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

SUMMARY OF DOCTORAL THESIS

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

Svalová slabost kriticky nemocných:
role mitochondriální dysfunkce v její patogenezi

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# LIST OF ABBREVIATIONS

| | |
|---|---|
| AA | Antimycin A |
| ADP | Adenosine Diphosphate |
| ATP | Adenosine Triphosphate |
| CIM | Critical Illness Myopathy |
| CIP | Critical Illness Polyneuropathy |
| cox | Cyclooxygenase |
| CS | Citrate Synthase |
| cyt c | cytochrome c |
| DMEM | Dulbeco´s Modified Eagle Medium |
| ECAR | Extracellular Acidification Rate |
| FAO | Fatty Acid Oxidation |
| FCCP | carbonyl cyanide-4-[trifluoromethoxy]phenylhydrazone |
| FFA | Fatty Free Acid |
| GPDH | Glyceraldehyde-3-Phosphate Dehydrogenase |
| GLUT-4 | Glucose Transporter 4 |
| HRR | High-Resolution Respirometry |
| IGF-1 | Insulin-like Growth Factor-1 |
| ICU | Intensive Care Unit |
| ICUAW | Intensive Care Unit Acquired Weakness |
| KHB | Krebs and Henseleit Bicarbonate medium |
| KCN | potassium cyanide |
| LA | Linolaeic Acid |
| MAS | Mitochondrial Assay Solution |
| 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) |
| NEFA | Non-Esterified Fatty Acids |
| OA | Oleic Acid |
| OCR | Oxygen Consumption Rate |
| OXPHOS | Oxidative Phosphorylation |
| PA | Palmitic Acid |
| rhGH | recombinant human Growth Hormone |
| SIRS | Systemic Inflammatory Response Syndrome |
| SSC | Surviving Sepsis Campaign |
| SR | Sarcoplasmic Reticulum |
| TMPD | Tetramethyl-p-Phenylenediamine |
| 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 nezjistili 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 ($300\mu M$) 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.

# INTRODUCTION

ICU-acquired weakness (ICUAW) is a major contributor to the failed long-term outcome in survivors of critical illness resulting in persistent functional disability and decreased quality of life years after patient´s hospital discharge. Critically ill patient may lose up to 10% of lean body mass (LBM) every week (1). An overall loss of 25% LBM is associated with difficulties in weaning patient of mechanical ventilation, increased incidence of ventilator associated pneumonia and delayed rehabilitation (2).

Unfortunately, no specific therapies exist. Presently, therapeutic interventions to attenuate ICUAW are limited to moderate glucose control (3) and early mobilization (4), (5). Therefore, deepening our understanding of its pathogenesis and identifying risk factors are important goals, as muscle-specific therapeutic targets are urgently needed.

Mitochondrial damage occurs early in the acute phase of critical illness and is followed by activation of mitochondrial biogenesis in survivors (6). Recently, increasing attention is paid to the role of mitochondrial dysfunction in sepsis and systemic inflammatory response syndrome (SIRS), which are the major risk factors for development of ICUAW (7).

## 1.1     ICUAW - background

ICUAW is defined as bilateral symmetrical weakness with no plausible cause other than critical illness, involving all extremities, with relatively spared facial grimace and completely preserved sensorium (8). Its prevalence may reach up to 96%, when a comprehensive diagnostic algorithm is used (9).

### 1.1.1     Risk factors

Sepsis and systemic inflammation are the main risk factors for ICUAW (9; 10). Other risk factors include the duration of mechanical ventilation and ICU length of stay (11), hyperosmolality and use of parenteral nutrition (12) as well as hyperglycaemia (13). The application of tight glycaemic control had been suggested as a promising prophylactic intervention.  However, the NICE-SUGAR trial (3) demonstrated that intensive glucose control (to target 4.5-6.0mmol/L) resulted in 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.

The contribution of corticosteroids and neuromuscular blocking agents overuse to the development of ICUAW has been extensively studied

but the results are inconclusive. Finally, the evidence that immobility is a critical risk factor for ICUAW remains indirect.

### 1.1.2    Pathophysiology

The pathophysiology of ICUAW is complex and is considered to be an integral part of a process leading to MODS.  Unfortunately, the mechanism has not so far been fully understood.

#### a) Skeletal muscle wasting

A catabolic state with progressive skeletal muscle wasting occurs rapidly in critically ill, particularly in patients with sepsis (12). In humans, a decreased protein synthesis in response to immobility is the prominent process underlying the acute muscle mass loss in critically ill (14) contrary to animal models of sepsis. 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 (15). As many of the critically ill become weak prior to any evidence of muscle wasting, the underlying mechanism is likely to be independent of a plain muscle wasting.

#### b) Reduction in force-generating capacity

Both, muscle and nerve are relatively inexcitable in septic patients (16), although nerve histology is often normal (17) suggesting functional pathology. *In vitro* studies showed changes in $Na^+$ channel functions in humans (18). Interestingly, serum from ICUAW patients can reduce a force generation in permeabilised muscle fibers by blocking the $Ca^{2+}$ release at the level of sarcoplasmic reticulum (SR) and have deleterious effect on muscle membrane excitability *in vitro* (19).

In the acute phase of critical illness, the nerve structure is preserved, while electrophysiological abnormalities occur (e.g. distal sensory-motor axonopathy). According to Bolton and colleagues (20), in the absence of neuronal ultrastructural changes microvascular alteration in the endoneurial capillaries is the key event in the development of critically ill polyneuropathy (CIP). Toxic factors including glucose may directly penetrate the leaky endoneurial membrane resulting in increased generation and deficient scavenging of reactive oxygen species in neurons (21) (22) and contribute to bioenergetic failure (see below).

The severity of CIP correlates with the duration of ICU stay and serum glucose levels (23), which is important for clinical practice. Additionally, the toxic oxidative effect of parenterally administered lipids (nutrition overload) can worsen the neural microvasculature damage (24). Early administration of parenteral nutrition to the critically ill has been shown to be harmful (25), as was the aggressive glutamine supplementation (26).

### c) Bioenergetic failure

Efforts to improve systemic tissue $O_2$ delivery in septic patients during initial fluid resuscitation resulted in 16% absolute reduction in 28-day mortality rate in patients with severe sepsis or septic shock (27). However, efforts to improve systemic $O_2$ delivery later in the course of sepsis and to supranormal levels failed to prevent multiorgan dysfunction (28). Finally, the hypothesis emerged that the energy metabolism in cells is deranged because of the inability of cells to utilize available $O_2$ (29).

The combination of tissue hypoxia, increased oxidative stress and insulin resistance accompanying sepsis and critical illness leads to mitochondrial dysfunction and impaired ATP production (so called bioenergetic failure (30)).

Mitochondrial dysfunction has been associated with increased severity of illness and mortality in patients with septic shock (31), (32). A reduction in total mitochondrial activity was observed in patients with severe sepsis (33). 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 (6). Naturally, the loss of normal mitochondrial function results in an inability of cells to meet their energy needs, which in turn accelerates muscle fatigability and weakness (34), (35), (36).

## 1.2 Experimental approach to skeletal muscle metabolism

Previous functional metabolic studies on skeletal muscle fibers, isolated mitochondria (37) or human myoblasts were extremely time-consuming when intended to test more than a few experimental manipulations (i.e. Clark electrode, radionuclide-labeled substrates) (32) (38). Recently introduced multi-well, plate-based assay (XF24 extracellular flux analyzer Seahorse Bioscience) simultaneously measures 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 (39), (40) and we extended its applicability to the population of human myoblasts cultured in vitro (51).

On top of that, we first adopted high resolution respirometry to homogenates of human skeletal muscle and validated this method against isolated muscle mitochondria (75).

## 2    AIMS

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

- To introduce and validate the measurement of mitochondrial functional indices by extracellular flux analysis (XF24) on an in vitro model of human skeletal muscle (i.e. cultured myoblasts and myotubes).

- To explore whether mitochondrial dysfunction of acute critical illness persists until protracted phase of critical illness in patients with ICUAW

- To explore whether and how manipulations with substrates in the extracellular environment influence the function in mitochondria.

### HYPOTHESIS

ICU-acquired weakness is associated with impaired mitochondrial function in an ex vivo model of human skeletal muscle.

# 3 MATERIALS AND METHODS

## 3.1 Patient characteristics

- *Control subjects.* For all studies, control muscle biopsy samples were obtained from metabolically healthy patients undergoing elective hip replacement surgery indicated for degenerative disease. We excluded patients with serious mobility impairment using compensatory aids or wheelchairs with the exception of walking stick. Next we excluded patients with neuromuscular disorders in their medical history and patients with diabetes mellitus or with another diagnosed endocrinopathy.

- *Critically ill patients.* All patients who were receiving mechanical ventilation for more than 2 weeks were screened for eligibility. Out of 22 eligible ICU-patients approached, only 8 consented for muscle biopsy. Detailed characteristics of study subjects are given in table 1. ***Inclusion criteria:*** age > 18 years, invasive mechanical ventilation for > or = 2 weeks, MRC score <48 points (41) of muscle weakness, any surgical or other invasive procedure requiring anaesthesia is necessary.

| No | Diagnosis | Sex | Age | APACHE II | Biopsy Day | MRC Score | Survived |
|----|-----------|-----|-----|-----------|------------|-----------|----------|
| 1 | Septic shock, bronchopneumonia | M | 70 | 22 | 15 | 20 | N |
| 2 | Aspiration pneumonia | M | 80 | 15 | 29 | 23 | Y |
| 3 | Sepsis | M | 60 | 31 | 40 | 25 | N |
| 4 | Cardiogenic shock | F | 65 | 27 | 41 | 4 | Y |
| 5 | CHF + CAP | F | 68 | 10 | 27 | 8 | Y |
| 6 | Chest trauma + HAP | M | 62 | 14 | 17 | 18 | Y |
| 7 | CABG, GI bleed | M | 68 | 23 | 25 | 16 | Y |
| 8 | CAP | F | 60 | 15 | 30 | 23 | Y |
| Mean±SD | | - | 67±7 | 20±7 | 28±9 | 17±8 | - |

**Table 1: Study subject characteristics;** *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.*

### 3.2 Skeletal muscle tissue homogenate

Biopsies of vastus lateralis muscle were obtained by an open surgical technique (∼500-1000 mg) in controls or by 5mm Bergstrom needle (42) in critically ill, collected into 5 mL of ice-cold Biopsy Preservation Solution, stored on ice and processed for muscle homogenate within 10 minutes. All steps of the homogenate preparation were performed on ice. Connective tissue, fat and blood vessels were carefully 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/100mg of muscle Ww, to obtain 10% homogenate), muscle fibres were homogenised by 4 – 6 strokes in Elvhjem-Potter teflon/glass homogeniser. Whole procedure took about 10 minutes. Thereafter, homogenate has been filtered through cheesecloth.

#### 3.2.1 High-resolution respirometry on skeletal muscle homogenates

High-resolution respirometry (HRR) uses polarographic measurement of oxygen consumption by a Clark electrode. It has recently been adapted to tissue homogenates (43), (44). We performed 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 in respirometer Oxygraph 2K (Oroboros Instruments, Innsbruck, Austria). Two assays were performed in parallel (45) in 2 chambers of the respirometer by serial addition of substrates and inhibitors by a Hamilton syringes. Oxygen concentration and flux were simultaneously recorded and analyzed by Dat lab software. Oxygen solubility factor used for calibration was 0.93 for K medium and 0.87 for MAS, respectively (46). Average initial O2 concentration was 210 - 220 nmol/ml.

***a) Analysis of global mitochondrial functional indices*** was performed in sequential steps (final concentrations in respirometry chamber are given in brackets): malate (2,5mM) + glutamate (15mM) => ADP (1mM) => cyt c (10μM) => succinate (10mM) => oligomycin (1μM) => FCCP (0.7μM) => AA (4μM). Non-mitochondrial respiration was $O_2$ consumption measured after addition of AA and subtracted from other values. Capacity of oxidative phosphorylation (OXPHOS, or 3p respiration) was $O_2$ consumption rate when substrates for both Complexes I (malate,glutamate) and II (succinate), abundant ADP and cyt c were present. 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. Additionally, the addition of cyt c allows for testing preservation of outer mitochondrial membrane

integrity during homogenisation, with value <20% is considered acceptable (47). In our subjects it was 13±6% in ICU and 11±8% in control patients.

***b) Functional analysis of individual respiratory complexes*** was performed using sequential addition of: malate (2.5mM) and glutamate (15mM) => ADP (1mM) => cyt c (10μM) => rotenone (3μM) => succinate (10mM) => malonate (5mM) => glycerol-3-phosphate (5mM) => AA (4μM) => ascorbic acid (10mM) and TMPD (200μM) => KCN (1mM). Complex I activity was calculated as the decrease in $O_2$ consumption after its inhibitor rotenone, Complex II activity as a decrease after addition of malonate. Complex III activity was determined as an increase of $O_2$ 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 $O_2$ consumption after addition of Complex IV substrates ascorbate/TMPD after Complex III had been inhibited by AA.

Finally, lysates from all samples were frozen to -80°C for later analysis of CS activity and protein content assay.

### 3.2.2 Western blotting

We performed western blotting to determine the relative content of mitochondrial proteins in skeletal muscle homogenate from study subject and controls respectively. Samples containing 6 μg of proteins were mixed with sample buffer and denatured by heating at 45°C for 15 min and western blotting were performed as described previously (48).

### 3.2.3 Spectrophotometric analysis

To validate our results from HRR, we performed classical spectrophotometry to assess the individual activities of respiratory complexes by a different technique (49).

### 3.3 Human skeletal muscle cell culture

Muscle biopsies were obtained as described previously and transported in 5 ml of ice-cold Dulbeco´s Modified Eagle Medium (DMEM) with low glucose (5.56mM), supplemented with glutamine (final 0.5mM), penicillin-streptomycin (P/S) solution (100μg/ml) and fungizone (250μg/ml) and processed immediately for satellite cell isolation (50) with a few modifications (51). Cells were grown on Petri dish in basal growth medium containing DMEM with low glucose and the media were changed every 2 or 3 days until cells reached ~80%–90% confluence.

At 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. At day 1, either experiment was conducted on myoblasts or growth medium was switched to a differentiation medium, which was exchanged every 2-3 days and cells were differentiated for 7 days prior to experimentation.

### 3.3.1 Immunofluorescence staining and cell viability

Desmin immunofluorescence staining was used to verify purity of the cell line (52). Cell viability was measured using the CellTiter96 MTS assay (Promega, Madison, WI) as previously described (53) on myoblasts from primary culture at baseline and then as required (e.g. on myoblasts from primary culture after exposure to a range of glutamine concentrations).

### 3.3.2 Glutamine

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 1). Human myoblasts (n=9) were cultured in basal growth medium supplemented from day 0 with glutamine. Cells were divided into six groups according to final glutamine concentration in medium. Six different glutamine concentrations were studied: 0, 100, 200, 300, 500 or 5000µM respectively. Cells were treated with trypsin, manually counted, and reseeded at days 5, 10, 15 and 20, and then proliferation curves were compiled. Next, a subset of myoblasts was reseeded 20000/well at day 15 into a 24-well gelatin-coated plate (Seahorse Biosciences, Billerica, MA) and differentiated 7 days prior to experimentation. At day 20, a second subset of myoblasts was seeded into another Seahorse plate. In both Seahorse plates, cells were continually exposed to six different glutamine concentrations. On day 21, the bioenergetic profile measurement was 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.
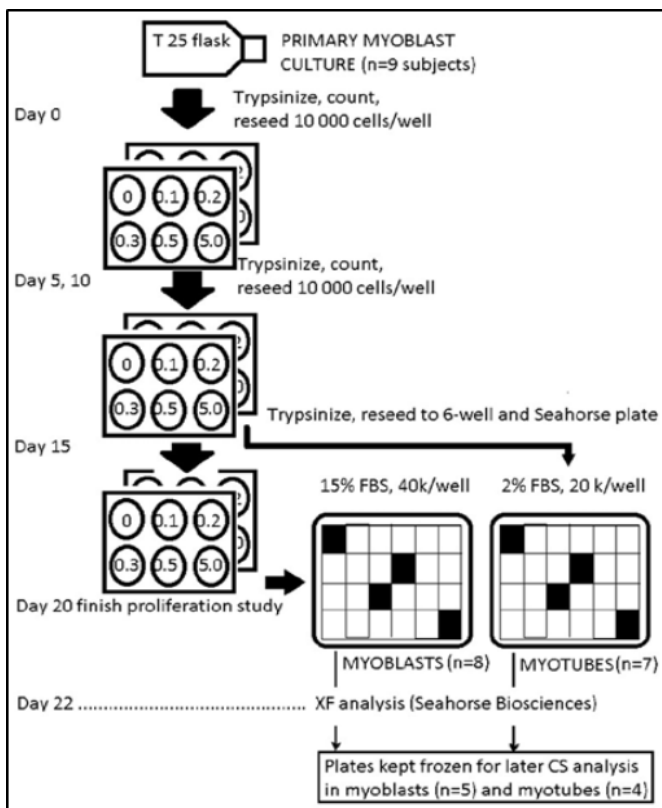
**Figure1:** *Schematic representation of the study design. CS: citrate synthase; FBS: fetal bovine serum.* (51)

### 3.3.3    Extracellular Flux Measurement

The XF Extracellular Analyser uses fluorescent sensors to measure real-time extracellular fluxes: oxygen consumption rate (OCR) and extracellular acidification rate (ECAR). The measurement is done above a layer of seeded cells in the bottom of a well of a customized microplate (54, 55). The measurement is performed at baseline (phase 1) and can be repeated every few minutes. Injector ports can deliver any combination of up to 4 substrates or inhibitors. The dynamics of extracellular fluxes then allows assessing indices of mitochondrial function.

## a) Bioenergetic profile experiment (Stress test)

We performed in each subject the standard bioenergetic profile experiment to assess 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 by addition of three defined compounds: an ATPase inhibitor oligomycin (1µM, phase 2), an inner membrane uncoupler FCCP (1µM, phase 3) and a complex III inhibitor antimycin A (4 µM, phase 4). Oxygen consumption after exposure to antimycin A is considered non-mitochondrial. 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). To reflect possible differences of cell number or mitochondrial content, we normalized basal OCR to the activity of citrate synthase (CS).
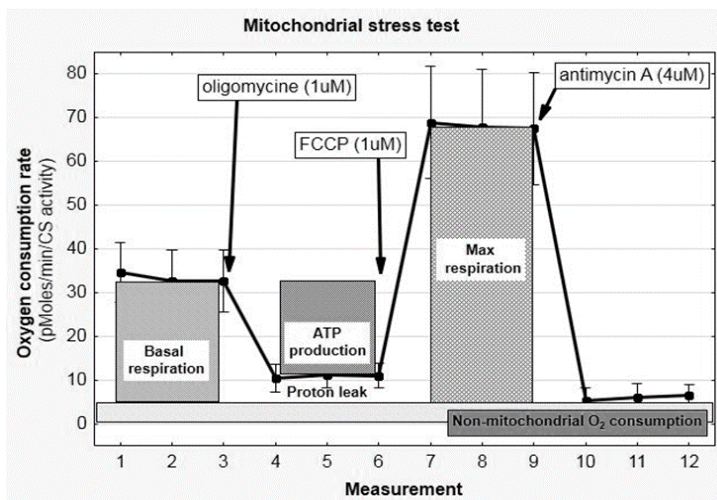


**Figure2:** *Bioenergetic profile experiment in ICUAW myotubes, pooled data from all subjects were used to construct this graph (means, 95% conf. int.)*

## b) Fatty acid oxidation assay

Fatty acid oxidation assay (FAO) performed using the XF Analyser detects changes in oxidative respiration resulting from the exogenous addition of fatty acids to muscle cells in culture in nearly real-time (55). FAO

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assay was performed on myotubes using 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 addition of etomoxir.

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.

### 3.3.4    Extracellular environment manipulation

In a subgroup ICUAW (n=4) and controls (n=4) myoblasts were differentiated into myotubes for 7 days and thereafter was the differentiation medium exchanged for six various exposition media in tri-to tetraplicates (see Table 2). Exposition lasted from day 8 to day 13. On the day 13, the stress test was performed.

| 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) |

**Table 2: Exposition to FFA – characteristics of experimental groups**; medium A (with glucose, without FFA), medium B (medium A with galactose, 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)

# 4    RESULTS and DISCUSSION

None of the existing methods to explore mitochondrial function was able to help us to answer research questions outlined above. We first adopted high resolution respirometry to homogenates of human skeletal muscle and validated this method against isolated muscle mitochondria. In addition, we adopted the protocol of extracellular flux analysis for the use in human myotubes.

## 4.1    HRR on human skeletal muscle homogenate

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. In order to avoid limitations of oxygen flux we maintained $O_2$ concentration above 90nmol/ml.

We excluded unintended influence of muscle biopsy technique (open surgery vs. needle biopsy). We did not find 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).

When homogenates (HOM) which had to wait 60-90min on ice for mitochondria to be prepared (56), were directly compared with mitochondria (MIT) isolated from the same subjects (n=5), they clearly showed signs of mitochondrial damage. However, on freshly prepared homogenate (HOM1) these signs were absent, when the same experiment was repeated (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).

Comparing with the standard technique of respirometry in isolated mitochondria, we have shown that HRR in muscle homogenate is simpler to perform and more robust, and additionally, we can study mitochondria in cytosolic context. Homogenisation causes only minor damage to outer

mitochondrial membrane and respiratory complexes and the results are identical regardless whether the sample was obtained by needle or by open surgical technique. We have shown that in order to get reliable results, 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.

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

We adopted the protocol of extracellular flux analysis for the use in human myotubes and 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.

## 4.3     Glutamine effect on mitochondrial function in muscle cells

Our group was the first to provide the evidence that glutamine can cause mitochondrial uncoupling and impair ATP generation in human skeletal muscle (51). This generates hypothesis that glutamine effect on mitochondrial metabolism may have contributed to disappointing clinical outcomes seen with aggressive glutamine supplementation (26,57).

Myoblast proliferation rate was maximal and exponential at 0.3mM of glutamine, which is close to the concentration in patients with protracted critical illness in the absence of glutamine supplementation (58). Surprisingly, supra-physiological concentrations of glutamine (5000 µM) as recommended for in vitro cell cultures (59) including human myoblasts (60) do not bring any additional benefit in terms of myoblast proliferation.

The most interesting finding is the uncoupling effect of high doses of glutamine whereas another global indices of mitochondrial function are not influenced. Myotubes cultured at 0.2–0.3mM glutamine used 80%–90% of basal OCR for ATP synthesis, while we observed increased mitochondrial uncoupling to ~30%–40% at both extremes of studied concentrations.

Glutamine - induced uncoupling may increase substrate metabolism and energy expenditure and glutamine supplementation in critically ill patients has been shown to increase insulin-mediated glucose disposal (61) and fat oxidation (62). However mitochondrial uncoupling may also be a result of an uncontrolled leak resulting from glutamine-induced mitochondrial damage, as possible mitochondrial toxicity of glutamine was previously reported by Groening et al (63).

If the mitochondrial uncoupling is due to increased nonspecific proton leak or controlled leak through UCP is not clear from our data and needs to be explored in further studies. If "mitochondrial toxicity" of glutamine is confirmed, this would shed new light onto recent data from large randomized controlled trials on glutamine supplementation. 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.

## 4.4    Mitochondrial function in muscle of patients with ICUAW

This is the first study to demonstrate mitochondrial dysfunction in skeletal muscle of patients with protracted critical illness. First of all, we observed an overall ~50% reduced capacity to synthetize ATP by aerobic phosphorylation (OXPHOS). Using HRR, 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 (Table 3).

Pertaining to the relation between the capacity of OXPHOS and concentrations of individual respiratory complexes we found that OXPHOS and depletion of Complex IV are strongly correlated. Complex III was also depleted, unlike complexes II and V. It is the concentration of the depleted complex IV that was limiting for the mitochondrial function, in keeping with data from Levy (64), 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.

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. Even though CS activity is widely used as a marker of mitochondrial content (32,65,66), it may become a subject of oxidative damage (67) 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.

| 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 3: high-resolution respirometry in homogenates; d**ata presented as median (interquartile range), p value as per Mann–Whitney U test.

Next, we studied functional capacities of individual complexes. Despite different methodology (HRR vs spectrophotometry) both techniques gave very similar results: we found no differences in the functionality of Complex I, which is known to be depleted in the acute phase of critical illness (32), 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 significantly increased functional capacity of complexes II and III/GPDH in the critically ill as compared to control subjects (threefold and twofold respectively, p <0.01).

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 (32) and upregulation of complex II at a later stage can be a compensatory response or an attempt to bypass dysfunctional complex I.

It has been shown that GLUT-4 dependent transport is dysfunctional in patients with ICUAW (68) and pyruvate dehydrogenase is inhibited (69). Skeletal muscle in protracted critical illness may consequently suffer from starvation of carbohydrate-derived substrate for citric acid cycle. On the contrary, free fatty acids are elevated in critically ill (62, 70) and intracellular lipid droplets accumulate early in diaphragmatic and biceps muscle in brain-dead donors (71).

Relative up-regulation of Complex II in the context of mitochondrial dysfunction may thus represent an adaptive response to insulin resistance (72) and preferential oxidation of lipids over carbohydrates. Interestingly, according to Mracek et al (73) glycerol requires respiratory complexes distal to Complex I to be converted to glyceraldehyde-3-phosphate, 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.

Last but not least, in the follow up study we used a well-established technique of extracellular flux analysis of cultured myotubes and studied the capacity of skeletal muscle cells to oxidise fatty acids in patients with ICUAW.

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

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 resulted in a corresponding increase of respiratory chain capacity in ICUAW myotubes, whilst we were not able to detect similar effect on the myotubes from healthy controls.

This finding may represent a functional adaptation of remaining mitochondria in mitochondria-depleted skeletal muscle to insulin resistance (74). In turn intracellular glucose starvation forces muscle to use an alternative energy substrate. Free fatty acids are elevated in plasma of critically ill (62,70) and may serve as an alternative energy source. The fact that 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.

Taken together, we have shown in an *in vitro* model of skeletal muscle of patients with ICUAW there is a trend to 55% reduction of mitochondrial content. After adjustment to mitochondrial depletion, there is no difference in mitochondrial functional indices, but a strong trend to 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.

# 5    CONCLUSION

- 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 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 accounting for mitochondrial depletion, 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 similar trend to reduction of mitochondrial content (non-significant, p=0.051) and no difference in mitochondrial functional indices comparing to skeletal muscle tissue homogenates.
- We found a significant increment of capacity to oxidise fatty acids in ICUAW myotubes compared to 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.
- 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 rhGH 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 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 HRR of human skeletal muscle tissue homogenates and adopted protocol of extracellular flux analysis for the use in human myotubes.

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# 7     ANNEXES

## I.     Publications with IF that formed the basis of the Thesis

**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. JPEN J Parenter Enteral Nutr. Forthcoming 2016. ISSN 0148-607.     **IF 3.151**

**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**

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