Clinical Study Protocol

Project No: NU21J-06-00078

Skeletal muscle regeneration in survivors of critical illness: How to prevent satellite cell failure? (SATELLITE)

Short title: Satellite cell physiology in critical illness

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Investigator(s)	*See separate listing*
Protocol Date	*20th December 2022*
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NCT	*(pending)*

SIGNATURE PAGE

Principle investigation declaration:

I hereby declare that this clinical trial will be conducted in accordance with the principles of Good Clinical Practice and Czech law. This study is exploratory in nature and no investigational products (shall it be drugs or medical devices) are tested in this trial. I also declare, that this study has been investigator initiated and supported exclusively from Czech Health Research Council, project No NU21J-06-00078.

Dr Adela Krajcova

Signature: 

Date: 20.12.2022

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LIST OF ABBREVIATIONS

AE Adverse Event
ICU Intensive care unit
ICUAW Intensive Care Acquired Weakness
ECOG Eastern Cooperative Oncology Group performance status
eCRF Electronic Care Report Form
FiO_2 Fraction of oxygen in inspired air
F-up Follow up
LFT Liver function tests MV
Mechanical ventilation
PEEP Positive end-expiratory pressure
PIP Peak inspiratory pressure
pCO_2 Partial pressure of carbon dioxide in arterial blood
pO_2 Partial pressure of oxygen in arterial blood
SAE Serious Adverse Event
SOFA Sequential Organ Failure Assessment

STUDY PERSONNEL

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NARRATIVE ABSTRACT

Modern intensive care enables patients to survive insults that in the past would not have been survivable. Nonetheless, increased number of survivors suffer from failed functional outcomes associated with prolonged muscle weakness and fatiguability. Whilst alterations of skeletal muscle biology that occur during critical illness slowly disappear over the period of months, muscle weakness remains. Recent pilot studies have shown that muscle weakness is associated with loss and alteration of satellite skeletal muscle cells, which are supposed to proliferate and repair damaged muscle tissue. The pathogenesis of this phenomenon has not been fully understood. In this project, we will study function and structure of satellite cells and their organelles (particularly mitochondria) using both classical bioenergetics and advanced microscopic techniques. Satellite cells will be isolated from biopsies taken from critically ill patients with developed muscle weakness in the acute and protracted phase of a disease and after 6 months. In time points, an ultrasound examination of muscle mass will be performed, and metabolism will be assessed using insulin clamps and calorimetry. In a series of in vitro experiments, we will test also effect of nutritional and anabolic factors and drugs, commonly used in ICU, on satellite cells. In a control arm, cells will be isolated from skeletal muscle of volunteers undergoing elective hip replacement surgery. Results of this study could significantly contribute to understanding of mechanisms leading to ICU acquired muscle weakness and to identify therapeutic strategy in future.

Key words: intensive care unit-acquired weakness; critically ill; skeletal muscle regeneration; satellite cells

BACKGROUND

Skeletal muscle weakness is a frequent complication of critical illness associated with an increased morbidity and mortality [1]. Altered muscle strength and functional ability occur mostly in patients with sepsis or extensive inflammatory response who require prolonged duration of mechanical ventilation and hospital stay[2]. In patients surviving intensive care, muscle weakness remains a long-term complication leading to significantly decreased quality of life[3,4]. The mechanisms underlying this phenomenon have been extensively studied in both animal models and critically ill patients. Proteolytic muscle degradation, electrical alterations, and excitation-contraction decoupling are well known factors contributing to development of muscle weakness in the acute phase[5]. Even more importantly, recent studies have shown that failed long-term functional outcome in critically ill is associated with decreased satellite cell content[5]. Satellite cells are localized beneath the basal lamina surrounding each multinucleated myofiber[6]. In adult muscle, satellite cells are normally quiescent, but they can be recruited rapidly after a muscle damage, providing the muscle with an ability to regenerate[7]. During injury, satellite cells are activated from the quiescent state (G0 phase) and enter the cell cycle (G1 phase)[8]. Most activated satellite cells then proliferate and are referred to as “myoblasts”. Myoblasts subsequently differentiate and express myogenic factor which allows them to fuse between themselves into multinuclear cylindrical myotubes and with the existing fibres to regenerate the damaged muscle tissue[6,8]. Some satellite cells return to the G0 state to replenish the pool of quiescent skeletal muscle cells[6,8]. These processes are regulated by key transcriptional factors such as Pax7 and myogenic regulatory factors[9,10]. Myotubes are highly metabolically active, rely on oxidative phosphorylation in a rich population of mitochondria which are structured in organized networks[11]. In contrast, myoblasts are more dependent on glycolysis and contain less mitochondria. Shortly after muscle injury, myogenic differentiation occurs[11] and mitochondrial mass, protein content and number of mitochondrial DNA copy increase[12,13]. Moreover, recent studies have shown that shape of mitochondria is also remodelled during myogenic differentiation[14,15]. Mitochondrial morphology is controlled by balanced processes of fusion and fission machinery[15,16]. Balanced fusion and fission events shape mitochondria to meet metabolic cues and to ensure removal of damaged organelles[16]. Mitochondrial morphology is therefore dramatically changed in response to various metabolic inputs[16] and it is tightly

regulated[15,16]. The morphology of mitochondria is altered in disease: mitochondria can shape into elongated tubules during mild stress and starvation. In contrast, acute severe stress frequently causes a dramatic mitochondrial fragmentation and decreased oxidative phosphorylation[16].

Recent studies performed on animal models have shown that some of the proteins responsible for the processes of fusion and fission are crucial for the proper muscle growth and function of mitochondria in muscle cells[15]. Therefore, alterations in mitochondrial dynamics often play a crucial role in skeletal muscle dysfunction and have been recently extensively studied in several myopathies[17]. Although it has been established that skeletal muscle regeneration parallels altered mitochondrial dynamics and biogenesis, the cells that mediate the remodelling of mitochondrial networks have not been yet thoroughly investigated[18]. Moreover, there is still lack of clear information about mitochondrial dysfunction in skeletal muscle weakness after critical illness.

Our group already adopted a method of satellite cell isolation and culture from human vastus lateralis biopsies and tested nutritional influences[19]. We have found in a pilot experiment that bioenergetic profiles of myotubes from patients with protracted ICU stay differ from healthy control, mostly by mitochondrial mass deficiency and reliance of remaining mitochondria on lipid-derived substrates[20]. In addition, we found out that 4 days of propofol exposure induces respiratory chain alterations (mostly fatty acid oxidation defect)[21], which we later managed to further characterize [22]. Long term significance of these findings to satellite cell ability to repair damaged muscle remains unknown.

STUDY HYPOTHESES AND AIMS

Hypothesis:

Critical illness induces damage to satellite cells in skeletal muscle that later impairs skeletal muscle structural and functional recovery and contributes to persistent weakness and failed functional outcome.

***Aims of the project*:**

1. We will compare structural and functional (incl. bioenergetic) characteristics of satellite cells in acute, protracted and post-ICU phase in critically ill patients vs. control subjects.

2. We will test the hypothesis that structural and/or functional alteration of satellite cells corelate with gross muscle mass and power in survivors of critical illness.

3. We will elucidate factors that influence bioenergetics and mitochondrial morphology of satellite cells (such as extracellular inflammatory milieu, drugs common in ICU as well as nutritional and anabolic factors).

METHODS

General methodological approaches

1. Clinical outcomes will be assessed by objective validated tests such as SF-36 questionnaire for the quality of life assessment, Medical Research Council Score of muscle power and 6 minutes walking test to measure aerobic performance.
2. Metabolic characteristics will be assessed at whole body level by hyperinsulinaemic euglycaemic clamps and at tissue level by vastus lateralis biopsies.
3. We will assess global mitochondrial indices of satellite cells by oxygen consumption measurement (production of ATP, uncoupling of inner mitochondrial membrane, maximal respiratory capacity of respiratory chain, glycolytic capacity and fatty acid oxidation). Simultaneously, we will measure production of reactive oxygen species and mitochondrial membrane potential. The changes will be assessed in various time intervals in the acute, protracted and post-ICU phase of critical illness.
4. We will analyze mitochondrial density, structure and organization of mitochondrial network in satellite cells, length of mitochondrial branches and connections in the acute, protracted and post-ICU phase of critical illness.

Design

Investigator initiated, prospective, controlled, cohort study with physiological end-points

Study subjects

Inclusion Criteria: All of the following must be present:

- Critically ill patients receiving mechanical ventilation, to be enrolled within 72 hours of admission, who are likely to need 7 days or more of ICU stay
- Sudden onset of disease, which can be determined in time (such as trauma, stroke, sudden cardiac arrest etc.)
- Informed consent signed by patient or patient's representative

Exclusion criteria: None of these shall be present

- Unlikely to survive 6 months
- Premorbid downslope functional trajectory or poor performance status (ECOG Gr. 3 or worse) or baseline functional status unknown
- Bleeding disorder (INR≥1.5 or PLT< that would preclude muscle biopsies)
- Known mitochondrial disease
- Endocrine crisis as a reason for admission
- Pregnant women

Control subjects: Elective hip surgery patients with very good to excellent performance status, only limited to joint pain (ECOG 0)

Informed consent procedure

All patients with capacity will be asked to provide a prospective written informed consent (See Appendix 3). For patients without capacity, a deferred consent procedure will take a place. In this case, an independent clinician will review and sign that the patient is lacking capacity and he/she fulfils all criteria to be enrolled to the study. The patient's next of kin will be informed about the study as soon as practical with the aid of information leaflet (Appendix 3). The patient will be asked to provide and sign informed consent as soon as he/she regains the capacity to do so. In case of consent refusal, patient's data will not be used in per-protocol analysis.

Study procedures

Eligible patients will be assessed at baseline, after 7 days and 6 months by clinical tests, metabolic tests and muscle biopsies as shown in table 1.

Test	**Baseline**	**Day 7 or ICU discharge**	**6 months follow up**	**Note: Control subjects**
Enrolment criteria, informed consent	**x**			**x**
Insulin clamps		**x**	**x**	**x**
Muscle biopsy	**x**	**x**	**x**	**X**
Functional performance test			**x**	**x**
Muscle mass by ultrasound	**x**	**x**	**x**	**x**

Table 1: Study procedures

Biopsies. Experimental group (= critically ill): Skeletal muscle samples (70 – 100 mg of muscle tissue) will be obtained from vastus lateralis muscle by needle biopsy technique (Bergström needle). *Control group (= orthopaedic patients):* Biopsies (> 100 mg of muscle tissue) will be taken from vastus lateralis muscle by open technique during surgery in control (hip replacement surgery)patients.

Isolation and cultivation of cells. Skeletal muscle cells will be isolated and cultured as previously described [19] (see **Figure 1**).

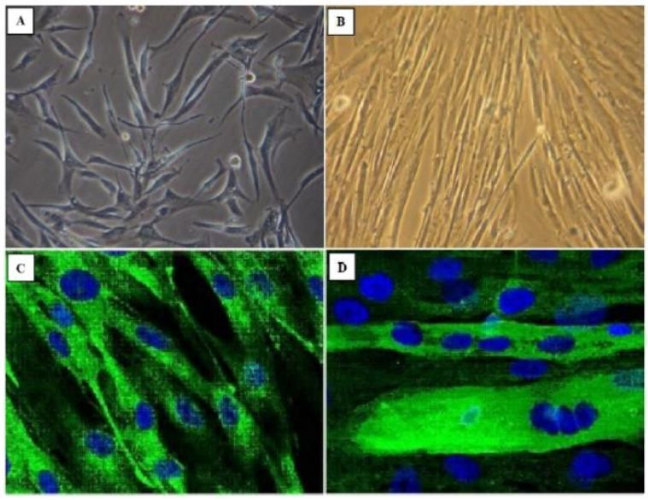

Figure 1. *Primary human skeletal muscle cells. Myoblasts (A) and differentiated myotubes (B) under binocular inverse microscope and appearance of myoblasts (C) and myotubes (D) stained for desmin (AB 907 rabbit anti–desmin polyclonal antibody [Millipore, Billerica, MA, USA]) under confocal laser scanning microscopy. Source: published data of main proponent* [19].

Bioenergetic profile. Mitochondrial metabolism will be assessed in living cells using Extracellular Flux Analyzer (Agilent Technologies Inc.) or high resolution respirometry (Oroboros O2k, Austria)[26], which simultaneously measures oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) with oxygen-sensing fluorophores and pH sensors in a real time. The technology uses a piston to reversibly enclose a small volume above the microlayer of cells seeded in a multi-well plate. During the experiment, oxygen consumption rate (~respiration) and proton excretion (~glycolysis) cause rapid and easily measurable changes in dissolved oxygen concentration and free protons in the microchamber. The changes are monitored at the baseline of experiment and after addition of up to 4 test agents. The technology enables us to determine basal respiration, ATP production, proton leak, maximal respiratory capacity (see **Figure 2**) and glycolysis or respiration linked to individual complexes of respiratory chain. Fatty acid oxidation will be measured by Extracellular Flux Analysis and independently by another method of measurement of radioactively labelled [1-^{14}C] palmitate oxidation using liquid scintillation [21].

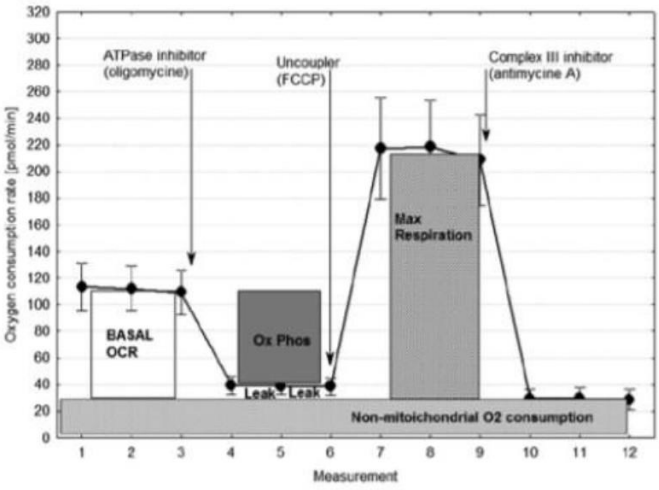

Figure 2. Principle of Extracellular Flux Analysis: oxygen consumption rate (OCR) measured at baseline and after addition of 1) oligomycin = ATP synthase inhibitor to distinguish ATP production and proton leak,

2) carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP) to maximally stimulate respiratory chain and 3) antimycin A and rotenone (AA/rot) to inhibit respiratory chain. Experiments were performed on myoblasts isolated from skeletal muscle (m. vastus lateralis] from orthopedic patients (n = 8).

Source: main author's published data [19].

Mitochondrial structure and membrane potential measurement. In parallel with OCR and ECAR analysis, mitochondrial structure will be determined. These parameters will be visualized and quantified in living cells via fluorescent microscopic tools in high resolution using Confocal Laser Scanning Microscopy with a 63x oil immersion objective (Leica TCS SP5 system, Leica Microsystems). Measurement will be based on accumulation of fluorescent dye inside mitochondria (MitoTracker™ Green staining) which enables to determine number and length of mitochondrial branches. Simultaneously, a positively charged dye tetramethylrhodamine ethyl ester will be used to image active mitochondria with excitation/emission maximum 549/575 nm[24]. Intensity of fluorescence will be normalized to levels measured after MitoTracker™ Green staining (see **Figure 3**).

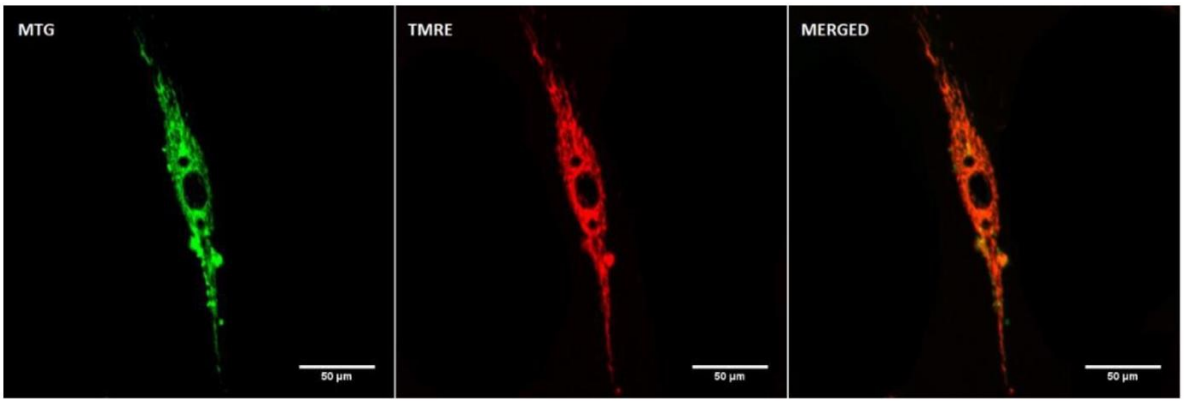

Figure 3. Satellite skeletal muscle cells after staining with MitoTracker™ Green (MTG; left), tetramethylrhodamine ethyl ester (TMRE; in the middle) and after staining of both agents (right). Positively charged dye TMRE (mitochondrial membrane potential dependent) is accumulated only in active mitochondria due to its

relative negative charge. Determination of mitochondrial membrane potential is performed after normalization to MTG fluorescence (mitochondrial membrane potential independent), which covalently binds to mitochondrial proteins by reacting with cysteine residues.

Acquired date will be analyzed using ImageJ™ (MacBiophotonics).

Power analysis and statistical plan

There is no formal primary outcome and hence no formal power analysis can be performed. The so far largest trials in the field have both 11 subjects with complete follow up [5,27] and in our trial we will have at least twice this number. Comparison of morphological and functional indices between the elective hip surgery patients (control cohort) and critically ill patients upon ICU admission will be determined using a Welch's unpaired T-test. Changes of these parameters in time (ie. from ICU admission to 6 months following ICU discharge) will be determined using a linear mixed-effects model with random intercept.

Direct access to source data/documents

All study procedures will be properly recorded in accordance with the rules on medical records. The patient documentation shall include eligibility criteria, record of informed consent procedure and all relevant information obtained during subsequent visits. The documentation must be kept to allow for an additional assessment of possible early and late side effects of the method.

Quality control and quality assurance

To ensure compliance with Good Clinical Practices and all applicable regulatory requirement.

Ethical Considerations

The final study protocol, including the final version of the Informed Consent Form, must be approved or given a favourable opinion in writing by in the Ethics Committee (EC) as appropriate. The Investigator is also responsible for informing the EC of any amendment to the protocol in accordance with local requirements. The investigator is also responsible for providing the EC with reports of any serious side effect of study procedures.

The study will be performed in accordance with the protocol, ethical principles that that have their origin in the Declaration of Helsinki WMA, and are consistent with Good Clinical Practice: Consolidated Guidelines and any applicable local regulation.

The Investigator(s) will ensure that subject (or the legal representative) is given full and adequate oral written information about the nature, purpose, possible risk and benefit of the study. Subject must also be notified that they are free to discontinue from the study any time. The subject (or the legal representative) should be given the opportunity to ask questions and allowed time to consider the information provided.

Voluntary written informed consent will be obtained before any study-related procedures are performed and the Investigator(s) must maintain the original, signed Informed Consent Form.

Data handling and recordkeeping

The team of investigators are allowed to inspect subject charts, study source documents, drug accountability records and other records relative to study conduct. The Investigator must maintain all documentation to the study for a period of 5years after the end of last study subject.

Publication Policy

We follow the authorship criteria established by the international Committee of medical Journal editors. Minimum criteria for authorship credit based on: Substantial contributions to conception and design, acquisition of data or analysis and interpretation of data. Drafting the article or revising it critically for important intellectual content and final approval of version to be published. To be considered, all potential authors must fulfil all the minimum criteria for authorship credit.

Failed functional outcomes in survivors of critical illness not only lead to individual suffering, but also represent a significant burden for the healthcare system and society. Results of our project will lead to identifying factors contributing to this debilitating condition at the level of satellite cells and thereby enable to adopt treatment strategies in ICU and generate hypotheses for trials with patient-centered clinical trials.

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INFORMED CONSENT FORM
for ICU patients or their relatives

Dear Sir or Madam,

our faculty hospital is currently involved in the research studying patients admitted to the intensive care unit (ICU), who developed a complication called "ICU-acquired muscle weakness". Given the fact, that you (or your relative) fulfilled all the inclusion criteria for recruitment in this project, we would like to provide more information about the study.

What is the purpose of research?

Modern intensive care can often save patients' live who would not been able to survive several decades ago. Unfortunately, more than 50 % of these patients, who survive critical illness, develop muscle weakness and fatigue. This problem can take more than months or even years and leads in significantly decreased quality of life. The mechanism of the ICU-acquired muscle weakness remains to be elucidated, but it is well known that muscle loss, changes in muscle metabolism and patients' immobility may play a role. Recent studies are also suggestive of disorders of muscle stem cells (also called "satellite cells") located inside the muscle that, under normal circumstances, have an ability to repair injured muscle tissue. Those patients, who survived critical illness, but developed muscle weakness, are suggested to have an abnormalities and dysfunction in these satellite cells. In our project, we focus on functional and structural changes of these cells and study also their sensitivity to the various nutritional and anabolic factors (e.g. insulin).

How will the research be conducted?

Income and outcome examinations (until 48 hours after ICU admission and subsequently after 7 days after recruitment). First, about 20 mL of blood will be taken from previously inserted venous catheter. A diagnostic ultrasound of your quadriceps femoris muscle will be performed. Then, a muscle tissue will be taken from your lateral part of quadriceps femoris muscle by a small needle biopsy technique. Upon a local anaesthesia (if needed), the muscle specimen will be removed (about 0.2 to 0.3 grams of tissue). Subsequently, we will quantify how sensitive you are to insulin (by glucose and insulin infusion administration whilst maintaining a normal glucose blood level). The levels of glucose are is measured in 5 min intervals from blood drop from inserted catheter. All above mentioned blood samples are taken from previously inserted catheter in the patients. In some cases, there is a need to insert one more peripheral venous catheter in your forearm.

Note: At those patients, who needed life-threatening acute surgery, the biopsy could be performed in operation room based on the consent from independent medical doctor. As soon as the patient becomes consciousness, and then in 7 days intervals or at ICU discharge, muscle strength will be assessed by physiotherapeutic. After 6 months, the study participant will be contacted on phone and asked for about 10-15 min interview to assess quality of life.

Are there any risks involved?

Muscle biopsy is a common technique where the risks (of bleeding or infection) are minimal (< 1 %). However, patients with an increased risk of bleeding are excluded from the study. During the application of local anaesthesia, there is a risk of allergic reaction. At some patients, the small leakage of fluid from the subcutaneous tissue can occur at the location of the wound. From a long-term perspective, a small scar (several milimeters) can remain at the site of biopsy.

Additional information:

Your genetic information (DNA) will not be analysed or archived in any way during the study.
When you consent this informed consent form, research will be conducted in Petri dish – thus, you will not be administered with any special medication/no special procedures in your treatment will be performed.

<u>Your possible participation in the study is completely voluntary and if you refuse it, it will have no effect on your further treatment. You can also cancel your participation in the study at any time and without giving reasons.</u>

The attending physician will give you any additional information and answer any further questions.

I hereby declare that I have read this informed consent form and all my questions have been answered. I further declare that I believe that Mrs./Mr. …….. would agree to participate in the study.

Date: …….. Name: …….. Relationship: ……. Signature: ……..

The discussion about participation in the study was guided by this person: ……..……

Continued informed consent of the patient:

I hereby declare that I have read this informed consent form and all my questions have been answered. Based on this:

☐ **I GIVE my consent to participate in the study.**

☐ **I DO NOT GIVE my consent to participate in the study.**

..... ….....
signature of the patient signature of the attending physician

..... ….....
signature of the witness, if the patient gave his consent orally date
Reason why he couldn't sign:
X muscle weakness
X other: .....

INFORMED CONSENT FORM
for orthopaedic patients

Dear Sir or Madam,

you have been asked to participate in the research studying patients admitted to the intensive care unit (ICU), who developed a complication called "ICU-acquired muscle weakness". These patients often have muscle fatigue and are supposed to have metabolic changes in their muscle. During their stay in ICU, they are biopted with a small needle to obtain muscle tissue specimen from quadriceps femoris muscle. The metabolism of their muscle is then studied in comparison with those patients, who have no muscle abnormalities or diseases. Given that you are planning to undergo elective hip replacement surgery, there is the opportunity to obtain the muscle sample from you by a simple, safe and easy procedure. Therefore, we would like to ask for your consent to this muscle sample removal.

What is the purpose of research?

Modern intensive care can often save patients' live who would not been able to survive several decades ago. Unfortunately, more than 50 % of these patients, who survive critical illness, develop muscle weakness and fatigue. This problem can take more than months or even years and leads in significantly decreased quality of life. The mechanism of the ICU-acquired muscle weakness remains to be elucidated, but it is well known that muscle loss, changes in muscle metabolism and patients' immobility may play a role. Recent studies are also suggestive of disorders of muscle stem cells (also called "satellite cells") located inside the muscle that, under normal circumstances, have an ability to repair injured muscle tissue. Those patients, who survived critical illness, but developed muscle weakness, are suggested to have an abnormalities and dysfunction in these satellite cells. In our project, we focus on functional and structural changes of these cells and study also their sensitivity to

the various nutritional and anabolic factors (e.g. insulin).

How will the research be conducted?

Satellite cells can be obtained from a tissue sample which is taken by muscle biopsy. A particle (5x5 mm size) is taken from the lateral part of the quadriceps muscle under general anaesthesia. Muscle biopsy is performed during surgery. The surgeon reveals the hip joint during surgery so that the muscle is exposed and it is therefore easy to remove a small particle from it with a special needle. The technique of the surgery does not change at all. The process of muscle biopsy will take up to 1 min. After the biopsy, there will be no differences in the next steps and routine procedures.
Subsequently, the tissue sample will be transferred to the university lab, where the satellite cells will be isolated and cultured in the growth medium supplemented with nutrients. The research will take place in Petri dish, perform structural and functional measurements and then the cells will be destroyed. At the same time, we will take your blood sample of about 20 mL (to analyse e.g. insulin levels, growth factors). We will also quantify how sensitive you are to insulin (by glucose and insulin infusion administration whilst maintaining a normal glucose blood level). In addition, diagnostic ultrasound of your quadriceps femoris muscle will be performed.

Are there any risks involved?

Muscle biopsy is a common technique where the risks are minimal. Theoretically – as with any surgical wound – there is a risk of bleeding and infection. However, patients with an increased risk of bleeding are excluded from the study.

Additional information:

Your genetic information (DNA) will not be analysed or archived in any way during the study.

When you consent this informed consent form, research will be conducted in Petri dish – thus, you will not be administered with any special medication/no special procedures in your treatment will be performed.

Your possible participation in the study is completely voluntary and if you refuse it, it will have no effect on your further treatment. You can also cancel your participation in the study at any time and without giving reasons.

The attending physician will give you any additional information and answer any further questions.

I hereby declare that I have read this informed consent form and all my questions have been answered. Based on this, I GIVE my consent to participate in the study.

..... ….....
signature of the patient signature of the attending physician

..... ….....
signature of the witness, if the patient gave his consent orally date
Reason why he couldn't sign:
X muscle weakness
X other: .....