen consumption after exposure to antimycin A is considered nonmitochondrial. After this is subtracted, OCR after oligomycin reflects proton leak through inner mitochondrial membrane and OCR after FCCP maximum respiratory chain activity. A decrease of OCR after oligomycin is a measure of ATPase activity (see Figure 2).

*(Figure 2)*

Fatty acid oxidation assay (FAO) consisted in serial addition of palmitate (stepwise addition to final concentrations of 120 and 240 μM), an uncoupler FCCP (1μM) and FAO inhibitor etomoxir (100μM). FAO rate was calculated as the decrease in OCR after addition of etomoxir. To reflect possible differences of cell number or mitochondrial content, we normalized basal OCR to the activity of citrate synthase (CS), which was measured using commercial kit from Sigma, as per manufacturer's instructions. For protein determination, we used the Bradford reagent according to the manufacturer´s instructions.

*Manipulating extracellular environment:* After having spent 7 days in standard differentiation media, myotubes from each subject were divided into four groups and exposed (in tri- or tetraplicates) to a range of FFA concentrations in extracellular environment: medium A (with glucose as a carbohydrate source, without FFA), medium B (medium A supplemented with PA:OA:LA in 1:1:3, 250 μM total FFA concentration), medium C (medium A supplemented with PA:OA:LA 2:1:2, 250 μM in total) and medium D (medium A supplemented with PA:OA:LA 2:1:2, 600 μM in total). Media supplemented with fatty acids were prepared freshly once a week and kept under argon atmosphere. Exposure to the media A-D lasted from day 8 to day 13. On the day 13, we repeated the test of mitochondrial functions. Assay medium and concentrations of uncoupler and inhibitors were identical to those used in experiment on myotubes before exposition to fatty acids.

*Cell viability:* We measured cell viability using CellTiter96 MTS assay (Promega, WI) as described by Cory (24). In brief, viable cells reduce the tetrazolinum dye, MTS, to purple formazan. The absorbance at 490 nm is then used as a measure of cell viability. Myotubes in all media survived exposure to experimental conditions for at least 10 days.

*Statistics:* Data are presented as medians (interquartile range). Kolmogorov-Smirnoff test was used for pair-wise comparisons and two way ANOVA for analysis of different extracellular environments, as appropriate. Statistica 8.0 (StatSoft Inc., USA) was used for all calculations and $p<0.05$ is considered significant.

## Results

### *Mitochondrial density*

In myotubes from ICU patients there was a trend to a reduction of mitochondrial density expressed as CS activity per well (4.10 [3.34-4.83] vs 5.89 [5.45-8.03] nmol/ml.min; p= 0.051).

### *Global mitochondrial functional indices*

After adjustment to mitochondrial content (i.e. the activity of citrate synthase), global mitochondrial indices were not different between ICU patients and controls. Respiratory chain capacity (RCC) was 56 [38-83] vs 31 [30-36] pmol.nkat$^{-1}$.s$^{-1}$ in ICU vs. control (p=0.36). ATP synthesis rate was 77.1 [72.5-79.2] % vs 81.1 [66.9-84.1] % of baseline oxygen consumption rate (p=0.89). Most notably, there was no sign of increase mitochondrial uncoupling in ICU patients. Proton leak through inner mitochondrial membrane was 22.9 [20.8-27.5] % vs 18.8 [15.9-33.1] % of baseline oxygen consumption rate (p=0.89).

### *Anaerobic glycolysis*

The rate of anaerobic glycolysis measured as extracellular acidification rate adjusted to total protein content was not different between ICU patients and controls either at baseline (0.46 [0.24-0.57] vs 0.36 [0.21-0.41] pmol. µg$^{-1}$.s$^{-1}$; p=0.39) or after inhibition or aerobic ATP production with oligomycine (0.75 [0.46-0.83] vs 0.61 [0.42-0.75] pmol. µg$^{-1}$.s$^{-1}$; p=0.39).

### *Fatty acid oxidation*

Fatty acid oxidation adjusted to mitochondrial density was in ICUAW patients significantly higher compared to controls (18.6 [14.2-26.9] vs 11.8 [14.2-26.9] pmol. nkat$^{-1}$.s$^{-1}$; p=0.015), see Figure 3.

*(Figure 3)*

### *Exposure to free fatty acids*

As a follow-up to the previous experiments we exposed the human myotubes to four media with a different concentration and composition of FFA (details in methods) to simulate metabolic situation in protracted critical illness. Six days of exposure to increased FFA concentration resulted in a corresponding increase of respiratory

chain capacity in ICUAW myotubes, whilst there was no effect on the myotubes from healthy controls (see Figure 4). Mitochondrial density (measured as the activity of citrate synthase) remained lower in ICUAW myotubes and was not influenced by FFA exposure. Even though there is some signal of increased mitochondrial uncoupling in ICUAW myotubes and that exposure to FFA may decrease it, these trends were not significant. There were no differences in anaerobic glycolysis (measured as ECAR) between ICUAW and control myotubes, either (data not shown).

*(Figure 4)*

## 4. Discussion

Dysfunctional aerobic ATP synthesis has been proposed as a contributing factor to the development of ICUAW (13) (12) (14), and our group was the first to demonstrate mitochondrial dysfunction in the homogenates of quadriceps muscle obtained from such patients (1). Because we found a 3-fold increase in functional capacity of complex II in homogenates of skeletal muscle in patients with ICUAW as compared to metabolically healthy controls and because complex II is fed mainly by electrons from fatty acid oxidation (FAO), in this follow up study we asked, whether the capacity to oxidise fatty acids is also increased in patients with ICUAW. FAO is a complex process, requiring activation of FA in the cytosol, highly regulated transport to mitochondrial matrix and finally coordinated action of four FAO enzymes with citric acid cycle and the respiratory chain. Studying muscle homogenates (25), permeabilized muscle fibres (26), (27) or isolated mitochondria (23) requires artificial disruption of cellular membranes, which may disrupt the delicate and complex mechanism of FAO and induce bias. We therefore decided to use intact cells in our study. Muscle biopsy sample contains satellite cells, which can be isolated, cultured in vitro and differentiated to myotubes. In patients with diabetes (28), (29), obesity and other diseases (20) (30) myotubes have been shown to retain metabolic phenotypic characteristics of the muscle they had been derived from. In order to study mitochondrial functional indices and fatty acid oxidation we used a well-established technique of extracellular flux analysis, which we had previously adopted for the use in human myoblasts and myotubes (19).

In myotubes from patients with ICUAW there was a trend to a reduction of mitochondrial density (expressed as CS activity per well) to 69% of values seen in

healthy controls (p=0.051), despite the same number of cells seeded into each well. This corresponds with the finding of similar mitochondrial depletion found in skeletal muscle homogenates of patients with ICUAW (1) and suggests perseveration of metabolic features of the donor muscle in the derived myotubes. In line with the results obtained by studying homogenates, after adjustment to mitochondrial content there is no difference in global mitochondrial functional indices between patients with ICUAW and healthy controls, nor there are signs of increased mitochondrial uncoupling.

However, the most interesting finding of our study is that FAO capacity in myotubes derived from patient with ICUAW was 157% of that in myotubes from healthy controls (p=0.015) and that 6 days of exposure to free fatty acids was able to significantly increase respiratory chain capacity in ICUAW myotubes, but not in myotubes derived from healthy controls. The effect is most apparent for the mixture of palmitic:oleic:linoleic acids in 2:1:2 ratio (p=0.049 and p=0.038 for 250 resp. 600 μM; Fisher LSD test), which is naturally occurring during activation of endogenous lipolysis (31), as opposed to 1:1:3 ratio seen with an infusion of standard Intralipid solution (32). This finding may represent a functional adaptation of remaining mitochondria in mitochondria-depleted skeletal muscle to insulin resistance (33). However, in a group of 9 elective hip replacement surgery patients (Krajčová A - unpublished data), who were either obese (BMI>30 kg/m$^2$) or had type 2 diabetes, and therefore can be considered insulin resistant, FAO adjusted to CS activity was 13.1 (11.0-13.9), i.e. 110% of value seen in metabolically healthy controls in our study, but only 74% of values seen in ICUAW. This suggests that FAO activation in weak critically ill patients is even more pronounced than in patients with metabolic syndrome. Transport of glucose into muscle fibre is a GLUT-4-dependent process and in patients with ICUAW it has been demonstrated that insulin fails to activate this process (5). In turn intracellular glucose starvation forces muscle to use alternative energy substrates. Free fatty acids are elevated in plasma of critically ill (34), (15) and may represent such alternative. The fact that increased availability of fatty acid can significantly increase respiratory chain capacity in ICUAW, but not in control myotubes (which are able to use glucose without limitation), is in support of this explanation. It should also be noted that oxidation of fatty acids, unlike other nutrients, feeds 50% of electrons to respiratory chain via complex II and bypasses complex I. Complex I has been shown to be inhibited in acute critical illness in both

rodent models (12) and in humans (35), (36). In turn, increased fatty acid oxidation may be an adaptation to long-term dysfunction of complex I which might have been present during the acute phase of critical illness (35).

The main weakness of our study is that we only studied very limited number of subjects, which increases risk of type II error (i.e. not being able to detect differences, which do exist). This was due to the fact that obtaining consent for muscle biopsy in this non-therapeutic study turned out to be very difficult. We did not perform biopsies in patients with protracted critical illness without ICUAW, and hence we are unable to distinguish, which metabolic alterations are attributable to critical illness, which to ICUAW and which changes are non-specific adaptation to mitochondrial depletion and altered metabolic situation. It should be stressed that the mean LOS in our study group was 39 days and biopsies were performed in ICU patients who had been ventilator dependent for more than 2 weeks (mean 28 days), which decreases generalizability of our result to general ICU population. In light of this, our results should be interpreted with caution and the study considered as a proof-of-concept one. On the other hand, the good correlation of results obtained by extracellular flux analysis in myotubes with those obtained by high-resolution respirometry in homogenates (1), confirms the feasibility of this methodology in the critically ill; and we consider this to be an important result of this study.

In conclusion, we have shown in an *in vitro* model of skeletal muscle of protracted critically ill patients with ICUAW there is a relative increase of fatty acid oxidation capacity. Long-term exposure to free fatty acids of these myotubes, in contrast to myotubes from healthy subjects, leads to an increase in the capacity of the respiratory chain. Further studies are needed to evaluate whether similar changes are achievable by nutritional manipulations in vivo and whether improved mitochondrial function would translate to improved functional outcomes in ICU survivors.

**Abbreviations**

AA: Antimycin A; Asc: ascorbate; ATP: adenosine triphosphate; BMI: body mass index; BT: blood temperature; CABG: coronary artery bypass grafting; CAP: comunity-acquired pneumonia; CS: citrate synthase; DMEM: Dulbecco's Modified Eagle's medium; ECAR: extracellular acidification rate; FAO: fatty acid oxidation; FFA: fatty free acid; FBS: foetal bovine serum; FCCP: Carbonyl cyanide-4-

(trifluoromethoxy)phenylhydrazone; GLUT-4: glucose transporter type 4; HAP: hospital-acquired pneumonia; ICU: Intensive Care Unit, ICUAW: Intensive Care Unit-acquired Weakness; INR: International Normalized Ratio; KCN: potassium cyanide; LA: linoleic acid; LOS-ICU: length of stay in intensive care; MRC: Medical Research Council score of muscle power; MTS: (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) dye; OA: oleic acid; OCR: oxygen consumption rate; PA: palmitic acid; RCC: respiratory chain capacity; TMPD: *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine;

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**Competing interests**

The authors declare no competing interests.

**Statement of Authorship**

Kateřina Jiroutková processed muscle samples, participated in the culturing of human skeletal muscle cells, performed XF analysis and contributed to the study design. Adéla Krajčová and Jakub Žiak participated in the culturing of human skeletal muscle cells, performed XF analysis and contributed to the design of the study. Michal Fric, Jan Gojda and Valér Džupa obtained informed consents, performed the biopsies and contributed equally to the conception of the study, whilst Martin Kalous contributed to the study design. Jana Tůmová and Jan Trnka contributed to the aquisition and interpretation of the data. František Duška conceived of the study, participated in its coordination and performed statistical analysis. All authors drafted the manuscript, critically revised the manuscript, agree to be fully accountable for ensuring the integrity and accuracy of the work, and read and approved the final manuscript.

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## Figures

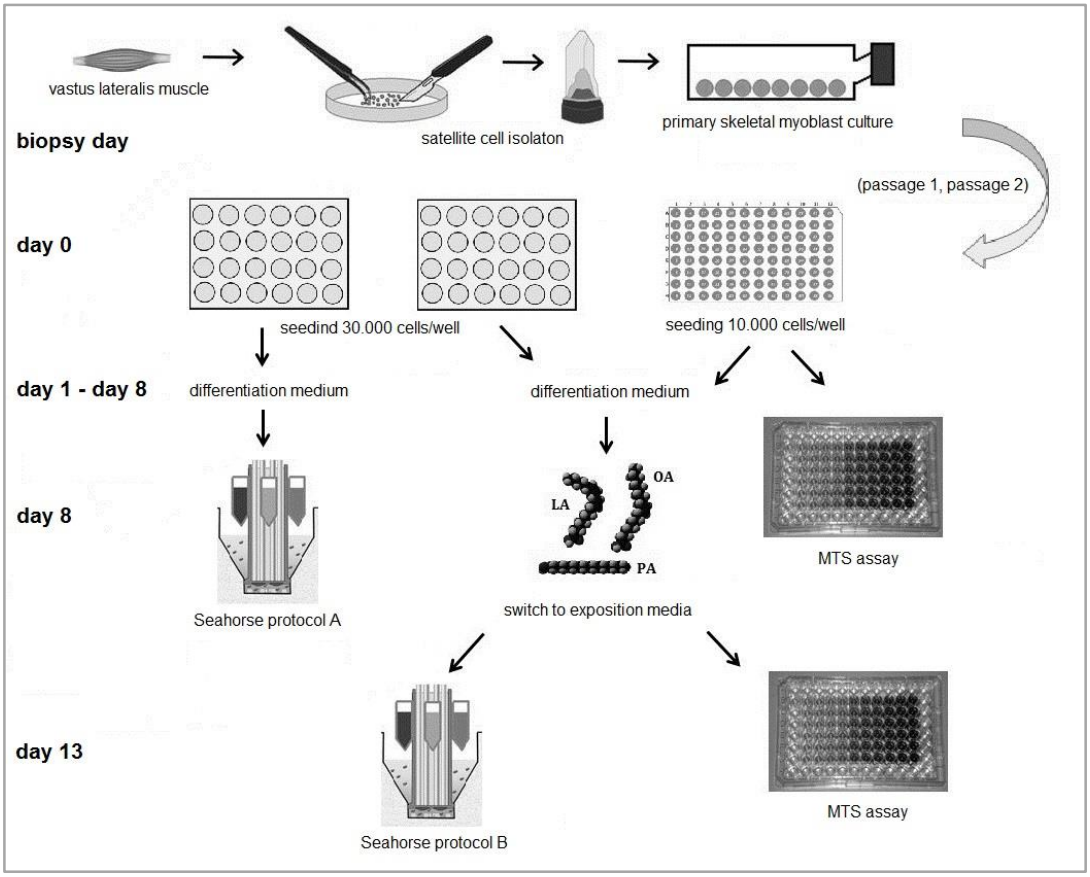


*Figure 1: Schematic representation of the study design.*

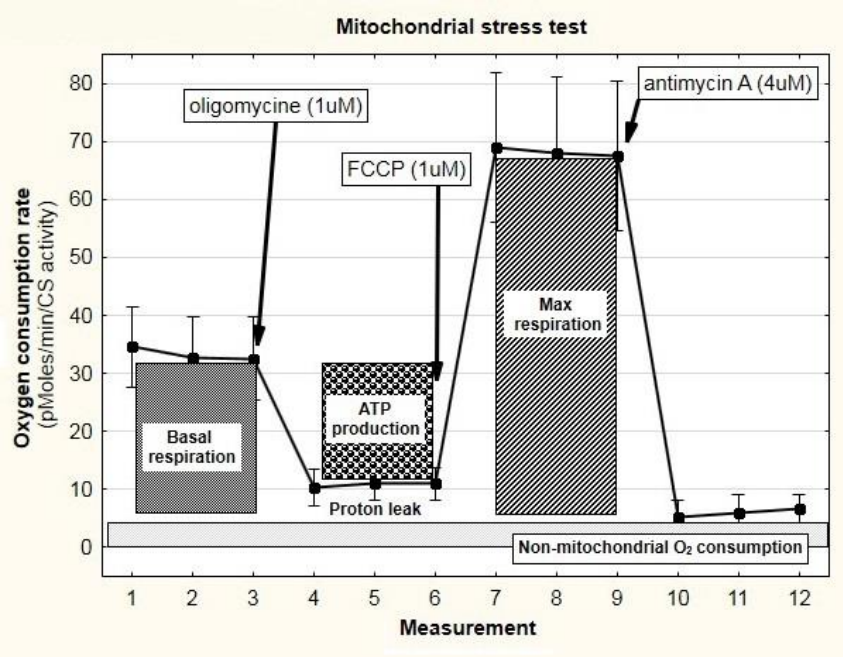


*Figure 2: Principle of extracellular flux analysis of mitochondrial metabolism. See text for details. Bioenergetic profile experiment in ICUAW myotubes, pooled data from all subjects in this study were used to construct this graph (means, 95% conf. int.) Note: FCCP = carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone; ATP = adenosine 5'-triphosphate;*

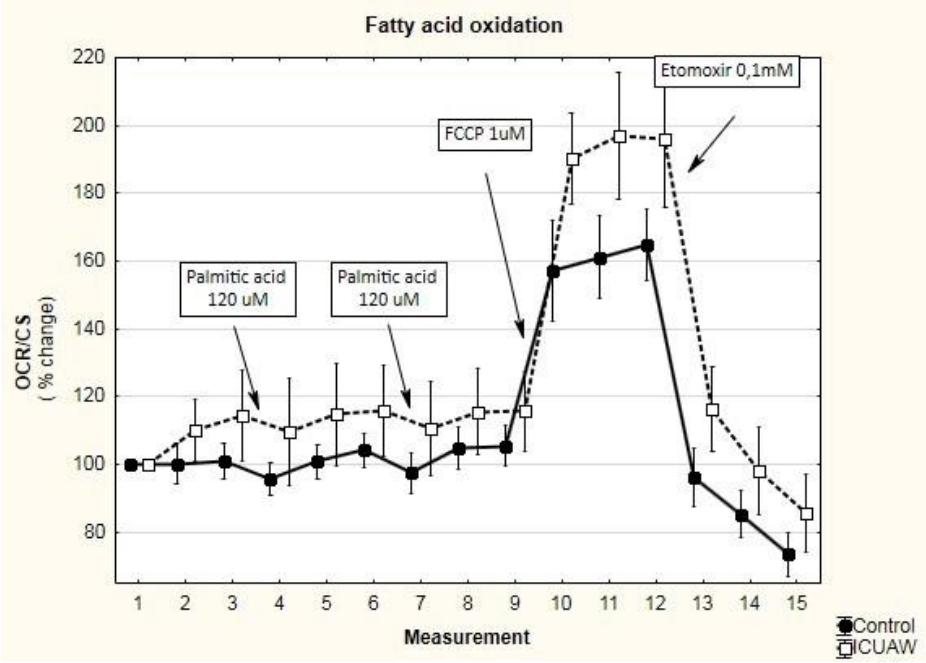


*Figure 3: Fatty acid oxidation in myotubes from ICUAW patients compared to controls. Data are presented as % change in OCR (mean and 95% confidence intervals) adjusted to mitochondrial density; measurement 1 (baseline) = 100%; Note: CS = citratesynthase; FCCP = carbonyl cyanide–4-[trifluoromethoxy] phenylhydrazone; OCR = oxygen consumption rate;*

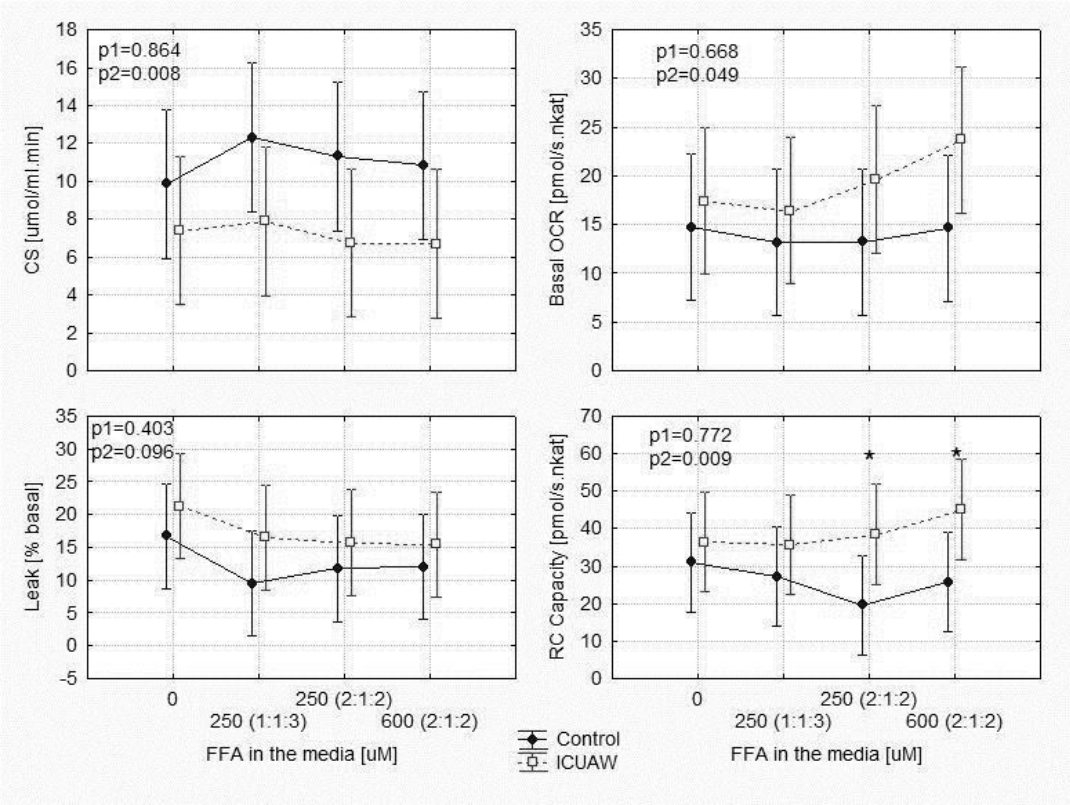


*Figure 4: Effect of myotubes exposure to different free fatty acid (FFA) concentrations. Data presented as mean and 95% confidence intervals. Note: p1=effect of environment; p2=control vs. ICUAW (2-way ANOVA), * = p<0.05 between ICUAW and Control as per Fisher LSD post hoc test; CS=citrate synthase; RC=respiratory chain; OCR=oxygen consumption rate; 1:1:3 or 2:1:2 refer to ratio of palmitic : oleic : linoleic acids.*

**Table 1**

| No. | Diagnosis | Survived | Age | APACHE II | Biopsy Day | MRC Score | LOS-ICU | Exposure Experiment |
|---|---|---|---|---|---|---|---|---|
| 1 | Septic shock, BP | N | 70 | 22 | 15 | 20 | 34 | N |
| 2 | Cardiogenic shock | Y | 65 | 27 | 41 | 4 | 45 | Y |
| 3 | CHF+CAP | Y | 68 | 10 | 27 | 8 | 30 | Y |
| 4 | Chest trauma+HAP | Y | 62 | 14 | 17 | 18 | 48 | Y |
| 5 | CABG, GI bleed | Y | 68 | 23 | 25 | 16 | 43 | N |
| 6 | CAP | Y | 60 | 15 | 30 | 23 | 35 | Y |
| Mean±SD | | - | 66±4 | 19±6 | 26±9 | 15±7 | 39±7 | - |

Table 1: Study subject characteristics. Note: APACHE II=Acute physiology and chronic evaluation II score, BP=bronchopneumonia, CABG=coronary artery bypass grafting, CAP=community-acquired pneumonia, CHF=congestive heart failure, GI=gastrointestinal, HAP=hospital-acquired pneumonia, MRC= Medical Research Council score of muscle power, LOS ICU = length of stay in intensive care. Survival means survival to discharge from hospital.

# Supplementary material

To paper: Jiroutkova K at al.: *Mitochondrial function in an in vitro model of skeletal muscle of patients with protracted critical illness and ICU-acquired weakness*

Content:

## 1. Media formulation:

**Growth medium**

| formulation (stock) | final | 20ml | 100 ml |
|---|---|---|---|
| DMEM (no gln, no phenol red, +pyr, +glc) | - | 16,1 ml | 81 ml |
| Foetal Bovine Serum (FBS) | 15% | 3,5 ml | 17,5 ml |
| Penicilin/Streptomycin solution (P/S) | 1% | 200 µl | 1 ml |
| Fungizone (250 µg/ml) | 0,05 µg/ml | 4 µl | 20 µl |
| Dexamethasone (1 mg/ml) | 0,4 µg/ml | 8 µl | 40 µl |
| Fetuin (5 mg/ml) | 50 ng/ml | 200 µl | 1 ml |
| L-Glutamine (200 mM) | 0,5 mM | 50 µl | 250 µl |
| Insulin (add separately) (1 mg/ml) | 10 µg/ml | - | - |
| hEGF, hFGF (add separately) | 10ng/ml, 1ng/ml | - | - |

**Differentiation medium**

| formulation (stock) | final | 50ml | 100ml | 150ml |
|---|---|---|---|---|
| DMEM (no gln, no phenol red, +pyr, +glc) | - | 46,4 ml | 93 ml | 139,5 ml |
| Penicilin-streptomycin solution | 1% | 0,5 ml | 1 ml | 1,5ml |
| Horse serum | 2% | 1 ml | 2 ml | 3 ml |
| Glucose (500 mM) | 25 mM | 1,97 ml | 3,9 ml | 5,85 ml |
| Insulin (add separately) (1 mg/ml) | 10 µg/ml | - | - | - |
| L-Glutamin (200 mM) | 0,5 mM | 0,125 ml | 250 µl | 375 µl |

**KHB medium**

| formulation (stock) | final | 20 ml | 100 ml | 150 ml |
|---|---|---|---|---|
| NaCl (1000 mM) | 111 mM | 2,22 ml | 11,1 ml | 16,65ml |
| KCl (1000 M) | 4,7 mM | 94 µl | 0,47 ml | 0,705ml |
| MgSO4 (100 mM) | 2 mM | 400 µl | 2 ml | 3ml |
| Na2HPO4 (100 mM) | 1,2 mM | 240 µl | 1,2 ml | 1,8ml |
| water | - | 17 ml | 85 ml | 127,5ml |
| + glc (add at the day of experiment) | 2,5 mM | - | - | - |

| | | | | |
|---|---|---|---|---|
| (500 mM) | | | | |
| +carnitine (with glucose) (50 mM) | 0,5 mM | - | - | - |

Keep sterile in a fridge. After adition of glc and car, heat to 37°C and check pH, must be7,4. Carnitine (50mM) is stable just few days at -20°C !!

**XF Seahorse medium**

| ormulation (stock) | final | 50 ml | 100ml | 150ml |
|---|---|---|---|---|
| XF assay medium | | 49,1 ml | 98,2 ml | 147,3 ml |
| Pyruvate (100 mM) | 1 mM | 0,5 ml | 1 ml | 1,5 ml |
| Glucose (0,5 mol/l) | 4 mM | 0,4 ml | 0,8 ml | 1,2 ml |

Check pH, must be 7,3 at 37°C.

**Medium A (growth medium glucose)**

| formulation (stock) | final | 20ml | 100ml |
|---|---|---|---|
| DMEM (no gln, no phenol red, +pyr +glc) | - | 16,1 ml | 81 ml |
| FBS | 15% | 3,5 ml | 17,5 ml |
| Penicilin-streptomycin solution | 1% | 200 µl | 1 ml |
| Fungizone (250 ug/ml) | 0,05 ug/ml | 4 µl | 20 µl |
| Dexamethasone (1 mg/ml) | 0,4 ug/ml | 8 µl | 40 µl |
| Fetuin (5 mg/ml) | 50 ng/ml | 200 µl | 1 ml |
| L-Glutamine (200 mM) | 0,5 mM | 50 µl | 250 µl |
| Insulin (separately) (1 mg/ml) | 10 µg/ml | - | - |

Filter and store at +4°C up to 1 month.

**Transport medium – medium for collecting human muscle biopsy**

| formulation (stock) | final | 20ml | 100ml |
|---|---|---|---|
| DMEM (no gln, no phenol red, +pyr +glc) | - | 19,7 ml | 98,7 ml |
| Penicilin-streptomycin solution | 1% | 200 µl | 1 ml |
| fungison (250 ug/ml) | 0,05µg/ml | 4 µl | 20 µl |
| glutamine (200 mM) | 0,5 mM | 50 µl | 250 µl |

Filter and store at +4°C up to 2 month.

## 2. Fatty acids preparation:

We used slightly modified Cousin procedure [Cousin, 2001], as described in [Patkova, 2014]. Protocol below is for preparation of 1 fatty acid.

*Materials:*
falcon tubes (50 ml, 15 ml), water bath (2x) with heating plate and stirrer, argon, parafilm, ice,
*Reagents:*
fatty acid (Palmitic acid is a white powder, Oleic and Linoleic are liquids. Store PA at room temperature, OA and LA after opening in -20°C)
BSA (powder); PBS; 0,1M NaOH
*Procedure:*

1. if working with OA or LA, fill all falcon tubes by Argon before procedure
2. prepare 20 ml of 15% BSA in PBS (1,5 g BSA in 10 ml)
3. slowly heat to 50 - 55°C and mix BSA until dissolving (takes about 15 minutes)
4. dissolve fatty acid in 0,1M NaOH by continuous mixing and heating to 70°C to a final concentration of 100 mM (takes about 30 minutes) (prepare 2 ml)
5. mix palmitate with 15% BSA at 50°C for 30 min - final stock solution should be about 5 mM
6. adjust pH to 7.4 after cooling to room temperature.
7. sterilize by filtration and store in aliquots in -20°C

We measured final concentration using the NEFA (non-esterified fatty acids) kit (Randox Laboratories, UK).

## 3. Sodium palmitate for Seahorse experiment preparation:

We prepared Sodium palmitate according to original protocol from Seahorse Bioscience. See http://www.seahorsebio.com/resources/tech-writing/protocol-bsa-palmitate.pdf for detail procedure.

## 4. Myotubes experiments:

### 4.1 Human myoblasts isolation – detailed protocol

We used Thompson's protocol (1996) slightly modificated after David Mott's Lab in Phoenix (2001) and by us. Unless state otherwise, all chemicals were from Sigma-Aldrich (St. Louis, MO)
**Materials and reagents:**
DMEM (low glucose, pyruvate, phenol red) by GIBCO (NY, USA), HBSS Ca2+ and Mg2+ free, 2,5% Trypsin (GIBCO), EDTA, Collagenase, Type I (239 units/mg), BSA, FBS (GIBCO), Pen-Strep, Fungizone, Glutamine 200mM (GIBCO), HEPES, Fetuin, Gelatine solution type B (2% in H2O), 60mm sterile petri dishes, T-25 flasks, scissors, forceps, 50 ml tubes, centrifuge, shaking waterbath at 37°C
**Reagent preparation:**
2% EDTA:

- 10,8 ml of 0,5 M EDTA

- 89,2 ml distilled water
- store at +4°C

1620 units/ml Collagenase
- 1 g collagenase
- 147 ml distilled water
- filter sterilize and aliquot
- store at -20°C

1% BSA
- 500 mg BSA
- 50 ml water
- filter sterilize, aliguot, store at +4°C

0.25% Trypsin/ 0,68% collagenase solution
- 10 ml of 2,5% Trypsin
- 2,5 ml of 2% EDTA
- 10 ml of 1620 units/ml collagenase
- 10 ml of 1% BSA
- 67,5 ml distilled water
- aliquot and store at -20°C

For Transport medium and Basal growth medium formulation see above.

Protocol:

Note: if you have enough tissue, it is better to prepare at least 2 flasks at once. Keep muscle always in medium. Coat T-25 flask with ~ 1 – 2 ml of 2% gelatine, put the flask into 37°C incubator for 30 min, then aspirate the rest of fluid and dry the flask in sterile box at least for another 30min.

1) Prepare in the box – 1x (2x) T-25 flask, 3 Petri dishes, forceps and scissors (sterile)
2) Prepare/thaw media (Transport medium, Trypsin/collagenase solution, HBSS, Basal growth medium with 5 mM glutamine)
3) Collect at least 150 mg of skeletal muscle tissue (we usually worked with 200 mg) into cold transport medium (~10 ml).
   **Keep on ice until ready to process (!)**
4) Transfer muscle to a sterile petri dish (with ~ 2 ml transport media) and remove fat and connective tissue with forceps (on ice)
5) Transfer prepared muscle to a next sterile petri dish. Mince the tissue using sterile curved forceps and curved scissors until muscle is in tiny pieces (~ 0,5 x 0,5 mm) at room temperature. Add more transport medium if necessary (~ 3 – 5 ml final)
6) Transfer muscle and medium into 50 ml tube using a 25 ml pipet. Rinse petri dish with about 15 ml of HBSS to collect every piece of muscle and transfer into the same tube and add HBSS to final volume ~ 25 – 35 ml.
7) Centrifuge 350 x g, 5 min, 25 °C
8) Remove the supernatant and add HBSS to pellet into final volume of 20 – 25 ml, swirl gently
9) Centrifuge 350 x g, 5 min, 25°C
10) If supernatant is still pink, do a third wash (repeat steps 8 and 9)
11) Remove supernatant, resuspend the pellet in 5 ml of trypsin/collagenase solution. Wrap the tube into parafilm. Trypsinize 30 min in 37°C shaking water bath

12) Add 0,75ml of FBS after 30 min of trypsinization. Using a pipette, aspirate gently the cell suspension up and down 3 – 5 times. There will be undigested tissue left
13) Centrifuge 350 x g, 10 min, 25°C
14) Remove the supernatant and resuspend the pellet in 2 ml of basal growth medium. Still there are some pieces of tissue
15) Transfer all the cells and tissue to a 60 mm non-collagen ciated petri dish. Incubate 1 hour at 37°C CO2 incubator. Fibroblasts will attach to the dish.
16) After 1 hour of incubation, transfer the cells to a T-25 gellatin-coated flask. Still there are little pieces of tissue. Use 0,5 ml of media for washing the residual pieces of tissue from the bottom of a petri dish.
17) Incubate at 37°C, 5% CO2 for 24 hours without disturbing,
18) After 24 hours, gently add 2,5 ml basal growth media with insulin, EGF and FGF to the flask. Change media every 3 days – centrifuge the old media (with pieces of tissue) for 5 min, 25°C, 350 x g. Resuspend the pellet in fresh growth media and transfer to the flask containing attached cells.
19) When cells are 25% confluent, just change media normal way (now you can remove all left pieces of tissue)

### 4.2 Human myotubes preparation:

Myoblasts were seeded on a gelatine coated XF-24 culture plate (30 000/well) or 96-well plate (10 000/well) at the day 0 in cultivation medium and grown to confluency overnight. At the day 1, medium was switched to differentiation medium and cells were differentiated until day 8. Insulin was added separately into each well.

### 4.3 Viability assessing in human myotubes – MTS test:

Myotubes viability was measured using the CellTiter96 MTS assay (Promega) to evaluate if there are changes in viability after cultivation with 600 uM palmitic:oleic:linoleic acids in 2:1:2.
Phenazine methosulfate (PMS) and [3-(4,5-dimethyl-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) was mixed in 1:20 ratio and stored at -20°C. Primary myoblasts (control group) from T-25 flasks were trypsinized and seeded in tetraplicates in 96-well gelatin-coated plate ($10^4$cells/well) in a basal growth medium with no glutamine. After three hours, cells were washed by PBS. MTS/PMS solution was diluted 1:5 in a basal growth medium without glutamine, insulin, EGF and FGF and added to washed cells. After 1,5 hour, MTS was reduced into a coloured formazan and absorbance was record at 490 nm using an ELISA plate reader. Higher absorbance shows greater viability [Cory et al., 1991]. There were no differences between various lengths of exposure (3 – 10 days, pilot experiment). Cell viability was also assessed before and after exposition.

### 4.4 Overview of experiments using human myoblasts:

Experiments in human myoblasts after isolation were done on p1 or p2 (Seahorse without exposition) and on p2 (Seahorse after exposion + MTS). After

cells in primary culture (p1) grown to confluency in T-25 flask (n=8), part of the cells was seeded to XF-24 plate and the second part to T-75 flask. If the primary cell culture was not growing well, cells were passaged into T-75 flasks before they had grown to confluency (n=5) and all cells were used from p2 for all experiments.
Seahorse protocols see below, MTS assay was mentioned above. 600 000 cells was used for each Seahorse experiment (20 wells x 30 000 cells) and 210 000 cells for each MTS test (7 x triplicates, 10 000 cells/well) – at all, 1 410 000 cells from each patient.

| Day | Seahorse before exposion | Day | Seahorse after exposion | Day | MTS |
|---|---|---|---|---|---|
| 0 | seeding | 0 | seeding | 0 | seeding |
| 1 | ME – different. medium | 1 | ME – different. medium | 1 | ME – different. medium |
| 2 | | 2 | | 2 | |
| 3 | ME – different. medium | 3 | ME – different. medium | 3 | ME – different. medium |
| 4 | | 4 | | 4 | |
| 5 | ME – different. medium | 5 | ME – different. medium | 5 | ME – different. medium |
| 6 | | 6 | | 6 | |
| 7 | | 7 | | 7 | |
| 8 | **Seahorse – protocol A** | 8/1 | ME – exposition media | 8<br>8/1 | **MTS** + ME – exposition media |
| | | 9/2 | | 9/2 | |
| | | 10/3 | | 10/3 | |
| | | 11/4 | ME – exposition media | 11/4 | ME – exposition media |
| | | 12/5 | | 12/5 | |
| | | 13/6 | **Seahorse – protocol B** | 13/6 | **MTS** |

*ME: medium exchange*

### 4.5 Exposition to FFA:

Medium formulation (growth medium glucose, medium A) and fatty acid preparation see above.

| Group | Medium | Fatty acids | Comment |
|---|---|---|---|
| A | medium A | - | |
| B | medium A | PA:OA:LA = 1:1:3, total 250µM | „ideal“ composition of fatty acids, physiological concentration |
| C | medium A | PA:OA:LA = 2:1:2 total 250µM | physiological composition and concentration |
| D | medium A | PA:OA:LA = 2:1:2 total 600µM | pathological concentration |

*PA: palmitic acid, OA: oleic acid, LA: linoleic acid*

Protocol:

1) Coat 24-well Seahorse plate and 96-well plate with gelatine – 30 min in incubator (37°C), aspire, 30 min drying in sterile box.
2) Seed 30 000 cells/well (Seahorse) to XF-24 well plate (4 wells are cells free, blank control), seed cells in 150 µl of media, about 30 minutes outside incubator, then about few hours inside, then gently add 100 µl media/well.
3) Seed 10 000 cells/well (MTS) to 96-well plate – 7 triplicates – seed cells in 200µl of media.
4) Incubate overnight.
5) Change the media – grown to differentiation medium (500µl + 5µl insulin/well for 24-well plate and 200µl + 2µl insulin/well for 96-well)
6) Let cells differentiate into myotubes.
7a) 24-well plate – on the day 8, change the medium for appropriate exposition media in each group (500µl media + 5µl insulin/well)
7b) 96-well plate – on the day 8, measure an MTS in the first triplicate. Change the medium for appropriate in each group for next 6 triplicates (200µl media + 2µl insulin/well)
8) On the day 13, measure Stress test using Seahorse analyzer and MTS assay

### 4.6 Seahorse protocol A (Stress test, FAO assay)

The day before assay:

- for a Stress test: prepare XF assay medium (see media formulation) and be sure you have pH=7,3 at 37°C (important) – adjust with 0,1 M NaOH
- prepare stock KHB (without adding substrates and carnitine)
- turn on the Seahorse analyzer, temperature 37°C, also hydrate the cartridge – 1 ml of XF calibrant solution in each well, keep in 37°C incubator without CO2 overnight.
- prepare a protocol for measurement

| | | |
|---|---|---|
| calibrate probes | | |
| equilibrate | | |
| Loop 3 times | mix | 1 min |
| | wait | 2 min |
| | measure | 2 min |
| **inject port A** | | |
| Loop 3 times | mix | 2 min |
| | wait | 2 min |
| | measure | 3 min |
| **inject port B** | | |
| Loop 3 times | mix | 2 min |
| | wait | 2 min |
| | measure | 3 min |
| **inject port C** | | |
| Loop 3 times | mix | 1 min |
| | wait | 2 min |
| | measure | 3 min |
| **inject port D** | | |
| Loop 3 times | mix | 1 min |
| | wait | 2 min |
| | measure | 3 min |

The day of assay:

- for a FAO assay: prepare KHB medium by adding glucose and carnitine to stock KHB, pH 7,4 at 37°C (adjust with NaOH). Carnitine preparation: prepare max. 0,5ml of 50mM solution (about 3 – 6 mg, Mr carnitine = 162g/mol). Keep stock carnitine compound bottle wrapped in parafilm.

Groups:

| Port | XF assay medium | XF assay medium | XF assay medium | KHB medium | KHB medium | KHB medium |
|---|---|---|---|---|---|---|
| A | oligomycin | oligomycin | oligomycin | PA | PA | PA |
| B | FCCP | FCCP | FCCP | PA | PA | PA |
| C | AA | AA | AA | FCCP | FCCP | FCCP |
| D | (medium) | (medium) | (medium) | etomoxir | etomoxir | etomoxir |

Prepare substrates and inhibitors. Keep stock solutions on ice (except palmitate acid(PA)):

| medium | substrates /inhibitors | port | working | stock | volume (µl) | vol. media (µl) |
|---|---|---|---|---|---|---|
| XF medium | oligomycin | A | 1µM | 5mM | 1,15 | 748,85 |
| | FCCP (XF) | B | 1µM | 5mM | 1,24 | 748,76 |
| | AA | C | 4µM | 20mM | 1,45 | 748,55 |
| | medium | D | - | - | - | 75 µl/port |
| KHB medium | PA | A | 120µM | 1mM | 690 | 60 |
| | PA | B | 120µM | 1mM | 744 | 6 |
| | FCCP (KHB) | C | 1µM | 5mM | 1,45 | 748,55 |
| | etomoxir | D | 0,1mM | 50mM | 16 | 734 |

Procedure:

1) Prepare at least 30 ml XF media, 20 ml KHB media, heat to 37°C.
2) Heat the stock sodium palmitate to 37°C.
3) Prepare eppendorf tubes for substrates/inhibitors, unfreeze the rest stock solutions and keep them on ice.
4) Check myotubes under microscope.
5) Wash the cells – columns 1,2,3 by XF medium, columns 4,5,6 by KHB medium – aspire the old medium, add 1 ml of a fresh one and aspire (leave 50µl in each well).
6) Add medium to each well to fill to 500µl (columns 1,2,3 XF, columns 4,5,6 KHB).
7) Incubate 50 – 60 min at 37°C without CO2 (good timing is important – see steps below).
8) Dilute substrates and inhibitors as mentioned above in a table. Use appropriate medium!!!
9) Pipette substrates/inhibitors to ports of loading cartridge, 75µl everywhere – load the cartridge and start calibration of the probes.
10) Before the end of assay, prepare lysis buffer with protease inhibitor: 3600µl buffer + 48µl inhibitor.
11) After the assay, aspire carefully the medium, wash cells with cold PBS and add 152µl of buffer/inhibitor solution per well. Wrap the plate in parafilm and freeze in -80°C for later analysis of protein content and CS activity.

## 4.7 Seahorse protocol B (Stress test after exposition to FFA)

The day before assay:

Prepare an XF assay medium (see media formulation) and be sure you have pH=7,3 at 37°C (important) – adjust with 0,1 M NaOH.

Turn on the Seahorse analyzer, temperature 37°C. Also hydrate the cartridge – 1 ml of XF calibrant solution in each well, keep in 37°C incubator without CO2 overnight. Prepare a protocol for measurement (adapted from C. Aguer, Toronto):

| | | |
|---|---|---|
| calibrate probes | | |
| equilibrate | | |
| Loop 3 times | mix | 4 min |
| | wait | 2 min |
| | measure | 2 min |
| **inject port A** | | |
| Loop 3 times | mix | 2 min |
| | wait | 2 min |
| | measure | 4 min |
| **inject port B** | | |
| Loop 3 times | mix | 2 min |
| | wait | 2 min |
| | measure | 2,5 min |
| **inject port C** | | |
| Loop 3 times | mix | 2 min |
| | wait | 2 min |
| | measure | 3 min |

The day of assay:

| Port | group 1 | group 2 | group 3 | group 4 |
|---|---|---|---|---|
| A | oligo | oligo | oligo | oligo |
| B | FCCP | FCCP | FCCP | FCCP |
| C | AA | AA | AA | AA |

Prepare substrates and inhibitors. Keep stock solutions on ice.

| medium | substrates /inhibitores | port | working | stock | volume (µl) | vol. media (µl) |
|---|---|---|---|---|---|---|
| XF medium | oligomycin | A | 1µM | 5mM | 3 | 1947 |
| | FCCP | B | 1µM | 5mM | 3,2 | 1947 |
| | AA | C | 4µM | 20mM | 3,8 | 1946 |

Procedure is the same as for Stress test done before an exposition to FFA (see above).

## 5. Supplementary Table S1

| Case | $paO_2/FiO_2$ | Noradrenalin dose (µg/kg/min) | WBC ($10^9$/l) | CRP (mg/L) | BT (°C) | Insulin dose (IU/kg/d) | Blood glucose (mmol/L) | Feeding (kcal/kg/d)1 |
|---|---|---|---|---|---|---|---|---|
| 1 | 229.0 | 0.03 | 32.0 | 91.6 | 36.4 | - | 6.65 | EN (20.9) |
| 2 | without arterial line, FiO2 0,3, PEEP 6, SpO2 99% | 0.08 | 14.1 | 26.0 | 36.3 | - | 4.38 | EN (19.5) |
| 3 | 201.9 | 0 | 9.5 | 12.5 | 36.2 | 0.6 | 10.53 | EN+PN (27.3) |
| 4 | 210.0 | 0 | 8.7 | 123.7 | 37.3 | - | 7.1 | EN (21.3) |
| 5 | 229.3 | 0 | 6.9 | 41.7 | 37.3 | - | 5.1 | EN (24.2) |
| 6 | 234.0 | 0 | 8.3 | 28.4 | 36.3 | - | 6.9 | EN (23.5) |

*Table S1: Patient characteristics on biopsy day; in all subjects rehabilitation therapy was performed twice daily according to local ICU practice; IU (International Unit), EN (enteral nutrition), PN (parenteral nutrition)*

## 6. Supplementary Figure S1

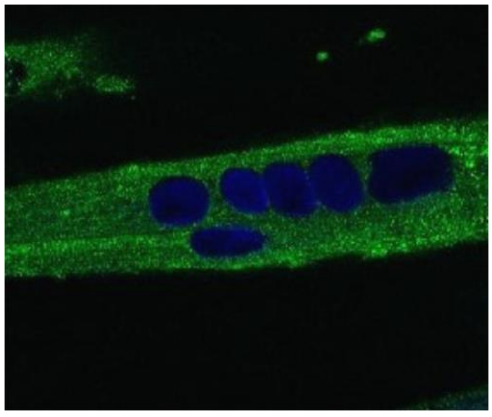

*Figure S1: myotube stained with AB 907 rabbit anti–desmin polyclonal antibody [Millipore, Billerica, MA], 10x2% BSA, Prolong Gold*

## 7. References


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# Supplement 2

**JIROUTKOVÁ, K**., KRAJČOVÁ, A., ŽIAK, J., FRIC, M., WALDAUF, P., DŽUPA, V., NĚMCOVÁFüRSTOVÁ, V., KOVÁŘ, J., ELKALAF, M., TRNKA, J., DUŠKA, F.

**Mitochondrial function in skeletal muscle of patients with protracted critical illness and ICU-acquired weakness.**

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Critical Care

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# Mitochondrial function in skeletal muscle of patients with protracted critical illness and ICU-acquired weakness

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## Abstract

**Background:** Mitochondrial damage occurs in the acute phase of critical illness, followed by activation of mitochondrial biogenesis in survivors. It has been hypothesized that bioenergetics failure of skeletal muscle may contribute to the development of ICU-acquired weakness. The aim of the present study was to determine whether mitochondrial dysfunction persists until protracted phase of critical illness.

**Methods:** In this single-centre controlled-cohort ex vivo proof-of-concept pilot study, we obtained vastus lateralis biopsies from ventilated patients with ICU-acquired weakness (n = 8) and from age and sex-matched metabolically healthy controls (n = 8). Mitochondrial functional indices were measured in cytosolic context by high-resolution respirometry in tissue homogenates, activities of respiratory complexes by spectrophotometry and individual functional capacities were correlated with concentrations of electron transport chain key subunits from respiratory complexes II, III, IV and V measured by western blot.

**Results:** The ability of aerobic ATP synthesis (OXPHOS) was reduced to ~54 % in ICU patients ($p<0.01$), in correlation with the depletion of complexes III (~38 % of control, $p = 0.02$) and IV (~26 % of controls, $p<0.01$) and without signs of mitochondrial uncoupling. When mitochondrial functional indices were adjusted to citrate synthase activity, OXPHOS and the activity of complexes I and IV were not different, whilst the activities of complexes II and III were increased in ICU patients 3-fold ($p<0.01$) respectively 2-fold ($p<0.01$).

**Conclusions:** Compared to healthy controls, in ICU patients we have demonstrated a ~50 % reduction of the ability of skeletal muscle to synthetize ATP in mitochondria. We found a depletion of complex III and IV concentrations and relative increases in functional capacities of complex II and glycerol-3-phosphate dehydrogenase/complex III.

## Background

Generalized inflammation and multi-organ failure in the acute phase of critical illness are accompanied by impairment of mitochondrial morphology [1] and function of skeletal muscle [2–5] and other organs [6, 7]. The extent of mitochondrial functional impairment correlates with disease severity, intracellular ATP depletion and outcomes [2]. It appears that the inability to meet cellular ATP demand is caused by a global depletion of functional mitochondria, as the reduction of respiratory complex content [4] or activities [3] is proportional to the reduction of citrate synthase activity (a measure of mitochondrial content) with the exception of septic non-survivors, in whom a disproportional reduction of complex I activity has been demonstrated [2]. Moreover, the ability to replenish functional mitochondria is an independent predictor of survival of critical illness [4].

Little is known about mitochondrial function in patients who do survive the acute phase of disease, but fail to wean from mechanical ventilation and enter a protracted phase of critical illness. We hypothesized that bioenergetics failure would be present in the skeletal muscle of patients with weaning failure and ICU-acquired weakness as a result of mitochondrial uncoupling and/or depletion. We performed muscle biopsies in such patients, measured concentrations and activities of

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key proteins of the respiratory chain and assessed mitochondrial function in the cytosolic context by high-resolution respirometry in fresh skeletal muscle homogenates [8].

## Methods

### Overview of study design

We performed *vastus lateralis* muscle biopsies in eight patients with protracted critical illness and in eight metabolically healthy control subjects undergoing hip replacement surgery. From the sample (150–200 mg) we prepared a homogenate, which was divided into two parts: the first part was immediately used for respirometry analysis (Protocols 1 and 2), whilst the second part was mixed in 1:1 with a lysis buffer and protease inhibitor, deeply frozen and kept at −80 °C for subsequent analysis of respiratory complex individual concentrations (by western blot) and activities (by spectrophotometry).

### Study subjects

Study subjects (age 66.6 ± 6.6 years, proportion male/female 5/3, body mass index (BMI) 27.1 ± 5.4) were recruited in a general ICU with 22 ventilated beds and a 10-bed medical ICU at Kralovske Vinohrady University Hospital in Prague. Control subjects (age 61.4 ± 15.8 years, proportion male/female 4/4, BMI 26.6 ± 3.1) were age-matched metabolically healthy patients undergoing elective hip replacement surgery for degenerative disease, in the Department of Orthopedics of the same hospital. All patients gave prospective informed consent. In patients unable to sign the form due to muscle weakness, the consent procedure was witnessed and assented by the next of kin. The University Hospital Ethical Review Board reviewed both the protocol and the consent form and approved the study. We included patients who had been ventilator-dependent for more than 2 weeks and scored <48 points in the Medical Research Council (MRC) score of muscle weakness [9] (scale 0–60 points where 0 means most severe weakness and 60 normal muscle power, an objective measure of muscle weakness). We excluded patients with pre-existing neurological disease, those with severe coagulopathy (platelets <50 G/L or international normalized ratio (INR) >1.5) precluding muscle biopsy and patients receiving steroids in higher than substitution doses. Out of 22 eligible ICU patients approached, only 8 consented for muscle biopsy.

Characteristics of study subjects are given in Table 1. Further details about the clinical course of their critical illness preceding the biopsy, including nutrition and glucose control, are in Additional file 1.

### Muscle biopsies and sample treatment

Unless stated otherwise, all chemicals were obtained from Sigma-Aldrich (St Louis, MO). For a full detailed description of the methods, a list of media and buffers and the step-by-step protocol, see Additional file 1.

Muscle biopsies were taken by 5 mm Bergstrom needle [10] from the *vastus lateralis* muscle approximately 10 cm above the knee. In order to minimize patients' discomfort, biopsies from ICU patients were taken under brief general anesthesia, which was required for a routine clinical procedure unrelated to the study (e.g., changing a central line). The sample was collected into 5 mL of relaxing solution BIOPS containing 10 mM $CaK_2$-EGTA, 7.23 mM $K_2$-EGTA, 20 mM imidazole, 20 mM taurine, 50 mM K-MES, 0.5 mM dithiothreitol, 6.56 mM $MgCl_2$, 5.77 mM ATP and 15 mM phosphocreatine adjusted to pH 7.1. The biopsies were kept on ice until further processing.

### High-resolution respirometry on skeletal muscle homogenates

High-resolution respirometry uses polarographic measurement of oxygen consumption by a Clark electrode. This method has been adapted to tissue homogenates

**Table 1** Study subject characteristics

| Subject | Diagnosis | Age | APACHE II | Biopsy day | MRC score | LOS-ICU, days | Survived |
|---|---|---|---|---|---|---|---|
| 1 | Septic shock, bronchopneumonia | 70 | 22 | 15 | 20 | 34 | N |
| 2 | Aspiration pneumonia | 80 | 15 | 29 | 23 | 71 | Y |
| 3 | Sepsis | 60 | 31 | 40 | 25 | 92 | N |
| 4 | Cardiogenic shock | 65 | 27 | 41 | 4 | 45 | Y |
| 5 | CHF + CAP | 68 | 10 | 27 | 8 | 30 | Y |
| 6 | Chest trauma + HAP | 62 | 14 | 17 | 18 | 48 | Y |
| 7 | CABG, GI bleed | 68 | 23 | 25 | 16 | 43 | Y |
| 8 | CAP | 60 | 15 | 30 | 23 | 35 | Y |
| Mean ± SD | | 67 ± 7 | 20 ± 7 | 28 ± 9 | 17 ± 8 | 50 ± 21 | - |

Survival means survival to discharge from hospital. *APACHE II* Acute physiology and chronic health evaluation II score; *MRC* Medical Research Council score of muscle power, *LOS ICU* length of stay in intensive care, *CHF* congestive heart failure, *CAP* community-acquired pneumonia, *CABG* coronary artery bypass grafting, *HAP* hospital-acquired pneumonia, *GI* gastrointestinal, *N* no, *Y* yes

[11] including those obtained from human skeletal muscle needle biopsy samples and validated against permeabilized muscle fibers [12] and isolated mitochondria [8]. In brief, connective tissue, fat and blood vessels were gently removed; the skeletal muscle fibers were dried by gauze and weighed on a calibrated scale (= wet weight, Ww). After addition of K media (1 mL/100 mg of muscle Ww), muscle fibers were homogenized by 4–6 strokes in the Elvhjem-Potter teflon/glass homogeniser. Respirometry was performed at 30 °C without preoxygenation with 0.2 mL of 10 % homogenate and 1.9 mL of K media in the respirometer Oxygraph 2 K (Oroboros Instruments, Innsbruck, Austria). K medium contains TrisHCl (10 mM), KCl (80 mM), $MgCl_2$ (3 mM), $KH_2PO_4$ (5 mM), ethylenediaminetetraacetic acid (EDTA) (1 mM), BSA (0.5 mg/ml) and water at pH 7.4. Oxygen concentrations were kept above a predetermined K90 at all times (See Figure S1 in Additional file 1). Two assays were performed in parallel in two chambers of the respirometer by serial addition of substrates and inhibitors with a Hamilton pipette.

Protocol 1 (see Fig. 1): analysis of global mitochondrial functional indices on homogenates was performed by serial addition of malate (2.5 mM) + glutamate (15 mM), ADP (1 mM), cyt c (10 μM), succinate (10 mM), oligomycin (1 μM), FCCP (0.7 μM), and antimycin A (4 μM). Non-mitochondrial respiration was oxygen consumption measured after addition of antimycin A and subtracted from other values. Capacity of oxidative phosphorylation (OXPHOS, or 3p respiration) was oxygen consumption rate when substrates for both complexes I (malate, glutamate) and II (succinate), abundant ADP and cytochrome c were present. The respiratory chain capacity (state 3u) was measured after uncoupling with FCCP. ATP synthesis rate was defined as the decrease in oxygen consumption after addition of oligomycin when substrates for complex I and II were present. The addition of cytochrome c allows for testing preservation of outer mitochondrial membrane integrity during homogenisation, with values <20 % considered acceptable [13]. In our subjects the values were 13 ± 6 % in ICU and 11 ± 8 % in control patients.

Protocol 2: functional analysis of individual respiratory complexes. We used serial additions (final concentrations in respirometry chamber) of malate (2.5 mM) and glutamate (15 mM); ADP (1 mM); cytochrome c (10 μM) rotenone (3 μM), succinate (10 mM), malonate (5 mM), glycerol-3-phosphate (5 mM), antimycin A (4 μM), ascorbic acid (10 mM) and tetramethyl-p-phenylenediamine (TMPD, 200 μM) and KCN (1 mM). Complex I activity was calculated as the decrease in oxygen consumption after its inhibitor rotenone, complex II activity as a decrease after addition of malonate. Complex III/glycerol-3-phosphate dehydrogenase (GPDH) activity was determined as an increase of oxygen consumption after addition of glycerol-3-phosphate after both complexes I and II had been inhibited by rotenone and malonate, respectively. Complex IV activity was measured as the increase of oxygen consumption after addition of complex IV substrates ascorbate/TMPD after complex III had been inhibited by antimycin A. See Fig. S2, Additional file 1.

Spectrophotometric analysis of individual activities of respiratory complexes has been described in detail elsewhere [14]. In brief, frozen sample was thawed and homogenized and then exposed to three further cycles of rapid freezing thawing. Complex I assay was performed in an assay mixture composed of 25 mM potassium phosphate, 3.5 g/l BSA, 2 mM EDTA, 60 μM dichlorophenollindophenol (DCIP), 70 μM decylubiquinone, 1 μM antimycin A and 0.2 mM reduced nicotinamide adenine dinucleotide (NADH), pH 7.8. Changes in absorbance were followed at 600 nm. Rotenone sensitive activity was calculated by subtracting the activity of wells with 10 μM rotenone. Complex II activity was measured in an assay mixture containing 80 mM potassium

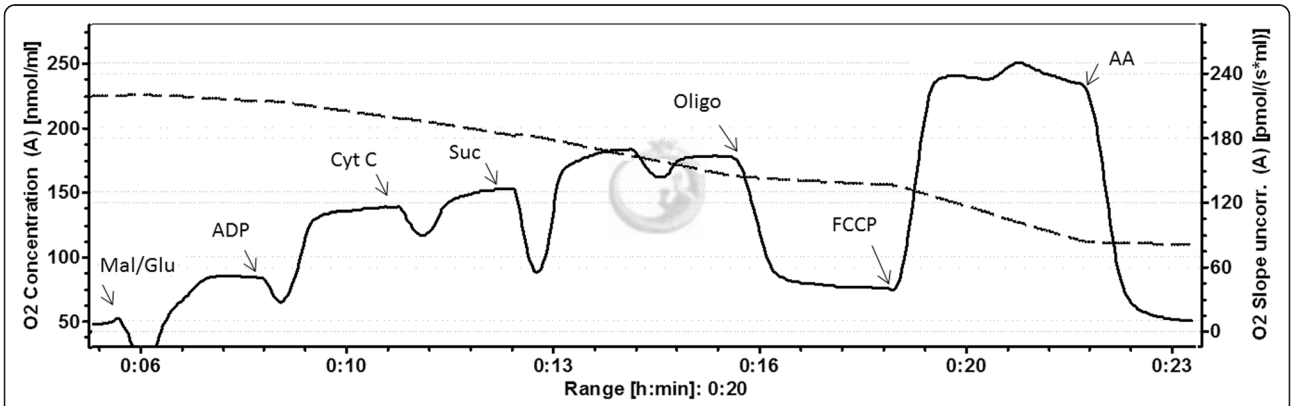


**Fig. 1** An example of high-resolution respirometry assay in a homogenate of skeletal muscle, Protocol 1. *Solid line* represents oxygen consumption rate, dashed line oxygen concentration. *Mal/Glu* malate/glutamate, *suc* succinate, *oligo* oligomycin, *FCCP* uncoupler, *AA* antimycin A

phosphate, 1 g/l BSA, 2 mM EDTA, 10 mM succinate, 80 μM DCIP, 50 μM decylubiquinone, 1 μM antimycin A and 3 μM rotenone, pH 7.8. Changes in absorbance were followed at 600 nm. Malonate sensitive activity was calculated by subtracting the activity of wells with 20 mM malonate. Complex III activity was measured in an assay mixture containing 50 μM ferricytochrome c, 25 mM potassium phosphate, 4 mM sodium azide, 0.1 mM EDTA, 0.025 % Tween20 and 50 μM decylubiquinol, pH 7.4. Changes in absorbance were followed at 550 nm. Antimycin A sensitive activity was calculated by subtracting the activity of wells with 10 μM antimycin A. Complex IV activity was measured in an assay buffer containing 30 mM potassium phosphate and 25 μM of freshly prepared ferrocytochrome c, pH 7.4. Changes in absorbance were followed at 550 nm. The absorbance of samples oxidized with 10 μl of 0.5 M potassium hexacyanoferrate (III) was subtracted from all measurements, and then the natural logarithm absorbance was plotted against time and compared to untreated control. Citrate synthase activity was measured using a commercial kit from Sigma, as per manufacturer's instructions [14].

### Western blots

Samples containing 6 μg of proteins were mixed with sample buffer and denatured by heating at 45 °C for 15 minutes. SDS-PAGE and western blotting were performed as described previously [15]. Briefly, proteins were separated on 12 % polyacrylamide gels at 120 V and then blotted onto a 0.2 μm nitrocellulose membrane (Protran BA83, Schleicher-Schuell, Dassel, Germany) for 3 h at 0.25 A. The membranes were blocked in 5 % weight/volume BSA in Tris-buffered saline for 30 minutes at room temperature. The washed membranes were probed with primary antibody cocktail Anti-human Total OxPhos Complex Kit at 4 °C overnight (dilution 1:175, # 458199, Life Technologies), containing primary antibodies against complex I (18 kDa), complex II (29 kDa), complex III (core 2; 48 kDa), complex IV (cytochrome c oxidase (COX) II subunit, 22 kDa) and $F_1F_O$ATPase (F1α; 45 kDa) subunits. After washing, the membranes were incubated for 2 h at room temperature with mouse horseradish peroxidase-conjugated secondary antibody (dilution 1:6600; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Protein bands were visualized with an enhanced chemiluminescence detection system (Thermo Fisher Scientific, Rockford, IL, USA) using Carestream Gel Logic 4000 PRO Imaging System (Carestream Health, New Haven, CT, USA). To demonstrate equal loading, the membrane was stripped and reprobed with anti-GAPDH antibody (dilution 1:1000, # ab9485, Abcam, Cambridge, UK). Densitometry was performed using the Carestream v5.2 program (Carestream Health). Data were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and referenced to an internal standard (a control patient sample was present on every immunoblot).

### Statistics

Data are presented as median (interquartile range). The Man–Whitney *U* test was used for all comparisons. Statistica 8.0 (StatSoft Inc., USA) was used for all calculations and $p < 0.05$ was considered statistically significant.

## Results

### Relative content of mitochondrial proteins

In ICU patients compared to controls, there was a significant reduction of core 2 subunit of complex III (median content in ICU patients was approximately 38 % of that in controls, $p = 0.02$) and COX2 subunit of complex IV (approximately 26 %, $p < 0.01$). No differences were detected in subunits of $F_1F_O$ATPase (approximately 109 %, $p = 0.89$) or complex II (approximately 90 %, $p = 0.99$). (see Fig. 2). We were unable to determine the content of subunits of complex I (the signals were bellow detection limits in both ICU and control patients).

### Global indices of mitochondrial function in skeletal muscle homogenates (Protocol 1)

In the skeletal muscle of patients with protracted critical illness (ICU) compared to control subjects (control), there was a reduction in citrate synthase (CS) activity per muscle wet weight (median 0.25 (IQR 0.16–0.28) vs 0.34 (IQR 0.28–0.43) nkat/mg Ww, $p = 0.03$). In keeping with this, the capacity of OXPHOS and of the respiratory chain were significantly reduced in ICU patients (approximately 54 % and 52 % of that in controls, $p < 0.01$ and $p = 0.03$) when expressed per muscle wet weight. OXPHOS normalized to muscle wet weight was correlated with the activity of CS ($r^2 = 0.53$, $p = 0.01$), the content of COX II subunit of respiratory complex IV ($r^2 = 0.39$, $p = 0.03$) and there was a trend towards a correlation to core 2 subunit of complex III ($r^2 = 0.29$, $p = 0.06$), but no relations at all were seen to concentrations of complex II ($r^2 = 0.04$, $p = 0.50$) or $F_{1\alpha}$ subunit of $F_1F_O$ATPase ($r^2 = 0.02$, $p = 0.67$).

After adjustment to CS activity, the differences in mitochondrial functional indices between ICU patients and control subjects disappeared (see Table 2). Of note, there was no difference in the degree of uncoupling of inner mitochondrial membrane between ICU patients and controls.

### Analysis of function of individual respiratory complexes

Protocol 2: by using sequential addition of substrates and specific inhibitors of the respiratory chain complexes, we were able to determine maximum electron

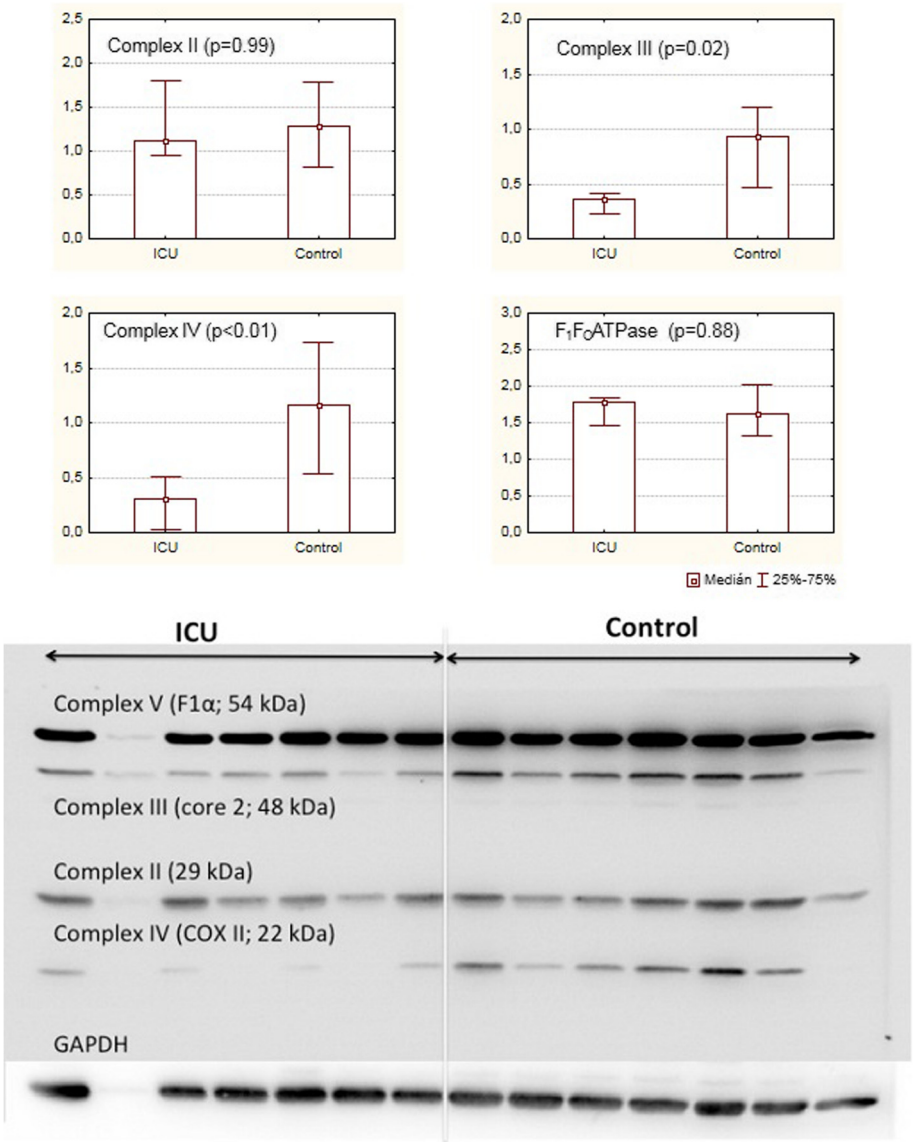


**Fig. 2** Concentrations of functional subunits of respiratory complexes in arbitrary units and an example of an immunoblot membrane. Data are presented as medians, *vertical bars* represent interquartile ranges. *GAPDH* glyceraldehyde 3-phosphate dehydrogenase, *COX* cytochrome *c* oxidase

fluxes through them. Oxygen consumption rates were adjusted to the activity of CS (see Fig. 3, upper row). Functional capacity of complexes I and IV were not different between ICU patients and the control group. Surprisingly, the capacity of complex II was demonstrated in the ICU group to be approximately 300 % of the capacity in controls ($p$ <0.01). The capacity of complex III/GPDH was also significantly ($p$ <0.01) higher in ICU patients as compared to controls. Very similar results were obtained when the individual activity of respiratory complexes was measured by spectrophotometry (Fig. 3).

Last we asked whether there is a relationship between the capacity of OXPHOS (or state 3p as determined in Protocol 1) and specific functional capacity of individual respiratory complexes (as determined in Protocol 2). Complex I ($r^2 = 0.33$, $p = 0.04$), and even more strongly complex IV ($r^2 = 0.65$, $p$ <0.01) correlated to OXPHOS, whilst complexes II and III/GPDH did not ($r^2 = 0.08$, $p = 0.36$ and $r^2 = 0.12$, $p = 0.28$, respectively). See Fig. S3 in the Supplementary material.

## Discussion

This study is the first to demonstrate mitochondrial dysfunction in skeletal muscle of patients with protracted critical illness. In the skeletal muscles of these patients we observed approximately 50 % reduction in the ability to synthetize ATP by aerobic phosphorylation per mg of muscle wet weight ($OXPHOS/W_w$) which correlated

**Table 2** Mitochondrial functional indices measured by high-resolution respirometry in homogenates

| Parameter | | Per muscle wet weight (pmol/s.mg Ww) | | | Per CS activity (pmol.nkat-1.s-1) | | |
|---|---|---|---|---|---|---|---|
| | | ICU (n = 7) | Control (n = 8) | *P* | ICU (n = 7) | Control (n = 8) | *P* |
| OXPHOS (3p) | | 7.6 (5.0–8.8)* | 13.9 (11.3–17.9) | <0.01 | 31 (28–36)* | 37 (32–74) | 0.15 |
| RC capacity (3u) | | 8.6 (6.7–10.5) | 16.4 (13.0–20.6) | 0.03 | 41 (37–44) | 42 (37–98) | 0.46 |
| Non-mito OCR | | 0.8 (0.6–1.5) | 0.8 (0.6–1.3) | 0.91 | 4 (3–5) | 2 (1–4) | 0.16 |
| $F_1F_o$ATPase | Absolute | 6.1 (4.8–7.6)* | 12.6 (9.2–13.0) | <0.01 | 26 (26–30)* | 33 (29–49) | 0.46 |
| | % OXPHOS | 81 (77–83)* | 84 (80–89) | 0.36 | 81 (77–83)* | 84 (80–89) | 0.36 |
| Proton leak | Absolute | 1.3 (1.0–1.4)* | 2.2 (1.3–3.6) | 0.10 | 8 (5–9)* | 7 (4–11) | 0.95 |
| | % OXPHOS | 19 (17–23)* | 16 (11–20) | 0.36 | 19 (17–23)* | 16 (11–20) | 0.36 |
| Complex I | | 4.8 (4.0–6.1) | 6.7 (5.5–8.6) | 0.19 | 23 (22–35) | 23 (18–26) | 0.35 |
| Complex II | | 4.6 (2.9–6,5) | 1.5 (0.8–3.8) | 0.06 | 23 (20–28) | 8 (3–14) | <0.01 |
| Complex III/ GPDH | | 1.5 (1.1–1.9) | 0.8 (0.4–1.3) | 0.12 | 7.4 (6.0–9.3) | 1.8 (1.2–3.9) | <0.01 |
| Complex IV | | 15.5 (13.0–19.5) | 19.7 (15.3–27.5) | 0.30 | 88 (69–99) | 49 (40–113) | 0.12 |

Data presented as median (interquartile range), *p* value as per Mann–Whitney *U* test. *N = 5 for ICU patients. *GPDH* glycerol-3-phosphate dehydrogenase, *Non-mito OCR* non-mitochondrial oxygen consumption rate, *OXPHOS* oxidative phosphorylation, *RC* respiratory chain

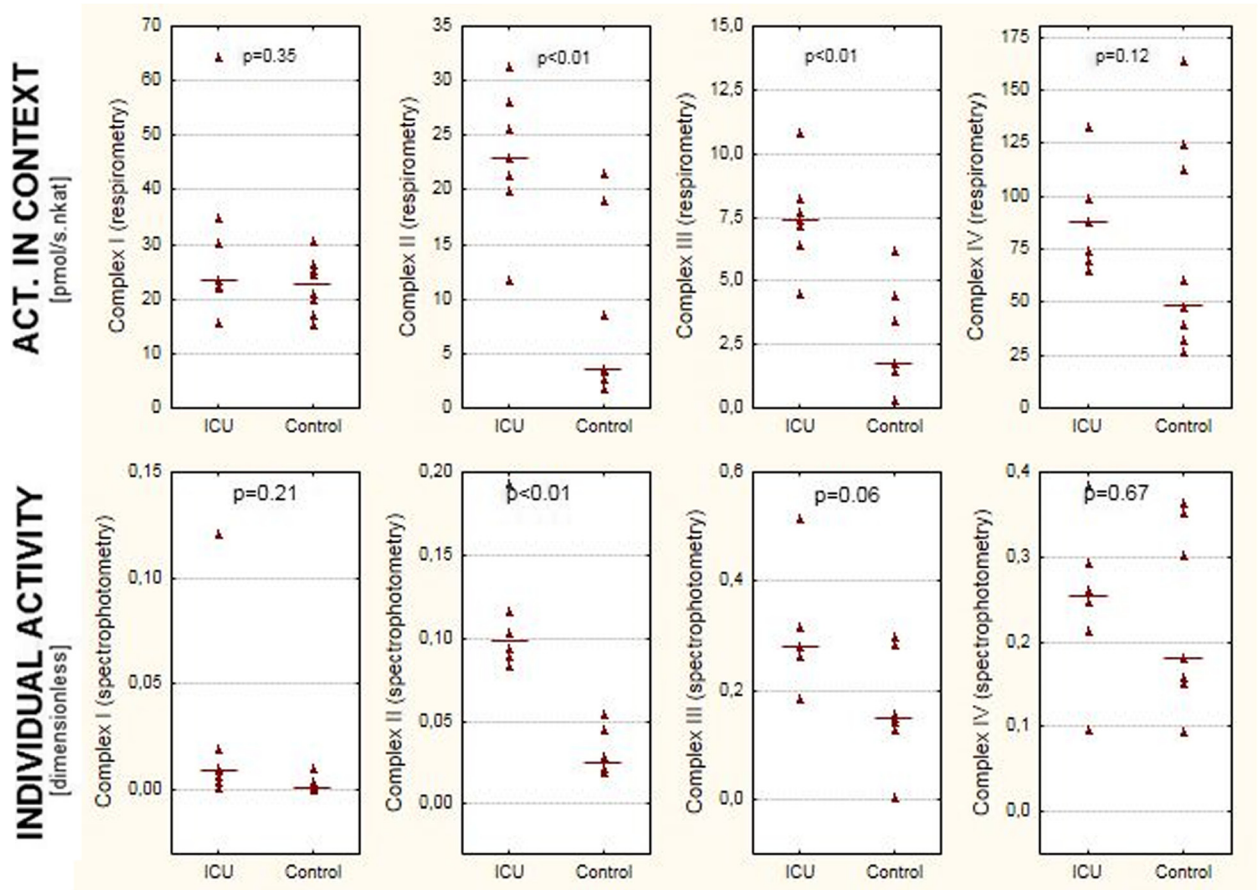


**Fig. 3** Activity of individual respiratory complexes adjusted to mitochondrial content (citrate synthase activity) measured by two independent methods. *Upper row* complex activity in cytosolic context determined by high-resolution respirometry in skeletal muscle homogenates. *Lower row* spectrophotometric analysis of the activity of individual respiratory complexes. *Lines* represent medians

with the concentration of depleted complex IV. Complex III was also depleted, unlike complexes II and V. When OXPHOS was adjusted to citrate synthase activity (OXPHOS/CS), the differences between ICU patients and control subjects disappeared and OXPHOS/$W_w$ strongly correlated with citrate synthase activity. The obvious interpretation of these results is that mitochondria are depleted in ICU patients, whilst complexes II and V are relatively abundant in remaining functional mitochondria. A similar disproportionality of the concentrations of respiratory complexes has been described in skeletal muscle during aging [16] and oxidative stress [17]. Even though citrate synthase activity is widely used as a marker of mitochondrial content [2, 18–20], it may become a subject of oxidative damage [21] and therefore it may not reliably reflect the mitochondrial density. Because we have not used an alternative method of measuring mitochondrial content (e.g., electron microscopy), we cannot say whether the depletion of complexes III and IV occurred in isolation or as part of mitochondrial depletion. It is the concentration of the depleted complex IV (and possibly complex I) that was limiting for the mitochondrial function, in keeping with data of Levy [22], who demonstrated the relation of complex IV dysfunction to bioenergetics failure in acute sepsis. Contrary to our hypothesis, there was no sign of increased mitochondrial uncoupling in ICU patients.

In order to explore the functional capacity of individual complexes, we performed a respirometry protocol in which we used specific substrates and inhibitors of individual complexes. If expressed per muscle wet weight (Table 2), we saw a trend towards increase in functional capacity of respiratory complexes II and III, whilst that of complexes I and IV tended to be non-significantly reduced to approximately 70 % of values seen in control subjects, and correlated with OXPHOS. After adjustment for citrate synthase activity, complexes II and III were increased significantly (threefold and twofold respectively, $p$ <0.01) and complexes I and IV were not different (Fig. 3). High-resolution respirometry measures the changes in oxygen consumption in fresh intact tissue homogenates after addition of respiratory substrates and inhibitors [11]. The sample contains intact mitochondria in a cytosolic context and it is believed that this approach better reflects physiological alterations occurring in vivo [23]. The technique has been calibrated against permeabilized muscle fibers [12] and isolated mitochondria [8]. When using this method for measuring the functional capacity of individual complexes one must bear in mind that the rate-limiting step can in theory appear downstream of the complex that is being analyzed. Complexes III and IV are under physiological conditions able to accommodate the flux of electrons from both complexes I and II and it is therefore unlikely that they become rate-limiting when fed by electrons from either complex I or II in isolation. For testing complex III we used glycerol-3-phosphate as a substrate whilst complexes I and II had been blocked. By doing so we avoided the risk of downstream limitation (i.e., at complex IV), but on the other hand, the rate-limiting step may be at the level of GPDH, which is functionally a part of the glycerol phosphate shuttle rather than the respiratory chain.

With these limitations of respirometry in mind, we repeated the measurements of individual complex activities by a different technique. Classical spectrophotometry is a well-established method [2, 3, 18], which assesses the activities of respiratory complexes by using artificial complex-specific substrates after the organelle structure has been destroyed by repeated freezing and thawing. This means that the measured activity of each complex is independent of the functionality of other complexes. As demonstrated in Fig. 3, both methods gave very similar results and confirmed the increased functional capacity of complexes II and III/GPDH in the critically ill as compared to control subjects.

Complex II (succinate dehydrogenase) normally drives electrons from succinate oxidation to fumarate in the citric acid cycle (CAC) via flavin adenine dinucleotide (FAD) to the respiratory chain. CAC itself is heavily dependent on reoxidation of NADH by complex I as it produces three molecules of NADH per one molecule of $FADH_2$. Eventual increase in NADH/NAD+ ratio inhibits CAC. Similarly, aerobic glycolysis produces 2NADH/molecule of glucose during the conversion to pyruvate and a further 2NADH by converting pyruvate to acetyl-CoA, which is oxidised in CAC. However, during oxidation of fatty acid and carbon skeletons of branched chain amino acids, reduced coenzymes $FADH_2$ and NADH are produced in a 1:1 ratio. Of all catabolic pathways, fatty acid oxidation is thus least dependent on the functionality of complex I. In the acute phase of critical illness complex I seems to be predominantly impaired [2] and upregulation of complex II at a later stage can be a compensatory response or an attempt to bypass dysfunctional complex I. Insulin resistance is a well-known feature of critical illness [24, 25] and it has been shown that GLUT-4 dependent transport is dysfunctional in patients with ICUAW (weakness developing in a critically ill patient without an identifiable cause other than nonspecific inflammation) [26] and pyruvate dehydrogenase is inhibited [27]. Skeletal muscle in protracted critical illness thus may suffer from starvation of carbohydrate-derived substrate for CAC. On the contrary, free fatty acids are elevated in the critically ill [24, 28] and intracellular lipid droplets accumulate early in diaphragmatic and biceps muscle in brain-dead donors [18]. Branched-chain amino acids (BCAA) derived from muscle protein degradation are deaminated in skeletal

muscle and their carbons are oxidized in a similar way to fatty acid oxidation. Relative upregulation of complex II in the context of mitochondrial dysfunction may thus represent an adaptive response to insulin resistance [29] and preferential oxidation of lipids and BCAA over carbohydrates. Glycerol-3-phosphate can be formed from glycerol derived from lipolysis [30], and it requires respiratory complexes distal to complex I to be converted to glyceraldehyde-3-phosphate [31], a glycolytic intermediate. Upregulation of complex III/GPDH seen in our ICU patients may reflect the increase in intracellular lipid turnover in the skeletal muscle of these patients.

However, the lack of correlation between OXPHOS and both functional capacities and relative abundance of complexes II and III/GPDH suggests that they may play other functions, which are not directly related to aerobic ATP production. It has been recently shown that cells accumulate succinate during hypoxia [32–34] or inflammation [35]. When oxygenation is restored, rapid re-oxidation of succinate produces electron flux, which downstream complexes are unable to absorb, and which is redirected backwards to complex I, generating excessive amounts of reactive oxygen species [36, 37]. Relative redundancy of the activity of complexes II and III over complex I observed by us in protracted illness could be an adaptation against cell damage when intracellular succinate levels are fluctuating.

Indeed our study has many limitations. First, our data are derived from a small group of highly selected subjects. We found it very difficult to consent patients for the biopsy in this non-therapeutic study. With such a small number of subjects there is always a risk of type II error, i.e., that we were unable to detect changes that were present. High inter-individual variability in the concentration and functionality of respiratory complexes (see Fig. 3) is well-known [2, 22], and further complicates the interpretation of data. Biopsies were performed in ICU patients who had been ventilator-dependent for more than 2 weeks (mean 28 days) and suffered from muscle weakness. We have selected this cohort of patients with muscle dysfunction in order to maximize the chances of observing any alteration of bioenergetics in a non-respiratory muscle, which seems to be less affected, even in the acute phase of critical illness, when compared to the diaphragm [18, 38] or intercostal muscles [3]. As a result, it remains unclear whether the changes in mitochondrial metabolism described above are consequences of prolonged immobility [39–41], the critical illness, or whether they occur only in patients who are weak. Of note, our control subjects were ambulatory elective hip surgery patients and it is unknown whether their potentially reduced mobility affected the mitochondrial function of skeletal muscle. In light of this, our pilot study should be treated as a proof-of-concept study and the results interpreted with caution.

## Conclusions

In conclusion, we have demonstrated mitochondrial dysfunction in the quadriceps muscle of patients with protracted critical illness compared to metabolically healthy age-matched control patients undergoing hip replacement surgery. There was approximately 50 % reduction in the capacity for aerobic ATP synthesis per mg of muscle wet weight, in correlation with significant reductions in functional subunits of complexes III and IV. When accounting for the activity of citrate synthase, which we used as a marker of mitochondrial content, there was no difference in global mitochondrial functional indices. We have shown a significant increase in the functional capacity of complexes II and III/GPDH. This can be possibly explained by metabolic adaptation to insulin resistance or succinate fluctuation, but exploring these hypotheses warrants further studies.

## Additional file

**Additional file 1: Supplementary material.** (DOCX 115 kb)

#### Abbreviations

ATP: adenosine triphosphate; BCAA: branched-chain amino acids; BMI: body mass index; BSA: bovine serum albumin; CABG: coronary artery bypass grafting; CAC: citric acid cycle; CAP: community-acquired pneumonia; CHF: congestive heart failure; COX: cytochrome *c* oxidase; CS: citrate synthase; Cyt c: cytochrome c; DCIP: dichlorophenollindophenol; EDTA: ethylenediaminetetraacetic acid; FAD: flavin adenine dinucleotide; FCCP: carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone; GAPDH: glyceraldehyde 3-phosphate dehydrogenase; GPDH: glycerol-3-phosphate dehydrogenase; HAP: hospital-acquired pneumonia; ICUAW: Intensive Care Unit - acquired weakness; kDa: kiloDalton; LOS ICU: length of stay in intensive care; MRC: Medical Research Council score of muscle power, $NAD^+$, nicotinamide adenine dinucleotide; OXPHOS: oxidative phosphorylation; TMPD: N,N,N',N'-tetramethyl-p-phenylenediamine; Ww: wet weight.

#### Competing interests

The authors declare no competing interests.

#### Authors' contributions

KJ processed muscle samples, participated in the respirometry analysis and contributed to the study design. AK and JZ helped to design the study, processed muscle samples, performed (together with MK) the respirometry analyses. ME and JT (together with AK) performed spectrophotometric analysis of respiratory complexes. VD, MF and JG obtained informed consents and performed the biopsies, whilst VFN and JK carried out the western blots. FD conceived of the study, participated in its coordination and performed the statistical analysis. All authors wrote their parts of the manuscript, revised the first draft and then read and approved the final version of the manuscript.

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# Supplement 3

ZIAK, J., KRAJCOVA, A., **JIROUTKOVA, K.**, NEMCOVA, V., DZUPA, V., DUSKA, F.

**Assessing the function of mitochondria in cytosolic context in human skeletal muscle: adopting high resolution respirometry to homogenate of needle biopsy tissue samples.**

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# Assessing the function of mitochondria in cytosolic context in human skeletal muscle: Adopting high-resolution respirometry to homogenate of needle biopsy tissue samples

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ABSTRACT

Using skeletal muscle homogenates for respirometry has many advantages, but the main challenge is avoiding the damage to outer mitochondrial membrane (OMM) and complex I. By optimising the amount of muscle and careful titration of substrates and inhibitors we developed a new protocol and compared it to isolated mitochondria. We found acceptable damage to OMM (~10–15% increment of oxygen flux after addition of cytochrome c) and to complex I (~70% of electron flux). Homogenate retained ~90% of phosphorylation capacity of isolated mitochondria. The use of fresh homogenate was crucial as mitochondrial function declined rapidly after 2–3 h of cold storage.



## 1. Introduction

The function of mitochondria in skeletal muscle is altered in human diseases ranging from rare inborn mitochondrial myopathies [DiMauro et al., 2013] to very common diseases such as type 2 diabetes [Schapira, 2012; Blake and Trounce, 2014] or sepsis [Brealey et al., 2002; Galley, 2011]. There is an increasing need for a reliable and simple method allowing ex vivo assessment of mitochondrial function in small (~100 mg) samples, which are typically obtained by Bergström needle biopsy [Bergström, 1975; Tarnopolsky et al., 2011]. Such small samples allow for the measurement of activities of individual complexes of respiratory chain by spectrophotometry [Pecinová et al., 2011; Votion et al., 2012; Pesta and Gnaiger, 2012] or their concentration by blotting techniques [Wittig et al., 2007; Nabben et al., 2011; Smith et al., 2014]. Protocols for functional assessment of mitochondrial function based on measurement of $O_2$ consumption are developed for isolated mitochondria [Chance and Williams, 1956], permeabilised muscle fibres [Saks et al., 1998 Jul] or for the use of human cultured myoblasts [Krajcova et al., 2015]. The yield during mitochondria isolation is low (20–40% [Tonkonogi and Sahlin, 1997]) and isolated mitochondria may not represent the whole mitochondrial population in a cell, potentially introducing bias [Palmer et al., 1985; Ferreira et al., 2010; Picard et al., 2010]. Recently, Pecinová et al., 2011; Velasco et al., 2012–2013 developed a technique of assessing mitochondrial function by the use of high-resolution respirometry on non-muscle tissue homogenates. The preparation of homogenates was straightforward and preserved functional mitochondria very well (<5% response to addition of cytochrome c). Larsen et al., 2014 first used the high resolution respirometry on homogenates of human skeletal muscle needle biopsy samples and compared them with permeabilised muscle fibres. This work showed good reproducibility and reliability of measurement of most mitochondrial functional indices, but also revealed damage to outer mitochondrial membrane and complex I, which led to a reduction to maximum respiratory capacity in skeletal muscle homogenates.

In this study we aimed to overcome these drawbacks and to improve the existing protocol for high-resolution respirometry on human skeletal muscle homogenates.

## 2. Methods

We performed a series of experiments on human skeletal muscle biopsy samples aiming to modify original protocol developed by Pecinová et al., 2011; Larsen et al., 2014. Before the final protocol was developed and its internal validity tested and compared with isolated mitochondria, we had to find optimal concentration of homogenate as

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well as of uncouplers, substrates and inhibitors. Lastly, we asked whether muscle biopsy technique (open vs. needle) could influence the results.

### 2.1. Study subjects

For all studies, we used muscle biopsy samples obtained from metabolically healthy patients undergoing hip surgery at the Department of Orthopaedic Surgery of Kralovske Vinohrady University Hospital in Prague. We excluded patients who were not ambulatory, used mobility aids, had neuromuscular abnormalities or diabetes mellitus on any treatment but diet. All patients gave written prospective informed consent. University Hospital Ethical Committee reviewed both the protocol and the consent form and approved the study. Overall, during the protocol development, we obtained a biopsy from 71 patients, but most of these have been used for preliminary or unsuccessful experiments. Data reported here are from n = 16, 8 men, 8 women, aged 60 ± 15 years (range 35–84).

### 2.2. Muscle biopsy and sample handling

Muscle biopsies were taken from the vastus lateralis muscle by open surgical technique (sample ~300 mg) or by a Bergström needle (sample ~100 mg) as soon as the muscle was exposed during the surgery. Tissue samples were collected into 5 ml of cold BIOPS solution, stored on ice and processed immediately after the transport to mitochondrial laboratory located ~7 min walk from operation theatre.

### 2.3. Preparation of homogenate and mitochondria

All steps of the preparation were performed on ice. After removing from BIOPS, muscle tissue was gently blotted by sterile gauze and eventual fat or connective tissue was removed using scissors and tissue forceps. Muscle sample was then weighted using analytical scale (1 μg readability), and cut into small pieces. One milliliter of K medium was added for each 100 mg of muscle to obtain 10% homogenate. Muscle tissue was disrupted by 4–5 strokes in a Potter-Elvehjem Teflon/glass homogenizer. Pestle was powered by a simple electric motor (1150 rpmi). Whole procedure took about 10 min. Thereafter, homogenate has been filtered through cheesecloth. Mitochondria were prepared as described before [Rasmussen et al., 1997]. In order to reduce variability, a single operator (JZ) was preparing samples for measurement in all subjects.

### 2.4. High resolution respirometry

Oxygen consumption was measured at 30 °C using an Oroboros Oxygraph-2k (Oroboros, Austria) with two 2 ml chambers allowing for parallel measurements [Pesta and Gnaiger, 2012]. Oxygen concentration and flux were simultaneously recorded and analysed by Dat lab software. Oxygen solubility factor used for calibration was 0.93 for K medium and 0.87 for MAS, respectively [Gnaiger, 2001]. Average initial $O_2$ concentration was 210–220 nmol/ml. Reagents were added into the closed chamber by using Hamilton syringes. Unless stated otherwise, we used the following final concentrations of reagents in the chamber: malate 2.5 mM and glutamate 15 mM (mal/glu, substrates for complex I), adenosine diphosphate 1 mM (ADP), cytochrome c 20 μM (cyt c), succinate 10 mM (suc, as substrate for complex II), oligomycin 1 μM (oligo, ATPase inhibitor), Carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone 0.6 μM (FCCP, an uncoupler), antimycine A 4 μM (AA, complex III inhibitor).

### 2.5. Protocol optimization measures

In order to find optimal amount of homogenate in a chamber, we dissolved 600, 400, 200, 100 or 50 μL of 10% homogenate, respectively, in K medium to final volume of 2.1 ml and tested oxygen fluxes after stepwise addition of glu/mal, followed by ADP, cyt c, suc, oligo and AA. Titration of ADP and oligomycin was performed by adding 0.25 mM increments and of FCCP in 0.1 μM increments (up to 10 μM). We also determined minimum oxygen concentration in the chamber below which oxygen flux becomes oxygen-dependent. After addition of mal/glu/succ and ADP to muscle homogenate, we simultaneously recorded oxygen concentration and flux in time and calculated Km, which is the oxygen concentration where oxygen flux is half-maximal. C90 and C95 are oxygen concentrations at which oxygen flux is reduced to 90 and 95% of maximum. Optimization experiments were performed on n = 4, with the exception of oligomycin titration, which was done on n = 2. Based on the results of these experiments we developed final respirometry protocol, which used 200 μL of 10% homogenate (containing 20 mg of wet muscle weight, $W_w$) in a final volume of 2.1 ml and a serial addition of mal/glu (2.5/15 mM), ADP (1 mM), cyt c (20 μM), suc (10 μM), oligo (1 μM), FCCP (0.6 μM), and antimycine A (4 μM). See Section S1 of Supplementary material for detailed step-by-step protocol. This sequence allowed for calculation of following mitochondrial function indices: Residual oxygen consumption (ROX) is oxygen consumption rate (OCR) after addition of AA and refers to non-mitochondrial oxygen consumption. Phoshorylating capacity (CI + IIp, state 3) is oxygen consumption rate when abundant ADP, substrates for complexes I and II, and cyt c are present. Electron transfer system capacity (ETS, state 3u) is oxygen flux in the presence of substrates for complex I + II, cyt c and an uncoupler. Complex I phosphorylation ratio (CI) is the contribution of electron flux via complex I to ETS, calculated as OCR after addition of ADP and cyt c to mal/glu divided by ETS (dimensionless, range 0–1). Damage to inner mitochondrial membrane (% cyt c) is defined as percent of increase in oxygen consumption after addition of abundant cytochrome c, when both ADP and mitochondrial substrates are present. Proton leak (L, state 4) is defined as oxygen consumption after ATPase was inhibited by oligomycin. Flux control ratio (FCR) is defined as (CI + IIp)/ETS and coupling control ratio (CCR) as L/(CI + IIp). Where appropriate, oxygen fluxes were corrected by subtracting ROX and normalized to the activity to citrate synthase (CS), which was used as a measure of mitochondrial content in a sample [Srere, 1996]. Whilst CI + IIp is used for calculation of dimensionless values (e.g. CI or FCR), the term OXPHOS is reserved for CI + IIp normalized to CS activity and expressed in pmol·ml/s·nkat. CS assay kit (Sigma) was used according to manufacturer's instructions. 150 μL of M-PER Mammalian Protein Extraction Reagent (Thermo Scientific, USA) was added to 150 μL of homogenate (or mitochondria) with 5 μL of Protease inhibitor cocktail. Lysates from all samples were frozen to −80 °C until processing.

### 2.6. Testing the influence of open vs. needle biopsy technique

When comparing these two techniques, the surgeon was asked to obtain two muscle samples from the same subject (n = 6). When vastus lateralis muscle was exposed during the surgery, one sample was taken by open technique using a scalpel or scissors and second sample from the same muscle by using 5 mm Bergström needle attached to a suction catheter. The surgeon involved in the study (VD) was trained in the use of Bergström needle. Further processing of both samples was identical and respirometry measurements were run in parallel, each chamber containing one sample of homogenate.

### 2.7. Comparison of homogenates with isolated mitochondria and durability experiments

To assess reliability and reproducibility of results, in 5 subjects we divided open biopsy sample into two parts and performed both methods in parallel and in duplicates. After the muscle tissue was blotted by gauze and cleaned from fat, the sample was cut into two parts, which were both immediately processed. Homogenate was prepared

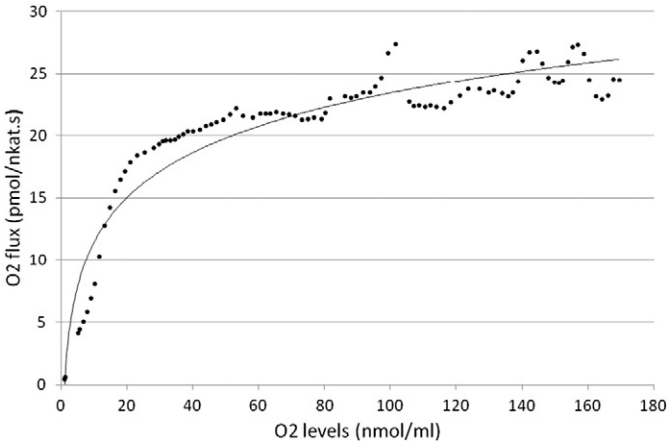


**Fig. 1.** The dependency of oxygen flux on oxygen concentration after addition of substrates for complexes I and II, ADP and cyt c.

as described above and stored on ice until mitochondria were ready. Mitochondria were prepared as described before [Rasmussen et al., 1997], which took 60–90 min. Mitochondrial yield was calculated as a percent of CS activity in isolated mitochondria compared to whole homogenate [Tonkonogi and Sahlin, 1997]. The CS activity obtained from 1 mg of wet weight of muscle was 13 ± 6 pkat in isolated mitochondria, compared to 34 ± 10 pkat in homogenate, demonstrating mitochondrial yield of 39 ± 12%. Mitochondria (MIT) and homogenates (HOM) were then measured in duplicates in two parallel chambers allowing us to calculate coefficient of variation (CV, see below). Identical protocol was used, each measurement took ~30 min. As the mitochondria are stable only for an hour on ice, they were measured first. It took up to 180 min from homogenate preparation until the end of homogenate measurement. In further 7 subjects we performed respirometry immediately after homogenate was prepared (HOM1). During durability experiments, we performed 2 measurements of the same sample: immediately after the homogenate was prepared and after 120–180 min of storage on ice. For these we used samples from 3 subjects of HOM1 and 4 additional subjects.

### 2.8. Buffers and reagents

Unless stated otherwise, all reagents were obtained from Sigma. Biopsy preservation solution (BIOPS) [Veksler et al., 1987; Letellier T, 1992; Votion et al., 2012] contains CaK2EGTA (2.77 mM), K2EGTA (7.23 mM), Na2ATP (5.77 mM), MgCl2 (6.56 mM), Taurine (20 mM), Na2Phosphocreatine (15 mM), imidazole (20 mM), DTT (0.5 mM) and MES (50 mM), pH 7.1 at 0 °C. K medium is composed of KCl (80 mM), Tris HCl (10 mM), KH2PO4 (5 mM), MgCL2 (3 mM), EDTA (1 mM), BSA (0.5 mg/ml), pH 7.4 at 30 °C. Mitochondrial assay solution (MAS) [Rogers et al., 2011] contains 220 mM mannitol, 70 mM sucrose, 10 mM KH2PO4, 5 mM MgCl2, 2 mM HEPES, 1 mM EGTA and 0.2% BSA. KCl buffer is composed of KCl (100 mM), Tris (50 mM), MgSO4 (5 mM), EDTA (1 mM), pH 7.4. ATP medium was prepared by adding of ATP (1 mM final) and BSA (0.5% final) into KCl buffer. Proteinase medium (P medium) consists of ATP medium and Trypsin (2.5 mg/ml). MS medium contains 225 mM mannitol and 75 mM sucrose.

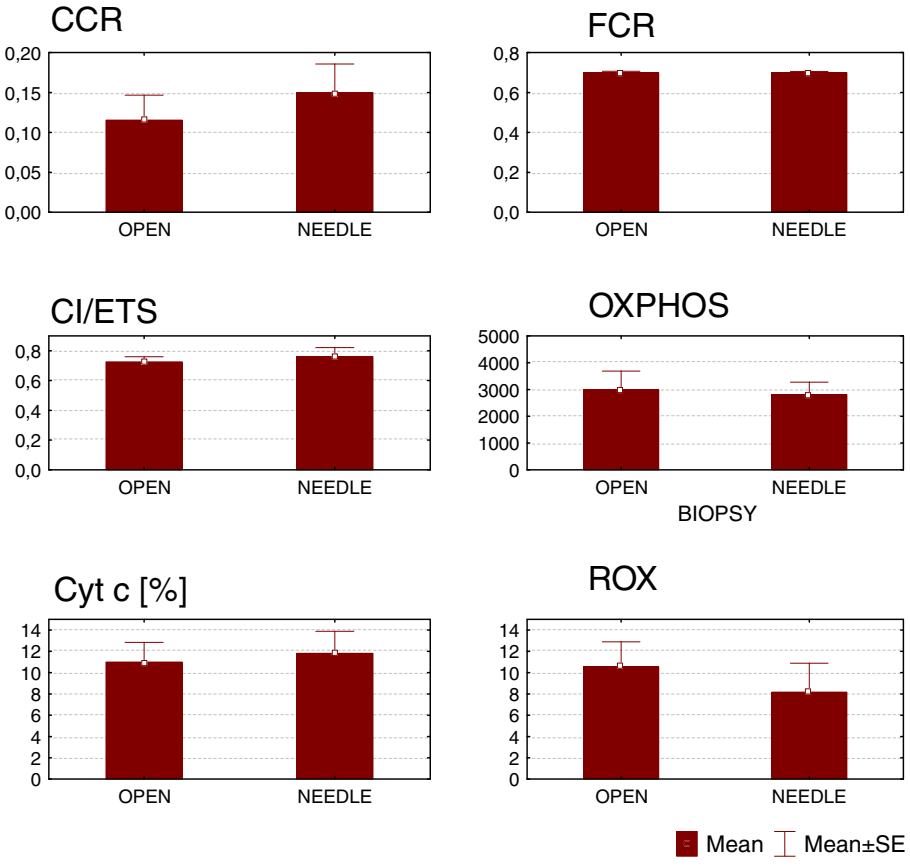


**Fig. 2.** Comparison of mitochondrial functional indices of human skeletal muscle homogenates obtained by open surgical vs. needle biopsy. Note: CCR = coupling control ratio (leak/CI + IIp), FCR = flux control ratio, CI/ETS = proportion of electron flux via complex I to electron transfer system capacity, OXPHOS [pmol·ml/nkat·s] = oxidative phosphorylation capacity normalized to citrate synthase activity, cyt c [%] = increase in oxygen flux after addition of cyt c, ROX [pmol/s] = unadjusted non-mitochondrial oxygen consumption.

### 2.9. Statistics

Before processing, the data distribution was checked for normality by inspecting histograms visually and by Kolmogorov–Smirnov test ($p > 0.2$). Values are reported as mean ± standard deviation (SD). For comparisons we used two-tailed Student t-test or one-way ANOVA with Tukey post-hoc test, where appropriate. Differences at p-value < 0.05 were considered significant. Whenever the measurements had been performed in duplicates, we calculated average values of two measurements for every sample before processing data further, with the exception of calculation of the coefficient of variation between duplicate measurements (CV). CV was calculated as CV [%] = 100 * SD/mean.

## 3. Results

### 3.1. Protocol optimisation

#### 3.1.1. Optimal concentration of homogenate

There were no significant differences in outer membrane damage (cyt c 14.2 ± 1.2; 11.2 ± 6.4; 13.2 ± 4.7 and 8.4 ± 4.6% for 100, 200, 400 and 600 μL, respectively, p = 0.70, ANOVA), complex I preservation (CI/ETS 0.72 ± 0.04; 0.82 ± 0.09; 0.82 ± 0.10 and 0.87 ± 0.09, p = 0.31 ANOVA) or other mitochondrial function indices among different amounts of homogenate. However, the use of 600 or 400 μL led to rapid oxygen exhaustion, whilst with 50 uL oxygen fluxes were too low (see Section S2 of Supplementary material, Fig S1 and S2). Therefore, we decided to use 200 μL of 10% homogenate for the rest of experiments.

#### 3.1.2. Titration of substrates and inhibitors

In preliminary experiments, addition of pyruvate (10 mM) to glu/mal mixture caused either decrease in OCR or no change (not shown) and we decided to use standard substrates in above-mentioned concentrations. FCCP yielded maximal flux without detectable inhibition between 0.6–0.7 μM with a decrease of ORC above 1 μM (See Fig. S3 and S4 in Supplementary material). Similarly, we observed no additional increase of oxygen flux above 1 mM of ADP and no additional decrease of oxygen flux above 1 μM of oligomycin. ADP titration demonstrated stability of ADP-stimulated oxygen fluxes for up to 40 min (See Fig. S4 in Supplementary material). These concentrations are used in the final protocol. In order to avoid limitations of oxygen flux by oxygen concentration, we determined $O_2$ concentration at which the oxygen flux reduced to 50% (Km), 90% and 95% of maximal rate. These were 11.8; 78 and 98 nmol/ml (Fig. 1). Thus, in all later experiments we aimed to maintain oxygen concentration above 90 nmol/ml at all times.

### 3.2. Influence of biopsy technique

We have not found any significant differences in mitochondrial functional indices (p = 0.41–0.99 t-test, Fig. 2) between needle and open biopsy techniques in human skeletal muscle homogenates. Outer mitochondrial membrane was mildly disrupted in both (increments after the addition of cyt c were 13.0 ± 6.0 vs. 12.7 ± 6.0%, p = 0.93 in needle and open biopsies respectively).

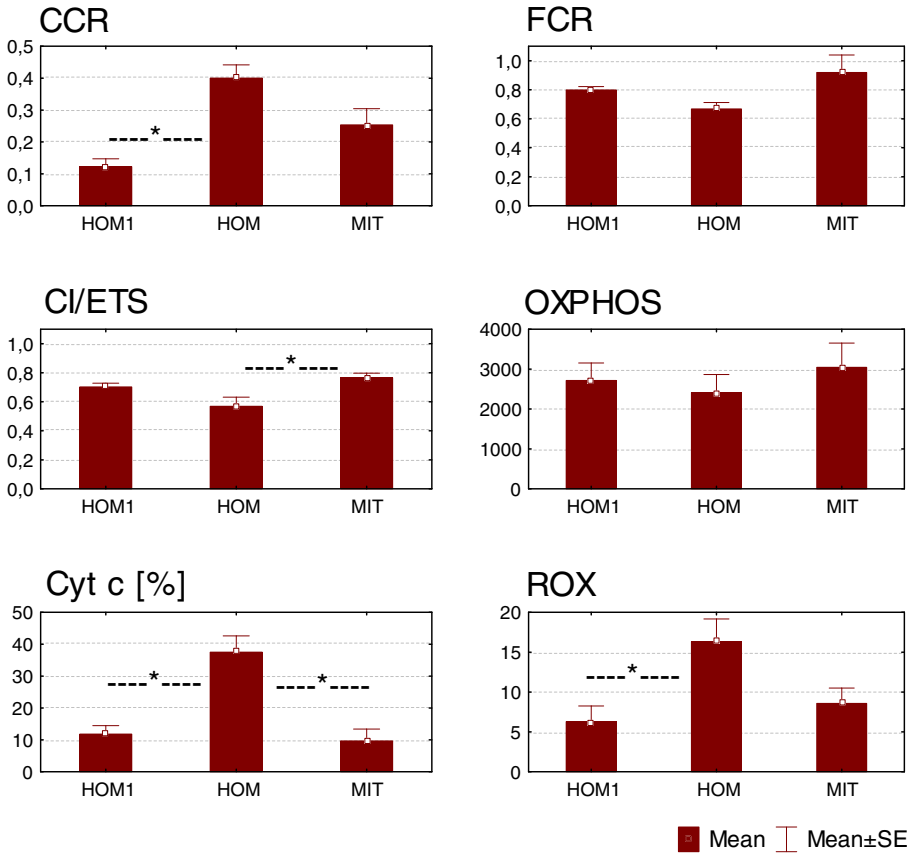


**Fig. 3.** Comparison of fresh homogenate (HOM1, n = 6) with homogenates (HOM) and isolated mitochondria (MIT) obtained from the same subjects (n = 5) and measured in parallel. Asterisk indicates a significant (p < 0.05) difference by Tukey post-hoc test. Note: CCR = coupling control ratio (leak/CI + IIs), FCR = flux control ratio, CI/ETS = proportion of electron flux via complex I to electron transfer system capacity, OXPHOS [pmol·ml/nkat·s] = oxidative phosphorylation capacity normalized to citrate synthase activity, cyt c [%] = increase in oxygen flux after addition of cyt c, ROX [pmol/s] = unadjusted non-mitochondrial oxygen consumption.

**Table 1**
Mitochondrial functional indices in fresh homogenate (HOM1) with homogenates (HOM) and isolated mitochondria (MIT) obtained from the same subjects and measured in parallel. Values with asterisk represent unadjusted oxygen fluxes in mitochondria from 20 mg of muscle wet weight; others were normalized to citrate synthase (CS) activity. Paired samples (HOM–MIT) were also tested by paired t-test and # means significant difference (at $p < 0.05$). Note: CI + IIp = respiration after addition of substrates for complexes I and II, ADP and cyt c. ETS = electron transfer system capacity measures after addition of substrates for complexes I and II, cyt c and an uncoupler.

| N | HOM1 | HOM | MIT | p-Value (ANOVA) |
|---|---|---|---|---|
| | 6 | 5 | | – |
| CI + IIp* [pmol/s] | 128 ± 44 | 65 ± 36# | 102 ± 47# | 0.061 |
| ETS* [pmol/s] | 165 ± 61 | 108 ± 60 | 125 ± 92 | 0.410 |
| ETS/CS [pmol·ml/nkat·s] | 3515 ± 1534 | 4072 ± 2013 | 3301 ± 1944 | 0.357 |
| LEAK* [pmol/s] | 21 ± 14 | 42 ± 23 | 28 ± 21 | 0.201 |
| CI/CS [pmol·ml/nkat·s] | 2045 ± 902 | 1841 ± 1119 | 2737 ± 1235 | 0.357 |

### 3.3. Protocol validation

#### 3.3.1. Comparison of high-resolution respirometry in homogenates with isolated mitochondria

When homogenates (HOM), which had to wait for mitochondria to be prepared, were directly compared with mitochondria (MIT) isolated from the same subjects (n = 5), they clearly showed signs of mitochondrial damage. These were absent when the same experiment was repeated on freshly prepared homogenate from different subjects (HOM1, n = 6). The main damage in HOM is of outer mitochondrial membrane (cyt c 38 ± 12 vs. 12 ± 6 vs. 10 ± 9% in HOM vs. HOM1 vs. MIT, $p < 0.001$ ANOVA), but there is also apparent leak through inner mitochondrial membrane (CCR 0.40 ± 0.10 vs. 0.12 ± 0.06 vs. 0.24 ± 0.11, p = 0.001, ANOVA), as well as damage to complex I (CI/ETS 0.57 ± 0.15 vs. 0.70 ± 0.06 vs. 0.76 ± 0.08, p = 0.011, ANOVA). For other mitochondrial respiratory indices see Fig. 3; and Table 1. Of note, the coefficient of variation for OXPHOS was quite low in HOM and HOM1 (5.2 ± 5.1 and. 4.6 ± 7.0%) as compared to MIT (12.4 ± 7.6%, p = 0.11, ANOVA). As the only difference between HOM and HOM1 measurement techniques was the delay between homogenate preparation and the respirometry (in case of HOM), we further explored the influence of homogenate storage on ice on respirometry results.

#### 3.3.2. Durability experiments

In 7 subjects we compared measurement on freshly prepared sample with the same sample stored on ice for 120–180 min. Individual data (shown in Fig. 4.) demonstrate a trend to outer and inner mitochondrial membrane damage, impairment of complex I with a decrease in OXPHOS capacity. There is also notable inter-individual variability in a response of homogenate to storage on ice.

## 4. Discussion

In this study we developed a protocol for the use of high-resolution respirometry on homogenates of human skeletal muscle samples obtained by needle biopsy. By comparing it with the standard technique of respirometry in isolated mitochondria we have shown that it is possible to enjoy its advantages, if certain limitations are kept in mind. When developing the protocol we adopted some aspects of methodology from protocols designed for homogenates of non-muscle samples [Pecinová et al., 2011; Kondrashova et al., 2001; Raffaella et al., 2008] or skeletal muscle samples of animals [Votion et al., 2012]. The only existing study on human skeletal muscle homogenates [Larsen et al., 2014] identified the main challenge of the technique, i.e. the damage of the very fine structure of complex I and to outer mitochondrial

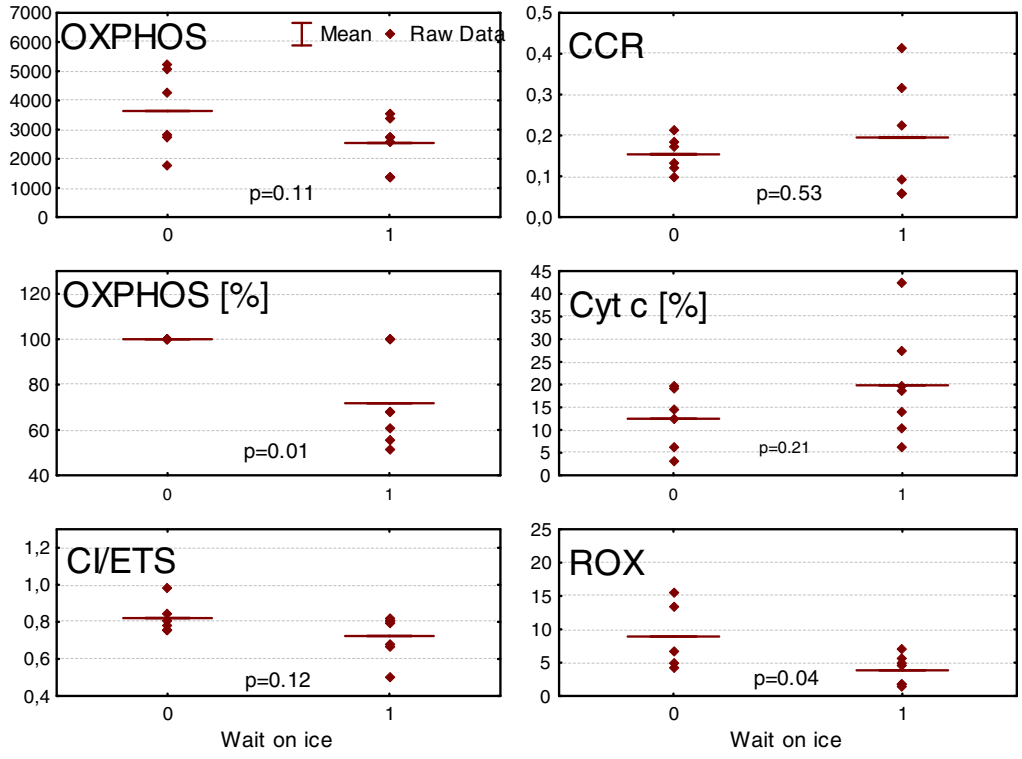


**Fig. 4.** Mitochondrial functional indices in fresh homogenates (left) and after 120–180 min of storage (right). Diamonds represent individual data, horizontal lines are means. Note: OXPHOS [pmol·ml/nkat·s] = oxidative phosphorylation capacity normalized to citrate synthase activity, CCR = coupling control ratio (leak/CI + IIs), OXPHOS [%] = relative change of OXPHOS when value of fresh homogenate is 100%, cyt c [%] = increase in oxygen flux after addition of cyt c, FCR = flux control ratio, CI/ETS = proportion of electron flux via complex I to electron transfer system capacity, ROX [pmol/s] = unadjusted non-mitochondrial oxygen consumption.

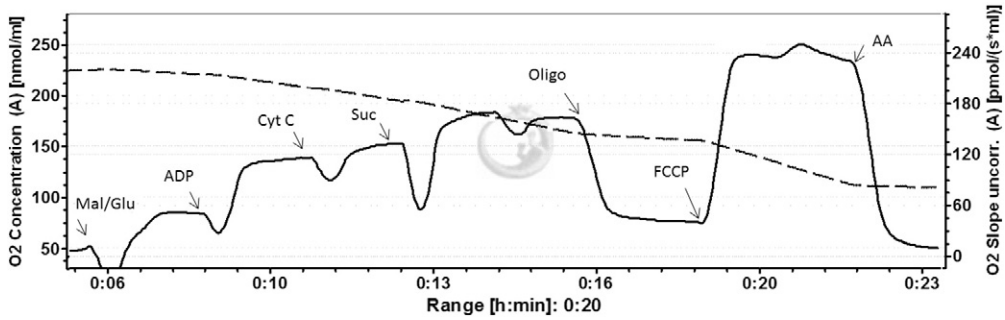


**Fig. 5.** Typical respirometry experiment on homogenate of human skeletal muscle. Solid line represents oxygen flux (right y-axis), dashed line represents oxygen concentration (left y-axis). Concentrations of substrates and inhibitors were used as described in Methods.

membrane. Whilst Larsen et al., 2014 focused on the technique of homogenisation, we paid attention to other details. In order to avoid adverse effects of hyperoxia on homogenate, we did not use preoxygenation and instead carefully titrated the amount of muscle, which enabled us to perform measurements at optimal oxygen fluxes without limitation by oxygen concentration. The extent to which eventual drop of oxygen concentration limits oxygen flux is much higher in permeabilised muscle fibres (Km ~40 nmol/ml) as compared to isolated mitochondria (Km <1 nmol/ml [Pesta and Gnaiger, 2012]), where Km is $O_2$ concentration at which the oxygen flux reduced to 50% of maximal rate. We determined Km of muscle homogenates to be ~12 nmol/ml. Despite using ~10 times more muscle tissue in a chamber compared to Larsen et al., 2014, we were able to run all experiments above safety limit of 90 nmol/ml $O_2$, in most cases without the need of chamber opening. Titration of substrates and uncouplers in separate experiments allowed for omitting stepwise additions of ADP and FCCP in the final protocol, which further shortened the measurement time to <30 min (see Fig. 5 for a typical experiment, and Fig. S5 in Supplementary material).

By these measures we achieved a protocol in which outer mitochondrial membrane functional damage measured as increase of oxygen flux after addition of cyt c was reduced to a range of 10–15%. This value is lower than obtained in Larsen's study (>20% [Larsen et al., 2014]) and comparable to isolated mitochondria (~10%). Damage to complex I was also reduced, as CI/ETS ratio in our protocol was ~0.70 in homogenate as compared to ~0.76 in isolated mitochondria and ~0.4 in Larsen's homogenates. In line with this, phosphorylation capacity adjusted to CS activity (OXPHOS) is in homogenates ~90% of the capacity of isolated mitochondria. Mitochondrial coupling tended to be even better in homogenates as compared to isolated mitochondria (CCR 0.12 ± 0.06 vs. 0.25 ± 0.11, $p = 0.052$), but this is because uncoupling in our mitochondria is higher than reported by other authors [Rogers et al., 2011]. Reproducibility measured as CV of duplicate measurement of OXPHOS was better in homogenates (~5%) as compared with isolated mitochondria (~12%), in keeping with Larsen's results [Larsen et al., 2014].

We cannot specify which change (or combination of changes) was responsible for these improvements, but our results clearly demonstrate that crucial for the quality of results is homogenisation-to-respirometry time. The results described above are only obtained if the respirometry measurement is performed immediately after the homogenisation of the sample. Whilst human skeletal muscle sample is stable on ice for up to 24 h [Skladal et al., 1994], homogenate is not. Only after 120–180 min we demonstrated signs of inner and outer membrane decomposition and damage to complex I resulting in a decrease in OXPHOS and increased decoupling. These changes occur in very variable and unpredictable way (Fig. 4), so the use of freshly prepared homogenates is of upmost importance. Moreover, our data highlight the importance of reporting homogenisation-measurement times in studies using this technique.

High-resolution respirometry has also been applied on isolated non-human [Picard et al., 2008 Aug; Kuznetsov et al., 2008] or human [Larsen et al., 2014] permeabilised skeletal muscle fibres. This method offers excellent preservation of intracellular mitochondrial architecture, but it is technically challenging (takes 1–2 h [Kuznetsov et al., 2008]) and the results may be more prone to inter-operator variability. In this study, we did not compare homogenates with isolated muscle fibres, but data from Larsen et al., 2014 demonstrated coefficient of variability CV ~11%. The use of hyperoxygenation is unavoidable with permeabilised fibres and puts the sample at risk of oxygen toxicity. The use of homogenates is more simple (preparation time ~10 min) and robust (CV ~5%), but despite perseveration of cytosolic context, intracellular mitochondrial architecture is indeed compromised.

Bergström needle biopsy [Bergström, 1979] is a standard procedure for obtaining samples from non-surgical patients or healthy volunteers. For practical application of the technique it is important to note, that biopsy technique itself does not introduce any systematic bias as all functional indices in needle biopsy samples were very close to 100% of values of samples obtained by open biopsy from the same subjects (Fig. 2). It has already been shown [O'Sullivan et al., 2006] that morphological results of both biopsy techniques are identical, as well as are activities of key enzymes of energy metabolism [Hayot, 2005]. Our data are first to demonstrate that functional mitochondrial indices are not different either.

The main limitation of this study is that we were unable to directly compare the newly developed protocol with isolated mitochondria using samples from the same subjects. This would only be possible by dividing the biopsy sample in operation theatre into two pieces stored in separate test tubes with BIOPS, enabling cold storage of muscle rather than homogenate. We have not done this and instead we compared a group of different subjects (HOM1) with measurements of isolated mitochondria (MIT). Inter-individual variability of mitochondrial functional indices induces potential bias into the results, as our patients' cohort was quite heterogeneous. Moreover, the fraction of isolated mitochondria (39% in our study) may not be representative for whole mitochondrial population in a sample [Palmer et al., 1985; Adhihetty et al., 2005 Oct; Ferreira et al., 2010]. Even though we have not seen any significant differences between MIT and HOM1, should there be any, we would not be able to explain what the reason for the difference is. It also means that our results are not generalizable to populations with primary and secondary mitochondrial diseases non-homogenously affecting mitochondrial population of a cell. Even though it can be speculated that using homogenates avoids isolation bias, this technique must first be validated against appropriate standard (eg. permeabilised muscle fibres) in future studies.

In conclusion, we developed a protocol for the assessment of mitochondrial function using high-resolution respirometry of homogenates of human skeletal muscle. When compared to isolated mitochondria, this technique was simpler to perform and more robust.

Homogenisation caused only a minor damage to outer mitochondrial membrane and respiratory complexes. The results are identical regardless whether the sample was obtained by needle or open surgical technique. We have shown that in order to get reliable results, respirometry must be performed immediately after the homogenisation of the sample as the degradation of mitochondrial function occurs quickly.

## Acknowledgement

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.mito.2015.02.002.

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# Supplement 4

KRAJCOVA, A., ZIAK, J., **JIROUTKOVA, K.**, PATKOVA, J., ELKALAF, M., DZUPA, V., TRNKA, J. and DUSKA, F.

**Normalizing glutamine concentration causes mitochondrial uncoupling in an in vitro model of human skeletal muscle.**

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# Normalizing Glutamine Concentration Causes Mitochondrial Uncoupling in an In Vitro Model of Human Skeletal Muscle

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## Abstract

*Background*: Glutamine has been considered essential for rapidly dividing cells, but its effect on mitochondrial function is unknown. *Materials and Methods*: Human myoblasts were isolated from skeletal muscle biopsy samples (n = 9) and exposed for 20 days to 6 different glutamine concentrations (0, 100, 200, 300, 500, and 5000 μM). Cells were trypsinized and manually counted every 5 days. Seven days before the end of exposure, half of these cells were allowed to differentiate to myotubes. Afterward, energy metabolism in both myotubes and myoblasts was assessed by extracellular flux analysis (Seahorse Biosciences, Billerica, MA). The protocol for myoblasts was optimized in preliminary experiments. To account for different mitochondrial density or cell count, data were normalized to citrate synthase activity. *Results*: Fastest myoblast proliferation was observed at 300 μM glutamine, with a significant reduction at 0 and 100 μM. Glutamine did not influence basal oxygen consumption, anaerobic glycolysis or respiratory chain capacity. Glutamine significantly ($P = .015$) influenced the leak through the inner mitochondrial membrane. Efficiency of respiratory chain was highest at 200–300 μM glutamine (~90% of oxygen used for adenosine triphosphate synthesis). Increased glutamine concentration to 500 or 5000 μM caused mitochondrial uncoupling in myoblasts and myotubes, decreasing the efficiency of the respiratory chain to ~70%. *Conclusion*: Glutamine concentrations, consistent with moderate clinical hypoglutaminemia (300 μM), bring about an optimal condition of myoblast proliferation and for efficiency of aerobic phosphorylation in an in vitro model of human skeletal muscle. These data support the hypothesis of hypoglutaminemia as an adaptive phenomenon in conditions leading to bioenergetic failure (eg, critical illness). (*JPEN J Parenter Enteral Nutr*. 2015;39:180-189)

## Keywords

extracellular flux analysis; human myoblasts; bioenergetic failure; aerobic phosphorylation; glutamine

## Clinical Relevancy Statement

Hypoglutaminemia is a feature of a wide range of wasting diseases, and glutamine supplementation as a part of nutrition support seemed to be beneficial in most.[1] However, recent work suggests that critically ill patients with multiorgan failure do not benefit[2] or are harmed[3] by aggressive glutamine supplementation. Mitochondrial dysfunction present in critical illness (and absent in most other diseases) may be the key factor interfering with glutamine effects and explaining the differences in clinical outcome. In this study, we look, for the first time, at the interaction between extracellular glutamine concentration and mitochondrial function in human skeletal muscle cells. We demonstrate that at moderate hypoglutaminemia (300 μM), myoblast proliferation is fastest and adenosine triphosphate synthesis is most efficient, while severe hypoglutaminemia (<100 μM), normal (500 μM) or supranormal (5000 μM) glutamine levels lead to respiratory uncoupling and energy wasting in an in vitro model of human skeletal muscle.

## Introduction

Muscle mass regeneration, during recovery from wasting illnesses, is vitally dependent on the function of myoblasts.[4] These cells are generated by proliferation of normally quiescent satellite cells, which are attached to the muscle basement membrane. After stimulation by anabolic signals, satellite cells start to proliferate and differentiate to myoblasts. Myoblasts

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synthesize contractile proteins and mitochondria and, finally, fuse with muscle fibers. Differentiated cells ready for fusion with muscle fibers are called myotubes. Myotubes have been used as an in vitro model of human skeletal muscle, since their metabolic features are similar[5] to in vivo muscle fibers. As most rapidly dividing cell lines use glutamine as both nitrogen donor and energy substrate,[6] it could be hypothesized that hypoglutaminemia impairs the proliferation and energy metabolism of myoblasts. This could provide a possible mechanism of enhanced lean body mass recovery with glutamine supplementation seen in some trials,[7] since glutamine does not influence protein synthesis directly.[8] In this study, we ask how hypoglutaminemia, at the degree seen in catabolic illnesses, influences the rate of proliferation and energy metabolism in human myoblasts and myotubes. Cells were isolated from muscle biopsy samples and their energy metabolism was assessed after 20 days of exposure to 6 different glutamine concentrations, resembling various degrees of clinical hypoglutaminemia (0, 100, 200, and 300 μM), a normal glutamine concentration in human plasma (500 μM[9]), and a concentration commonly used in cell cultures (5000 μM[6,10-15]).

## Methods

### *Overview of Study Design*

See Figure 1. For each of 9 participants, we first isolated satellite cells from a vastus lateralis biopsy sample and created a primary culture of myoblasts. At the beginning of experiment (day 0), we divided cells into 6 groups and exposed them in duplicates to 6 different glutamine concentrations. Cells were trypsinized, manually counted, and reseeded at days 5, 10, 15, and 20. This allowed us to create proliferation curves. A subset of cells was reseeded at day 15 into a 24-well Seahorse plate (Seahorse Biosciences, Billerica, MA) and allowed to differentiate into myotubes during next 7 days. At day 20, myoblasts were seeded into another Seahorse plate. In both Seahorse plates, cells were still exposed, in tri- or tetraplicates, to 6 different glutamine concentrations before extracellular flux analyses were performed on both myoblasts (n = 8) and myotubes (n = 7). Seahorse plates were kept frozen for later analysis of protein content and citrate synthase activity in a subset of patients (n = 5 and 4 for myoblasts and myotubes, respectively).

### *Study Participants and Muscle Biopsy*

Note: Unless stated otherwise, all chemicals were obtained from Sigma-Aldrich (St Louis, MO).

For all studies, we used muscle biopsy samples obtained from patients (n = 9) undergoing elective hip replacement surgery at the Department of Orthopaedic Surgery of Kralovske Vinohrady University Hospital in Prague. The University Hospital Ethical Committee reviewed both the protocol and consent form and approved the study. All patients provided written prospective informed consent. We excluded the patient with diabetes mellitus. Muscle biopsies were taken from the vastus lateralis muscle by an open surgical technique (~300 mg) as soon as the muscle was exposed during surgery. Biopsy samples were collected into 5 mL of cold Dulbecco's modified Eagle's medium (DMEM) with glucose (5.56 mM), pyruvate (1 mM), and phenol red (GIBCO, Carlsbad, CA) supplemented with glutamine (5000 μM), penicillin-streptomycin solution (100 IU/mL–100 μg/mL), and fungizone (0.5 μg/mL) and immediately put on ice.[16]

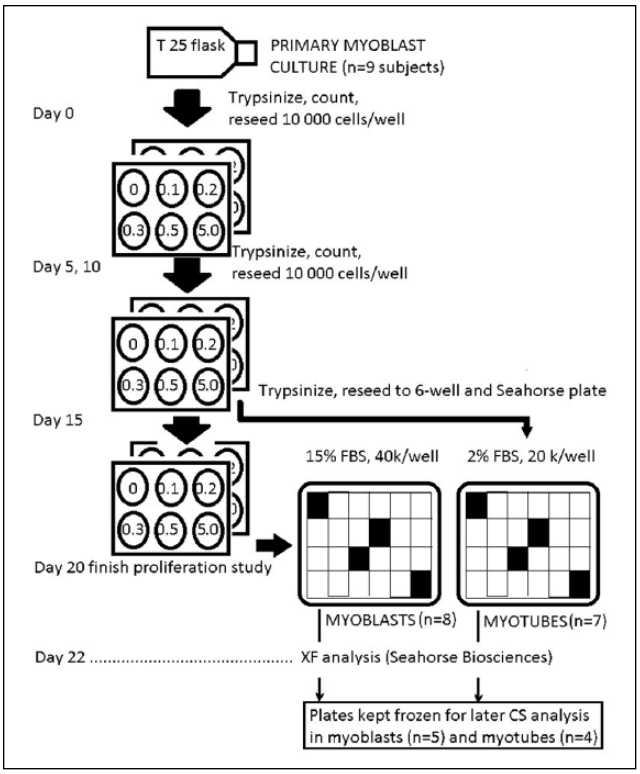


**Figure 1.** Schematic representation of the study design. CS, citrate synthase; FBS, fetal bovine serum.

### *Isolating and Culturing Human Myoblasts*

Satellite cells were isolated from muscle tissue as previously described,[17] with a few modifications. At least 150 mg of skeletal muscle tissue was minced and washed by Hank's balanced salt solution 2–3 times and cells were subsequently dissociated by incubation in 0.25% Trypsin/0.68% collagenase solution in a shaking water bath at 37°C for 30 minutes. Fetal bovine serum (FBS) was added to the suspension, and cells were collected by centrifugation at 350 *g*. Cells were subsequently preplated in an uncoated Petri dish for 60 minutes at 37°C with 5% $CO_2$ to remove fibroblasts and then transferred to gelatin-coated flasks (T-25) in DMEM with glucose (5.56 mM), pyruvate (1 mM), and phenol red (GIBCO). The medium was

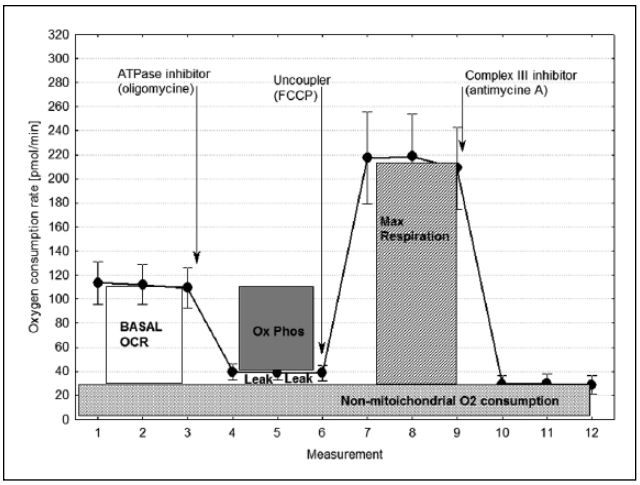


**Figure 2.** Principle of extracellular flux analysis of mitochondrial metabolism. Pooled data from myoblasts of all participants were used to construct this graph. Means, vertical bars represent 95% confidence intervals. FCCP, carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone; OCR, oxygen consumption rate; Ox Phos, oxidative phosphorylation. See text for details.

supplemented with 15% FBS, penicillin-streptomycin solution (100 IU/mL–100 µg/mL), fungizone (0.05 µg/mL), dexamethasone (0.4 µg/mL), fetuin (50 ng/mL), insulin (10 µg/mL), human growth factors (epidermal growth factor [EGF], 10 ng/mL; fibroblast growth factor [FGF], 1 ng/mL), and 5 mM glutamine. The plates were cultured at 37°C in 5 $CO_2$. The media were changed every 2 or 3 days until cells reached 80%–90% confluence.

## *Proliferation Rate Measurement*

Myoblasts from the primary culture were trypsinized and cells were seeded into a 6-well plate in basal growth medium–DMEM with glucose (5.56 mM) and pyruvate (1 mM) (GIBCO) supplemented with 15% FBS, penicillin-streptomycin solution (100 IU/mL–100 µg/mL), fungizone (0.05 µg/mL), dexamethasone (0.4 µg/mL), fetuin (50 ng/mL), insulin (10 µg/mL) and human growth factors (EGF, 10 ng/mL; FGF, 1 ng/mL) with 0, 110, 240, 370, 640, or 5000 µM glutamine, respectively. When preparing fresh media for all but the 5000-µM glutamine groups, we considered glutamine content in FBS and spontaneous glutamine breakdown with a half-time of 3 days at 37°C.[18] After solving the first-order kinetic equation ($C = C_0 \cdot e^{-kt}$, where C is the concentration at time t = 18 hours [half of the media-change interval], $C_0$ is the concentration at the beginning, and k is the first-order rate constant—in our case, 0.0104 $h^{-1}$) and subtracting the amount of glutamine in 15% FBS (17 µM[19]), the concentrations of glutamine in the middle of the media exchange interval should be 0, 100, 200, 300, and 500 µM. The 5000-µM group had 5000 µM in fresh media, since this is routine laboratory practice, and even after partial degradation, glutamine still remains highly redundant. The medium was changed every 2 or 3 days. At days 5, 10, and 15, cells were trypsinized, counted and reseeded. At day 20, cells were trypsinized, counted and seeded in a 24-well gelatin-coated Seahorse plate for flux analysis. As a measure of proliferation rate, we calculated the number of cells produced from a single cell during 5 days of culture. This number was calculated by dividing the number of cells at the end of the 5-day interval by the number of cells seeded into the well (ie, by 10,000).

*Cell viability* was measured using the CellTiter96 MTS assay (Promega, Madison, WI) as previously described.[20] Viable cells reduce the tetrazolinum dye, MTS, to purple formazan. The absorbance at 490 nm is then used as a measure of cell viability. We performed MTS on myoblasts from primary culture at baseline and after 20 days of exposure to different glutamine levels.

## *Extracellular Flux Analysis*

We used a Seahorse XF24 Analyzer (Seahorse Biosciences) for all experiments. The principle of the method is described elsewhere.[21] In brief: oxygen consumption rate (OCR) is measured in a microlayer, which sits above cells seeded in a 24-well microplate. Four wells were used as background control, with the remaining 20 wells for testing cells at 6 glutamine concentrations in tri- to tetraplicates. The measurement is performed at baseline (phase 1) and after the addition of up to 4 test agents. The dynamics of OCR allows the assessment of mitochondrial function. In our experiments, we used the sequence of an ATPase inhibitor oligomycine (phase 2), followed by an inner membrane uncoupler, FCCP (carbonyl cyanide-4-[trifluoromethoxy]phenylhydrazone, phase 3). Finally, we blocked the respiratory chain complex III with Antimycin A (phase 4). Oxygen consumption after exposure to Antimycin A is considered nonmitochondrial (see Figure 2). Basal OCR was calculated as $OCR_{Phase1} - OCR_{Phase\ 4}$. When comparing OCR before-and-after exposure with inhibition, the absolute cell number is irrelevant since the same population of cells is compared. Therefore, most parameters of energy metabolism are expressed as a percentage of baseline value. *ATP turnover* was calculated as $100*(OCR_{Phase1} - OCR_{Phase2})$/Basal OCR [%]. *Leak* through the inner mitochondrial membrane was calculated as $100*(OCR_{Phase2} - OCR_{Phase4})$/Basal OCR [%]. By analogy, *respiratory chain capacity* (or maximum respiration) was calculated as $100*(OCR_{Phase3} - OCR_{Phase4})$/Basal OCR. Extracellular acidification rate (ECAR) relates to lactate production[21] and is used as a measure of the rate of anaerobic glycolysis. Seahorse measurements were performed for myoblasts from 8 participants and myotubes from 7 participants.

To reflect possible differences of cell number or mitochondrial content, we normalized basal OCR to the activity of citrate synthase (CS) in a subset of participants (n = 5 for myoblasts and n = 4 for myotubes).

### Protein Content and CS Activity

Immediately after XF flux measurement, each well in a plate was washed with cold phosphate-buffered saline (PBS) and filled with 150 µL M-PER Mammalian Protein Extraction Reagent (Thermo Scientific, Waltham, MA) with 2 µL Protease inhibitor cocktail. Then the plate was stored at –80°C. On the day of the assay, plates were thawed and the cellular lysate was transferred into Eppendorf tubes and centrifuged at 15,000 *g* for 15 minutes at 4°C. The supernatant was collected for protein determination and CS activity assay. For protein determination, we used the Bradford reagent according to the manufacturer's instructions. CS activity was assayed in myoblasts from participants 2, 3, 4, 5, and 6 and myotubes from participants 1, 3, 5, and 6 using a CS assay kit (Sigma-Aldrich) with at least 8 µg of whole-cell extract protein, 0.3 mM acetyl-CoA, and 0.1 mM 5,5′-dithiobis-(2-nitro-benzoic acid). The reaction was started with adding 5 mM oxaloacetate to the mixture. CS activity of cells is stable 2–3 hours during storage on ice.[22] Enzyme activity was monitored by recording the changes in absorbance at 412 nm over 1.5 minutes at 30°C.

### Optimizing the XF Flux Analysis Protocol for Human Myoblasts

In a series of preliminary experiments, we first optimized the conditions of the measurement for human myoblasts by modifying existing protocols for C2C12 myoblasts[23] and human myotubes.[14] We started with a protocol for C2C12 myoblasts seeded at 20,000 cells/well, realizing that maximum OCR reached only the median of 76 or 65 pM/min after 1 or 2 µM FCCP, respectively, while OCR for the same number of C2C12 cells was approximately 10 times higher. In subsequent experiments, we increased the number of cells seeded per well and compared 20,000, 40,000, and 75,000 cells/well. With 40,000 cells, we achieved maximum OCR well above 100 pmol/min, while $pO_2$ remained above 5 kPa throughout the experiment. In control wells (without addition of inhibitors), OCR remained stable during 240 minutes of the experiment. We next compared the effects of 0.76, 1.0, and 1.5 µM oligomycine, achieving maximum inhibition of ATPase with 1.0 µM. This concentration was therefore used for the rest of the experiments. Similarly, we compared uncoupling effects of 0.5, 1, and 2 µM FCCP and achieved maximum OCR with 1 µM. Last, to verify the ability of 4 µm Antimycin A to inhibit the respiratory chain, we performed an experiment in which we added 0.6 M KCN (complex IV inhibitor) after Antimycin A. No additional decrease in OCR was seen with the addition of cyanide. In light of this, the final protocol we used for testing the influence of glutamine on mitochondrial function uses 40,000 cells/well in basal growth medium with 0, 100, 200, 300, 500, or 5000 µM glutamine. After 24 hours, we exchanged the medium for XF Assay Medium (Seahorse Biosciences) with 4 mM glucose and 1 mM pyruvate (pH 7.4) at 37°C. XF flux analysis was performed after a 60-minute incubation in a $CO_2$-free atmosphere. Baseline OCR was measured in 3 cycles: 3 minutes mixing, 2 minutes waiting and 3 minutes measuring. Then these 3 cycles were repeated after adding oligomycin (1 µM), FCCP (1 µM), and Antimycin A (4 µM).

### XF Flux Analysis of Human Myotubes

After 15 days of exposure to various glutamine concentrations, cells were trypsinized and seeded on a 24-well gelatin-coated Seahorse plate at 20,000 cells/well in a basal growth medium with 6 different concentrations of glutamine. After 24 hours, we exchanged the medium for a differentiation medium (DMEM) with glucose (25 mM), pyruvate (1 mM), 2% horse serum, penicillin-streptomycin solution (100 IU/mL–100 µg/mL), insulin (10 µg/mL), and the respective glutamine concentrations. Medium was exchanged every 2 days for the next 6 days, and on day 7, an XF assay was performed as described[14]; assay medium and concentrations of uncoupler and inhibitors were identical to those used for myoblasts.

### Statistics

All data were tested for normality of distribution. We used 1-way or factorial analysis of variance (ANOVA, with Tukey post hoc test where appropriate) for most comparisons. Proliferation data (number of daughter cells, N) were found to be log-normally distributed, and after logarithmic transformation, they were fitted with the normal distribution ($P > .2$ in the Kolmogorov-Smirnov test) and processed by factorial ANOVA. All calculations were performed in Statistica version 8 (StatSoft, Inc, Tulsa, OK). Differences at $P < .05$ were considered significant.

## Results

### Fastest Cell Proliferation Was Observed in 300 µM Glutamine

Cells across a range of glutamine concentrations showed exponential proliferation (see Figure 3), which was fastest in 300 µM, as demonstrated by mean numbers of cells formed from 1 ancestor cell during a 5-day cycle of culture (geometric means of all 4 culture intervals): 10 (0 µM glutamine), 15 (100 µM), 18 (200 µM), 25 (300 µM), 24 (500 µM) and 23 (5000 µM). Across all glutamine concentrations, we observed the proliferation rate to slow down after 10 days. However, cell viability as per the MTS test was not compromised ($P = .86$, data not shown). Influences of time and glutamine concentration were both significant at $P < .001$ in factorial ANOVA, but there was no interaction between them ($P = .83$).

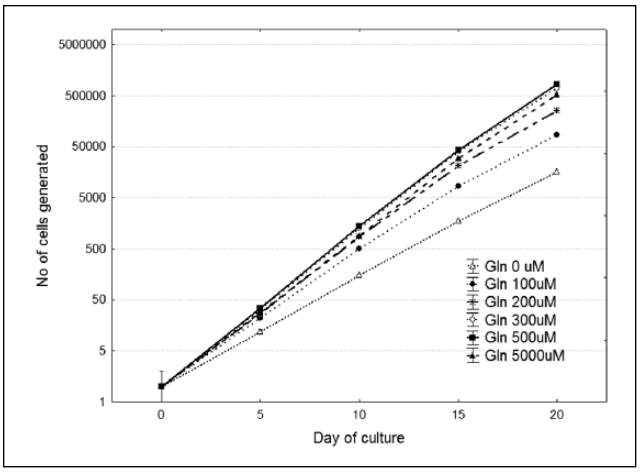


**Figure 3.** Proliferation rate of human myoblasts in relation to glutamine (Gln) concentration in the media. Mean number of cells formed from a single cell at day 0. Note log scale; confidence interval bars are not displayed for clarity.

## *Glutamine Deprivation Does Not Influence Mitochondrial Density but Tends to Decrease Protein Content*

Protein content was higher in wells containing myotubes, despite the fact that half the number of cells were seeded (20,000 myotubes and 40,000 myoblasts per well). In both cell lines, total glutamine deprivation tended to decrease total protein content per well by up to ~50% (Figure 4A) compared with cells cultured in 5000 μM glutamine. There was also a significant correlation between protein content in a well and glutamine in the media (Spearman $R = 0.46$ for myotubes and $R = 0.38$ for myoblasts; $P < .05$ for both). No such trend was seen for CS activity, which remained unchanged across glutamine concentrations (Figure 4B), and the differences in CS activity between cell lines roughly correspond with the fact that twice as many myoblasts were seeded per well compared with myotubes.

In turn, CS per protein ratio (a measure of relative mitochondrial density) tended to increase with severe glutamine deprivation (Figure 4C) as well as OCR/ECAR ratio (Figure 4D), reflecting the relative contributions of aerobic and anaerobic metabolism on ATP generation. Cells cultured in a standard "laboratory" concentration of glutamine (5000 μM) were highly oxidative, having an OCR/ECAR ratio of 11 ± 4 (myoblasts) or 9 ± 2 (myotubes). Severe glutamine deprivation tended to increase the OCR/ECAR ratio even further, reaching 28 ± 10 or 19 ± 9, respectively, in cells cultured in glutamine-free media.

## *Glutamine Does Not Influence Basal OCR*

After normalization to mitochondrial content (expressed as CS activity), basal respiration does not differ either between myoblasts and myotubes ($P = .53$) or across glutamine concentrations ($P = .87$) (see Figure 5A).

## *Influence of Glutamine on the Efficiency and Capacity of the Respiratory Chain*

After subtracting nonmitochondrial oxygen consumption, OCR at baseline reflects a sum of oxygen consumed for ATP synthesis and for energy dissipation due to leak of protons through the inner mitochondrial membrane. These 2 components are distinguished by measuring OCR after the addition of the F1F0 ATPase inhibitor oligomycine (Figure 2). The ATP synthesis rate normalized to mitochondrial content (CS activity) was not different either between cell lines ($P = .16$) or across glutamine concentration ($P = .63$; see Figure 5C). Leak of protons through the inner mitochondrial membrane normalized to CS activity (Figure 5D) was significantly higher in myotubes compared with myoblasts ($P < .001$) and was influenced by glutamine concentrations ($P = .041$). The relation of glutamine concentration to mitochondrial leak was further explored by looking at that percentage of basal OCR, which is not inhibited by oligomycine (Figure 6).

Myotubes cultured at 200–300 μM glutamine used 80%–90% of basal OCR for ATP synthesis and only 10%–20% of energy was dissipated as heat. At both extremes of glutamine concentration, we observed increased mitochondrial uncoupling to ~30%–40%, which decreased efficiency of the respiratory chain below 70% (see Figure 6). Myoblasts had significantly lower leak compared with myotubes ($P = .019$), which only increased with high glutamine concentrations (from ~10% to ~30%). Even though the effect of glutamine was significant in the overall model ($P = .015$), none of the differences were significant in the Tukey post hoc test (eg, $P = .053$ for the difference between 200 and 5000 μM in myotubes).

Spare respiratory chain capacity (maximum respiration) is measured as oxygen consumption after artificial uncoupling of the inner mitochondrial membrane (refer to Figure 1). If expressed as OCR normalized to mitochondrial content (Figure 5B), maximum respiration tends to be nonsignificantly higher in myoblasts compared with myotubes ($P = .13$), and it is not influenced by glutamine ($P = .94$). Similarly, when related to basal respiration, maximum respiration is 200%–300% and not different between cell lines or among glutamine concentration.

## *No Influence of Glutamine on Anaerobic Glycolysis, Glycolytic Capacity, or Nonmitochondrial Oxygen Consumption*

ECAR is used as a measure of anaerobic glycolysis as it mainly reflects the production of lactate.[21] There was no difference in anaerobic glycolysis in a range of glutamine concentrations in

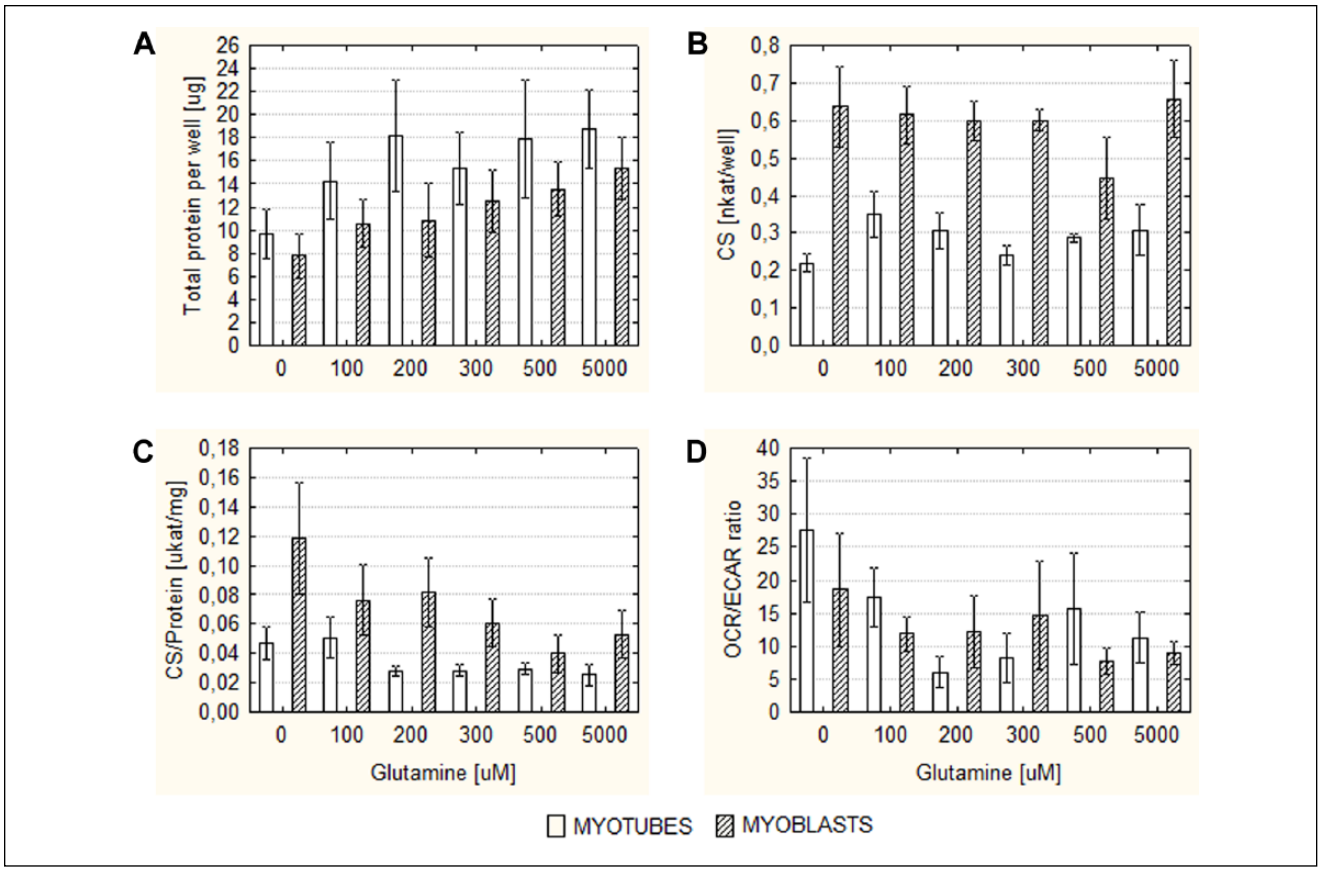


**Figure 4.** (A) Total protein content per well. (B) Citrate synthase (CS) activity per well. (C) CS per protein ratio. (D) Oxygen consumption rate (OCR) per extracellular acidification rate (ECAR) ratio. Means, vertical bars represent 95% confidence intervals.

either cell lines. After blocking ATP synthase with oligomycine, anaerobic glycolysis becomes the only source of ATP for the cell, and hence it increased to ~110%–170% above baseline. This response was unaffected by glutamine concentration, and neither was extra-mitochondrial oxygen consumption (data not shown).

### *Overall Metabolic Profile of Myoblasts and Myotubes in Relation to Glutamine in Culture Media*

Last, to get further insight into energy metabolism, we expressed oxidative phosphorylation, anaerobic glycolysis, and energy dissipation via proton leak in equivalents of ATP and compared those values (Figure 7). We assumed that 1 mol of $O_2$ of oligomycine-inhibitable OCR gives rise to 5 mol of ATP and that 1 mol of $H^+$ (or 1 mol of lactate) produced by cells equates to the synthesis of 1 mol of ATP.

Figure 7 again demonstrates the highly oxidative nature of both myotubes and myoblasts and the tendency of increased leak with higher glutamine concentrations, particularly in myotubes. ATP wasted by inner mitochondrial leak is not compensated by increased anaerobic glycolysis. Of note, these data were not normalized to CS activity (as doing so would make no sense for anaerobic metabolism), and therefore it is not possible to compare myotubes with myoblasts or perform any valid statistical analysis.

## Discussion

Rapidly dividing cells have been shown to be dependent on glutamine as a substrate for de novo nucleotide synthesis[24] and energy metabolism.[10,24,25] This has been demonstrated for lymphocytes,[26] enterocytes[27] and type II pneumocytes.[28] This study, for the first time, shows a similar dependency for human myoblasts. Surprisingly, supra-physiological concentrations of glutamine (5000 µM) as recommended by many protocols for in vitro cell cultures,[6,10-13] including human myoblasts,[14] do not bring any additional benefit in terms of myoblast proliferation. Furthermore, it may even be the case that concentrations as low as 300 µM glutamine, which is close to the concentration in patients with protracted critical illness in the absence of glutamine supplementation,[29] are optimal. Significant limitations of myoblast proliferation were observed at concentrations

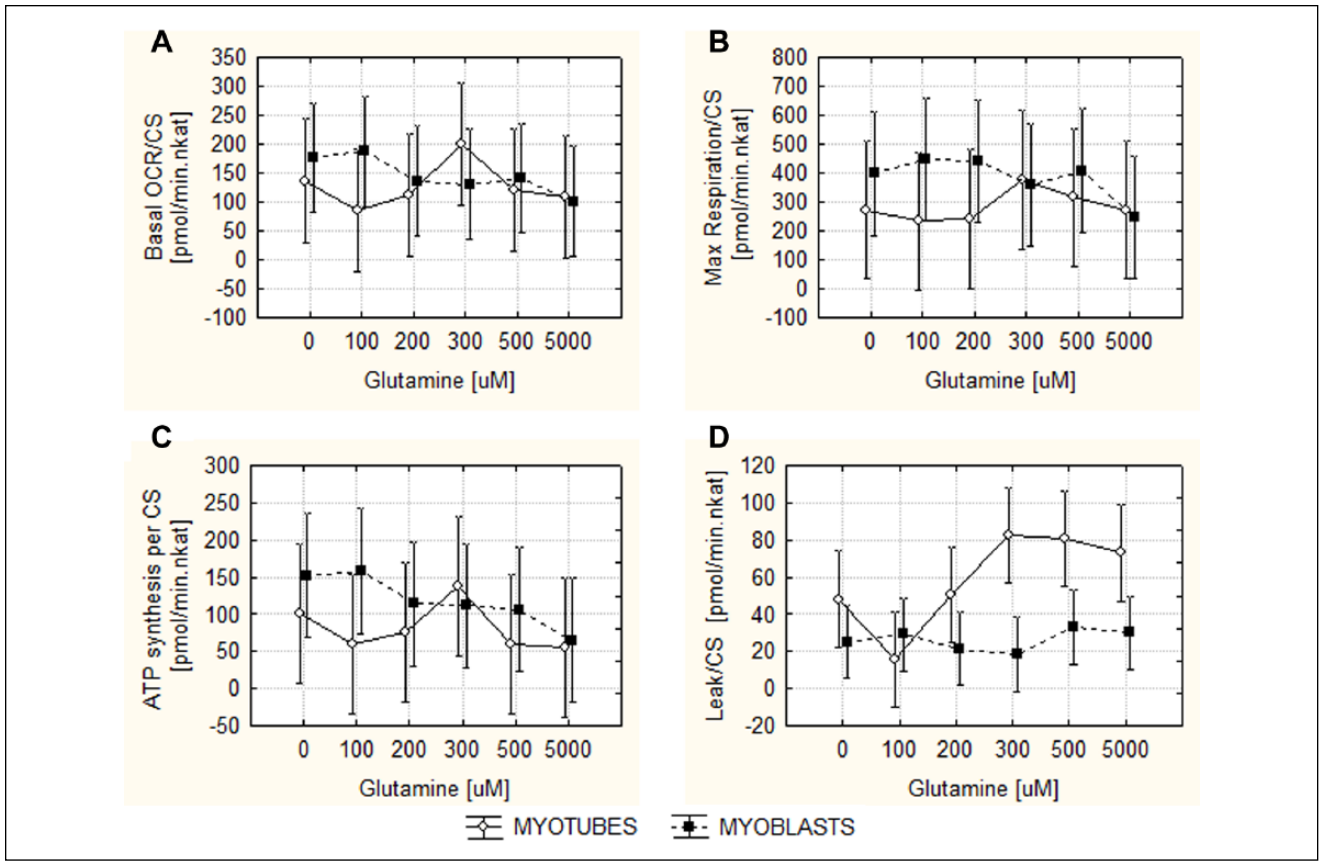


**Figure 5.** (A) Basal oxygen consumption rate. (B) Maximal respiration. (C) Adenosine triphosphate (ATP) synthesis rate. (D) Proton leak through the inner mitochondrial membrane. All values are expressed as the amount of oxygen used after adjustment to mitochondrial content in a well expressed as citrate synthase (CS) activity (pmol/min$^{-1}$/nkat$^{-1}$). Means, vertical bars represent 95% confidence intervals. OCR, oxygen consumption rate.

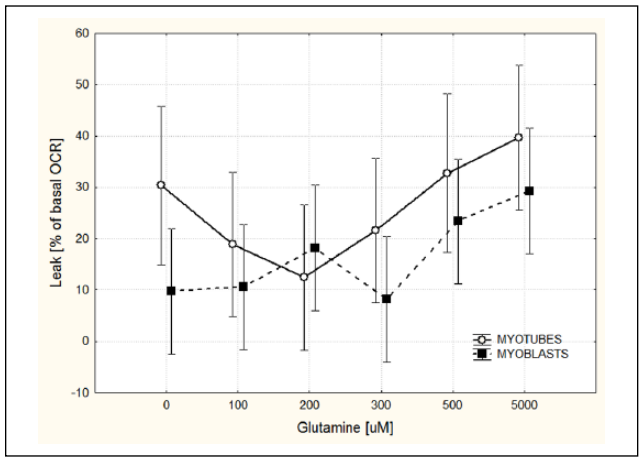


**Figure 6.** Proton leak through the inner mitochondrial membrane expressed as a percentage of the basal oxygen consumption rate. Means, vertical bars represent 95% confidence intervals. OCR, oxygen consumption rate.

≤200 μM glutamine, which is well below the range seen in wasting diseases[30] or in acute critical illness.[29,31,32] Although it is difficult to extrapolate in vitro data to whole-body physiology, our results suggest that restoring extracellular glutamine concentration to normal levels may not improve conditions for muscle regeneration. Even cells grown in glutamine-free media remained viable and did proliferate (Figure 3). A trend to a decrease in protein content per well (Figure 4A) without a change in CS activity (Figure 4B) in wells seeded with glutamine-deprived cells may reflect a lower number of cells (with more mitochondria), diminished total protein content in the same number of cells, or a combination of both. Nicklin et al[33] demonstrated, in various mammalian cell lines, that glutamine is an upstream activator of mTOR, a key regulator of protein synthesis.[34] This could represent a molecular mechanism as to how glutamine deprivation can alter intracellular protein content. Because the differentiation stage may vary among different glutamine concentrations, total protein content may not represent cell number, and we did not use it for normalization of OCR in metabolic studies. Instead, we adjusted OCR to CS activity—a marker of mitochondrial density.[22]

Myoblasts and myotubes cultured in a standard "laboratory" concentration of glutamine (5000 μM) were largely reliant on aerobic phosphorylation, having an OCR/ECAR ratio

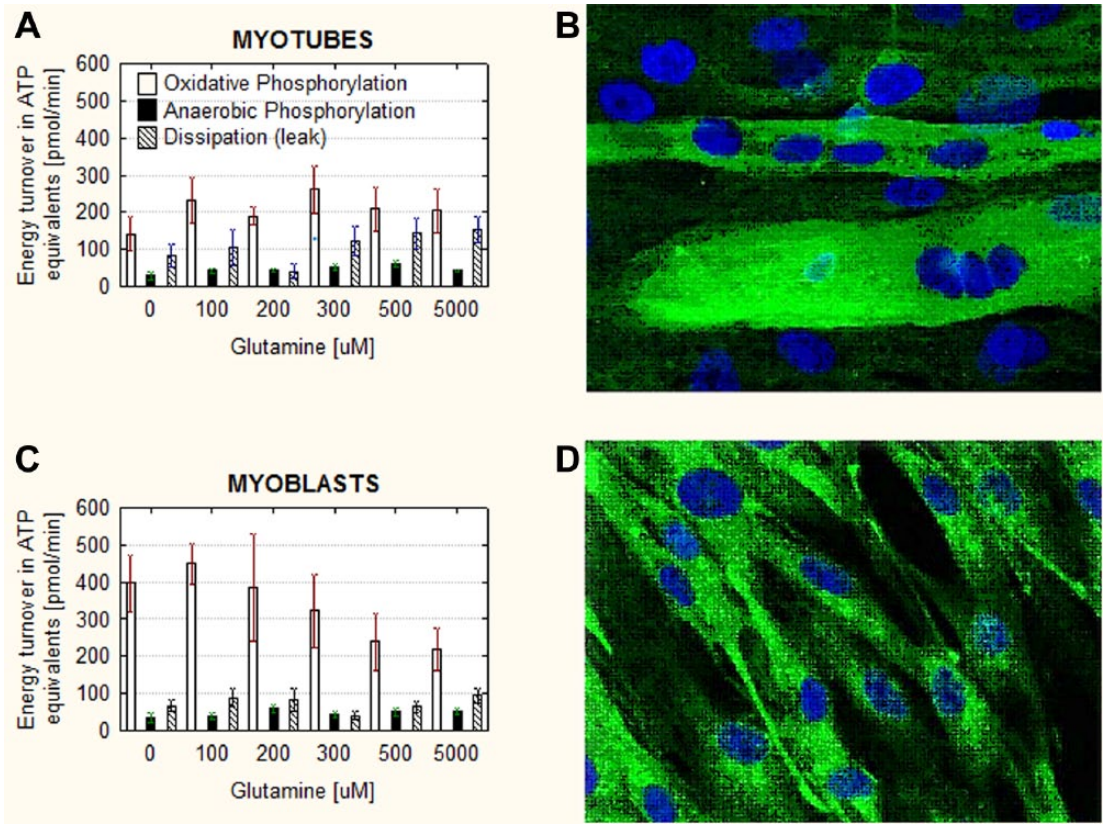


**Figure 7.** Energy profile (A, C) and the appearance of myotubes (B) and myoblasts (D) stained for desmin (AB 907 rabbit anti–desmin polyclonal antibody [Millipore, Billerica, MA]). See text for explanation. ATP, adenosine triphosphate.

~10, which is comparable to feline cardiomyocytes[35] or C2C12 myoblasts.[36] Much lower OCR/ECAR (typically between 1 and 5) ratios are reported for most cancer cell lines.[37] Figure 7 demonstrates a very small contribution of anaerobic phosphorylation to overall ATP turnover. A trend to a further increase of the OCR/ECAR ratio in glutamine-deprived cells may reflect the fact that pyruvate—instead of being converted to lactate—is turned into oxaloacetate. This anaplerotic reaction can replenish intermediates of the citric acid cycle when there is not enough glutamine to form 2-oxoglutarate via glutamate. However, we failed to demonstrate the inhibitory effect of glutamine deprivation on anaerobic glycolysis either at baseline (Figure 7) or after inhibition of aerobic ATP synthesis.

When looking at the effect of glutamine on energy metabolism of myotubes and myoblasts, the most interesting finding is the uncoupling effect of glutamine. Oxygen used in respiratory chain energizes both ATP synthesis and proton leak through the inner mitochondrial membrane. In both myoblasts and myotubes cultured at 200–300 μM glutamine, we observed highly coupled respiration with ~90% of basal OCR being used to drive ATP synthesis (Figures 5D and 6). Supra-physiological concentrations of glutamine (5000 μM) decreased the efficiency of the respiratory chain to ~60%–75% in both cell lines. In myotubes but not myoblasts, we saw a similar degree of uncoupling with extremely low glutamine (0 μM). When looking at absolute numbers, the uncoupling did not compromise aerobic ATP generation (Figure 5C) or alter basal OCR (Figure 5A), even though some trends to decrease aerobic phosphorylation are seen in myoblasts (Figures 5A and 7). Mitochondrial uncoupling can result either from inner mitochondrial membrane damage or controlled leak of protons through the family of uncoupling proteins (UCPs). Of these, UCP-2 and UCP-3 are found in skeletal muscle.[38] In cultured macrophages, colonocytes, and pancreatic β-cells, glutamine has been shown to increase translation of UCP-2 with the maximum induction seen at 0.4–1.0 mM.[39] It has been proposed[40] that UCP-3 and UCP-2 may also serve as uniporters of pyruvate from—and glutamine into—mitochondria, respectively. In this context, the need to transport abundant glutamine into mitochondria to join the citric acid cycle (via glutamate and 2-oxoglutarate) may have induced UCPs and caused the uncoupling seen with higher glutamine concentrations. Skeletal muscle is the main organ for glucose disposal in vivo, and glutamine-induced uncoupling should then increase substrate metabolism and energy expenditure. In line with this, glutamine supplementation in critically ill patients has been repeatedly shown to

increase insulin-mediated glucose disposal[41-43] and fat oxidation.[44] Iwashita et al[45] observed increased energy expenditure with glutamine in healthy volunteers. In this context, with glutamine supplementation and uncoupling, glucose and fat can be increasingly oxidized in skeletal muscle to energize futile cycles and heat generation.

However, in our study, we have not demonstrated any significant increases in basal OCR with glutamine-induced uncoupling, and it seems that increased leak was rather at the expense of aerobic phosphorylation (albeit its changes were not significant). This would suggest a rather uncontrolled leak resulting from glutamine-induced mitochondrial damage. Possible mitochondrial toxicity of glutamine was first noted by Groening et al.[46] When investigating the protective effect of glutamine on the activity of cytochrome c oxidase in septic rat myocardium, they found a trend to decreased oxygen extraction and diminished cyclooxygenase (COX) activity in sham-operated (nonseptic) hearts, despite higher COX levels with 0.75 g/kg glutamine compared with placebo. The authors mention the possibility of glutamine intracellular breakdown to glutamate and ammonia, which resulted in oxidative damage of mitochondria.[46]

It should be noted that muscle biopsies were taken from participants with hip arthropathy, which may have influenced muscle physiology. The culture media with different glutamine concentrations were not isonitrogenous, and we cannot rule out the possibility that the observed effects are due to different nitrogen content rather than glutamine itself. Moreover, the methods used do not allow deeper insight into observed changes of energy metabolism. Further studies should explore the effects of glutamine on intracellular ATP concentration, inner mitochondrial membrane potential, and expression of UCPs, as well as measure mitochondrial DNA oxidative damage in human nontumor cell lines. If "mitochondrial toxicity" of glutamine is confirmed, this would shed new light onto recent data from large randomized controlled trials on glutamine supplementation. Glutamine seems to be beneficial in chronic wasting diseases but harmful in acute critical illness with multiorgan failure.[3] Our data generate the hypothesis that the reason for the discrepancy of clinical outcomes with glutamine may lie in mitochondria: bioenergetic failure is a feature of critical illness, where altered and swollen mitochondria are not able to meet extreme ATP demands of tissues, leading to multiorgan failure and death. The hypoglutaminemia of critical illness may represent an adaptive phenomenon[47] by decreasing circulating mitochondrial toxin below what we consider safe levels. This may be protective of mitochondria at the expense of other glutamine-dependent processes. After the initial insult and following mitochondrial damage and repair, the beneficial effects of higher levels of glutamine may prevail, since these other glutamine-dependent processes may become more important in the subsequent rehabilitation phase. During the recovery phase after mitochondria are repaired and replenished in survivors[48] and also in chronic illnesses, the beneficial effect of glutamine may prevail.

In conclusion, in this study, we have developed a protocol for the use of extracellular flux analysis in cultured human myoblasts. We have shown that human myoblast proliferation is optimal at 300 μM glutamine, which is a level consistent with moderate clinical hypoglutaminemia. Increasing glutamine concentration above this level does not improve the proliferation rate and leads to significant uncoupling of the respiratory chain and a trend to impaired ATP synthesis in both myoblasts and myotubes. Glutamine does not influence respiratory chain capacity, the rate of anaerobic glycolysis, or non-mitochondrial oxygen consumption in human skeletal muscle cells.

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# Supplement 5

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PŘEHLEDOVÝ ČLÁNEK

# Svalová slabost kriticky nemocných

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**Souhrn**

**Nově vzniklá svalová slabost kriticky nemocných (ICUAW) je častou komplikací intenzivní péče s dalekosáhlým dopadem, především na její dlouhodobý výsledek. ICUAW je asociována se syndromem systémové zánětlivé odpovědi (SIRS) a lze na ni nahlížet jako na projev multiorgánové dysfunkce na úrovni periferního nervu a svalu. Na úrovni periferního nervu dochází k hyperpolarizaci membrány a poruše vedení vzruchu díky snížení počtu a změně vlastností $Na^+$ kanálů. Ve svalových vláknech je porušena fluktuace intracelulární koncentrace $Ca^{2+}$ v průběhu cyklu kontrakce-relaxace. Snižuje se též obsah kontraktilních bílkovin (proteolýza), jader (apoptóza) i mitochondrií, které jsou funkčně defektní (bioenergetické selhání). Postižení svalu u kriticky nemocného tedy zahrnuje jak funkční defekt (myopatii), tak úbytek tkáně (sarkopenii). Klinická diagnostika ICUAW je v podmínkách JIP/ARO obtížná a často opožděná, přesné určení podílu postižení nervové a svalové tkáně na svalové slabosti má spíše akademický význam, neboť specifickou terapii v současnosti neznáme. Prevence a terapie je individualizovaná a spočívá v minimalizaci vyvolávajících faktorů, adekvátní nutrici (prevence sarkopenie) a co nejčasnější mobilizaci kriticky nemocného.**
***Klíčová slova:*** **svalová slabost – kriticky nemocný – SIRS – polyneuromyopatie – sarkopenie**

**Abstract**

**Intensive Care Unit-Acquired Weakness**

**Intensive Care Unit-Acquired Weakness (ICUAW) is a common complication of intensive care that places significant impact on the long-term outcome. ICUAW is associated with systemic inflammatory response syndrome (SIRS) and is now considered an aspect of the multiple-organ failure syndrome at the level of peripheral nerves and muscles. The membrane of the peripheral nerve is hyperpolarized and is inexcitable at least in part due to the changes of both the number and features of the Na+ channels. Within the muscles, the intracellular $Ca^{2+}$ fluctuation pattern flattens during the cycle of contraction-relaxation. There is also a decrease in the contractile protein content, and in the reduction of the number of both the nuclei and mitochondria. Thus, the muscle involvement in ICUAW includes both functional changes (myopathy) and a net loss of muscle tissue (sarcopenia). The diagnosis of ICUAW is difficult and often delayed. Direct assessment of the extent to which the muscles and nerves contribute to weakness is often not worthy, as no specific therapy is yet available. The prevention and management of ICUAW now consist of avoiding risk factors, providing adequate nutrition and encouraging the culture of early mobilization of ICU patients.**
**Keywords: muscle weakness – critically ill – SIRS – polyneuromyopathy – sarcopenia**

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## Úvod

Díky pokroku intenzivní medicíny v posledních 50 letech bylo dosaženo mnoha úspěchů v péči o kriticky nemocné pacienty. Celková krátkodobá mortalita jednoznačně klesá, současně ale narůstá množství a spektrum komplikací, které vyplývají z vlastního prolongovaného kritického stavu, anebo jsou důsledkem intenzivní péče o nemocného. Hospitalizace na JIP/ARO se prodlužuje [1], což vede k navýšení nákladů na léčbu. Ve většině klinických studií v oblasti intenzivní péče se za kritérium úspěšnosti považuje propuštění z JIP nebo přežití 28., eventuálně 60. den. Ukazuje se však, že velká část nemocných, kteří jsou z JIP přeloženi na standardní oddělení, odloženě umírá [2] – nejčastěji na pneumonii, tromboembolické komplikace a jiné důsledky svalové slabosti a imobility. Klinické studie, které jsou potom podkladem pro hodnocení efektivity a bezpečnosti léčebných postupů v kontextu medicíny založené na důkazech, většinou odloženou mortalitu nezachytí, neboť jen málo z nich sleduje přežití a funkční výsledek léčby s delším časovým odstupem. Intenzivistu 21. století by mělo zajímat nejen to, zda kriticky nemocný přežije intenzivní péči, ale i v jakém stavu ji opouští. Deplece svalové hmoty (sarkopenie) a porucha funkce nervosvalového aparátu (neuromyopatie) vznikají již v průběhu terapie na JIP. Jsou hlavními příčinami rozvoje svalové slabosti kriticky nemocných, která komplikuje odvykání pacienta od ventilátoru, brzdí rehabilitaci a pokud přetrvává dlouhodobě, významně zhoršuje následnou kvalitu