Charles University in Prague
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Doctoral thesis

Insulin resistance in the critically ill and its treatment options

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Keywords
Insulin resistance; Functional proprioceptive stimulation; Skeletal muscle bioenergetics; Burn injury; Hypermetabolism; Muscle wasting

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 at the Department of Burns and its laboratory, Third Faculty of Medicine, Charles University, Prague, during my Ph.D. studies.

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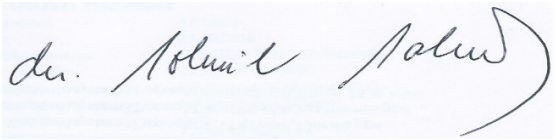

Prague, 09.09.2024

MUDr. Bohumil Bakalář

CONFLICT OF INTEREST STATEMENT

I have no competing financial relationships with any organisation 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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ABBREVIATIONS and ACRONYMS

AA	antimycin A
AcCoA	acetyl coenzyme A
ADP	adenosine diphosphate
AGEs	advanced glycation end products
Akt	serine/threonine kinase
APACHE II	Acute Physiology and Chronic Health Evaluation II
ATP	adenosine triphosphate
CIM	critical illness polymyopathy
CIP	critical illness polyneuropathy
COA	coenzyme A
DAMPS	danger-associated molecular patterns
DHEA	dehydroepiandrosterone
DHEAS	dehydroepiandrosterone sulphate
DNA	deoxyribonucleic acid
ESICM	European Society of Intensive Care Medicine
ESPEN	European Society for Clinical Nutrition and Metabolism
ET	electron transfer
FCCP	carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone)
FESCE	functional electrical stimulation-assisted cycle ergometry
FFA	free fatty acids
GH	growth hormone
GLUT	glucose transporters
GNG	gluconeogenesis
HDL	high density lipoprotein
HHR	high-resolution respirometry
ICU	intensive care unit
ICUAW	intensive care unit acquired weakness
IGFBP-1	insulin like growth factor binding protein 1
IGFBP-3	insulin like growth factor binding protein 3
IGF1	insulin-like growth factor 1
IL	interleukin
IR	insulin resistance
IRS	insulin receptor substrate
LDL	low density lipoprotein
MALA	metformin-associated lactic acidosis
MAPK	mitogen-activated protein kinases
mtDNA	mitochondrial deoxyribonucleic acid
mTOR	mammalian target of rapamycin
MuRF1	muscle RING-finger protein-1
NF-κB	nuclear factor κB
NMES	neuromuscular electrical stimulation
NTIS	non-thyroidal illness syndrome

OXPHOS	activated oxidative phosphorylation capacity
PAMPs	pathogen-associated molecular patterns
PEPCK	phosphoenolpyruvate carboxykinase
PI3K	phosphatidyl inositol 3-kinase
PICS	Persistent Inflammation, Immunosuppression, and Catabolism Syndrome
PH	pleckstrin homology
PPARα	peroxisome proliferator-activated receptor alpha
PRRs	pattern recognition receptors
RAGE	advanced glycation end products receptor
RCTs	randomized controlled trials
RING	really interesting new gene
PKB	protein kinase B
RCR	respiratory control ratio
RONS	reactive oxygen and nitrogen species
ROS	reactive oxygen species
ROX	residual oxygen consumption
rT3	reverse triiodothyronine
SGLTs	sodium glucose co-transporters
T3	triiodothyronine
T4	thyroxine
TAG	plasmatic level of triacylglycerols
TGFβ	transforming growth factor β
TMPD	tetramethylphenylenediamine
TNFα	tumour necrosis factor α
TSH	thyroid stimulating hormone
UCP1	uncoupling protein 1
V_{Max}	Maximal volume of utilised O_2
Ww	wet weight

SUMMARY

The purpose of this study was to evaluate the feasibility of functional proprioceptive stimulation and its impact on energy metabolism, insulin sensitivity, and skeletal muscle bioenergetics in adult patients with deep burns covering 30% or more of their total body surface area.

Patients received two 30-minute sessions of functional proprioceptive stimulation daily in addition to routine physical therapy or physical activity alone. Following the initial period, an additional two weeks were observed without intervention.

During the first 40 days after injury, patients' energy metabolism was measured using indirect calorimetry and nitrogen balance. Insulin sensitivity was evaluated through hyperinsulinaemic euglycaemic clamp, and insulin resistance was calculated using HOMA on days 10, 25, and 40 after injury. Skeletal muscle biopsies were taken on these days to assess mitochondrial activity and muscle cell bioenergetics.

Intervention with functional proprioceptive stimulation had no effect on energy expenditure, nitrogen balance, or insulin sensitivity. However, at the cellular level, it increased the ability of mitochondria to synthesize ATP by aerobic phosphorylation.

To date, the use of functional proprioceptive stimulation in patients with thermal trauma has not been published. This study demonstrates that this rehabilitation method was feasible and well-tolerated in burn patients. Compared to physical therapy alone, the twice-daily 30-minute moderate-intensity vibration mode in addition to usual physical therapy in patients with extensive burns did not change energy expenditure, insulin sensitivity, nitrogen balance, or energy substrate oxidation. At cellular level, the intervention improves the capacity of aerobic phosphorylation in skeletal muscle mitochondria.

Clinical effects remain to be demonstrated in adequately powered trials.

SOUHRN

Cílem této studie bylo posoudit proveditelnost funkční proprioceptivní stimulace a její vliv na energetický metabolismus, citlivost na inzulin a bioenergetiku kosterního svalstva u pacientů s rozsáhlými popáleninami. Studie byla provedena u dospělých pacientů s hlubokými popáleninami na 30 nebo více % celkového povrchu těla. Pacienti podstoupili dvě 30minutové aplikace funkční proprioceptivní stimulací denně jako doplněk k rutinní fyzikální terapii nebo k samotné fyzické aktivitě. Poté následovaly dalších 2 týdny bez intervence.

U nemocných byly v prvních 40 dnech po úrazu měřeny energetický metabolismus metodou indirektní kalorimetrie a dusíková bilance. 10., 25. a 40. den po úrazu byla hodnocena citlivost k inzulinu pomocí hyperinzulinového euglykemického clampu, a vypočtena rezistence na inzulin metodikou HOMA. V tyto dny byly také odebrány biopsie kosterního svalu ke zhodnocení mitochondriální aktivity a bioenergetiky svalové buňky.

Intervence funkční proprioceptivní stimulací neměla vliv na energetický výdej, dusíkovou bilanci ani citlivost na inzulin. Na buněčné úrovni však zvýšila schopnost mitochondrií syntetizovat ATP aerobní fosforylací.

U pacientů s termickým traumatem nebylo dosud použití funkční proprioceptivní stimulace publikováno. Studie prokázala, že tato rehabilitační přístrojová metoda byla u popálených proveditelná a dobře snášená. V režimu dvakrát denně 30 min. střední intenzitou vibrací neměla vliv na energetický metabolismus nebo citlivost na inzulin, ale významně zvyšovala syntézu ATP v kosterním myocytu.

Klinické účinky je třeba ještě prokázat ve studiích s dostatečnou kapacitou.

1 LITERARY INTRODUCTION

Hyperglycaemia in haemorrhagic shock was initially documented by Claude Bernard in 1877. Today, this manifestation in patients receiving intensive care is a predictable and regular discovery. Glucose metabolism undergoes changes immediately following a stress insult, such as infection, hypoxemia, circulatory failure, mechanical trauma, or thermal injury. Although the insults may differ, adaptive responses remain fairly consistent. Endocrine response activation immediately after the insult causes hyperglycaemia, insulin resistance and gluconeogenesis. At first, metabolic changes are brought about by an increase in catecholamines, glucagon and cortisol release. Subsequent alterations are primarily regulated by the inflammatory response and its mediators. If the said response extends for a long period (e.g. due to the emergence of secondary insults), the metabolic variations may result in extensive catabolism, which is connected to complications and an unfavourable prognosis.

1.1 Physiology of insulin action

Insulin maintains metabolic and haemodynamic equilibrium simultaneously. It is essential for physiological regulation of blood glucose, protein and lipid metabolism, growth, mitogenesis, cell proliferation and differentiation, reproduction, cognition, mood, learning and memory retention through its numerous functions on insulin target cells such as hepatocytes, myocytes, adipocytes, brain cells and endothelial cells, the primary sites of insulin's actions.

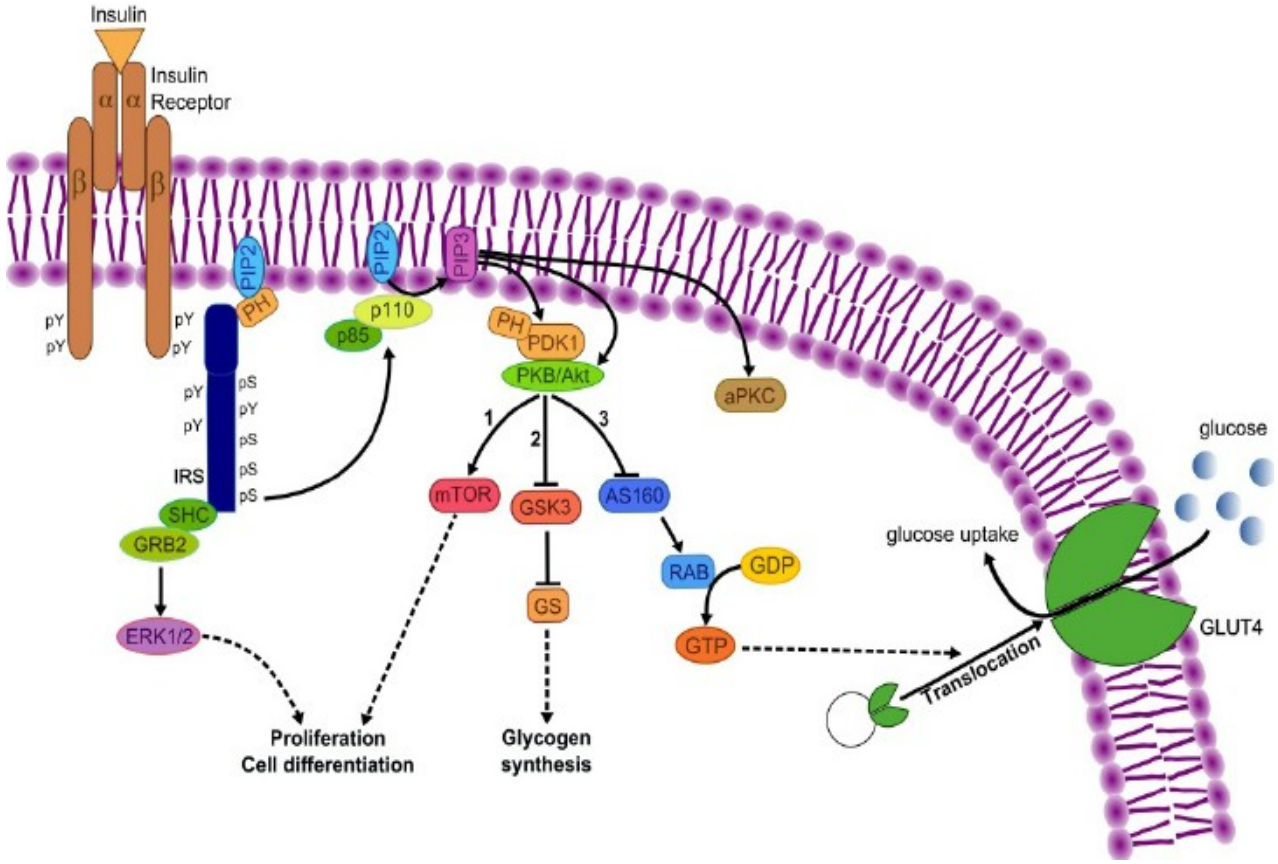
The action of insulin in target tissues is mediated by a complex cellular signalling mechanism, the components of which are present in virtually all cells of the human body. Insulin binds to a specific receptor situated on target cells. The insulin receptor is a member of the family of tyrosine kinase receptors. Binding of insulin results in the dimerization of receptors. The physical proximity of the intracellular parts of the receptors activates the tyrosine kinase, enabling the fused insulin receptor molecules to dephosphorylate each other's tyrosine residues. Specific domains of the insulin receptor that have phosphorylated tyrosine residues create binding sites for adaptor proteins. Adaptor proteins attach various proteins of signalling cascades to the activated receptor. The most significant adaptor proteins are insulin receptor substrates (IRS), particularly

the insulin receptor substrate which includes IRS-1 and 2. The IRS proteins lack catalytic activity and are comprised of a PH (pleckstrin homology) domain (phosphorylated-tyrosine-binding; this domain facilitates IRS binding to the insulin receptor), and a tyrosine-rich section that can bind other proteins when phosphorylated by the insulin receptor tyrosine and thereby transmit the signal forward. During this process, various intracellular proteins are targeted, with the aim of synthesising and activating metabolic enzymes, activating glucose transporters and influencing cell mitogenic activity and apoptosis.

The classical signalling pathway consists of two fundamental branches. The activation of the phosphatidyl inositol 3-kinase (PI3K) pathway is imperative for the metabolic actions of insulin. The process concludes with the relocation of insulin-sensitive glucose transporters (GLUT) from cytoplasmic storage vesicles to the plasma membrane, promoting glucose absorption. The mitogen-activated protein kinases (MAPK) pathway's activation promotes mitogenesis, triggers various nuclear transcription factors, heightening the expression of growth-related proteins and prompting hypothalamic neurons to produce neuropeptides that suppress appetite. The insulin receptor also translocates directly to the nucleus and creates transcriptional complexes, regulating gene expression (1, 2).

The proteoanabolic pathway of insulin action entails proteosynthesis dependent on serine/threonine kinase Akt/protein kinase B (PKB), which subsequently activates the mammalian target of rapamycin (mTOR) kinase. Through the formation of the mTORC1 complex, the mTOR pathway is induced by the insulin-like growth factor 1 (IGF1) and insulin through Akt/PKB or directly by amino acids and exercise, promoting muscle proteosynthesis (3).

Figure 1. Insulin signalling



Adapted from Alba Gonzalez-Franquesa, https://www.researchgate.net/figure/Insulin-signaling-pathway_fig1_241686360.

Activation of the PI3K pathway in endothelial cells leads to the release of nitric oxide (causing vasodilation), while activation of the MAPK pathway triggers the synthesis of endothelin-1 (causing vasoconstriction). As a result, the combined vascular effects contribute to the distribution of blood glucose to target organs and control capillary recruitment (4).

In hepatocytes and skeletal muscle cells, insulin promotes the synthesis of glycogen and the transport of amino acids, while also inducing protein synthesis. In adipose tissue, insulin enhances the expression and activity of fatty acid synthase.

In the brain, insulin serves two crucial functions: regulating food intake and cognitive functions, primarily memory (5). Inflammatory processes within the hypothalamus, where insulin receptors exist at a high density, impact local signalling systems and disrupt glucose and energy metabolism. Disruptions in insulin signalling within the brain could play a part in neurodegenerative ailments (6).

1.2 Glucose transport

Glucose can passively diffuse across the cytoplasmic membrane via transmembrane proteins, glucose transporters, or can be actively transported with sodium co-transporters known as SGLTs.

Currently, there have been 14 GLUTs and 12 SGLTs identified within the human anatomy (7). Most cells have multiple types of GLUT on their membrane with individual transporters serving distinct cell needs and possessing unique kinetics and regulatory capabilities. Some GLUTs demonstrate the capacity to transport other types of sugar. GLUT 1 and 3 are specifically designed to sustain basal glucose uptake in tissues that rely on glucose metabolism, such as the brain, blood cells, kidneys, and placenta. GLUT 1 is situated on the membrane of pancreatic β-cells and hepatocytes, whereas GLUT 2 allows glucose to pass from resorptive epithelia (renal cortex, enterocytes) into the blood. Insulin-dependent cells, such as myocytes and adipocytes - the largest tissues in the body - predominantly use GLUT 4 as transporters. Consequently, their role in quantitative glucose management is critical.

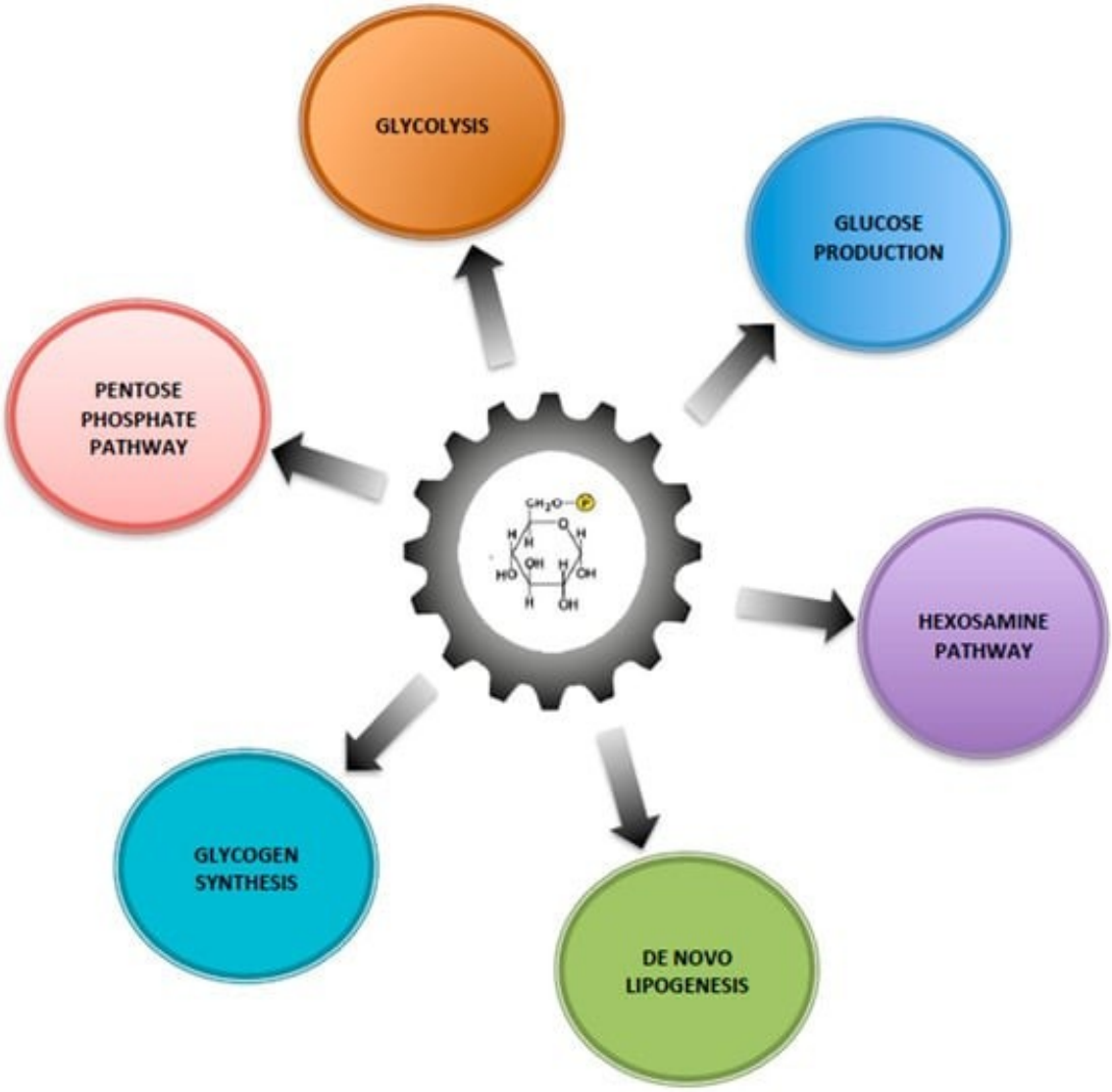
GLUT 4 is stored in vesicles located in the cytoplasm. Upon the binding of insulin to the receptor or as a result of muscle contraction, the cell membrane and vesicles will fuse together. This fusion leads to the externalization of the GLUT 4 binding site, facilitating glucose entry into the cell. Upon removal of stimulus, the transporter is then stored within the vesicle and may be recycled as required.

Upon entry into the cell, glucose undergoes phosphorylation to create glucose-6-phosphate. This process prevents glucose from escaping the cell and makes glucose-6-phosphate a crucial substrate for carbohydrate metabolism as it links various metabolic pathways such as glycolysis, the pentose phosphate pathway, glycogen synthesis, de novo lipogenesis, and the hexosamine pathway (refer to Figure 2) (8).

The maximum rate at which glucose can be oxidized, i.e. serve as an energy source, is 5 $mg.kg^{-1}.min^{-1}$ (9).

Most tissues require at least minimal amounts of glucose because of its non-energetic function as a precursor of many other molecules. In erythrocytes, leukocytes and lymphocytes, bone marrow, peripheral and central nervous system, renal medulla and transparent tissues of the eye, glucose is an essential source of energy.

Figure 2. Carbohydrate metabolism



Adapted from (8).

1.3 Pathophysiology of insulin resistance

Insulin resistance (IR) indicates the inability or inefficiency of the body to respond well to hormone insulin. The insulin signalling cascade is intricately regulated, employing a high level of sophistication. Various factors have been recognised as triggers of insulin resistance, such as obesity (10), acid-base balance (11), critical hyperglycaemia (12), low-grade inflammation (13), the overproduction of reactive oxygen species (ROS) (14), mitochondrial dysfunction (15), osmotic stress (16), impaired insulin signalling (17), fatty acids (18), and amino acids (19).

1.3.1 Central insulin resistance

Dysregulation of hepatic glucose output is the primary cause of hyperglycaemia in critically ill patients (20). When glycogen stores are depleted, glucose is chiefly produced by gluconeogenesis. The study of Dieter Mesotten et al. (21) examined the impact of intensified insulin treatment on phosphoenolpyruvate carboxykinase (PEPCK), a regulatory enzyme of gluconeogenesis, in patients who did not survive critical illness. Hyperinsulinaemia in the strict euglycaemia group was found not to inhibit PEPCK expression, while insulin like growth factor binding protein 1 (IGFBP-1) synthesis, which is commonly inhibited by insulin in healthy individuals, was unaffected by treatment. In both groups, IGFBP-1 levels were equally predictive of mortality as the APACHE II score.

An experimental sepsis model was adapted for dogs fed an artificial diet and confirmed significant insulin resistance in the liver while demonstrating the resistance of hepatic glycolysis to the insulin's stimulatory effect (22). Stress hyperglucagonemia is recognized as a primary cause of hepatic insulin resistance (23).

1.3.2 Peripheral insulin resistance

The clamp studies conducted on critically ill patients have indicated unchanged insulin-stimulated glucose oxidation, with a maximum of approximately 4 $mg.kg^{-1}$ of lean body weight per minute, but significantly reduced capacity for non-oxidative glucose metabolism - particularly glycogen synthesis in the muscle. These results suggest that the insulin signalling defect is distal to transport (GLUT-4 expression and its translocation to the membrane) and glucose phosphorylation (hexokinase II). In addition, glucose transport (GLUT-4 gene expression) is stimulated by intensified insulin treatment (24). The overall increase in glucose uptake is due to heightened retention in tissues that are constitutively insulin-independent (blood elements, lungs, spleen, brain, fibroblasts). It is also possible to increase glucose uptake in muscles independent of insulin. This is caused by increased expression of the transporter GLUT-1, which has already been demonstrated in regenerating skeletal muscle (25) and hypoxic cardiomyocytes (26).

1.4 Glycaemic clamp method

The development of the glycaemic clamp method began in the 1960s, and it was first introduced in the Czech Republic during the 1980s (27, 28). Glycaemic clamps have several applications, including metabolic and nutritional disorders, diabetes, and

endocrinology. Insulin and glucose levels interact in the body, affecting secretion and action. Clamp methods are currently regarded as the benchmark for testing insulin secretion and insulin resistance, enabling the evaluation of glucose regulation. The glycaemic clamp technique induces a constant level of glucose or insulin in the patient's bloodstream through glucose and insulin infusions. Glycaemic clamps are classified as hyperglycaemic, hypoglycaemic, or euglycaemic depending on the targeted glucose level. In this study, we opted for a euglycaemic clamp to maintain the glycaemic level equivalent to the patient's fasting glycaemia.

1.4.1 Euglycaemic insulin clamp

The aim of the euglycaemic insulin clamp (27) is to elevate the plasma insulin concentration rapidly, reaching a new plateau, and sustain it at roughly 100 µU/ml above the basal level for 120 minutes. If the plasma glucose concentration is not sustained at its euglycaemic level, this could lead to a significant and fast-onset risk of hypoglycaemia. Therefore, the investigation comprises an adjustable glucose infusion with a fixed dose of insulin.

The insulin infusate is prepared in isotonic saline and blood from the subject is added at a ratio of 2 ml per 50 ml of infusate to prevent insulin absorption on glassware and plastic surfaces. Crystalline insulin is diluted to a concentration of 300 $mU.ml^{-1}$. A 10-minute priming infusion is followed by a constant infusion of 40 $mU.m^{-2}$ surface area per minute for 110 minutes.

The glucose clamp technique is utilised to prevent hypoglycaemia by maintaining each individual at their own basal arterial plasma glucose concentration. The glucose infusion only commences four minutes after the initiation of insulin infusion and is set at 2.0 $mg.kg^{-1}.min^{-1}$ based on empirical evidence. It is then elevated to 2.5 $mg.kg^{-1}.min^{-1}$ after ten minutes.

Under steady-state plasma glucose conditions, it is essential that the amount of glucose infused equals the amount of glucose translocated out of the glucose space, which is referred to as glucose metabolized (M), given that endogenous glucose production is suppressed completely. Nonetheless, the glucose infusion needs adjustment before it can be equated to the amount of M. As the plasma glucose concentration cannot be maintained perfectly, a correction needs to be made. This "space correction" accounts for glucose that has been added to or removed from the glucose compartment. Therefore, when computing M for the time period of 20-40 minutes, plasma glucose concentrations at the start and end

of that interval are taken into consideration. To calculate the SC, in terms of $mg.kg^{-1}.min^{-1}$, use the following formula:

$$\underline{SC = (G2 - G1) \times 10 \times (0.19 \times \text{body wt})}$$
$$20 \times \text{body wt},$$

where body weight is in kilograms, and glucose concentrations at the end (G2) and beginning (G1) of the time period are measured in milligrams per decilitre. Calculation of glucose (mg) removed or added to the glucose space in the 20-minute period is determined by 10 multiplied by 0.19 multiplied by body weight (in kg) multiplied by the difference between G2 and G1. The final expression of the dimensions in $mg.kg^{-1}.min^{-1}$ is achieved through the terms 20 and body weight.

It should be noted that the space correction may have a significant effect, particularly if plasma glucose levels are unstable.

The calculation of M in this article is performed at 20-minute study intervals using the equation

$$M = INF – SC.$$

Here, INF represents the glucose infusion rate, and SC denotes the space correction. The values for all parameters are computed in units of $mg.kg^{-1}.min^{-1}$.

1.5 Catabolism

Studies of the anabolic response of muscle confirm that ageing is associated with reduced muscle proteosynthesis and a reduced response to anabolic stimuli compared to younger individuals. This situation is referred to as muscle anabolic resistance (29). Catabolic processes in muscle are accelerated by increased inflammatory activity, mediated mainly by cytokines (interleukins, tumour necrosis factor α – TNFα - and other regulatory molecules such as atrogin 1), damage caused by ROS (oxidative stress) and mitochondrial dysfunction. Proteocatabolism in the muscle occurs via activation of the ubiquitin-proteasome pathway with activation of myostatin and transforming growth factor β (TGFβ), which are present in the muscle extracellular matrix in an inactive form under resting conditions. Their activation stimulates transcription factors which, by binding directly to nuclear DNA, induce the synthesis of atrogin 1 and muscle RING-finger protein-1 (MuRF1, E3 ubiquitin protein ligase, muscle really interesting new gene

– RING - Finger 1). Ubiquitination of muscle proteins occurs, predisposing them to proteolysis by the 26S proteasome (30).

In addition to a reduction in the total amount of muscle mass, the quality (morphology and internal architecture) of the muscle also deteriorates. The loss is particularly significant in fast type II muscle fibres, with disorganisation of sarcomeric spaces, reduction in the number of satellite cells (impaired muscle regeneration), accumulation of fat between muscle fibres and within myocytes (myosteatosis) and microscopic denervation with a reduced number of motor units (loss of spinal motoneurons) and deterioration of neuromuscular junction function (31).

Inflammatory changes activate the nuclear factor κβ (NF-κβ) pathway and the production of inflammatory mediators, mainly interleukin 6 (IL6), interleukin 1 (IL1) and TNFα, which accelerate the loss of muscle mass and strength, among other negative effects (32). At the cellular level, the aforementioned protein-catabolic ubiquitin-proteasome pathway is activated, the IGF1-mediated anabolic effect is reduced, and muscle anabolic resistance is increased. All the pro-inflammatory mechanisms involved in the development of sarcopenia are not well understood. However, it is known that sustained elevation of TNFα is associated with the wasting syndrome - cachexia, sarcopenia and cell apoptosis. These changes are more pronounced in muscles with low anabolic activity and in the muscles of older people. Exercise - especially weight-bearing (resistance) exercise - significantly reduced plasma levels of TNFα and soluble TNF receptor 1 and could therefore be an effective intervention to reverse the proapoptotic setting (33). Also, increases in the pro-inflammatory cytokines IL6 and IL1 are associated with loss of muscle strength, impaired gait and physical disability (34).

Mitochondrial dysfunction resulting from damage to mtDNA through reactive oxygen and nitrogen species (RONS) is involved in cellular senescence and organ dysfunction during ageing. Furthermore, extended periods of muscle inactivity increase the production of RONS, which serve as important signalling molecules in muscle dysfunction and atrophy (35). Mitochondrial production of RONS is worsened by their own presence, meaning that the release of RONS from a limited number of mitochondria can lead to a surge of oxidation products in nearby mitochondria. This wave can subsequently spread throughout the cell (36). Research conducted on mice has demonstrated that sarcopenia is primarily caused by mitochondrial dysfunction. This can be attributed to impaired oxidative phosphorylation and reduced ATP production, as well

as decreased myocyte ability to repair mtDNA damage and eliminate damaged mitochondria through autophagocytosis (37).

1.6 Critical illness

The initial bodily response to a supraphysiological stimulus is a neuroendocrine response under brain control. This response's magnitude is directly proportional to the degree of the inciting insult. All bodily cells take part in or are impacted by the adaptive response. Following the stress stimulus, the sympathetic nervous system is stimulated, resulting in ten times the normal concentration of noradrenaline in the plasma and up to 50 times the usual concentration of adrenaline (38). The adrenal cortex secretes an elevated level of cortisol compared with the physiological circumstances, and the degradation rate of endogenous steroids decreases (39). The activation of the hypothalamic-pituitary axis results in the release of adenohypophyseal hormones into the bloodstream, accompanied by increased levels of vasopressin and angiotensin II.

This process triggers the release of recognizable molecular patterns, known as alarmins, from affected tissues. Innate immunity swiftly responds upon recognition of these patterns. These patterns are presented by exogenous microorganisms and their toxins known as pathogen-associated molecular patterns (PAMPs), as well as endogenous intracellular molecules, such as mitochondrial DNA and N-formyl peptide, which emerge from dying host cells after exposure to damage-induced insult, also known as damage-associated molecular patterns (DAMPs). As a result, the innate immune system and its pattern recognition receptors (PRRs) play a crucial role in the molecular pathway leading to inflammation. PPRs function as nutrient sensors, and in the presence of fatty acids, they trigger an inflammatory cascade that has an impact on insulin signalling and leads to the development of insulin resistance (40). When excessive amounts of alarmins are released, they can lead to a cytokine storm that has the potential to be lethal (41).

During the onset of the stress response, two distinct periods can be identified: the initial period characterised by metabolic instability and a significant increase in catabolism, which typically endures for two days after the injury occurs, and the subsequent period of between three to seven days, featuring marked muscle loss and stabilised metabolic disorders. This initial phase will either result in improvement and convalescence, or an extended period of inflammation and catabolic activity.

High levels of stress hormones are accountable for the majority of metabolic modifications during the initial acute phase. Glycogenolysis within the liver is triggered.

As a result of limited oxidation, centralized circulation leads to a decrease in skeletal muscle perfusion and temporarily reduces glucose consumption. Glucose is then transformed into lactate in the cell cytosol and discharged into the circulation. Furthermore, lipolysis is triggered, increasing the amount of free fatty acids (FFA) in plasma.

Typical metabolic characteristics of the late phase of the acute stage include gluconeogenesis (GNG) and insulin resistance (IR). GNG predominantly occurs in the liver, and to a lesser degree (typically 20-25%) in the renal cortex. However, when assessed per gram of tissue, GNG is higher in the kidneys. During extended fasting, the proportion of renal GNG in total production increases, with overweight individuals producing more glucose in their kidneys than in their liver during complete fasting. Metabolic acidosis significantly promotes renal GNG (42). The substrates for GNG consist of three- and four-carbon molecules, primarily lactate, pyruvate, glucoplastic amino acids (hepatocytes show preference for alanine, while the kidneys favour glutamine), and glycerol. GNG has the capacity to generate up to 10 g of glucose per kg of body weight on a daily basis (43).

Insulin resistance is an evolutionary adaptation that prioritises glucose supply to certain tissues to enhance survival chances. Insulin deficiency within dependent tissues, such as skeletal muscles, causes intracellular glucose deficiency while glucose toxicity may occur in non-insulin-dependent tissues, such as nerve tissue and blood cells. The feedback loop between glycaemia and insulin secretion is disrupted, leading to increased insulin levels. Hyperinsulinemia hinders ketogenesis in the liver. Free fatty acids are converted back to triacylglycerols in the cell cytosol, consuming ATP in these futile cycles (44). Thus, amino acids remain the sole energy source for specific tissues, particularly skeletal muscle. Additionally, myocytes release amino acids in large amounts into the bloodstream to meet the requirements of GNG (about 2-3 g of protein must be broken down to synthesize 1g of glucose) and acute phase protein synthesis. Muscular anabolic resistance increases due to the presence of PAMPs/DAMPs and cortisol, which can inhibit the effects of proteo-anabolic hormones such as insulin, IGF1, growth hormone, and sex hormones. As a result, body proteins are consumed, and autophagy occurs at an accelerated rate. Unfortunately, the condition is not reversible by the external supply of energy and nutrient substrates.

In favourable cases, inflammation tends to be self-limiting. The cessation and restoration of homeostasis is a meticulously coordinated biochemical activity. Specific

mediators activate pathways that indicate the physiological conclusion of the acute inflammatory phase, and resolvins, known as resolution mediators, commence control of the immune response's physiological pathways by the acute phase's conclusion (45). The extent of GNG declines, then amino acids are primarily utilized for protein synthesis and urea production drops, leading to a positive nitrogen balance.

If alarmins continue to flow persistently, the critical condition persists, which is known as PICS - Persistent Inflammation, Immunosuppression, and Catabolism Syndrome (46). This often arises due to secondary insults such as infections acquired in hospitals, repeated surgical interventions, organ failure, or an inadequate immune response. Immune response remains high and catabolism is increased, resulting in significant muscle atrophy. A cumulative loss of 25 % or more of the body's proteins leads to metabolic failure (47). The individual is unable to acquire the necessary quantity of energy internally, and as a result, there is a considerable decrease in basic metabolism, hypothermia, and healing complications, even with a shorter period of starvation (e.g. perioperatively). The individual becomes reliant on external nutritional support. A number of metabolic malfunctions can be illustrated even three years after recovery (48).¨

1.7 Consequences of insulin resistance in critically ill patients

1.7.1 Hyperglycaemia

In hyperglycaemia, glucose accumulates in certain cells, which can result in damage to their morphology and function (i.e. glucotoxicity). Moreover, intracellular glucose excess triggers the formation of advanced glycation end products (AGEs) through glycation of proteins, leading to alterations in their physical and chemical properties, including changes in solubility, charge, isoelectric point, and crosslinking. AGEs also engage with the immunoglobulin receptor (RAGE), thereby stimulating the generation of cytokines (49).

Oxidative stress can be induced when glucose undergoes autooxidation and generates reactive oxygen species such as superoxide, leading to the formation of more oxygen radicals. Superoxide triggers alternative glucose metabolism pathways, resulting in sorbitol build-up within cells, elevated pro-inflammatory and pro-coagulation factor expression, protein kinase C activation (leading to vasoconstriction, increased endothelial permeability, and leukocyte adhesion), and NF-κB activation (48).

Prolonged and intensified hyperglycaemia is linked to augmented morbidity and mortality. However, moderate hyperglycaemia (7 - 9.9 $mmol.l^{-1}$) is now deemed more favourable to strict control for critically ill patients, including those suffering from brain injury (50).

1.7.2 Dyslipidaemia

In critically patients, an altered lipid profile is observed, marked by a reduction in overall cholesterol concentration (both LDL and HDL decrease) and a rise in triglycerides (51). The liver of the critically unwell patients experiences an exceptional scenario - a simultaneous supply of high levels of glucose and free fatty acids. The insulin-induced stimulation of fatty acid synthesis via AcCoA-carboxylase seems to be more sensitive than the catabolic effects of glucose pathways. Malonyl-CoA, generated in the cytosol of hepatocytes, impedes transport and oxidation of fatty acids in the mitochondria, ultimately promoting the resynthesis of triacylglycerols. Intensified insulin therapy led to a partial normalization of LDL and HDL cholesterol, a reduction in elevated triglycerides, and had a significant effect on survival according to a multivariate logistic analysis (24). Possible explanations include the availability of higher cholesterol (for membrane integrity and steroid hormone synthesis) or a scavenging effect from the uptake of endotoxin by LDL or HDL particles (52).

1.7.3 Reduced proteoanabolic effects of insulin

Loss of muscle mass despite adequate nutritional support is a major problem in critically ill patients (53), especially during prolonged ICU stays. While insulin treatment appears to have an anabolic effect in animal models, in the Leuven study (54), doses of insulin that normalised glycaemia had no clinically apparent effect on protein metabolism. However, a higher protein content was found in the necropsies of the deceased in the intensive insulin treatment group.

1.8 Insulin resistance and polyneuromyopathy

Critically unwell patients often develop critical illness polymyopathy (CIM) and polyneuropathy (CIP) which delay ventilator weaning, hinder rehabilitation, and are linked to longer stays in hospital and the intensive care unit, as well as a higher mortality rate. This impairment during critical illness is primarily attributable to mitochondrial dysfunction, causing an impaired capacity to generate ATP. The energy deficit causes primary axonal

degeneration, especially distally, where there are axonal transport systems comprising energy-dependent structural proteins. Hyperglycaemia may worsen this issue by inducing neuronal mitochondrial dysfunction (55). Patients who died from critical illness had greater mitochondrial dysfunction in their skeletal muscle compared to patients who survived critical illness (56). Insufficient translocation of GLUT4 from the perinuclear spaces to the sarcolemmal membrane has been identified as a central mechanism that impairs glucose supply to muscle cells in patients with CIM (57). Critically ill patients have significant insulin resistance compared to healthy individuals, with patients suffering from CIM experiencing the most severe insulin resistance. Intensive insulin therapy has been found to significantly reduce the electrophysiological incidence of CIP/CIM (58).

2 PREVIOUS ATTEMPTS TO INFLUENCE INSULIN RESISTANCE AND PROTEIN CATABOLISM IN CRITICALLY ILL PATIENTS

2.1 Insulin administration

Blood glucose control with insulin is used frequently for critically ill adults and children. Insulin is considered an affordable and inexpensive anabolic hormone, too. This assumption has been confirmed in burn subjects using submaximal insulin doses (59). However, insulin has another anabolic non-protein effect that must be taken into account: it promotes lipogenesis, thereby resulting in the storage of triglycerides in adipocytes and of LDL in hepatocytes (60).

A wide variation in practice exists in blood glucose targets. Agus et al. showed in the HALF-PINT study (61) that critically ill children with hyperglycaemia did not benefit from tight glycaemic control targeted to a blood glucose level of 4.4 to 6.1 $mmol.l^{-1}$, as compared with a level of 8.3 to 10 $mmol.l^{-1}$. They concluded that in an enriched population of critically ill children with hyperglycaemia, the higher blood glucose target was associated with clinical outcomes that were similar to outcomes with the lower target, but carried a lower risk of hypoglycaemia.

A meta-analysis from 2018 (62) revealed that there were no obvious benefits of tight glycaemic control, compared with conventional glycaemic control in critically ill children. Tight glycaemic control did not reduce mortality rates and was not associated with decreasing infection rates. A significantly increased incidence of hypoglycaemia was seen in the tight glycaemic control groups.

As of 2015, there are no RCTs focused on optimal glycaemic control in critically ill patients. Current (2023) ESPEN recommendations (63) is to administer insulin when glycaemia is above 10 $mmol.l^{-1}$.

2.2 Metformin

The main risk of insulin administration is hypoglycaemia. Metformin, a biguanide used for more than 60 years in the treatment of type 2 diabetes, does not cause hypoglycaemia and is therefore considered a suitable candidate for the treatment of hyperglycaemia even in critically ill patients. It has pleiotropic effects in the body. The most significant effect is the partial inhibition of the mitochondrial respiratory chain Complex 1 (at therapeutic doses of approximately 40 % V_{Max}), which leads to a decrease in ATP production and a decrease in the ATP/ADP ratio in the cell cytosol. This activates the enzyme adenosine monophosphate protein kinase, a key cellular regulator of glucose and lipid metabolism. The cell begins to conserve energy and suppress ATP-dependent processes. The level of GNG (but also proteosynthesis) is reduced and hepatic FFA oxidation is increased. In muscle, GLUT 4 translocation to the plasma membrane is increased, thereby reducing IR and increasing insulin efficiency (64). In addition, metformin directly affects mitochondrial permeability transition pores, oxidative stress-sensitive channels whose opening signals cell death, and reduces the generation of oxygen free radicals (65). Metformin also has a stabilizing effect on gut microbiota and intestinal barrier integrity (66).

Metformin is excreted by the kidneys without being metabolized. Therefore, renal insufficiency can cause a risk of metformin accumulation, leading to inefficient mitochondrial metabolism. It is important to keep renal function in mind when prescribing metformin to minimize the risk of metformin-associated lactic acidosis (MALA) developing. Therefore, renal insufficiency can cause a risk of metformin accumulation, leading to inefficient mitochondrial metabolism. As a result, energy-deficient cells have to compensate for the lack of ATP by anaerobic glycolysis, leading to lactate acidosis. Predisposing factors for MALA include dysfunction of organs such as renal, hepatic, or cardiac, shock, systemic infection, dehydration, and a history of pre-existing metabolic acidosis (67). These factors are common in intensive care medicine and thus metformin administration to critically ill patients is currently *off-label*. Promising results have been obtained from studies with small subject numbers to date (68, 69). However, the identification of suitable patients, the timing of administration, and correct dosing remain the essential treatment strategies to be clarified (70). An additional drawback of potential

metformin therapy for critically ill patients is the impossibility of parenteral administration, which significantly reduces its efficacy when administered intravenously.

2.3 Growth hormone and IGF1

The use of growth hormone (GH) on critically ill patients in the late 20th century was extensively researched. Administration of GH significantly increases insulin resistance and leads to hyperglycaemia. Despite the potential for a decrease in protein catabolism due to its highly anabolic nature, hopes were not realised. A multicentre study in 1999 (71) illustrated elevated mortality rates in patients treated with recombinant GH (rGH). The primary criticism of this study is that participants received rGH at a dosage and frequency that deviated from the body's natural production, and they did not receive additional nutrients or amino acids during treatment (72). Individualised treatment of rGH may be advantageous and anabolic in low-dose pulse administration for patients with extensive burns, polytrauma, difficult healing, or selected paediatric patients (73). However, the use of rGH in critically ill patients is not currently advised (74).

IGF1 is synthesised by the liver in response to GH stimulation and plays a role in its effects such as sodium retention and lipolysis. IGF1 is recognized for its proteo-anabolic properties on the skeletal muscle, liver tissue, and the gastrointestinal tract mucosa (75). In a few small studies, IGF1 (administered as a recombinant analogue of mecasermin rinfabate, and as a complex of equimolar amounts of IGF1 and its binding protein IGFBP-3) has demonstrated favourable outcomes in the treatment of wound healing, as well as in the management of shock, sepsis, and burns (76). However, the use of IGF1 in critically ill patients has been discontinued alongside rGH. As a result, its applications have shifted beyond the context of critical care medicine.

2.4 Propranolol

Propranolol, a non-selective β-blocker, is given to decrease the hyper-metabolic response mediated by adrenergic hormones. It has been widely researched in burned children, where it resulted in increased proteosynthesis (77). However, there is limited research on propranolol use in adult patients. A meta-analysis examining the safety and effectiveness of propranolol in extensively burned children and adults (78) found no evidence of reduced mortality, infection rates, or hospitalization duration. The

administration of this treatment was linked to a noticeable reduction in blood transfusions and a decrease in heart rate among adult patients.

2.5 Anabolic steroid hormones

Dehydroepiandrosterone (DHEA), also referred to as androstenolone or prasterone, serves as a precursor to sex steroids and is among the most prevailing steroids found in the human body. Plasma levels of its metabolite dehydroepiandrosterone sulphate (DHEAS) are considerably higher than DHEA, acting as a hormone reservoir. The production of DHEA begins to surge at puberty (adrenarche) and remains high until the conclusion of the second decade of life. Subsequently, its concentrations decrease, typically falling to 1/10th of their previous maximum in the eighth decade (known as andropause).

DHEA, also referred to as the "fountain of youth," has numerous benefits, including the improvement of neutrophil function and immunomodulatory effects that fortify the immune system against viruses. Additionally, it increases IGF1 levels and insulin efficiency, reduces IR, and decreases hyperglycaemia at the metabolic level. In critically ill patients, increased glucocorticoid secretion leads to a shift in adrenal biosynthesis at the expense of DHEA. Furthermore, trauma patients experience a decline in DHEAS levels to unmeasurable levels. An elevated cortisol/DHEAS ratio is linked to greater morbidity and mortality.

The administration of DHEAS has resulted in increased survival rates among animals with trauma, sepsis, or burns. Human studies indicate that restoring the cortisol/DHEAS ratio enhances wound healing, bone remodelling, and the psychological state of patients. Gerontological diseases and individuals with immunodeficiency are the primary advocates for DHEA replacement. While DHEA administration can lead to a similar range of negative effects as other anabolic steroids (as described below), there is no strong evidence to support this, as acne is the most common side effect observed by researchers (79). It is puzzling, therefore, that the sole form of DHEA dosage officially available in the Czech Republic is vaginal beads. At present, researchers are not conducting any RCTs regarding DHEA or DHEAS supplementation in critical care patients.

Reduced testosterone levels are commonly found in critically ill patients (80), and supplementing it has a positive impact on nitrogen balance. Its effect can be either anabolic, increasing proteosynthesis in children, or anticatabolic, decreasing proteolysis in adults, without adversely impacting insulin resistance and glucose metabolism (81). Testosterone administration is linked to noteworthy negative outcomes, including a higher prevalence of

acute coronary syndromes and deep vein thrombosis, hepatotoxicity, and the development and promotion of prostate cancer growth, as well as causing virilization in women. This reduction in its indications has led to oxandrolone and nandrolone becoming more commonly used due to their greater anabolic potency and improved safety profile. A meta-analysis of three randomised controlled trials administering oxandrolone to extensive burns at various stages of burn disease (82) concluded that oxandrolone is effective in burns management:

1. has no effect on mortality;
2. has no effect on the number of infectious complications;
3. does not cause hepatotoxicity;
4. shortens the length of hospital stay;
5. leads to less weight loss and improves fat-free body mass gain;
6. reduces nitrogen loss.

However, in critically ill patients' general population, data with sufficient quality are lacking and therefore the potential use of oxandrolone outside the scope of post-acute medicine remains controversial. Individualised oxandrolone treatment may be beneficial for certain patients in intensive care, such as those with multiple trauma, type 2 diabetes, paediatric patients, and those experiencing difficult healing and prolonged catabolism, at some point during their illness (83). Objective evaluation suggests potential efficacy.

2.6 Fenofibrate

Fenofibrate is among the preferred drugs for treating dyslipidaemias. Its action mechanism involves activating lipoprotein lipase through the peroxisome proliferator-activated receptor alpha (PPARα) pathway. PPARα is a transcription factor expressed in high-oxygen-consumption tissues like the liver, striated muscle, heart, and kidney. Additionally, this factor is present in the endothelium and musculature of blood vessels, as well as in immune cells. Fenofibrate enhances fatty acid oxidation in mitochondria, while also exhibiting anticoagulant and anti-inflammatory properties (84).

Lipotoxicity occurs when the accumulation of triacylglycerols and diacylglycerol metabolites inside cells obstructs insulin signalling pathways at the level of IRS-1, leading to an increase in insulin resistance. The administration of fenofibrate produced a decrease in both central and peripheral insulin resistance, as well as an improvement in mitochondrial oxidative capacity, in children with extensive burns (85, 86). However, there is a dearth of similar research conducted on critically ill adults.

Therefore, the role of fenofibrate in the treatment of critically ill patients must be evaluated critically. Potential fenofibrate therapy in these patients has the drawback of being impossible to administer parenterally and poses risks such as accumulation in renal insufficiency and rhabdomyolysis. The latter is particularly hazardous to patients with hypoalbuminemia, renal insufficiency, and hypothyroidism, which are relatively common in intensive care medicine.

2.7 Thyroid hormones

During critical illness, plasma levels of triiodothyronine (T3) are generally low with elevated reverse rT3 and normal or slightly reduced levels of thyroxine (T4) and TSH (87). T his is termed non-thyroidal illness syndrome (NTIS). The decrease in T3 is directly related to muscle and bone catabolism, while low T4 levels are associated with an unfavourable prognosis for the patient (88). However, there is currently insufficient evidence to support the recommendation of thyroid hormone supplementation for critically ill patients with NTIS.

2.8 Muscular rehabilitation

The exercise opportunities for critically ill patients are restricted, causing muscle catabolism and the emergence of neuromyopathy, particularly in the elderly (89). Limited physical capabilities increase hospitalization durations, expense medical resources, reduce survival rates post-discharge, and negatively impact life quality (90-93). Nevertheless, a mere 25 % of ICU patients receive muscular rehabilitation (94).

There may be issues in how patient care is organised if rehabilitation cannot be coordinated with other procedures and the patient's schedule. The frequent use of sedatives hinders patient verticalisation and mobilisation. Furthermore, endotracheal tubes, femorally inserted venous and/or arterial catheters, and urinary catheters, which may become loose during rehabilitation, are among other factors that negatively impact prompt rehabilitation. Finally, a lack of funding for rehabilitation activities from health insurance providers is an additional challenge (95).

Types of interventions that should be considered for rehabilitation of the critically ill include passive mobilization, verticalization, bed cycle ergometry, assisted electrical muscle stimulation, hoist or tilt tables, active mobilization, and the use of technologies like robotics (96, 97), virtual reality (98, 99) and muscle vibration (100, 101).

Passive rehabilitation administered for 10 hours daily to mechanically ventilated (otherwise healthy) subjects (n = 7), improved muscle function scores by 35 % as compared to the non-rehabilitation group. However, it did not prevent proteolysis or muscle wasting (102).

Previous studies found that neuromuscular electrical stimulation (NMES) had minimal adverse effects and could potentially preserve muscle strength, reduce mechanical ventilation time, and shorten intensive care unit stays (103). Waldauf et al (104), n = 150, and Berney et al. (105), n = 162, used functional electrical stimulation-assisted cycle ergometry (FESCE) in mechanically ventilated ICU patients with different research protocols. Nevertheless, both teams have concluded that the addition of FESCE to usual care rehabilitation for ICU patients did not improve muscle strength upon hospital discharge or physical functioning after 6 months. Additionally, the incidence of cognitive impairment was practically identical between intervention and control groups in survivors at the 6-month mark.

Active movement triggers intracellular signalling that leads to GLUT4 externalization, reduced proteolysis, muscle growth, muscle glycogen synthesis, and a boost in local blood flow. At the systemic level, this modified myocyte metabolism is evident via a decline in insulin resistance, enhanced glucose clearance, and positive nitrogen balance (106). So, a possible alternative to insulin therapy could be engaging in physical exercise that boosts the metabolism of the contracting muscle, leading to a several-fold increase in glucose consumption by the muscle. This, in turn, enhances its insulin sensitivity and the anabolic effect of amino acids for up to 24 hours. However, the available data suggests that muscle mass decreases and muscle weakness develops rapidly, typically within a week. This occurs before the patient can be awakened from artificial sleep, and thus unable to engage in rehabilitation (58, 107).

Early rehabilitation has been suggested to facilitate the natural healing process. Although active rehabilitation and early mobilization of critically ill patients pose challenges to the treatment team, they are safe for ventilated patients (108), and can improve inspiratory and skeletal muscle strength (109). Protocolised rehabilitation and early mobilisation decrease the length of stay in the intensive care unit and the duration of ventilator dependency (110).

Hickmann et al. (111) showed that exercise during the first week of septic shock preserved muscle fibres, possibly by limiting excessive autophagy activation. The meta-

analysis by Fuke et al. (112) found that early rehabilitation only improved short-term physical outcomes in critically ill patients. On the other hand, Doiron et al. (113) assessed that there is currently low quality evidence for the effect of early mobilization in critically ill adults.

The ESICM recommendations have yet emphasised the importance of early mobilisation of patients in the ICU (114).

For severe burns, 'early' means starting rehabilitation within 14 days of injury (115). The critically ill burn patient is exposed to multiple insults that could potentially increase the risk of progressive debilitation, deconditioning and cognitive decline, ultimately leading not only to acute problems such as ICUAW and prolonged mechanical ventilation, but also to post-discharge functional limitations and reduced health-related quality of life. These issues have not been well studied in burn survivors.

There are important gaps in knowledge about exercise in the critically ill, including the timing, intensity, duration and frequency of each of these interventions. These settings depend to a large extent on the experience of the therapist and so can harm the patient if used inappropriately. Eggmann et al. (116) suggest that shorter and more frequent sessions may be preferable to achieve an adequate training response. Watanabe et al. (117) found out that a higher dose of rehabilitation was associated with a significant improvement in activities of daily living at discharge.

3 FUNCTIONAL PROPRIOCEPTIVE STIMULATION (FPS)

There is a large group of critically ill patients for whom active rehabilitation is impossible or very difficult. These are patients who are deeply sedated, have severe circulatory and respiratory failure, are in a prone position, have serious craniocerebral injuries, unstable fractures of the chest, spine or pelvis, or extensive burns. As an alternative to the common rehabilitation, these patients may be offered the use of focal vibrations of a certain frequency over certain muscle attachments, the so-called illusory movements or functional proprioceptive stimulation (FPS), which leads to the illusion of movement in the brain (118, 119) and thus indirectly, through activation of brain motor areas, stimulates the antidegenerative processes of the nervous system and muscles (120, 121).

FPS has been used for rehabilitation purposes since 1969, currently mainly for patients with stroke, spinal cord injury, Parkinson's disease, multiple sclerosis, dystonia and

hemineglect (122). Its use in critical care is in its infancy and there are no known recommendations regarding the timing, duration and intensity of the vibrations or most effective combinations of kinaesthetic illusions. Unlike other rehabilitation techniques, FPS can be performed easily on patients connected to devices such as ventilators, dialysis or extracorporeal circulation, in the prone position, or placed in a fluidised bed.

In our pilot study (123), the use of FPS in five extensively burned patients did not significantly affect monitored vital functions, and the attached vibrating units did not alter healthy or injured skin or skin grafts and did not negatively affect local healing.

In these circumstances, the use of FPS appears to be a suitable alternative as, unlike whole-body muscle vibration (124), it does not stress the cardiovascular or respiratory systems, does not increase tissue oxygen consumption and allows rehabilitation even in the case of extensive musculoskeletal injuries. However, it is not yet known how long it is appropriate to activate brain centres per session, how many sessions per day are optimal, from what time and at what intensity.

4 ANALYSIS OF RESPIRATORY CHAIN

The oxidative phosphorylation system is located on the inner side of the mitochondrial membrane and uses two electron carriers (coenzyme Q and cytochrome c) that allow electron transfer between complexes I-IV. Electrons are obtained by oxidation of reduced coenzymes NADH and FADH2 derived from metabolic processes, in particular the Krebs cycle. Electrons are transferred through complexes that differ in their redox potential, thus allowing the correct direction of electron flow, the final acceptor of which is oxygen. The generation of the macroergic ATP particle is provided by the ATP synthase complex, which uses the generated membrane potential resulting from the electron transfer.

To determine mitochondrial function, we can use spectrophotometry to measure the activity of respiratory chain complexes, high-performance polarography to measure oxygen consumption, oxidation of labelled substrates to determine the capacity of mitochondrial energy metabolism, or fluorescence methods to measure radical particle activity.

Spectrophotometry is an analytical method for measuring the properties of an analyte based on the absorption of light of different wavelengths. It uses monochromatic radiation that is absorbed by the analyte in solution and the intensity of the unabsorbed light is compared to the radiation source, with the amount of light absorbed being proportional to the concentration of the analyte. For this analysis, the muscle biopsy is processed into homogenate or isolated mitochondria. Measurement of the activity of the complexes is carried out using enzymatic reactions for the selected complex to determine its activity.

4.1 High-resolution respirometry

The pioneers of polarography, Chance and Williams, created the initial instrument based on the Clark electrode in 1955 (125). Its application was the measurement of isolated mitochondria. In 1989, Gnaiger built a precursor to the oxygraphy (126), which entered mass production three years after.

The well-known high-resolution respirometry (HRR) Oroboros 2k is a worldwide accepted method to investigate directives of mitochondrial physiology and pathophysiology. High-resolution respirometry is a technique used to investigate the bioenergetics of cells using a limited number of biological samples. Bioenergetic parameters can be determined from isolated mitochondria, permeabilized tissues and cells, or tissue homogenates. The HRR technique allows for determination of respiration, membrane potential and the production of oxygen radicals, calcium ions or ATP, with the use of a fluorometer. The

Oxygraph-2k utilises two enclosed chambers to assess oxygen consumption through oxygen polarographic sensors. These sensors feature an Ag/AgCl anode and a gold cathode, which are immersed in a KCl electrolyte solution. A teflon membrane covers the electrode, which oxygen traverses to the cathode where it undergoes reduction to water. At the anode, Ag oxidises, producing a current that is directly proportionate to the partial pressure of oxygen. The software converts oxygen consumption and expresses it in picomoles per second, relative to the amount of tissue measured. The instrument employs a Peltier heat pump to maintain constant temperature and glass-covered magnetic stirrers to ensure consistent environmental homogeneity, both measures that enhance measurement reliability.

By selecting an appropriate protocol, with subsequent addition of substrates and inhibitors, values are obtained to determine the bioenergetic parameters of the measured mitochondria (127). These parameters include determination of respiratory chain complex activity, ATP consumption, non-mitochondrial respiration, uncoupled respiration or oxidative respiration.

4.2 **The parameters of mitochondrial respiration**

Mitochondrial respiration is divided into four phases, LEAK respiration, OXPHOS respiration, electron transfer (ET) capacity and residual oxygen consumption (ROX). This division applies to mitochondrial preparations of the isolated mitochondria type, homogenates and permeabilized cells and tissues. These phases are obtained by titrating ADP, substrates and uncouplers, and specific inhibitors of metabolic pathways. In living cells, the phases are LEAK, ROUTINE, ET and ROX (128, 129).

Leak respiration is a result of protons leaking through the mitochondrial membrane, independent of ATP synthase, due to oligomycin inhibition. Two processes of proton leakage exist: basal leak, which is unregulated, and induced leak, which is catalysed by specific inter-membrane proteins and can be both activated and inhibited. Proton leak depends on the composition of the membrane's lipid bilayer and proteins. Anion-transporting proteins, like adenine nucleotide translocase, usually contribute to basal proton leak but not to its activity. Regulated proton leak, on the other hand, involves both adenine nucleotide translocase activity and uncoupling protein 1 (UCP1), which is mainly found in brown adipose tissue (130). Electron leakage can occur across the electron chain, mainly at complexes I and III, which serve as the primary oxygen radical generators.

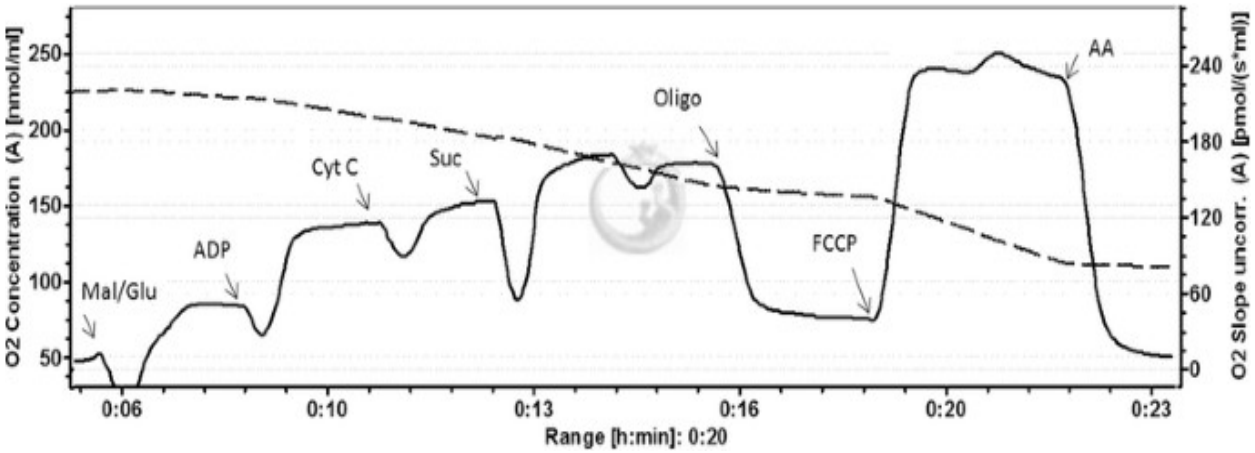
LEAK phase is measured with reduced substrates, in the absence of ADP or in the presence of inhibition of the phosphorylation system by the inhibitor oligomycin. Thus, no oxidative phosphorylation occurs and the oxygen flux corresponds only to electron leakage.

Oxidative phosphorylation is the oxidation of reduced substrates to oxygen in the electron transport chain chemically coupled to the phosphorylation of ADP to ATP. The parameter measured is the OXPHOS capacity, which gives the maximum coupled respiratory capacity of the mitochondria in the ADP-activated state, with saturating amounts of ADP, inorganic phosphate, oxygen, and reduced substrates. The electron transport capacity represents the respiratory capacity of electron transfer through the respiratory chain in the mitochondrion, measured as oxygen consumption in the uncoupled state, with an optimal concentration of uncoupler. For example, carbonyl cyanide-p-trifluoromethoxyphenylhydrazone can be used as an uncoupler and with gradual titration to achieve the maximum oxygen flux that determines ET capacity. By comparing the OXPHOS/ET capacity ratio, we obtain information about the reserve capacity of oxidative phosphorylation. If the ratio is equal to 1, the capacity of the respiratory chain is fully utilized and is not limited by oxidative phosphorylation. If the ratio is less than 1, respiration is not fully utilized and there is reserve capacity in oxidative phosphorylation. A ratio greater than 1 indicates the presence of arteficial compounds in the measured sample (129).

Oxygen consumption by residual particles is obtained after inhibition of total respiration, when only autooxidative particles such as haemochromogens or haematins are oxidised. The parameters of mitochondrial respiration, OXPOHOS, LEAK and ET are often adjusted by subtracting ROX respiration.

Respiration can be classified into five distinct permanent phases. The measurement process commences at phase 1, which involves adding a sample containing mitochondria to a medium that contains inorganic phosphate. After the addition of ADP, endogenous substrates are then consumed. The subsequent phase known as ROX corresponds to the consumption of endogenous substrates. Phase 3 occurs when substrates for complex I and II are added. Finally, in phase 4, respiration proceeds after ADP depletion or ATPase inhibition. Phase 5 takes place once all the oxygen in the ventricle has been consumed. Antimycin A is utilised to initiate phase 5 and to identify any residual oxygen consumption, as depicted in Figure 3.

Figure 3. Mitochondrial functional indices as determined by high resolution respirometry in skeletal muscle homogenate



Note: AA = Antimycin A; ADP = adenosine diphosphate; Cyt C = Cytochrome c; FCCP = Carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone; Mal/Glu = malate + glutamate; Oligo = Oligomycin; Suc = succinate.

5 HYPOTHESES AND OBJECTIVES

Hypotheses

The study hypotheses are defined as follows:

H1: Rehabilitation with FPS improves glucose uptake and reduces insulin resistance.

H2: Rehabilitation with FPS improves nitrogen balance in extensive burns.

H3: Early rehabilitation with FPS is associated with an augmentation in oxidative phosphorylation capacity.

Objectives

Based on these findings, we have defined the following objectives:

a. To comprehensively describe the mitochondrial function in the skeletal muscle of the extensively burned in both the late acute and prolonged phases of the disease.
b. To investigate the impact of early and late FPS rehabilitation on the bioenergetics of severe burns.

6 MATERIAL AND METHODS

6.1 Trial Design

We conducted a single center, randomized controlled cross-over study carried out in 10 level 3 bed intensive care unit at the Department of burns 3rd Medical Faculty of Charles University and University Hospital Kralovske Vinohrady, Prague, Czech Republic. All the procedures were in accordance with the ethical standards of the responsible institutional committee on human experimentation and with the Helsinki Declaration of 1975, as most recently amended. The Ethical Review Board of the Kralovske Vinohrady University Hospital approved the protocol on 26/06/2019 under the number EK-VP/47/0/2019, and the trial was *a priori* registered on ClinicalTrials.gov on 07/08/2020 under the number NCT04467619.

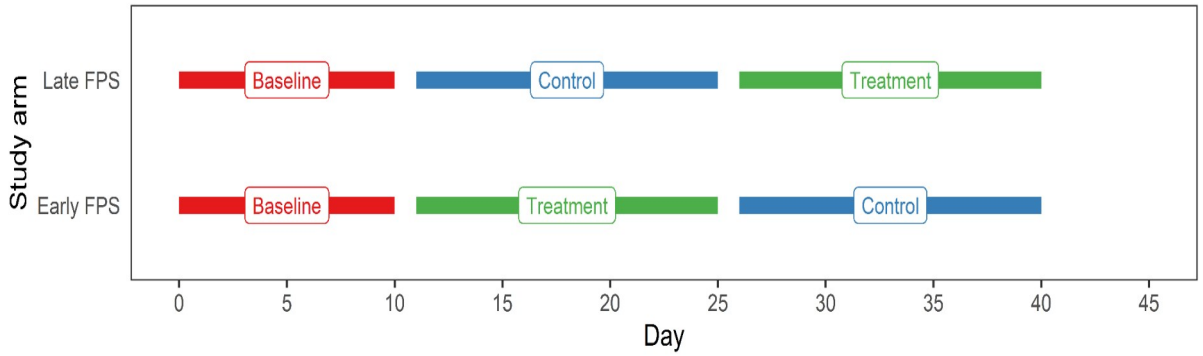
6.2 Study Participants

Study subjects were adult (>18 years) patients with deep (IIa, IIb and III) burns encompassing 30 % or more of their total body surface area (TBSA), admitted between 01/04/2021 and 31/03/2023. A prospective, written informed consent was obtained from all participants or their legal representatives. Patients with pre-existing neuromuscular disease, diabetes, on steroids (any dose), the presence of an implanted pacemaker or defibrillator, malignancy, leg amputation, those enrolled into another outcome-based study or those with terminal illnesses were excluded.

6.3 Study procedures

During the pre-screening phase, all patients with extensive burns received a standard guideline-driven burn care, which included regular surgical wound treatment, calorimetry-based goal-directed nutrition and standard physiotherapy started at day 5 and continued throughout and beyond the trial. Between the 5th and 10th day following admission, eligible patients were enrolled into the trial. After baseline metabolic studies, which included vastus lateralis biopsies and hyperinsulinemic euglycaemic clamp (vide infra), patients were randomized to either receive FPS in addition to standard physiotherapy or standard physiotherapy alone for the following two weeks. Afterwards, metabolic studies were repeated, and the allocated intervention were crossed over. After another two weeks, the third and last set of metabolic studies was performed. Randomization was performed by computer generated random sequence without the use of blocks or stratification. The overview of study design is in Figure 4.

Figure 4. Study design



6.4 Intervention

Functional proprioceptive stimulation was administered twice daily for 30 minutes by a dedicated, trained physiotherapist as per the manufacturer's instructions, using the Vibramoov device (Techno-concept Inc., France). The applicators were positioned bilaterally on the lower limbs, and the device was set to medium-intensity, multi-activity movements. The device replicates proprioceptors in a manner equivalent to active exercise, prompting a central nervous system response akin to volitional exercise. Patients were receiving standard physical therapy in addition to using the device.

6.5 Control group

Standard physiotherapy was administered by an independent team of physiotherapists uninvolved in the trial. For patients with extensive burns, the treatment normally involves non-active physiotherapy on the uninjured limb, utilization of the stretch reflex, and respiratory physiotherapy if necessary. This was the sole form of rehabilitation received by the control group. Early mobilization was introduced to eligible patients as soon as it became feasible.

6.6 Outcomes

A recent study (131) found energy expenditure in 43 patients with extensive burns to be 3845± 398 kcal.day^{-1}. In order to have 0.8 probability to detect an increase in REE by 15 % induced by FPS at $p<0.05$, twelve subjects are needed. Given the expected high dropout rate, we planned a priori to continue enrolment until the target of 12 patients had been reached. We monitored and reported protocol implementation (feasibility), and

adverse events. Exploratory outcomes of the study were insulin-mediated glucose disposal (an index of whole-body insulin sensitivity), nitrogen balance, rate of glucose and oxidations. And indices of mitochondrial functions.

6.7 Indirect Calorimetry and Calculations

Oxygen consumption and CO_2 production were measured daily between 8 and 9 am by indirect calorimetry (Q-NRG+, Cosmed, Italy) for 30 mins or until steady state was reached. We took care to use calibration gases closest to FiO_2 of inspired air, which had not been changed for 3 hrs. before the procedure. Leaks from the ventilator circuit or intercostal drains had to be 0.1 $L.min^{-1}$. In spontaneously breathing patients, we used a ventilated canopy system. Nitrogen excretion was estimated as the sum of nitrogen in urea, ammonia and creatinine excreted in urine plus 0.8 g/day attributed to nitrogen loss through skin and stool. We corrected for nitrogen loss by a change in urea in total body water. Energy expenditure (EE) was calculated (132). Carbohydrate and fat oxidation was calculated using Frayn equations (133), with the correction of nonprotein respiratory quotient according to the ratio of N excreted as ammonia.

Equations used for calculations of metabolic parameters:

RQ and VO_2:

$$RQ = \frac{VCO_2}{VO_2}$$

$$VO_2 = \frac{VCO_2}{RQ}$$

REE (kcal/day):

$$REE = 1440 * \left(3.941 * VO_2 + 1.106 * VCO_2\right)$$

$$REE = 1440 * \left(\frac{3.941 * VCO_2}{RQ} + 1.106 * VCO_2\right)$$

$$REE = 1440 * VCO_2 * \left(\frac{3.941}{RQ} + 1.106\right)$$

VCO_2 from REE and RQ (L/min):

$$VCO_2 = \frac{REE}{1440 * \left(\frac{3.941}{RQ} + 1.106 \right)}$$

Protein oxidation (g/day):

$$protein_{oxidation} = urinary_{nitrogen} * 6.25$$

Carbohydrates oxidation (g/day):

$$carbohydrate_{oxidation} = 1440 * \left(4.55 * VCO_2 - 3.21 * VO_2 \right) - 2.87 * urinary_{nitrogen}$$

Fat oxidation (g/day):

$$lipid_{oxidation} = 1440 * \left(1.67 * VO_2 - 1.67 * VCO_2 \right) - 1.92 * urinary_{nitrogen}$$

Adjusted for BSA g/day/1.73 m^2:

$$adj_{¿} = \frac{protein_{oxidation} * 1.73}{BSA}$$

$$adj_{¿} = \frac{carbohydrate_{oxidation} * 1.73}{BSA}$$

$$adj_{¿} = \frac{lipid_{oxidation} * 1.73}{BSA}$$

6.8 Euglycaemic Hyperinsulinaemic Clamps and Homeostasis Model Assessment

At the 10th, 25th, and 40th day after the injury, enteral and parenteral nutrition was interrupted at midnight till 10 a.m.; euglycaemic (goal blood glucose of 5 $mmol.l^{-1}$) hyperinsulinaemic (insulin infusion dose of 120 $mIU \cdot min^{-1} \cdot m^{-2}$) clamps procedures were performed at 08:00 am, as previously described (27). After adjustment for body surface area, glucose disposal rate during the last 30 mins of each clamp (M-value) represents a measure of insulin sensitivity. Of the glucose infused, indirect calorimetry allows us to calculate the proportion of glucose that is oxidized.

Insulin resistance was calculated from fasting insulin level and glycaemia according to the equation proposed by Matthews *et al*. (134) known as Homeostasis Model Assessment (HOMA).

$$IR_{HOMA} = (I_0 \times G_0) / 22.5$$

6.9 Skeletal muscle biopsies

Muscle biopsies were obtained from the vastus lateralis muscle under general anesthesia at the 10th, 25th, and 40th day after the injury during a routine surgical procedure unrelated to the study. A fresh homogenate was immediately prepared from the muscle sample (200 mg) for respirometry analysis, as described elsewhere (135). In brief: The sample was collected into 5 mL of ice-cold relaxing solution BIOPS. Connective tissue, fat and blood vessels were gently removed; the skeletal muscle fibres were dried by gauze and weighed on a calibrated scale and homogenized by 6-8 strokes in the Elvhjem-Potter teflon/glass. Immediately afterwards, high resolution respirometry was performed at 37°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). Immediately, two respirometry analyses were performed. Firstly, we analyzed global mitochondrial functional indices (by a standard substrate-uncoupler-inhibitor titration) and individual Complex I, II and IV functional capacities by serial addition of malate + glutamate, ADP, cytochrome c, succinate, ATP/ase inhibitor oligomycin, a potent uncoupler of mitochondrial oxidative phosphorylation (a protonophore) carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP), Complex I inhibitor rotenone, Complex IV inhibitor antimycin A, and ascorbate + tetramethylphenylenediamine (TMPD). Non-mitochondrial respiration (residual oxygen consumption, ROX) was subtracted from other values where appropriate (see Figure 3). Respiratory control ratio (RCR) as a measure of inner mitochondrial membrane coupling was calculated as state 3p/state 4. Secondly, the determination of palmitate oxidation (FAO) and Complex III functional capacity were performed by addition of palmitoyl-carnitine after priming with malate, cytochrome c and ADP. Afterwards rotenone was added and the decrement in oxygen flux resulting from inhibition of palmitate oxidation was calculated as FAO. Then, Complex III functional capacity was determined by the addition of freshly prepared duroquinol – an artificial Complex III substrate, followed by antimycine A to determine ROX. Oxygen fluxes reported are reflective of 20 mg of muscle tissue wet weight (Ww) in a chamber and were not normalized any further. Data from healthy controls were obtained from a previous study (136).

6.10 Statistical Analysis

For statistical data processing, the program R version 4.3.1with the graphical interface RStudio version 2023.06.2 was used. For data manipulation and graphical displays, the

collection of libraries for data science Tidyverse version 2.0.0 was used. Exploratory data analysis was performed for all parameters. Continuous data are presented as mean and standard deviation, and categorical data as counts and percentages. Loess regression was used to visualize the trend of parameters over time in the graphs. Unpaired t-test was used to compare changes in continuous parameters in the control and treatment phases. P values < 0.05 were considered statistically significant.

7 **RESULTS**

Out of the 34 patients admitted during the study period, we enrolled 25 patients. Complete datasets were obtained from 12 patients (See flowchart of the study at Figure 5). A summary of the baseline characteristics of those enrolled can be found in Table 1.

Figure 5. Flowchart of the study

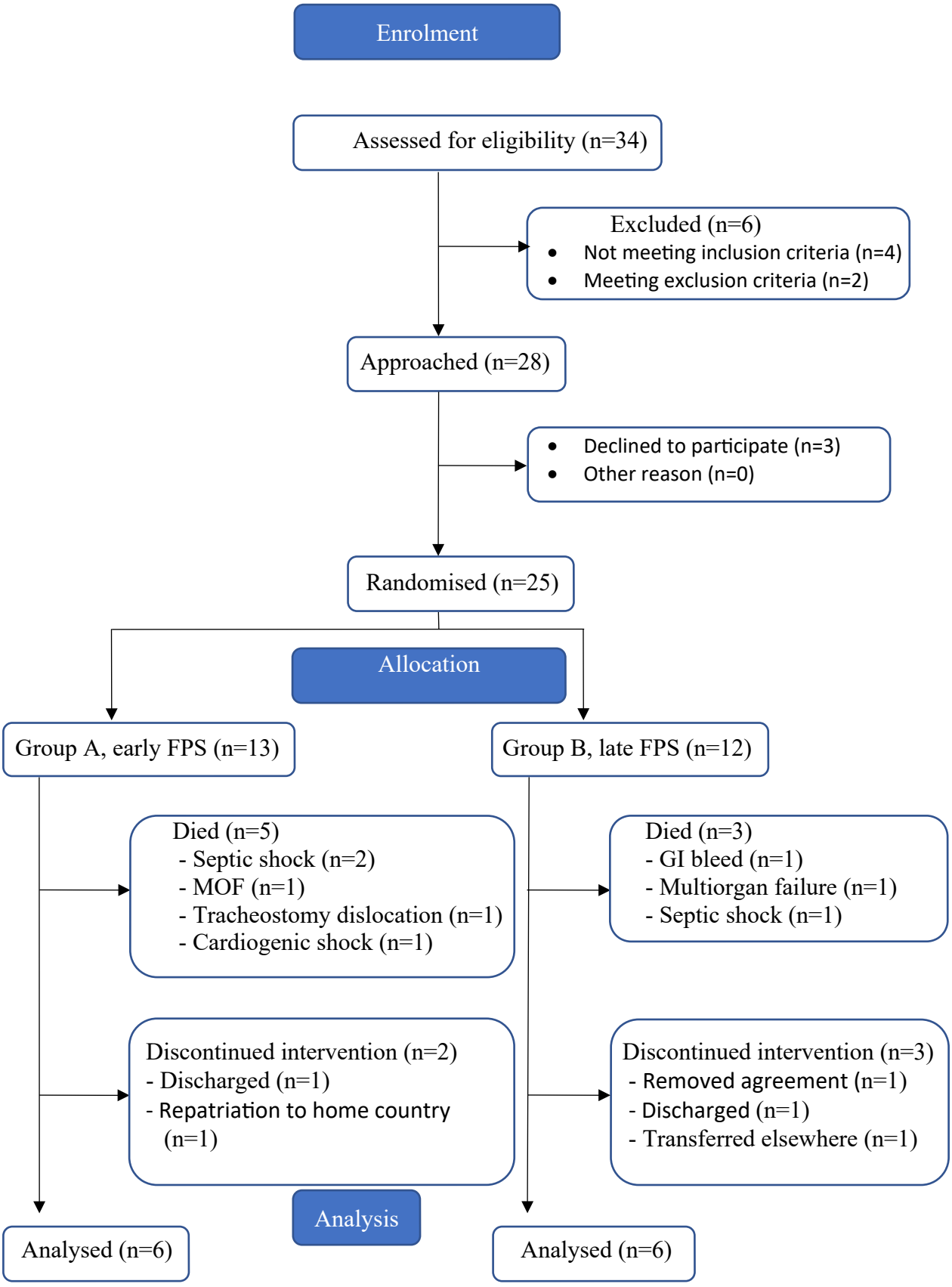


Table 1. Baseline Study Subject Characteristics.

Parameter	Group A (n=6)	Group B (n=6)
Age (y)	51 (40.8, 71.8)	41 (36, 60.3)
Gender (M/F)	5/1	6/0
Body Mass Index	28.8 (28, 32.8)	26.3 (24.9, 27.4)
Charlson Comorbidity Index (137)	4.5 (1.5, 6)	3 (0.3, 7.3)
Total Body Surface Area (%)	47.5 (37.5, 51.5)	42.5 (31, 66)
Revised Baux Score (%) (138)	49.5 (41.5, 58.3)	30 (26.3, 39.8)
APACHE II on admission (139)	14 (13.3, 16.3)	20.5 (16.3, 26.3)
Basal Metabolic Rate (kcal.24 h^{-1})	1943 (1804, 2096)	1808 (1767, 1998)

Note: APACHE = Acute Physiological and Chronic Health Score. Data presented as median (interquartile range).

There were no side effects of the FPS application nor complications of muscle biopsies. Clinical outcomes such as dynamics of SOFA score or length of stay did not differ among groups (see Tables 2 and 3).

Table 2. SOFA score (140)

	Day 10			Day 25			Day 40		
	Early FPS	Late FPS	*p*-value	Early FPS	Late FPS	*p*-value	Early FPS	Late FPS	*p*-value
SOFA score	6.5 (5.3, 7)	7 (6.3, 7.8)	0.10	3.5 (2.3, 5.5)	4 (3.3, 4)	0.41	1.5 (1, 3.5)	1.5 (1, 2.8)	0.41

Note: SOFA= Sepsis-related Organ Failure Assessment. FPS = FPS = Functional Proprioceptive Stimulation (Illusory Movements). Data presented as median (interquartile range).

Table 3. Length of hospital stay

	Early FPS	Late FPS	*p*-value
Length of stay (d)	86 (80.5, 90)	71 (57, 123.3)	0.38

Note: FPS = Functional Proprioceptive Stimulation (Illusory Movements). Data presented as median (interquartile range).

7.1 **Whole body energy metabolism**

In order to assess short term effects of FPS we recorded calorimetry data after each FPS session and compared with the measurements at baseline. There were no significant changes in energy expenditure, but both early and late application of FPS led to a significant decrease in RQ, reflecting a shift from carbohydrate to lipid utilization (Table 4).

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Table 4. Short term changes in whole body energy metabolism induced by 30 min. of FPS

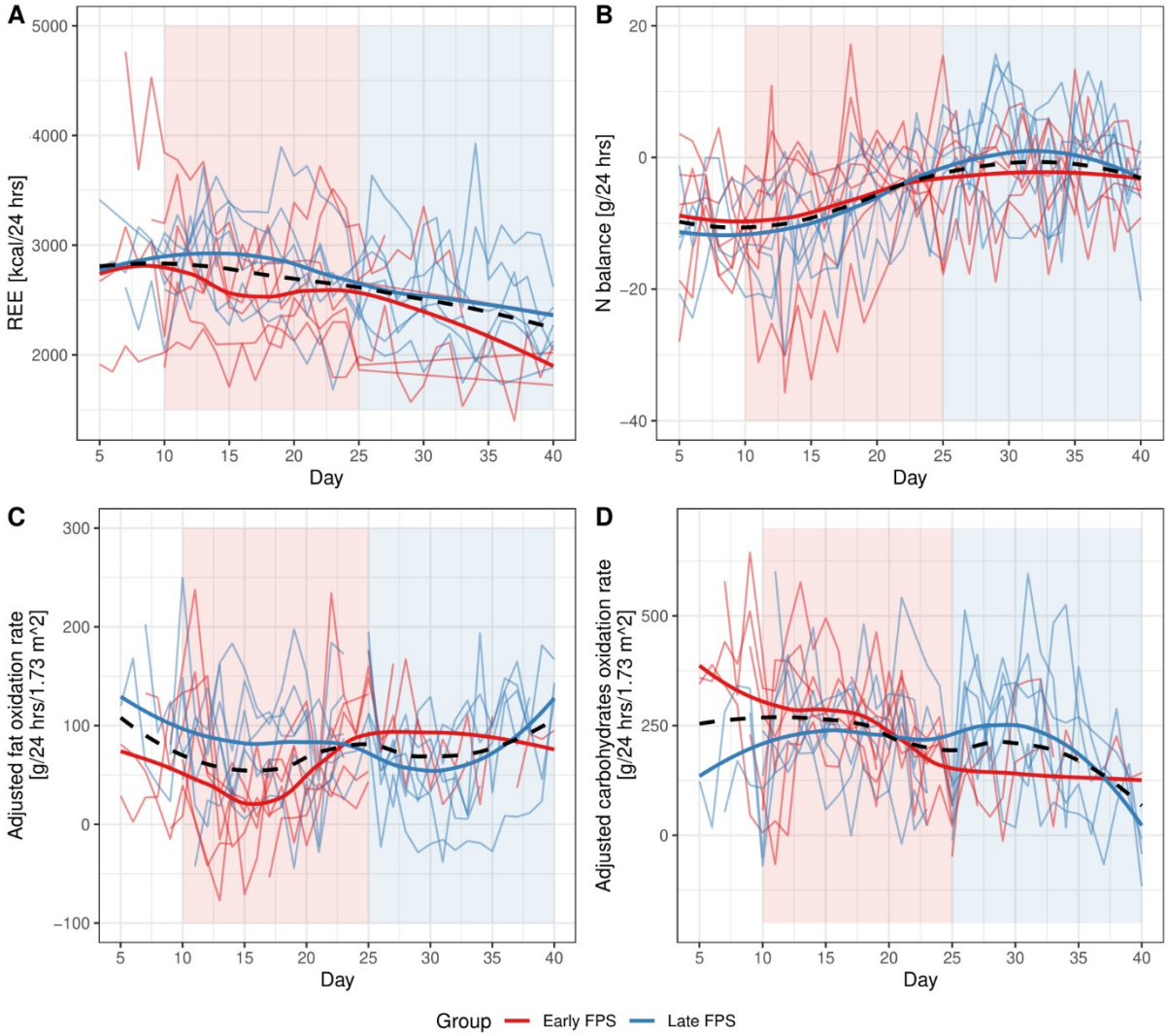
	Group Early FPS (n=6)			Group Late FPS (n=6)		
	before FPS	after FPS	*p*-value	before FPS	after FPS	*p*-value
Respiratory quotient	0.87 (0.85; 0.90)	0.82 (0.82; 0.84)	< 0.05	0.85 (0.83; 0.87)	0.83 (0.78; 0.85)	< 0.05
Energy expenditure [kCal.24 h^{-1}]	2645 (2299; 2851)	2636 (2285; 3036)	0.62	2466 (2229; 2785)	2611 (2338; 2778)	0.39
Glucose energy source [% of EE]	38 (23.1; 42.6)	27 (24.2; 28.1)	< 0.05	36 (28.5;43.1)	29 (15.1;36.3)	< 0.05
Lipid energy source [% of EE]	27 (16.4; 31.0)	40 (32.3; 43.0)	< 0.05	30 (27.4; 40.2)	40 (30.8; 55)	< 0.05
Protein energy source [% of EE]	36 (31.9; 40.6)	36 (32; 38.1)	0.21	32 (30.2; 36)	32 (28.5; 35.6)	0.54
VO_2 [ml.min^{-1}]	371 (318; 432)	370 (316; 436)	0.77	369 (332; 397)	385 (346; 404)	0.69
VCO_2 [ml.min^{-1}]	327 (293; 361)	306 (276; 346)	0.52	296 (266; 330)	298 (272; 320)	0.77

Note: EE – Energy Expenditure. FPS = Functional Proprioceptive Stimulation (Illusory Movements). Data presented as median (interquartile range).

In order to assess long term FPS effects compared to standard of care, we obtained indirect calorimetry morning recordings in 315 out of 360 total days in the study (12 % of the measurements were missed). Resting energy expenditure (REE) has been gradually decreasing over time with marked intra- and inter-individual variability and regardless of treatment group allocation (mean change induced by intervention -33 (95%CI -292;+227) kcal.day^{-1}, p=0.79, Figure 6A. There were no significant changes in N-balance or whole-body

carbohydrate and fat oxidation induced by the intervention (Figure 6 B-D). Detailed data on metabolic parameters are given in Tables 5, 6, and 7.

Figure 6. Resting Energy Expenditure (A), N-balance (B), Carbohydrate (C) and Fat Oxidation (D) throughout the study



Note: Thin lines represent individual data, thick line mean per group, dashed line mean of all patients. Red = early FPS, blue = late FPS.

Table 5. Metabolic parameters at fasting condition on the Day 10

	Day 10		
	Early FPS	Late FPS	*p*-value
Glucose disposal ($mg.kg^{-1}.min^{-1}$)	2.4 (2, 2.7)	2.4 (1.9, 2.9)	0.44
Nitrogen balance ($g.24\ h^{-1}$)	-9.4 (-12.3, -6.4)	-8.8 (-13.3, -6.1)	0.50
Respiratory quotient	0.79 (0.78, 0.84)	0.80 (0.73, 0.83)	0.34
Measured BMR (% of calculated BMR)	131 (118, 170)	144 (122, 164)	0.44
Glucose energy source (% of BMR)	12 (10, 37.3)	22 (4.8, 34.8)	0.44
Lipid energy source (% of BMR)	53 (27.8, 63)	44 (38.8, 71)	0.37
Protein energy source (% of BMR)	36 (29.8, 39.8)	25 (21.3, 31)	0.06

Note: BMR = Basal Metabolic Rate. Data presented as median (interquartile range).

Table 6. Metabolic parameters at fasting condition on the Day 25

	Day 25		
	Early FPS	Late FPS	*p*-value
Glucose disposal ($mg.kg^{-1}.min^{-1}$)	3 (2.5, 3.7)	3.1 (2.7, 3.7)	0.37
Nitrogen balance ($g.24\ h^{-1}$)	-4.5 (-8.9, -1.5)	-6.1 (-7.3, -4.9)	0.44
Respiratory quotient	0.77 (0.74, 0.82)	0.78 (0.76, 0.79)	0.44
Measured BMR (% of calculated BMR)	119 (116, 141)	135 (118, 143)	0.37
Glucose energy source (% of BMR)	4 (0.3, 22.8)	10 (1.5, 18.3)	0.44
Lipid energy source (% of BMR)	56 (41, 69.3)	57 (52, 67)	0.47
Protein energy source (% of BMR)	33 (29, 34.8)	30 (25.3, 39)	0.37

Note: BMR = Basal Metabolic Rate. Data presented as median (interquartile range).

Table 7. Metabolic parameters at fasting condition on the Day 40

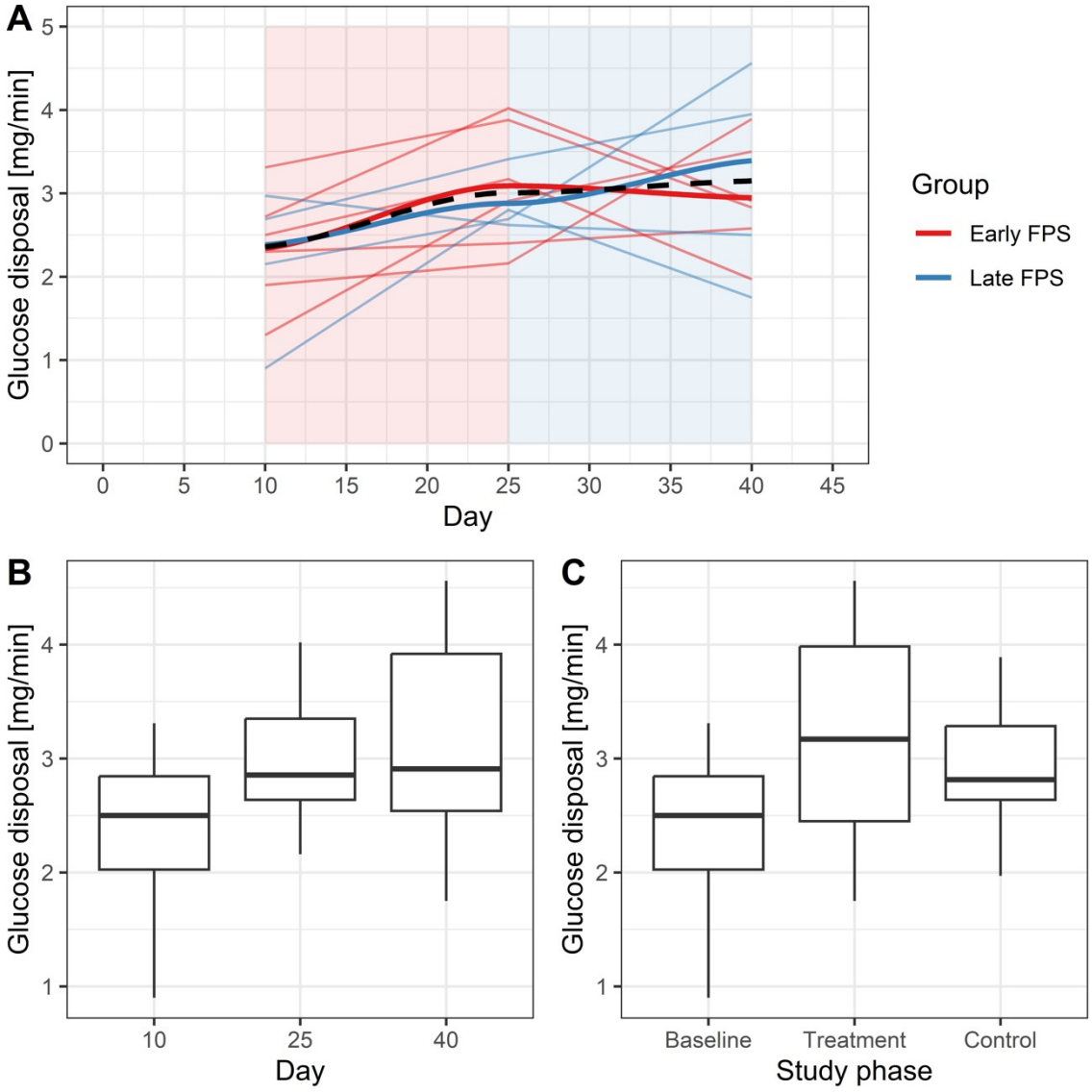
	Day 40		
	Early FPS	Late FPS	*p*-value
Glucose disposal ($mg.kg^{-1}.min^{-1}$)	2.9 (2.6, 3.4)	3.1 (2.6, 4)	0.44
Nitrogen balance ($g.24\ h^{-1}$)	-2.8 (-4.0, -1.2)	0.7 (-1.2, 2.8)	< 0.05
Respiratory quotient	0.81 (0.74, 0.83)	0.73 (0.72, 0.74)	0.09
Measured BMR (% of calculated BMR)	107 (100, 119)	113 (104, 129)	0.29
Glucose energy source (% of BMR)	21 (3.8, 29)	5 (0.5, 9.5)	0.19
Lipid energy source (% of BMR)	55 (44.3, 65.8)	58 (48.5, 66.5)	0.50
Protein energy source (% of BMR)	30 (20.3, 3.8)	35 (33.3, 38.8)	0.08

Note: BMR = Basal Metabolic Rate. Data presented as median (interquartile range).

7.2 **Insulin sensitivity and resistance**

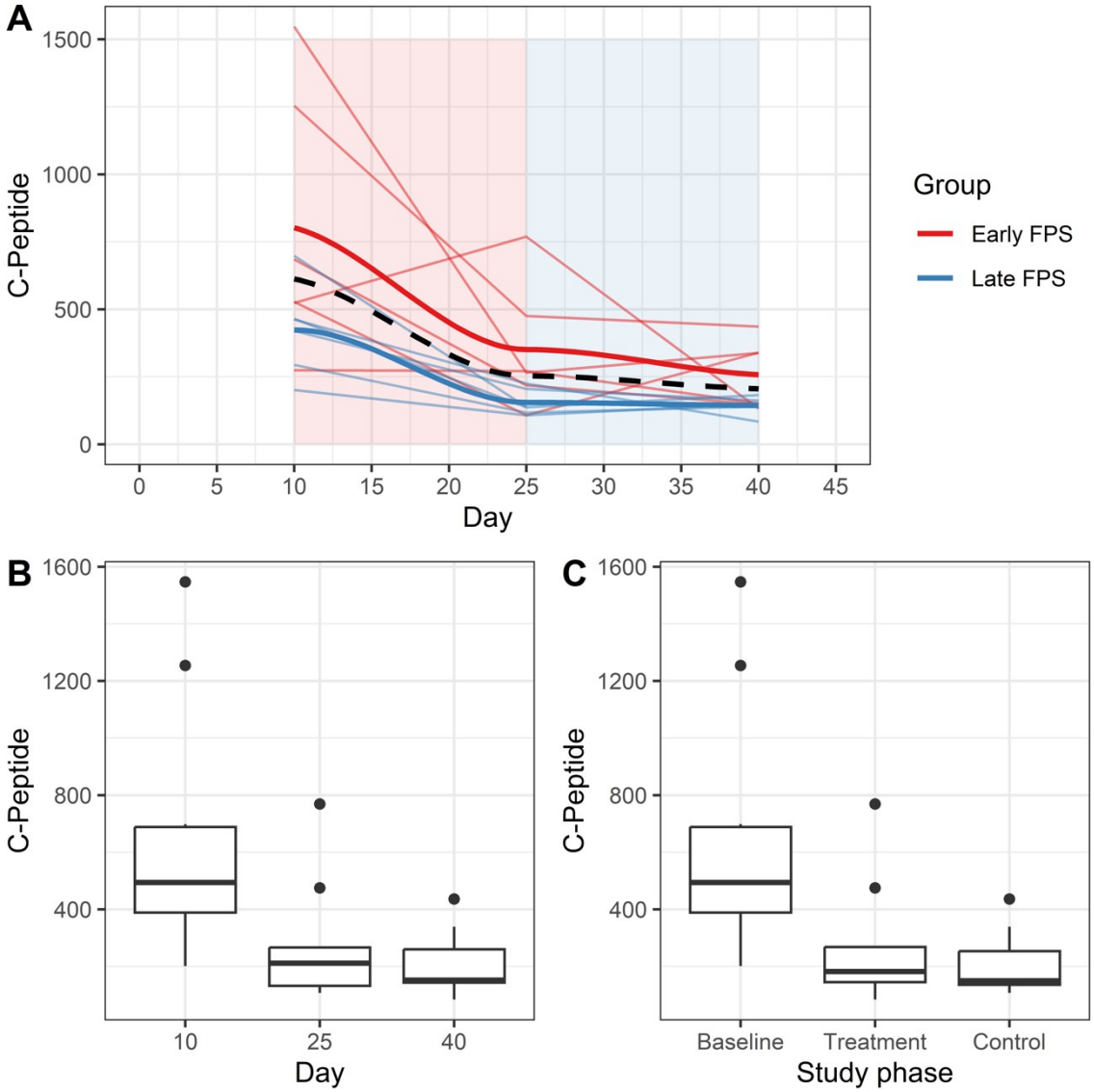
In line, there were no significant changes in insulin sensitivity induced by intervention insulin-mediated glucose disposal. The mean difference in glucose disposal induced by intervention was -0.33 (95% CI -1.18; +0.53) mmol.h^{-1}, p=0.43. Insulin sensitivity and endogenous hyperinsulinaemia normalizes in both groups over time during the course of burn disease (See Figures 7 and 8).

Figure 7. Insulin-mediated glucose disposal during euglycaemic hyperinsulinaemic clamp



Note: Thin lines represent individual data, thick line mean per group, dashed line mean of all patients.

Figure 8. Dynamics of C-peptide [µmol/L] as a measure of endogenous insulin secretion



Note: Thin lines represent individual data, thick line mean per group, dashed line mean of all patients.

Insulin resistance calculated as HOMA normalised in both groups between the Day 10 and 40, see Figure 9. There were no significant differences between groups on the Day 25 or 40.

7.3 **Plasmatic triacylglycerols (TAG)**

Changes in TAG can be seen in Figure 10. There were no significant differences between groups throughout the study.

Figure 9. Insulin resistance calculated as HOMA

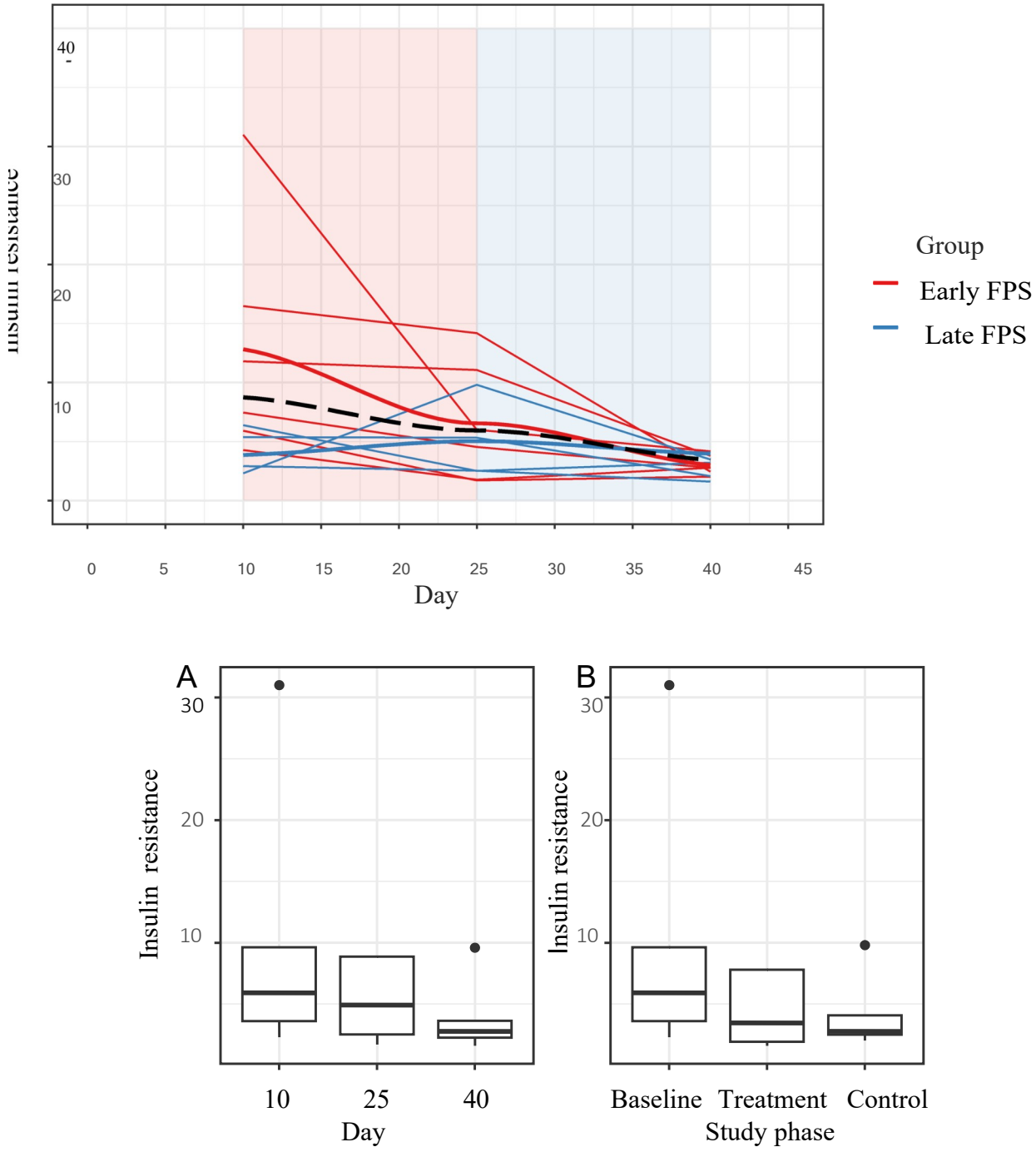
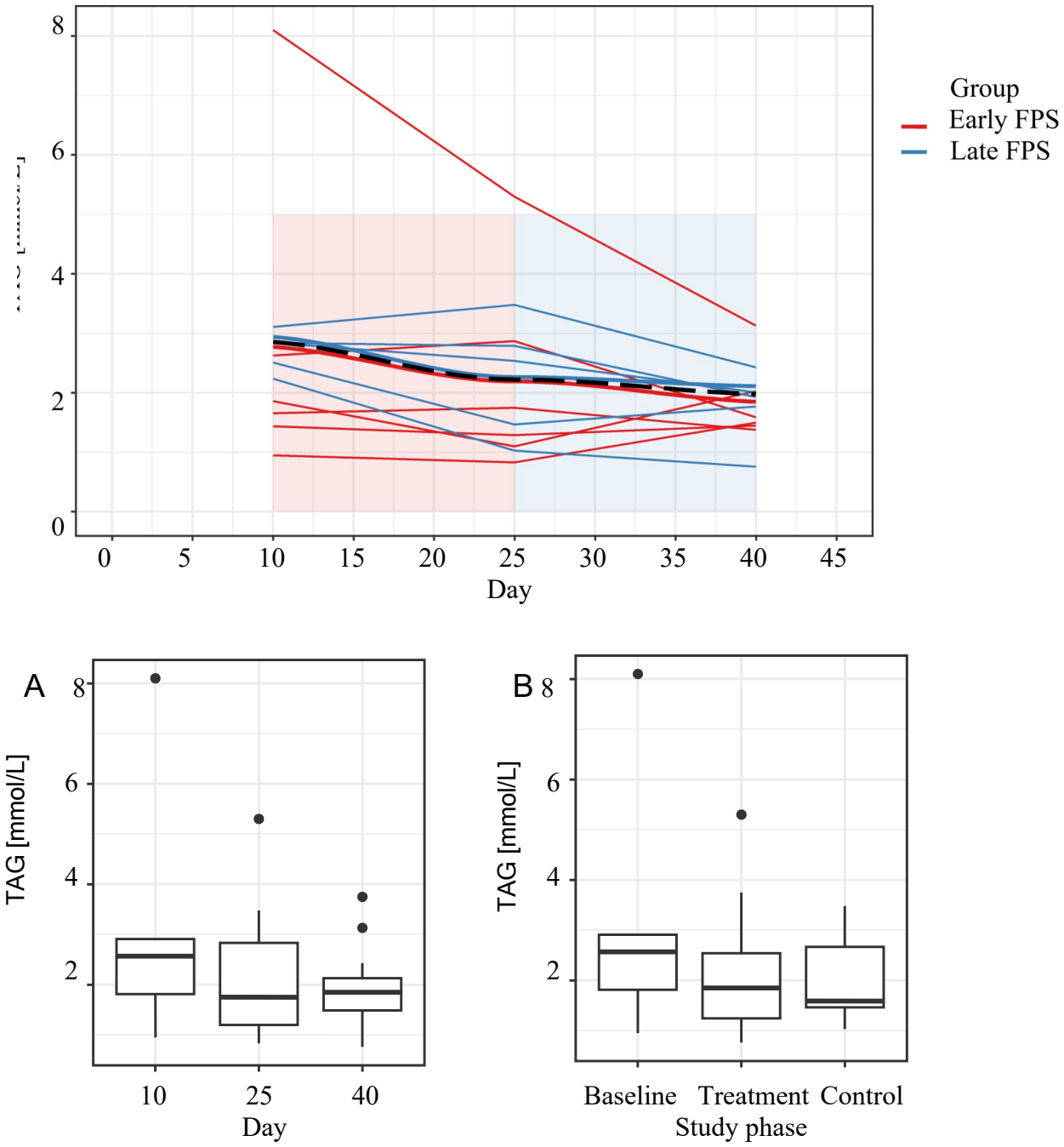


Figure 10. Changes in TAG



Note: Thin lines represent individual data, thick line mean per group, dashed line mean of all patients.

7.4 **Skeletal muscle bioenergetics**

High resolution respirometry of native skeletal muscle homogenates was successfully performed in 3 time points in all 12 patients. Integrity of outer mitochondrial membrane was well preserved after sample homogenization (with the increment of OCR after the addition of cyt c <20% in all samples). The capacity of oxidative phosphorylation (OXPHOS) at the start of the study was 191±53 pmol.min $^{-1}$.mg $^{-1}$ (74% of healthy volunteers) and tended to decline further over time in patients on standard treatment. Application of FPS significantly improved OXPHOS by 60 (95%CI 3;117) pmol.min $^{-1}$.mg $^{-1}$, p=0.041 (See Figure 9A). In line, oxygen consumption rate related to proton leak through inner mitochondrial membrane tended to be reduced by FPS: mean difference related to intervention -37 [95% CI -77;+3] pmol.min $^{-1}$.mg $^{-1}$, p=0.064, Figure 9B. There were no significant changes in functional capacities of electron transfer chain complexes I, II, IV nor in the capacity to oxidize fatty acids. See Figure 9 C-F.

Differences of skeletal muscle bioenergetics between groups in comparison to healthy controls on the Day 10, 25 and 40 were not significant, see Tables 8 - 10.

Table 8. Parameters of skeletal muscle bioenergetics in comparison to healthy controls on the Day 10

	Day 10		
Comparison to healthy controls (%)	Early FPS	Late FPS	*p*-value
ATP production	79 (70, 95)	69 (60, 93)	0.43
Complex I activity	88 (64, 119)	78 (62, 85)	0.28
Complex II activity	75 (59, 87)	54 (45, 64)	0.17
Complex IV activity	106 (94, 116)	73 (65, 89)	0.10
Fatty acid oxidation	110 (67, 133)	75 (70, 78)	0.23

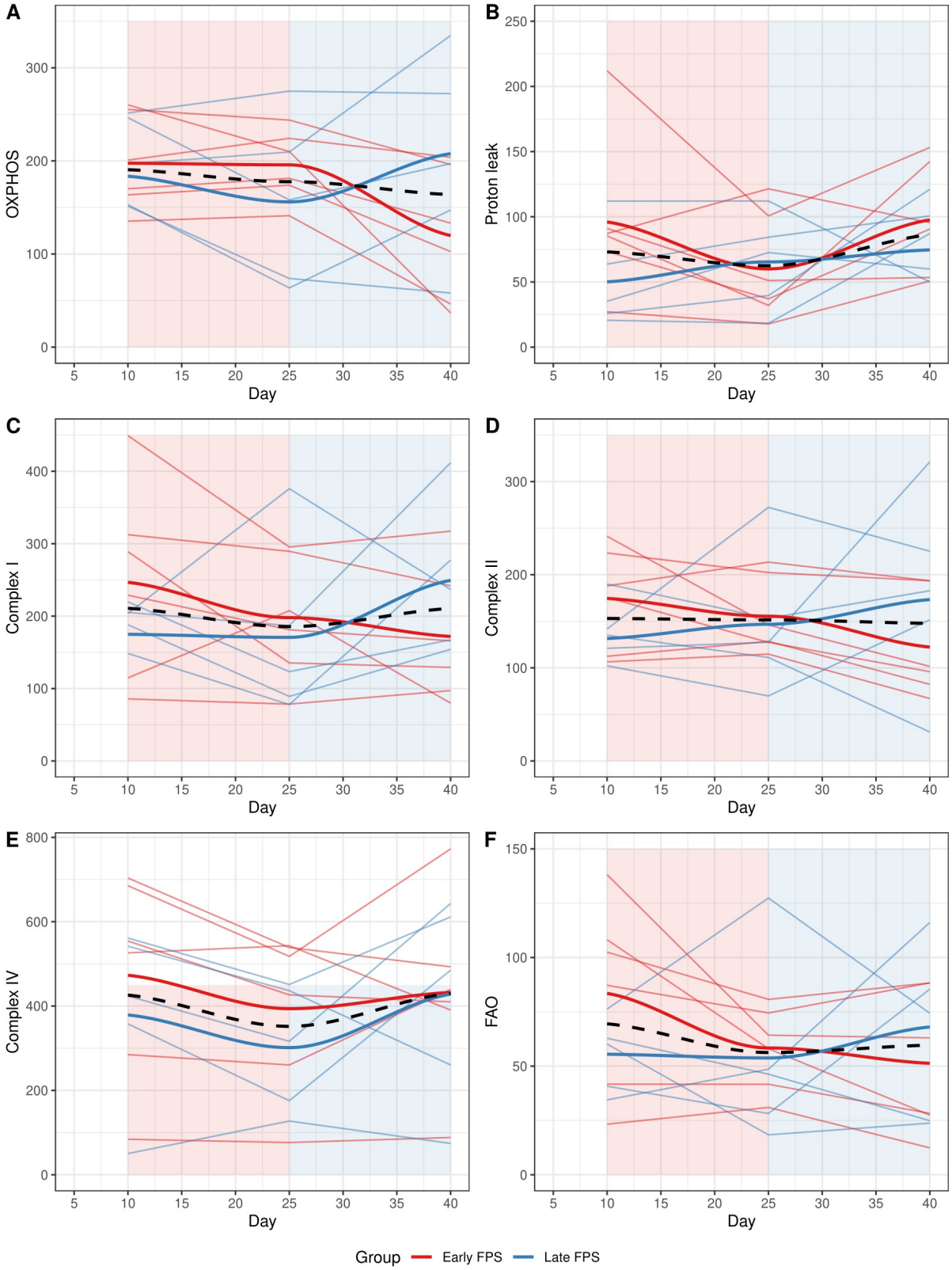
Table 9. Parameters of skeletal muscle bioenergetics in comparison to healthy controls on the Day 25

	Day 25		
Comparison to healthy controls (%)	Early FPS	Late FPS	*p*-value
ATP production	88 (70, 96)	73 (38, 97)	0.62
Complex I activity	71 (59, 98)	61 (39, 88)	0.77
Complex II activity	62 (54, 77)	59 (49, 73)	0.67
Complex IV activity	82 (74, 92)	77 (60, 91)	0.94
Fatty acid oxidation	81 (63, 100)	59 (42, 96)	0.72

Table 10. Parameters of skeletal muscle bioenergetics in comparison to healthy controls on the Day 40

	Day 40		
Comparison to healthy controls (%)	Early FPS	Late FPS	*p*-value
ATP production	64 (47, 79)	85 (63, 104)	0.23
Complex I activity	62 (35, 86)	96 (73, 106)	0.10
Complex II activity	43 (38, 70)	71 (57, 91)	0.35
Complex IV activity	76 (68, 83)	85 (80, 101)	0.43
Fatty acid oxidation	59 (35, 96	94 (47, 108)	0.72

Figure 11. Skeletal muscle mitochondrial functional indices



Note: Thin lines represent individual data, thick line mean per group, dashed line mean of all patients. Y axis = oxygen consumption in $pmol.min^{-1}.mg^{-1}$ of Ww. OXPHOS = oxidative phosphorylation; Proton leak = oxygen consumption rate related to uncoupling of inner mitochondrial membrane; FAO = fatty acid oxidation.

8 **DISCUSSION**

In this pilot study, we tested the biological effects of functional proprioceptive stimulation - an innovative rehabilitation intervention that produces afferent signals from proprioceptors to the central nervous system induced by vibrations instead of active movements. We chose to test it in the population most likely to potentially benefit from it: patients with extensive burns, who not only suffer from severe muscle wasting, but also have their mobility severely restricted for weeks due to the nature of their injuries. We found that although the intervention was feasible and well tolerated, it did not induce the expected metabolic response. At baseline, our patients were mildly hypermetabolic (REE ≅ 2500 kcal.24 h^{-1}, 136 % predicted) and suffered from severe muscle wasting (N balance ≅-10 g.24 h^{-1}, which means net loss of 63 g of protein or 320 g of lean body mass) and insulin resistance. Muscle wasting, insulin resistance and hypermetabolism slowly improved over time, with or without the FPS intervention. There was no increase in energy expenditure during and immediately after the FPS session, either. The only measurable difference was a consistent reduction in CO_2 production after the session, leading to a reduction of RQ by 0.03. This could be explained by a shift of substrate oxidation from carbohydrate to lipid oxidation or by transient hypoventilation after FPS, e.g. in case FPS itself induced hyperventilatory response and hypocapnia.

At cellular level, we found increased uncoupling of electron transfer chain and a mild reduction of oxidative phosphorylation capacity at baseline on average to 74 % of values seen in healthy volunteers, i.e. abnormalities consistent with critical illness. Interestingly, although FPS did not measurably improve the functional capacities of any of the complexes of electron transfer chain nor its ability to oxidize fatty acids, it improved the overall capacity of oxidative phosphorylation – most likely due to increased coupling of electron transport chain with ATP synthesis. Neurally mediated activation of metabolic processes has been described, e.g. in sprinters in splints just before the run race (141) or during electrical stimulation (142), and similar mechanism might play a role during illusory movements. We have not observed any reflection of these cellular changes in whole body metabolism, but our study was only powered to detect changes in REE of 15% or higher.

The increase in ATP production in muscle with FPS may also be explained by the activation of central mechanisms affecting skeletal muscle metabolism. This could provide a basis for further research focusing on combined stimulation of the brain as a central metabolic organ using afferent signalling (e.g. using FPS), efferent stimulation (e.g. using repetitive

transcranial magnetic stimulation (143)) and visual augmentation of movement (e.g. using virtual reality (144)) in critically ill patients whose ability to actively move is very limited or impossible.

The main strength of our study is a robust cross-over protocol in relatively homogenous population of patient with burns and thorough insight into skeletal muscle biology obtained by bioenergetic studies from serial muscle biopsies and hyperinsulinemic clamps. We gained in depth insight into the metabolic response to burn disease and its course during and after the transition from acute to protracted critical illness.

Indeed, this study has several limitations. Firstly, although cross-over design reduces the effect of confounders, we were unable to observe the natural course of burn disease without intervention applied earlier or later. Moreover, the onset and duration of metabolic effects of the intervention are unknown, and we cannot exclude carry-over effects. Secondly, the study was designed to measure the effect of the intervention on patient-centered clinical outcomes only. It is important to acknowledge that the absence of verifiable FPS effects should not be taken as evidence of the absence of clinical effects. Further, these clinical effects remain to be tested with sufficiently powered trials. Finally, as the dose-effect relationship remains uncharted, we recommend investigating more frequent or longer FPS sessions in future trials, given the intervention turned out to be feasible and well tolerated.

9 CONCLUSION

In conclusion, adding 2 sessions of 30 minutes daily of functional proprioceptive stimulation to usual physical therapy did not alter the energy expenditure, insulin sensitivity, nitrogen balance, or energy substrate oxidation in patients with extensive burns compared to physical therapy alone. However, it did improve the capacity for oxidative phosphorylation of skeletal muscle mitochondria, likely due to improved coupling.

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11 LIST OF PUBLICATIONS

1. Publications with IF that formed the basis of the Thesis

Bakalar B, Pachl J, Andel M et al. The high-dose alanyl-glutamine dipeptide administration attenuates insulin resistance in multiple trauma patients. *Clin Nutr* 20 (Suppl. 3):38, 2001. IF 2.317

Bakalar B, Pachl J, Andel M, Otahal M, Duska F, Pazout J, Fric M. Parenteral glutamine attenuates insulin resistance in multiple trauma patients. *Intens Care Med*, 29:2003 (Suppl. 1), S103. IF 2.71

Bakalar B, Duska F, Pachl J, Andel M. Glutamine increases oxidative rates of glucose disposal. *Intens Care Med*, 30:2004 (Suppl. 1), S60. IF 3.034

Bakalar B, Duska F, Pachl J, Fric M, Otahal M, Pazout J, Andel M. Parenteraly Administered Dipeptide Alanyl-Glutamine Prevents Worsening of Insulin Sensitivity in Multiple Trauma Patients. *Crit Care Med,* 2006;34(2):381-386. doi:10.1097/01.CCM.0000196829.30741.D4. IF 4.234 (Supplement 1)

Waldauf P, Urban T, Krajčová A, Jiroutková K, Blahutová B, Bakalář B, Řasová K, Grünerová-Lippertová M, Gojda J, Duška F. Can functional electrical stimulation–assisted cycle ergometry replace insulin infusion in patients? A nested substudy in a randomized controlled trial with 6 months' follow-up. *J Parenter Enteral Nutr*. 2022; 46: 249–253. IF 3.4 (Supplement 2)

Bakalář B, Švecová M, Duška F, Grünerová-Lippertová M, Urban T, Waldauf P, Zajíček R. Illusory movements for immobile patients with extensive burns (IMMOBILE): a randomized, controlled, cross-over trial. *Burns*, 2024. IF 3.2 (Supplement 3)

2. Publications without IF related to the topics of the Thesis

Duška F, Bakalář B, Anděl M. Growth hormone as an anabolic agent in the critically ill - History and perspectives. Diabetol Metab Endokrinol Vyziv. 2003;6(1).

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