Faculteit Geneeskunde en Gezondheidswetenschappen
Departement Revalidatiewetenschappen en Kinesitherapie

The effects of exercise on post-burn muscle wasting

De effecten van fysieke activiteit op spierafbraak na brandwonden

Proefschrift voorgelegd tot het behalen van de graad van
doctor in de medische wetenschappen
aan de Universiteit Antwerpen te verdedigen door:

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Antwerpen, 2024

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“Follow your dreams, believe in yourself and work hard for what you want to achieve, even in difficult times. It will be worth it.”

Nicola Spirig

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List of Abbreviations

4E-BP1/PHAS-1	Eukaryotic initiation factor 4E binding protein
AChR	Acetylcholine receptor
ADL	Activities of daily life
AKT/PKB	Protein Kinase B
AMP	Adenosine monophosphate/ 5'-adenylic acid
AMPK	AMP-activated protein kinase
ATP	Adenosine-5’-triphosphate
BCL	B cell lymphoma 2
Bnip3	BCL2 Interacting Protein 3
BW	Body weight
COX	Cytochrome c oxidase
CSA	Cross sectional area
DALYs	Disability-adjusted life-years
ECM	Extracellular matrix
EDL	Musculus Extensor Digitorum Longus
eEF2	Eukaryotic elongation factor 2
eIF2B	Translation initiation factor 2B
eIF3f	Eukaryotic translation initiation factor 3 subunit F
eIF-4E	Eukaryotic translation initiation factor 4E
FOXO1/3	Forkhead box protein O 1/3
GSK-3β	Glycogen synthase kinase 3β
IFN-γ	Interferon-γ
IGF-1	Insulin like growth factor-1
IL-1/6	Interleukin-1/6
IR	Infrared
IRS-1	Insulin receptor substrate 1

KLF15	Krüppel-like factor 15
LC3	microtubule-associated protein 1 light chain 3
mRNA	Messenger Ribonucleic acid
mtDNA	mitochondrial DNA
mTORC1/2	Mammalian target of rapamycin complex ½
MURF-1	Muscle-specific RING-finger 1
Atrogin-1/ MAFbx	Muscle atrophy F-box
MyHC	myosin heavy chain
MyoD	Myoblast determination protein 1
NF-κB	Nuclear factor-κB
Ns	non-significant
PGC1-α	Peroxisome proliferator-activated receptor-gamma coactivator 1-α
PI3K	Phosphoinositide-3-kinase
PIP3	Phosphatidylinositol (3,4,5)-trisphosphate
PIP3	phosphatidylinositol (3,4,5)-trisphosphate
PVDF	polyvinylidene difluoride
QOL	Quality Of Life
REDD1	Regulated in Development and DNA Damage Responses
REE	Resting Energy Expenditure
RIPA	Radioimmunoprecipitation assay buffer
RNA	Ribonucleic acid
S6K1/p70s6k	Ribosomal protein (rp)S6 kinase-1
SDH	succinate dehydrogenase
SEM	Standard Error of Means
SMAD2/3	Mothers against decapentaplegic homolog 2/3
SOL	Musculus Soleus
STAT3	Signal transducer and activator of transcription 3
TBS(-T)	Tris-Buffered Saline(-Tween)
TBSA	Total Body Surface Area

TGF-β	Transcription growth factor-β
TNF-α	Tumor necrosis factor α
TPS	Total protein stain
TRAF6	TNF receptor associated factor 6
TRIM32	Tripartite Motif Containing 32
UPS	Ubiquitin Proteasome System

Chapter 1

General introduction

Burn injuries are a major public health problem worldwide, affection millions of people annually. Worldwide burns of any cause, including flames, liquids, hot solids, chemicals or electrocutions, are the fourth most common form of trauma, causing an estimated 180 000 deaths every year. The epidemiology of burns is complex and varies widely across different regions and populations. A higher incidence in low- and middle-income countries causes burns to be the leading cause of disability-adjusted life-years (DALYs) lost [1,2]. Ranging from minor wounds to life-threatening thermal injuries, burns are a leading cause of morbidity and have a large socioeconomic impact [2]. Non-fatal burns cause extensive tissue damage and profound physiological disturbances, leading to a range of complications. Hence, the complex nature of burns' pathology extends beyond its immediate physical impact and affects various aspects of patient's general health, including devastating long-term physical, psychosocial and financial impacts [3].

The impact of burns depends on the degree and extent of the injury. Depth of burn injuries is categorized into different degrees *(Figure 1.1, A)*. First-degree burns are superficial, affecting only the top layer of skin and typically manifest as redness and mild pain, often healing without scarring. Hence, the term 'first-degree burn' can be somewhat misleading, as it primarily denotes an inflammatory reaction rather than a true burn wound with extensive tissue damage. Second-degree burns can manifest as either superficial or deep partial thickness injuries, extending deeper into the skin and resulting in the formation of blisters and more significant pain. Third-degree burns, the most severe, damage all skin layers and may involve underlying tissues, resulting in insensitivity to pain due to nerve destruction. Fourth-degree burns extend into muscles and bones, posing life-threatening risks [4]. The extent of a burn injury on the other hand, is assessed using the total body surface area measurement (TBSA) (*Figure 1.1, B)*. TBSA represents the percentage of the total body surface affected by burns. Based on the TBSA, burns are typically categorized into a severity scale. Burns encompassing <10%TBSA are considered a minor burn, 10-20% TBSA a moderate burn and ≥20%TBSA a severe burn [5]. The depth and severity of a burn injury, but also the presence of inhalation injuries and the age of the patient determine the course of treatment, the potential for scarring, and long-term consequences [6,7].

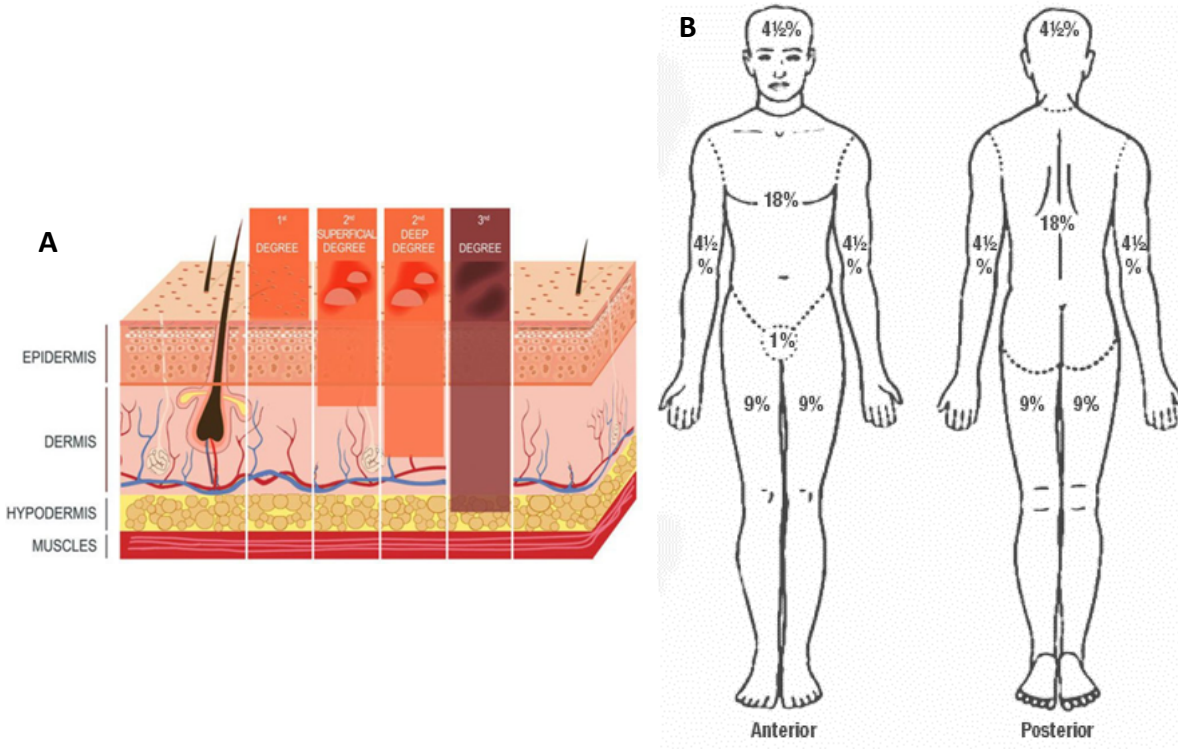

Figure 1.1 An overview of the classification of burn depth (A) and a body diagram for estimated percentage of total body surface area burned (TBSA) (B). The body surface is sectioned into areas with numerical values to determine the total %TBSA burned. (A) from elitelv.com; (B) from UWHealth.org

Current treatment strategies involve a multifaceted approach and are guided by the degree and TBSA of the burn, aiming at minimizing tissue damage, preventing infection and optimizing wound healing. Minor burns can often be managed without hospitalization, in contrast to moderate to severe burns, in which different approaches to facilitate healing are crucial, that also include pain management, infection control and nutritional support. Burn treatment requires a multidisciplinary team of healthcare professionals and personalized care plans [8].

Over the past decades, mortality from severe burn injuries has decreased substantially due to advances in preventive measures, medical care and surgical management [8]. As a result, new complications have emerged as persistent health threats affecting the lives of burn survivors for years after the initial injury [9]. Survivors often experience long-term physical and psychological consequences, including chronic pain, scarring, joint contractures, reduced mobility and muscle atrophy (also known as muscle wasting) [10]. The focus of medical care and scientific research concerning burns has shifted from the acute management to improving long-term physical functionality and quality of life of burn survivors [11].

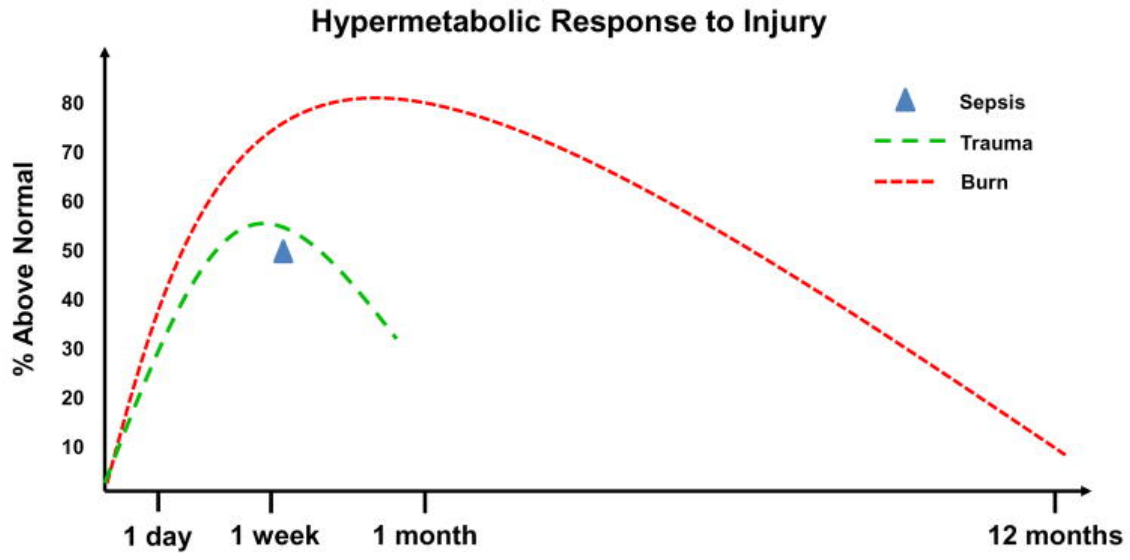

Figure 1.2 Long-term hypermetabolic response to injury in septic patients (sepsis), blunt trauma (trauma), or severe burns (burn). From Porter et al. (2016) [7]

The systemic sequelae of a severe burn injury (≥20% TBSA) are the result of the activation of a complex pathophysiological stress response [12]. During the first 24 hours post-burn ('ebb phase'), the most prominent feature is a lower resting energy expenditure (REE) to preserve energy depletion for vital organ survival [13]. During the subsequent 'flow phase', which persists for months to years after a burn injury, an increased REE ensues *(Figure 1.2)* [14]. During this phase, a persistent and strong inflammatory and humoral stress response induces a state of whole-body hypermetabolism characterized by an increased oxygen consumption, gluconeogenesis and lipolysis and most importantly, an increased proteolysis and a decreased mitochondrial energy production *(Figure 1.3)* [15,16,17]. The latter two are responsible for a significant progressive loss of skeletal muscle mass (muscle wasting) which, in the clinical situation, is aggravated by hospitalization-induced bed rest [18,19,20]. Hence, a complex interplay arises by the extensive tissue damage and the hypermetabolic response initiated by the burn injury itself, but also by prolonged periods of immobility and the impact of clinical treatments. The decrease in skeletal muscle mass and strength following a burn injury not only results in reduced physical function but also impacts patient's recovery and prognosis. As such, quality of life diminishes and healthcare usage heightens, which in turns escalates the economic load on both the individual and the broader societal framework [21]. Since skeletal muscle accounts for approximately 40% of total body mass and provides critical functions in metabolism, energy expenditure, strength and locomotor activities, postburn skeletal muscle wasting is a serious postburn comorbidity warranting attention [7,10,22]. Skeletal muscle wasting has been reported in other diseases, such as cancer, kidney dysfunction, heart failure and aging, as well, but its occurrence in burn patients presents a unique clinical challenge due to its complex pathophysiology [22,23].

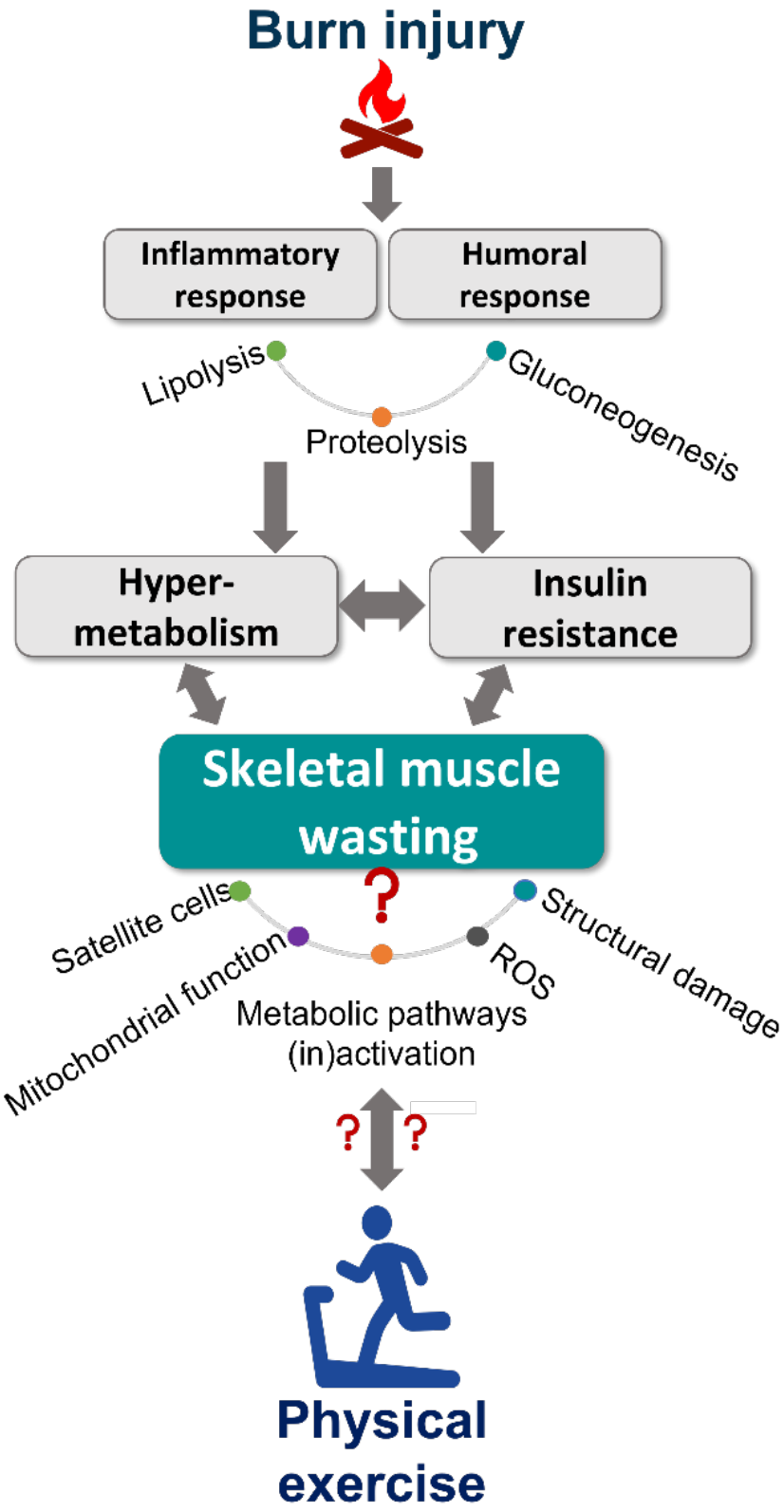

Figure 1.3 Schematic overview of stress-responses after burn injury, leading to hypermetabolism, insulin resistance and muscle wasting.

Skeletal muscles consist of distinct muscle fiber types, each characterized by a specific amount of MyHC isoforms and metabolic activity. Muscle fiber types are categorized in two main categories: slow-twitch (type I) and fast-twitch (type II), further divided into type IIa, IIb or IIx [24,25]. These differences result in varying contraction speeds, with type 1 fibers being primarily involved in endurance actions (e.g. postural activation) whereas type 2 fibers are primarily involved in fast actions (blinking eys, lifting weights), and different susceptibility to pathological signals [24]. In the human body, each individual skeletal muscle is typically composed of a mix of the fiber types in varying proportions. However, in rat specific muscles are primarily composed of either slow (m. Soleus (SOL)) or fast (m. Extensor Digitorum Longus (EDL)) muscle fibers (*Figure 1.4)* [26]. This unique composition allows the separate study of disease or therapy effects on the distinct fiber types. As such, research has shown that denervation and immobilization affect slow-twitch fibers more, while fast-twitch fibers are more prone to atrophy induced by sepsis, chronic heart failure or cancer cachexia [25]. Moreover, previous studies suggest that after burns, the different muscle fibers respond differently to the systemic effects of the burn injury as well, with fast-twitch muscles experiencing more pronounced protein breakdown compared to slow-twitch ones [27]. Therefore, understanding the fiber-type-specific effects of burn injury could offer crucial insights into the pathology and potential treatment strategies.

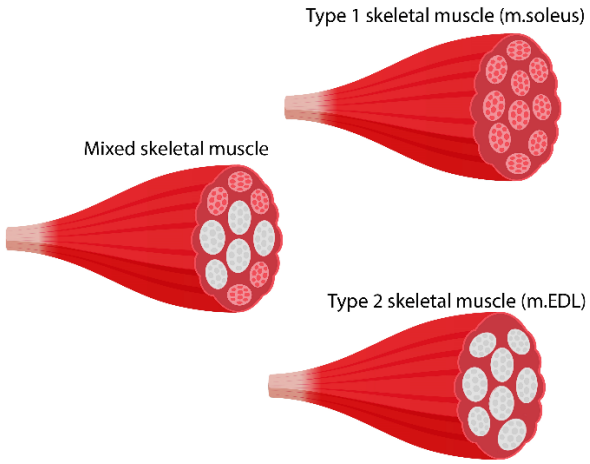

Figure 1.4 Most muscles are composed of a mix of muscle fiber types with different properties. To investigate specific fiber-specific effects, the rat model offers a unique possibility in that in rats and mice some muscles consist primarily of type 1 (m.soleus) or type 2 fibers (m.EDL).

Studies investigating the underlying processes of muscle wasting post-burn, and the potential differential effects in both muscle types, are scarce, leaving a significant knowledge gap in our understanding of this critical aspect of burn care. Research in other cachectic populations has yielded valuable insights into muscle wasting [23]. These insights provide a starting point for similar research within the burn population to understand muscle wasting in the context of post-burn recovery more successfully. Our research is constructed based upon the present insights regarding muscle wasting, together with already known systemic consequences of burn injuries.

Currently, an effective treatment to counteract skeletal muscle wasting post-burn is not available. Pharmacological and nutritional aids only show limited efficacy in counteracting post-burn muscle wasting [28]. Apart from their limited effects, issues such as adverse effects, cost benefits, ease of administration and monitoring are limiting the use of combination therapies, which are the most likely to maximally attenuate post-burn muscle wasting. Physical exercise on the other hand, has been shown to exert systemic anabolic factors, while simultaneously counteracting negative effects of prolonged disuse. Although physical exercise rehabilitation is already used as an early treatment strategy in other cachectic conditions, its use in burn rehabilitation is still limited and varies sharply between different burn centers [30,31,32]. To date, the main focus of burn rehabilitation is on restoring functionality, preventing hypertrophic scars and maintaining a good range of motion in the limbs. Exercise training, to specifically counteract muscle wasting, is a component that only, if at all, gets attention long after hospital discharge [33]. Even though post-burn, physical exercise as a clinical intervention may provide the greatest benefits to burn patients, positively impacting both molecular and functional level [21]. The incorporation of exercise doesn't only target the negative effects of postburn disuse, but also interact with numerous metabolic pathologies. In other cachectic diseases, exercise training has shown to target specific signaling pathways, helping in increasing protein synthesis and attenuating protein breakdown [30]. Consequently, the subsequent decrease of skeletal muscle atrophy is associated with improved quality of life, reduced hospitalizations and prolonged survival [34]. Acknowledging the profound significance of

physical exercise, given its potential to slow, prevent, or reverse muscle wasting, indicates that physical exercise should be considered a cornerstone in the treatment of skeletal muscle wasting. Nevertheless, the lack of studies in this area has limited the understanding of the numerous molecular alterations that exercise training may have on skeletal muscle wasting post-burn. Hence, recommendations on the use of exercise as part of the metabolic management are vastly underutilized due to a knowledge gap regarding the mechanisms involved in the metabolic changes following severe burns and the effects of exercise [33].

Filling these research gaps is essential for the development of more effective strategies for managing and mitigating muscle loss in burn survivors, ultimately enhancing their overall recovery and quality of life. Hence, we conducted an in-depth review of the state-of-the-art on post-burn muscle wasting. In addition, our focus was on elucidating the molecular processes involved and describing the effects of exercise on these mechanisms post-burn.

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Chapter 2

Outline and aims

Since long-term survival following severe burns has increased significantly in the last decades, the focus of burn rehabilitation has shifted from mere survival to improving the long-term consequences, including muscle wasting. Despite current research advancements, an effective therapy that prevents or reverses muscle loss is not yet available [1]. Recommendations on the use of exercise as part of the metabolic management are vastly underutilized due to a lack of knowledge based on scientific research regarding the mechanisms involved in the metabolic changes following severe burns [2]. Proving possible beneficial effects of exercise and refuting possible negative consequences may lead to an increase in understanding and consequently increase the use of early physical exercise programs to prevent post-burn muscle wasting. This in turn may increase the overall quality of life of burn survivors, and reduce the social burden associated with these devastating injuries. However, essential to the development of novel therapeutic rehabilitation approaches is a comprehensive understanding of the patho-physiological process(es) of skeletal muscle wasting after burns. Currently, the molecular processes associated with postburn muscle wasting have not been fully unraveled yet, hence also the effects of exercise remain unexplored.

We hypothesize that the implementation of an early physical exercise protocol can effectively mitigate skeletal muscle wasting within a severe burns rat model through restoration of the skeletal muscle protein balance.

The **main aim** of this thesis was to unravel the effects of physical exercise on the cellular and molecular pathways of skeletal muscle wasting in a rat model with severe burns (40%TBSA). To unravel the scientific knowledge gaps, specific aims were set up.

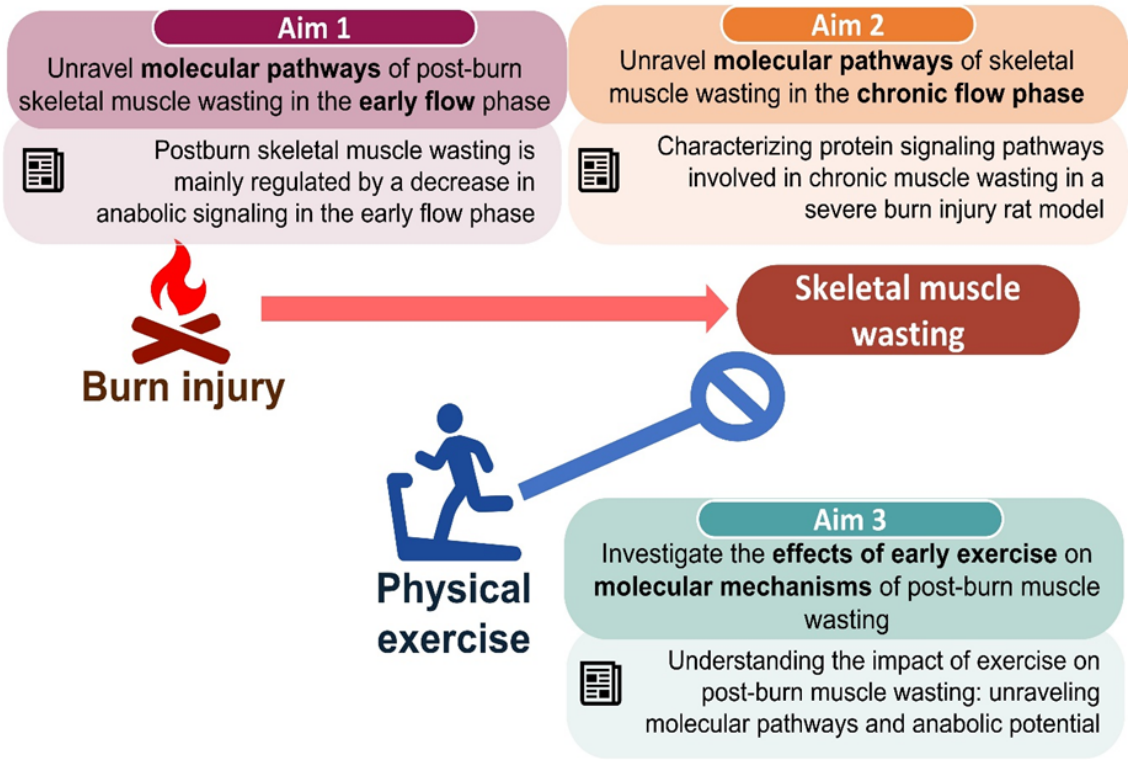

Figure 2.1: Conceptual overview of the research aims

Aim 1: Unravel molecular pathways of post-burn skeletal muscle wasting in the early flow phase

To gain a better understanding of the role of the skeletal muscle in both the short-term and long-term devastating effects of burns, detailed fundamental knowledge of the signalling pathways involved is crucial. Fundamental experimental studies, essential to unravel the contributions of various pathways that lead to disturbances in protein balance after burns, are still scarce, as elaborated in **Chapter 3**. Such information will serve as a critical basis for further animal and human research to eventually explore the effects of potential therapeutic interventions by targeting specific aspects of the signalling pathways responsible for post-burn muscle mass regulation. Therefore, our first objective was to unravel the molecular pathways of skeletal muscle wasting in the early flow phase, using a rat burns model.

In **Chapter 4,** we examined the impact of mild (10% TBSA) and severe (40% TBSA) burns on skeletal muscle wasting during the early flow phase. We analyzed *ex vivo* the underlying cellular and molecular pathways of skeletal muscle wasting in mild and severely burned rats at 10 days post-burn, with special emphasis on documenting the differences in type 1 and type 2 dominant muscles (musculus Soleus (SOL) and musculus Extensor Digitorum Longus (EDL), respectively). We collected anthropomorphic data, evaluated muscle morphology, and measured mitochondrial oxidative activity and apoptosis using the appropriate techniques. Furthermore, we analyzed various signaling proteins associated with skeletal muscle wasting, including crucial proteins involved in both anabolic and catabolic pathways.

Based on previous literature, we hypothesize that 10 days post-burn, muscle wasting will be present across all muscles, more pronounced in severely burned animals compared to mildly burned animals. We expect that during this phase post-burn, atrophy is attributed to a disrupted protein balance due to increased catabolic protein signaling in the soleus muscle (SOL), and continued catabolic protein signaling in the extensor digitorum longus (EDL), together with sustained decreased anabolic signaling in both muscles (*Figure 2.2)*.

Aim 2: Unravel molecular pathways of post-burn skeletal muscle wasting in the chronic flow phase

Our second objective was to unravel the molecular pathways of skeletal muscle wasting in the chronic flow phase, using a rat burns model. We aimed to investigate whether the mechanisms we found to be involved in **Chapter 4** continued to exert detrimental effects on skeletal muscle during the late flow phase. Understanding the molecular processes during the chronic phase is essential since the negative effects of severe burns are known to persist for months or even years post-burn [3]. Such insights can have major implications for future treatment options. In **Chapter 5**, we examined the effects of severe burns on the molecular pathways of skeletal muscle wasting at 40 days post-burn. We evaluated

anthropomorphic data, muscle morphology and protein signaling pathways in both predominantly type 1 m. Soleus and predominantly type 2 m. Extensor Digitorum Longus. Additionally, we analyzed several myokines, cytokines secreted by muscle contraction and involved in the regulation of metabolism in both the muscle and other organs, to identify possible therapeutic targets [4].

We hypothesize that severe burns continue to induce skeletal muscle atrophy 40 days post-burn, resulting from a net negative protein balance with increased levels of catabolic signaling proteins and decreased levels of anabolic signaling proteins *(Figure 2.2).*

Aim 3: Investigate the effects of an early exercise program on molecular mechanisms of post-burn muscle wasting

Early exercise interventions have demonstrated the ability to prevent muscle wasting in other wasting diseases like cancer and critical illness [5,6]. Currently, early exercise regimens to reduce the hypermetabolic, hypercatabolic and insulin responses in the early flow phase of burns, are vastly underutilised in clinical practice [7]. The lack of fundamental understanding of the physiological effects of exercise hinders its clinical use by the fear of worsening the hypermetabolic state. Therefore, our last objective was to assess whether an early exercise program in severely burned rats can counteract skeletal muscle wasting and unravel its impact on the cellular and molecular pathways found to be involved in post-burn muscle wasting in **Chapter 5**. In **Chapter 6,** severely burned and sham rats were trained five days a week for five weeks using a progressive treadmill training protocol. *Ex vivo* analysis of skeletal muscle samples was used to compare the results of exercise groups with the control groups, concerning morphology, protein signaling pathways and myokine expression. This fundamental animal research project can provide evidence that exercise in the early phases post-burn is effective to prevent muscle wasting and may lay the foundation for further research.

We hypothesize that implementing a progressive aerobic training protocol during the initial flow phase post-burn will attenuate muscle pathophysiological changes associated with muscle wasting. We expect that the exercise program will partially prevent weight loss seen after burn by preserving muscle mass through modulations in signaling pathways regulating protein balance. We specifically hypothesize that the post-burn exercise program will increase anabolic signaling and decrease catabolic signaling in both the Soleus and Extensor Digitorum Longus muscles, resulting in a positive protein balance (*Figure 2.2*).

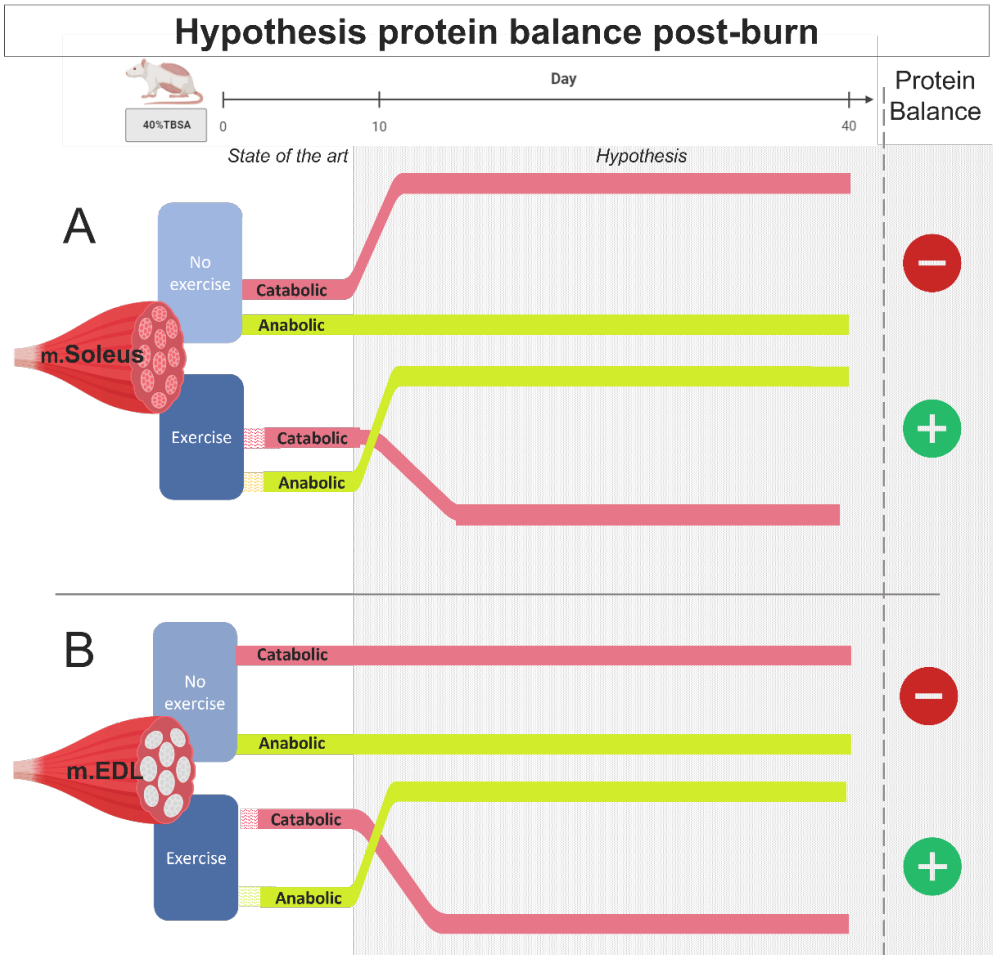

Figure 2.2 Graphical presentation of a hypothesis of the evolution of anabolic and catabolic signaling proteins post-burn based on literature and scientific information of other cachectic populations. This hypothesis presents the most likely evolution over time of anabolic and catabolic protein signaling pathways with and without exercise in type 1 dominant musculus Soleus (A) and type 2 dominant musculus Extensor Digitorum Longus (EDL) (B) after a severe burn injury (40%TBSA). The curves are semi-quantitative, with the first part (during the first week) based on previous literature, while the curves in the dashed area represent purely hypothesized evolution. In the last column, the hypothesized protein balance at day 40 is shown (based on these pathways).

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Chapter 3

Molecular mechanisms of postburn muscle wasting and the therapeutic potential of physical exercise

Dombrecht D, Van Daele U, Van Asbroeck B, Schieffelers D, Guns PJ, Gebruers N, Meirte, J and van Breda, E. Molecular mechanisms of post-burn muscle wasting and the therapeutic potential of physical exercise. J Cachexia Sarcopenia Muscle. 2023;14(2):758-70

1 Abstract

After a severe burn injury, a systemic stress response activates metabolic and inflammatory derangements that, among other, leads to muscle mass loss (muscle wasting). These negative effects on skeletal muscle continue for several months or years and are aggravated by short- and long-term disuse. The dynamic balance between muscle protein synthesis and muscle protein breakdown (proteolysis) is regulated by complex signaling pathways that leads to an overall negative protein balance in skeletal muscle after a burn injury. Research concerning these molecular mechanisms is still scarce and inconclusive, Understanding of which, if any, molecular mechanisms contribute to muscle wasting is of fundamental importance in designing of therapeutic interventions for burn patients as well. This review not only summarizes our present knowledge of the molecular mechanisms that underpin muscle protein balance, but also summarizes the effects of exercise on muscle wasting post-burn as promising strategy to counteract the detrimental effects on skeletal muscle. Future research focusing on the pathways causing post-burn muscle wasting and the different effects of exercise on them is needed to confirm this hypothesis and to lay the foundation of therapeutic strategies.

2 Introduction

During the last decades, skeletal muscle has gained scientific and clinical interest as metabolic tissue, in addition to its well-known prominent role in movement. Evidence is accumulating that active skeletal muscle contributes to overall health by acting as an important metabolic secretory organ [1, 2]. The secretome of skeletal muscle acts in an endocrine, paracrine, or autocrine manner affecting muscle itself but other organs as well. One of the key metabolic roles of skeletal muscle is its huge reservoir of proteins, serving as pool of amino acids available to use in critical situations [3]. Accounting for approximately 40% of total body mass, skeletal muscle is a highly important tissue in the regulation of overall protein balance [4].

Following an injury, the human body reacts by activating a pathophysiological stress response. This metabolic stress response develops in two phases, i.e. the “ebb” and “flow” phase, first described by Cuthbertson et al [5]. The ‘ebb’ phase, the first 24-48hours after the injury, is characterized by an increased secretion of catabolic hormones such as catecholamines and cortisol leading to a lower resting energy expenditure (REE) to preserve energy for vital organ functions [6]. The subsequent “flow phase” on the other hand, is characterized by long lasting inflammatory and humoral immune responses leading to an increased REE, persisting for months or even years after the injury, and having major detrimental effects on skeletal muscle [6, 7].

After a burn injury, this hypermetabolic flow phase behaves proportional to the total burned surface area (%TBSA) and is characterized by an increased gluconeogenesis, lipolysis, and proteolysis. Consequently, a decrease in muscle mass (muscle wasting) develops, further aggravated by forced bedrest, multiple surgeries, and medication during hospitalization and/or inactivity due to pain and fatigue afterwards [7-9]. Muscle wasting impacts a burn patient’s life both in the short and long term after the injury. 10-15% loss of lean body mass leads to increased infection rates, delays in wound healing, reduced

muscle strength and function, contributes significantly to higher morbidity and mortality rates and affects activities of daily living and quality of life [10, 11].

In comparison with muscle wasting in elderly (sarcopenia), in certain diseases (e.g. cancer cachexia) and in critically ill patients, muscle wasting in burn patients is poorly understood [12-14]. Moreover, in critical ill patients early (in hospital) mobilization is an important component of usual care, with main goal of diminishing the amount of muscle wasting [15]. In contrast, in burn patients the focus during early rehabilitation is primarily directed towards wound healing, preventing hypertrophic scars and maintaining limb mobility and less towards physical measures to prevent muscle wasting [16, 17]. Hence, more in-depth knowledge of the underlying processes of muscle wasting, and the potential beneficial effects of physical exercise, is mandatory to understand and substantiate the clinical importance of (early) exercise rehabilitation in burns as well.

This paper provides an overview of the current understanding of molecular processes leading to post-burn muscle wasting and possible counteracting effects of physical exercise on muscle mass homeostasis. We will shed light on molecular mechanisms controlling muscle mass, by describing pathways leading to muscle catabolism post-burn, and provide an overview of preventive effects of exercise on muscle wasting in burns.

3 Molecular mechanisms of skeletal muscle mass regulation post-burn

Skeletal muscle mass loss can be triggered by a number of situations in which skeletal muscle serve as a reservoir of amino acids readily available to maintain protein synthesis in vital tissues and organs in need [3]. In response to such pathophysiological conditions, sometimes in combination with disuse, molecular signaling pathways modulate a shift in protein balance ultimately leading to skeletal muscle mass loss [14, 18]. Regulation of skeletal muscle protein balance is complex, recent evidence emphasizes its multifaceted aspect including both gene transcription, translation and protein breakdown [19]. Either a

lack of skeletal muscle activity, pathological catabolic stimuli, or the combination of both leads to skeletal muscle atrophy. Although different in origin, the effects and downstream signaling pathways are similar [18]. In the previous decades, signaling pathways of skeletal muscle loss are for a large part unraveled in various conditions but remain relatively unknown in burn patients.

After a burn injury, skeletal muscle wasting is a prominent hallmark of the systemic stress response, characterized by a disturbed protein balance, mainly as the result of increased proteolysis rather than a decrease in protein synthesis (Figure 3.1) [20, 21]. During the catabolic state post-burn amino acids from skeletal muscles are redistributed to supply the burn wound with substrates necessary for wound healing and to produce energy to fulfill the increased needs of post-burn hypermetabolism [22]. Despite the necessity of this proteolytic process, which continues even after wound closure, the overall outcome is that more proteins are broken down into amino acids than are actual needed, resulting into unwanted and non-functional muscle wasting. Besides, driven by the excessive release of free amino acids post-burn, protein synthesis is upregulated, and a portion of the amino acids are transferred to the liver contributing to an increase in gluconeogenesis [23-25].

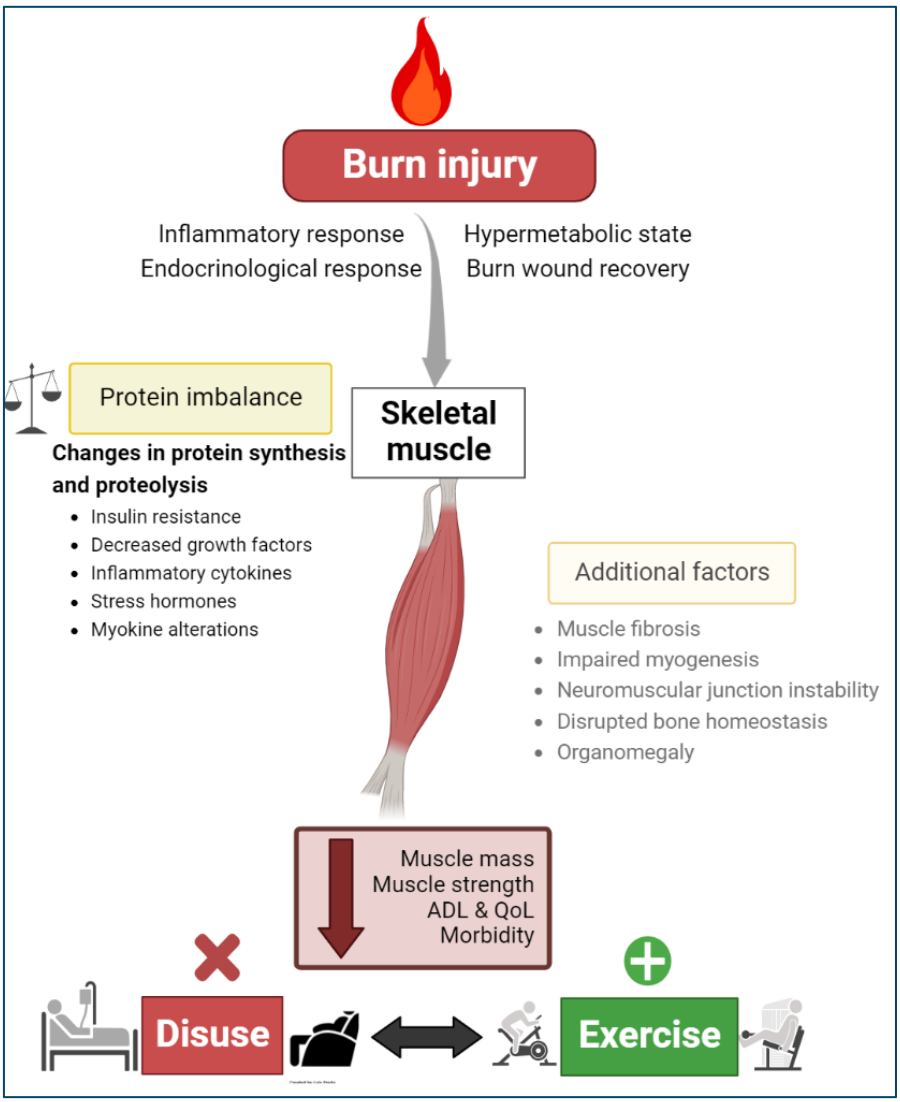

Figure 3.1: Main processes leading to skeletal muscle wasting after a burn injury and additional factors contributing to post-burn muscle wasting. ADL, activities of daily life; QoL, quality of life

Post-burn it has been shown, as for other diseases such as cancer cachexia and critical ill patients, that the main mechanisms controlling muscle wasting are the Ubiquitin Proteasome System (UPS) and autophagy [12]. The latter including mitophagy, leading to mitochondrial dysfunction. In the following paragraphs we will describe in short the mechanisms of protein synthesis and protein breakdown and their corresponding signaling pathways, each time followed by the current knowledge concerning the specific role of these pathways after burns. Lastly, we will elaborate the possible effects of exercise on muscle wasting post-burn.

3.1 Protein synthesis post-burn

Protein synthesis in skeletal muscle is mainly regulated by the PI3K-AKT ('phosphoinositide-3-kinase'-'AKT' ('Protein kinase B' (PKB)) pathway downstream of anabolic hormones phosphorylating mTORC ('mammalian target of rapamycin complex') and its downstream targets, leading to protein translation (figure 3.2). In determining the anabolic or catabolic state of a muscle, growth factor stimulated PI3K-AKT pathway is one of the most important factors (figure 3.2). Insulin and insulin like growth factor-1 (IGF-1) are anabolic hormones regulating protein synthesis by recruiting insulin receptor substrate 1 (IRS-1), which in turn phosphorylates PI3K [26]. Phosphorylation of lipids at the cell membrane leads to formation of phosphatidylinositol (3,4,5)-trisphosphate (PIP3) that recruits AKT. Upon activation, AKT phosphorylates a set of substrates that block apoptosis and induce protein synthesis, gene transcription and cell proliferation [3, 27]. Among others, AKT activates mTORC, having a central function in integrating a variety of growth signals and in many cellular metabolic processes [3][more details in [26]]. Two mTORC complexes exist, i.e., mTORC1 and mTORC2 [28]. The mechanisms of mTORC2 are not fully unravelled, but in vivo research in mice did not reveal a structural impact on skeletal muscle by mTORC2 inhibition [29]. mTORC1 on the other hand, has been shown to regulate protein translation initiation in skeletal muscle. Decreased levels of mTORC1 are associated with decreases in protein synthesis and a decreased muscle mass [30]. Therefore, this review will focus on mTORC1. mTORC1 increases skeletal muscle protein synthesis by inhibition of autophagy and initiation of protein translation. Two important downstream target proteins of mTORC1 are 'ribosomal protein (rp)S6 kinase-1' (S6K1), or p70s6k, and 'eukaryotic initiation factor 4E binding protein' (4E-BP1), or PHAS-1 [30]. mTORC1 stimulates S6K1 as stimulator of protein translation and inhibits 4E-BP1 as negative regulator of eIF-4E ('Eukaryotic translation initiation factor 4E') protein initiation factor [26]. Besides, downstream of AKT, GSK-3β (glycogen synthase kinase 3β) is phosphorylated and inhibited, activating 'translation initiation factor 2B' (eIF2B) and regulating translation initiation [31].

Other factors modulate protein synthesis pathways as well. For instance, energy stress activates the AMP-activated protein kinase (AMPK), a key regulator of energy homeostasis with a critical role as energy sensor during exercise and metabolic disorders [32]. AMPK is involved in both anabolic and catabolic pathways, maintaining energy stores, and ensuring a balance between energy supply and demand, whereas in skeletal muscle AMPK inhibits mTORC1 and 'Eukaryotic elongation factor 2' (eEF2) activity [33]. Growing evidence shows that also mechanical and nutritional factors affect protein synthesis. Contraction of skeletal muscle leads to activation of AKT and stimulation of mTORC1, which will be elaborated later in this review, whereas amino acid leucine for example directly stimulates the mTORC1-signalling pathway, increasing skeletal muscle protein synthesis [34, 35].

During the post-burn flow phase these molecular signaling pathways are affected by metabolic stress responses, but simultaneously increased muscle protein breakdown rates lead to availability of free amino acids stimulating muscle protein synthesis [36]. Reduced rates of protein synthesis post-burn are caused by endocrine disturbances, leading to decreases in anabolic hormones such as insulin and IGF-1. During the post-burn catabolic state, a sustained impaired IGF-1 signaling and insulin resistance has been observed leading to a decreased activation of the PI3K-AKT signaling pathway. This decreased PI3K-AKT signaling directly results in increases of GSK-3β activity, causing decreases in protein translation [37]. Besides, a decrease in PI3K-AKT dependent activation of mTORC1 results in a decreased protein synthesis through limited activation of its different downstream target proteins [37, 38]. In addition, an increased ratio of AMP to ATP is observed in response to a burn injury. This increase leads to activation of AMPK and is possibly contributing to a decreased protein synthesis. AMPK is known to inhibit eEF2 signaling (another target protein of mTORC1) and it has been shown that the initiation of protein translation is diminished through decreases in eEF2 signaling [39]. Although it is reasonable to suggest that such decreases in protein translation pathways are also valid in burn patients, evidence has not yet been confirmed.

Increases in protein synthesis post-burn are mainly caused by activation of mTORC1, activated independently of the PI3K-AKT pathway [37]. An accelerated protein breakdown post-burn increases the availability of free intracellular amino acids which activate mTORC1 independent of PI3K-AKT. Consequently, this mTORC1 upregulation leads to an elevated basal activation of S6K1, one of the target proteins of mTORC1 and a stimulator of protein translation, contributing to an increase in protein synthesis post-burn [37].

Although overall protein synthesis is slightly elevated in skeletal muscle post-burn, this is insufficient to compensate for the profoundly increased protein breakdown, leading to a net loss of proteins from skeletal muscle and eventually to muscle wasting [36, 40]. Further research is needed to fully elaborate the contribution of different pathways leading to increased protein synthesis rates post-burn.

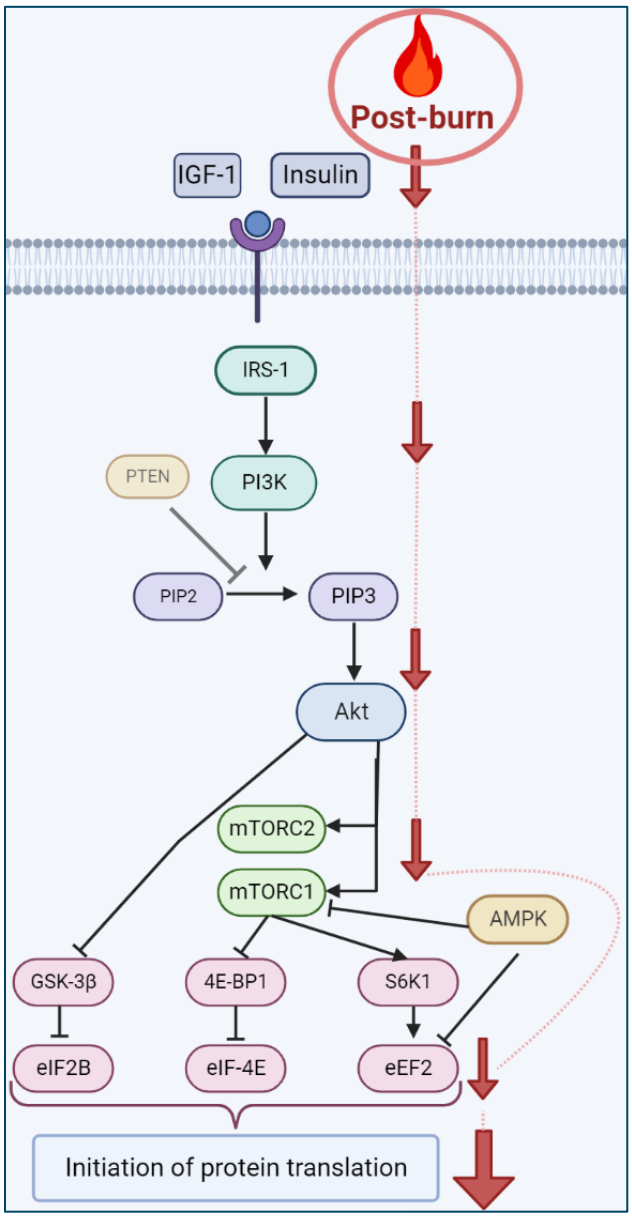

Figure 3.2 PI3K-AKT ('phosphoinositide-3-kinase'-'AKT') signaling pathway of protein synthesis in skeletal muscle and the additional effects during the flow phase after a burn injury. Upon IGF-1 ('Insulin like growth factor-1) or Insulin binding to its receptor, IRS-1 ('insulin receptor substrate 1') activates PI3K, generates PIP3 ('phosphatidylinositol (3,4,5)-trisphosphate') at the plasma membrane, facilitating phosphorylation of AKT. Subsequently, downstream mTORC1/2 ('mammalian target of rapamycin complex 1/2') are activated, stimulating S6K1 ('ribosomal protein (rp)S6 kinase-1') (and consequently eEF2 ('Eukaryotic elongation factor 2')) and inhibiting 4EBP1 ('eukaryotic initiation factor 4E binding protein') (consequently inhibiting eIF-4E ('Eukaryotic translation initiation factor 4E') resulting in the initiation of protein translation

3.2 Protein breakdown post-burn

In atrophying muscle two key systems controlling intracellular protein degradation are activated, i.e., the ubiquitin proteasome system (UPS) and autophagy (see [41] for a detailed review). Evidence is accumulating that these interconnected systems play a crucial role not only in conditions leading to muscle wasting, but in maintaining a healthy overall skeletal muscle homeostasis as well [42]. Without a basal activation of both processes, damaged proteins and abnormal organelles accumulate and impede normal functioning of the cells, whereas overactivation leads to degradation of healthy cell components, cell death and consequently muscular damage [42-44]. The UPS is mainly responsible for degradation of myofibrillar proteins i.e., α-actin, myosin heavy chains and troponin I. Target proteins are degraded into amino acids in several consecutive steps (figure 3.3, or [45]).

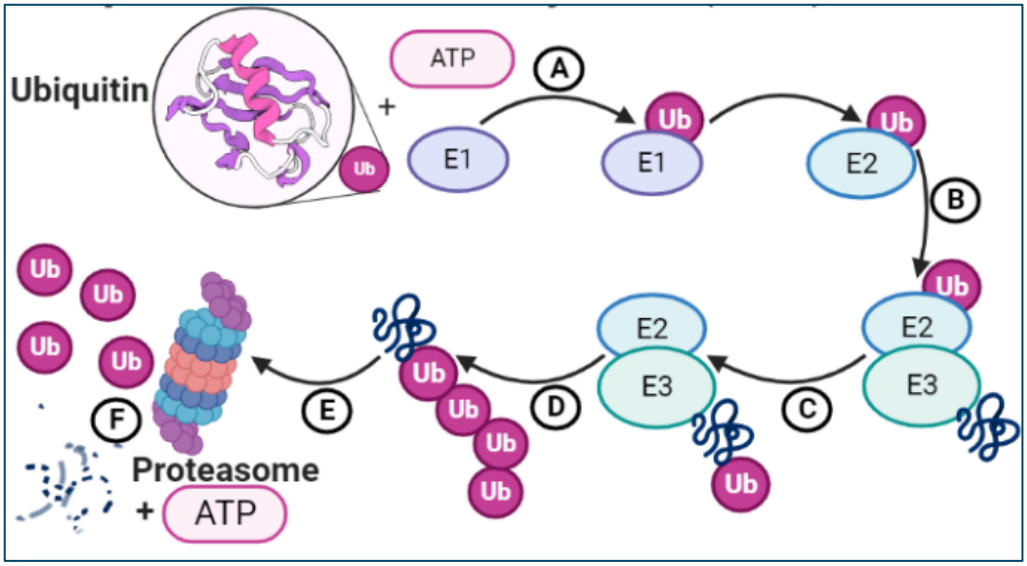

Figure 3.3*:* The ubiquitin proteasome system (UPS). A) E1 enzyme activates the ubiquitin; B) Conjugating E2 enzyme transports the activated ubiquitin to ligase enzyme E3; C) The activated Ubiquitin meets and interacts with the protein substrate ; D) a poly-ubiquitin chain is formed by multiple cycles of conjugation of ubiquitin to the target substrate ; E) The poly-ubiquitin chain is transported to the proteasome (26S proteasome complex) and binds through ubiquitin receptors; F) The poly-ubiquitin chain is removed, the protein is unfolded and broken down into amino acids and ubiquitin is recycled via deubiquitinating enzymes.

Autophagy on the other hand, results in selective degradation of muscle organelles i.e., mitochondria and cytoplasmatic constituents, following different cellular stress signals (energy stress, hypoxia, chemicals, toxins, etc.). [13, 42, 44, 46]. Activation of the autophagy-lysosome system in muscle cells has already been described for several catabolic conditions linked to muscle atrophy. During starvation for instance, lysosomal macro-autophagy (figure 3.4) represents the major proteolytic pathway that puts the body in a negative protein balance [47].

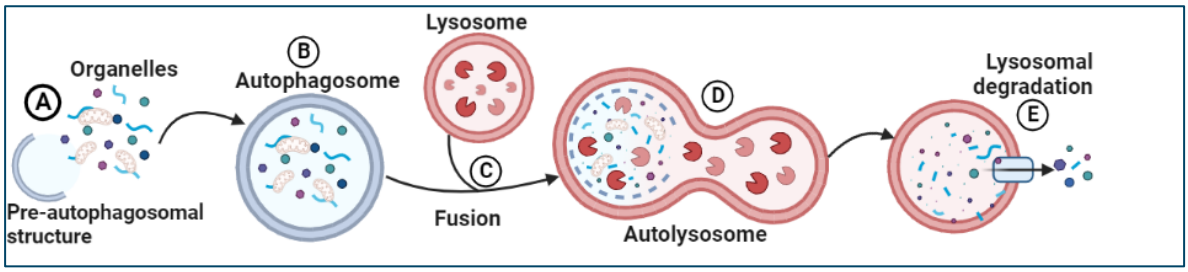

Figure 3.4*:* Autophagy: cytoplasmic organelles are sequestered by pre-autophagosomal structures (A) to generate autophagosomes (B), double membrane-bound vesicles. Next, autophagosomes fuse with lysosomes (C) to become autolysosomes (D). Finally these sequestered components are degraded by lysosomal degradation (E).

The UPS and autophagy are mediated by specific genes, increased in atrophying muscle, called atrogenes [48]. Under this definition of atrogenes, muscle specific E3 ligases have emerged, each with a specific affinity to certain proteins [18, 49]. The two most important E3 atrogenes are MURF-1, regulating the half-life of sarcomeric proteins and thus targeting proteins involved in contractile and structural functions, and Atrogin-1/MAFbx, targeting the degradation of MyoD and eIF3f (‘Eukaryotic translation initiation factor 3 subunit F’), both crucial in protein synthesis and regeneration[50]. Less elaborated are E3 atrogenes Trim32, regulating the degradation of thin filaments of muscles i.e., α-actinin and desmin, and Fbxo40, involved in regulating the IGF-1/insulin pathway [42, 51]. Specific autophagy-related atrogenes upregulated during muscular atrophy are Beclin, LC3 and Bnip3[52]. Expression of atrogenes, activity of the UPS, autophagy and subsequent proteolysis leading to muscle atrophy is regulated by various intracellular signaling pathways.

In several pathological conditions associated with skeletal muscle wasting intracellular factors are released that mediate the action of a variety of signaling molecules, triggering a cascade of processes like the UPS and autophagy. The release of those different intracellular factors is context dependent and varies between pathologies [51]. After burns, the UPS has been shown to be the main mechanism associated with accelerated breakdown of myofibrils leading to muscle wasting, but also activation of the autophagy-lysosome system in muscle cells has been described during post-burn muscle wasting [53, 54]. Both processes are activated as a result of alterations in cellular signaling pathways by the systemic pathophysiological stress response during the post-burn flow phase [55].

3.2.1 *Insulin resistance and growth factors*

In healthy muscles, the PI3K-AKT signaling pathway downstream of insulin and IGF-1 is responsible for regulating protein synthesis, but also for inhibiting excessive proteolysis by inactivating forkhead box protein O transcription factors (FOXO) (figure 3.5) [26, 56, 57]. FOXOs are responsible for the activation of the atrogene transcription program by activating atrogene transcription factors MURF-1 and Atrogin-1, increasing the activity of the UPS that subsequently leads to proteolysis. Hence, in healthy muscles, FOXO-inhibition by the PI3K-AKT pathway prevents excessive proteolysis [57]. In fasting or catabolic diseases, the PI3K-AKT-mTOR pathway is downregulated due to decreased insulin and/or IGF-1 levels. Both an increase in FOXO-mediated proteolysis and an associated decrease in protein synthesis make the PI3K-AKT-mTOR pathway a major player in skeletal muscle atrophy [13].

After burns, the hypermetabolic flow phase causes both insulin resistance and impaired IGF-1 signaling, resulting in a decrease in PI3K-AKT activation. Subsequently, a decrease in activation of mTORC1 results in decreased protein synthesis and increased proteolysis [37, 38]. Moreover, an associated reduced inhibition of FOXOs results in increased activation of atrogene transcription factors MuRF-1 and Atrogin-1, increasing activity of the UPS. Activated FOXOs lead to an upregulation of not only the UPS but of autophagy as well [48, 58]. Besides, studies have shown that after burn injury there is an upregulation of the

autophagy-related gene LC3, leading to an increase in FOXO transcription factors, and thus to an increase in autophagy [48, 59]. Hereby, FOXO-mediated proteolysis is initiated increasing protein breakdown and contributing to the disturbed protein balance leading to post-burn muscle wasting. Besides, FOXOs also negatively regulate muscle mass through other genetic programs i.e. those for muscle cell growth, differentiation and metabolism, further affecting post-burn muscle wasting [60].

3.2.2 *Inflammatory stress response*

Nuclear Factor kappa B (NF-κB), a key regulator of inflammatory responses, is another important transcription factor activating the atrogene transcription program and contributing to muscle wasting [61]. NF-κB is stimulated by catabolic pro-inflammatory cytokines like tumor necrosis factor α (TNF-α), Interleukin-6 (IL-6), Interleukin-1 (IL-1) (figure 3.5). In healthy muscles cytokine levels remain relatively stable, limiting activity of NF-κB. In catabolic diseases on the other hand, release of cytokines is profoundly increased, leading to increased activation of NF-κB. The exact atrophy promoting mechanisms of NF-κB are not fully unraveled yet, but recently Thoma et al. showed an interesting interplay between NF-κB and increased levels of MURF-1 and Atrogin-1, leading to protein degradation by activation of the UPS [62]. Besides, NF-κB targets two other genes, MyoD and cyclin D, responsible for muscle cell differentiation [63].

During the post-burn flow phase, an inflammatory stress response is activated and release of pro-inflammatory cytokines commences. Elevated concentrations of cytokines TNFα, IL-6 and IL-1 and interferon-γ (IFN-γ) increase expression of NF-κB and thus expression of atrogenes after burns (figure 3.5) [61, 64, 65]. Vice versa, NF-κB also contributes to the regulation of release of these cytokines, resulting in a self-perpetuating loop of NF-κB activation [62]. In addition, inflammatory signaling of IL-6 has been reported to stimulate the Signal transducer and activator of transcription 3 (STAT3) pathway, aggravating myofibrillar protein loss and atrophy of skeletal muscle by increasing caspase-3, myostatin and atrogene expressions [66]. Literature is inconclusive concerning stimulation of the STAT3 signaling pathway after burns specifically. Although IL-6 expression has repeatedly

shown to be upregulated in both serum and skeletal muscle of burn patients, this did not always translate in higher levels of STAT3 expression within the muscle [64, 67, 68]. Hence, the catabolic state after a burn injury further triggers muscle wasting by the release of several pro-inflammatory cytokines, increasing expression of NF-κB and activating the UPS, but further research is needed to elaborate other pathways involved, like the STAT3 pathway [61, 64].

3.2.3 *Upregulation of stress hormones*

Transcription factors upregulating proteolysis can be activated by glucocorticoids as well by interfering with different signaling pathways affecting muscle mass loss [69]. Recently, Shimizu et al. unraveled part of the mechanisms of glucocorticoid mediated skeletal muscle atrophy [69]. Glucocorticoids activate the UPS by regulating the expression of two target genes encoding KLF15 and REDD1 ('Krüppel-like factor 15' and 'Regulated in Development and DNA Damage Responses'). These glucocorticoid-targeted genes interfere with the PI3K-AKT pathway, inhibiting mTOR activity and as such leading to an increase in FOXO activation, which in turn upregulates the expression of atrogin-1 and MURF-1 [70]. Inhibition of mTOR also leads to a decrease in protein synthesis, accentuating the anti-anabolic activity of glucocorticoids [69]. Glucocorticoids also activate inflammatory signaling pathways through adaptor protein TRAF6, coupling inflammatory signals to down-stream transcription factor networks, which in turn lead to expression of NF-κB, increasing UPS activation and decreasing MyoD genes[71]. In healthy situations, glucocorticoid release is limited. However, in many pathological conditions associated with muscle mass loss a general stress response leads to a strong increase of glucocorticoids. By interfering with the above mentioned signaling pathways, transcription factors are activated, and skeletal muscle atrophy is stimulated. Besides their effect on the PI3K-AKT and NF-κB pathways, glucocorticoids can also cause muscle wasting by altering the muscle production of myostatin [72].

Also burns lead to alterations in endocrinologic responses, resulting in a hormonal disbalance and the release of stress hormones like glucocorticoids [73]. The interaction of

glucocorticoids with the different signaling pathways, as described above, leads in the post-burn flow phase to an increase in 1) FOXO-mediated proteolysis, 2) UPS activation by NF-kB, 3) myostatin concentrations, and 4) to a decrease in MyoD genes [74].

3.2.4 *Myokine alterations*

Myostatin, a muscle derived myokine that acts in an endocrine, autocrine and/or paracrine manner, is of specific interest in the control of muscle mass [75]. As a negative regulator of muscle mass i.e., increases in myostatin inhibit muscle growth and its expression has been shown to be enhanced by FOXO and NF-κB. Through the transcription factors SMAD2 and SMAD3, myostatin increases the concentration of MURF-1 and Atrogin-1, and upregulates the UPS (figure 3.5) [75, 76]. Interestingly, myostatin has also been shown to cross-react with the PI3K-AKT pathway, leading to a downregulation of protein synthesis [77]. Since the specific mechanisms for myostatin secretion are not fully unraveled further research is warranted.

The endocrinologic response during the post-burn flow phase, leads to an increase in myostatin concentrations. Increases in myostatin levels lead to a further activation of atrophying programs through SMAD2 and SMAD3 and decreases in protein synthesis by interfering with the PI3K-AKT pathway [74]. However research concerning myokines involved in post-burn muscle wasting is still scarce and needs further exploration.

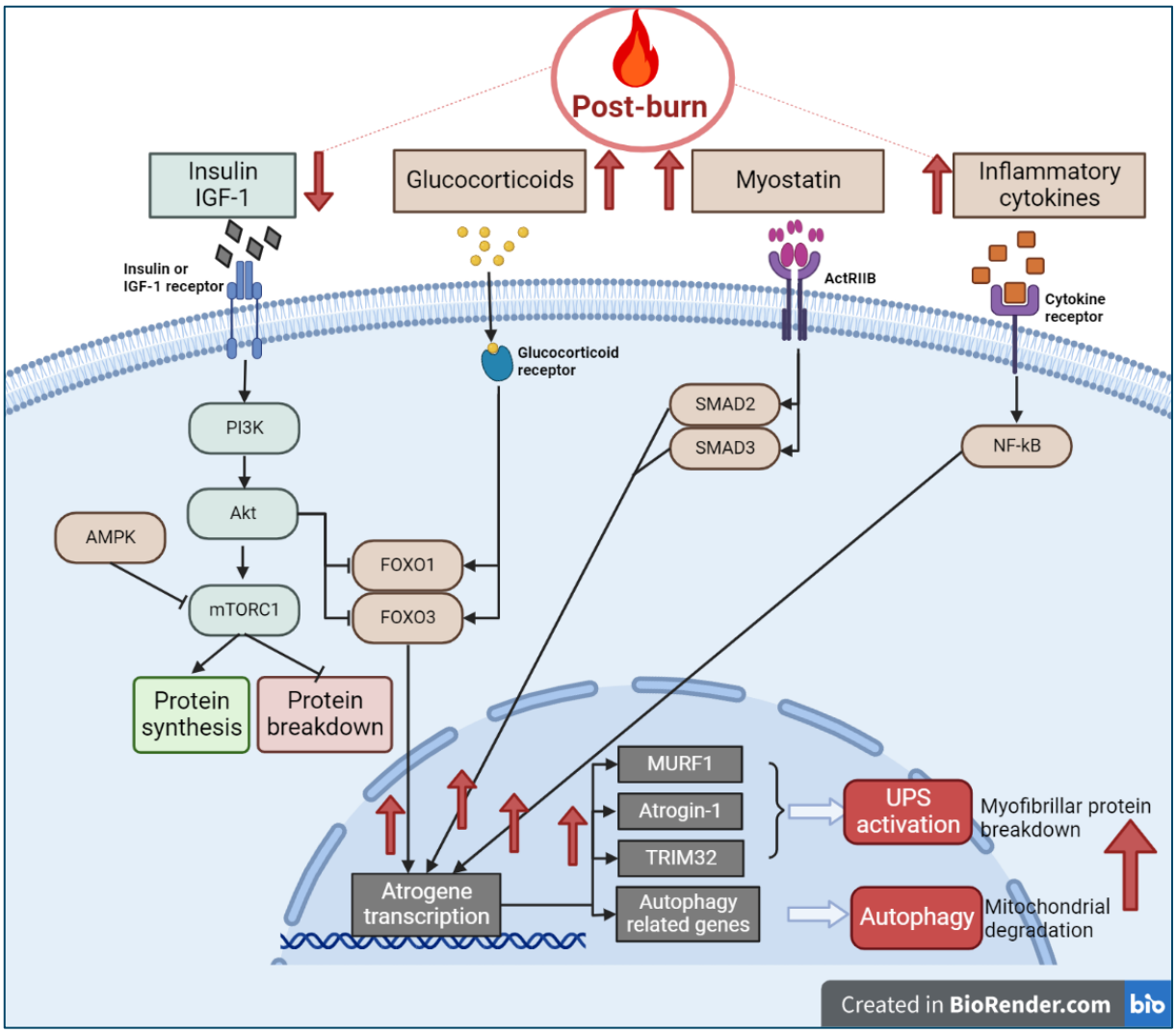

Figure 3.5 Intracellular signaling pathways regulating protein breakdown in skeletal muscle and the additional effects during the flow phase after a burn injury. The atrogene transcription program is activated by 1) decreases in insulin or IGF-1 ('insulin like growth factor-1') signaling, decreasing the activity of the PI3-Akt-mTOR ('phosphoinositide 3-kinase-Akt-mammalian target of rapamycin') pathway, increasing expression of transcription factors FOXO1/3 ('forkhead box protein O 1/3'); 2) increases in glucocorticoid release resulting in increases of FOXO1/3; 3) increased amounts of inflammatory cytokines leading to higher NF-κB ('nuclear factor-κB') expression; 4) transcription factors SMAD2 and SMAD3, stimulated by myostatin. The atrogene transcription program consequently leads to proteolysis via increased activity of MURF-1 ('muscle-specific RING-finger 1'), atrogin-1, TRIM32 ('Tripartitie Motif Containing 32') and autophagy related genes, stimulating the Ubiquitin Proteasome System (UPS) and Autophagy

3.3 Additional factors contributing to post-burn muscle wasting

3.3.1 *Increased muscle fibrosis and impaired myogenesis*

Apart from a protein imbalance, muscle homeostasis is affected by alterations in the interstitial environment surrounding myofibers as well [78]. In normal physiological conditions, the strength of the extracellular matrix (ECM) is maintained by a dynamic process of collagen degradation and synthesis [79]. In cachectic conditions however, a more rigid phenotype is created by pathogenic collagen synthesis, induced by transcription growth factor-β (TGF-β) and myostatin [79]. TGF-β induces excessive collagen formation and accumulation of the ECM by stimulating SMAD3, whereas myostatin increases fibroblast expansion and collagen production within the interstitial space of skeletal muscle [80, 81]. Muscle fibrosis, stimulated by TGF-β signaling, is observed post-burn as well, decreasing force transmission and strength [74, 78]. Apart from the decreases in muscle size, as mentioned above, increased TGF-β, myostatin and SMAD3 eventually lead to accumulation of collagen tissue between muscle fibers post-burn [74, 80].

Activation and differentiation of skeletal muscle satellite cells is essential for adequate tissue repair. During long term recovery protein synthesis is promoted by myonuclear addition via the fusion of satellite cells into existing myofibers [82, 83]. Post-burn, it has been shown that satellite cells and myoblasts are activated in an attempt to regulate the balance between muscle atrophy and the maintenance of muscle mass. Instead, a decrease in myogenic fusion and differentiation of muscle cells is seen after severe burn injury [83-85]. Increases in MyoD and decreases in myogenin show an altered regulation of the myogenic program after a burn injury [83, 85]. Consequently, muscle recovery is impeded by decreases in the number of myonuclei per myotube and a decreased differentiation and fusion of myoblasts [83]. Despite an increasing knowledge, the number of studies investigating in vivo changes in myogenesis post-burn is still scarce.

3.3.2 *Ineffective neurotransmission and neuromuscular junction instability*

Muscle mass and muscle function preservation is also dependent on the physiological structure of neuromuscular junctions and the efficiency of transmission signaling between nerves and skeletal muscle [86]. A major component of the neuromuscular junction that is necessary to produce a normal action potential is the acetylcholine receptor (AChR), located in the post-synaptic membrane. Stability of this post-synaptic membrane is affected by changes in different pathways and mitochondrial dysfunctions [87] . In muscle wasting conditions the atrogene MURF-1 is increased in neuromuscular junctions where it is involved in autophagosomal degradation of the AChR [88]. In addition, neuromuscular junction stability is affected by changes in mTORC1 signaling in postsynaptic muscle fibers, contributing to muscular atrophy [86, 89]. After a burn injury an abundance of inflammatory cytokines and chemokines induces changes in these pathways and mitochondrial dysfunction, inducing neuron degeneration and peripheral motor neuropathies and leading to ineffective neurotransmission [90, 91]. These effects are worsened by a lack of retrograde signaling from muscle to nerve due to immobilization, increasing degeneration of spinal cord ventral horn neurons [90, 92]. Consequently, for several years after a severe burn injury, ineffective neurotransmission contributes to post-burn muscle wasting [90].

3.3.3 *Organ cross-talk organomegaly and disrupted bone homeostasis*

Long term (co)morbidities after a burn injury are also affected by organomegaly and bone loss, which either directly or indirectly aggravate muscle wasting by the interesting concept of organ-crosstalk [93]. In experimental studies organomegaly often masks the severity of skeletal muscle wasting by elevating total body weight. After a burn injury, organomegaly is regarded as a common comorbidity, increases in liver and spleen masses are usually observed [68]. Interestingly, both liver and skeletal muscle protein metabolisms are regulated by similar inflammatory factors involved in the post-burn flow phase [94]. Increased serum cytokine levels lead to a shift of amino acids and free fatty acids from mainly skeletal muscles to peripheral tissues, like the liver and burn wounds [95]. In burn

wounds, these are used as substrates for healing, but in the liver an increased amount of intrahepatic fat and increased gluconeogenesis is seen, further stimulated the anaerobic metabolism of fibroblasts in the burn wounds, resulting in hepatocyte hypertrophy [96]. As a consequence, the liver keeps producing inflammatory proteins for 6 to 9 months after burn, contributing to the negative consequences post-burn. Preventing such liver enlargement may result in a reduction of complications post-burn [96, 97].

Crosstalk between skeletal muscle and bone has been reported as well. The systemic inflammatory response and associated production of endogenous glucocorticoids post-burn have detrimental effects on the bone [98]. It has been shown that from day one post-burn, a loss of bone mineral density takes place [68, 98]. Bone act as a paracrine organ producing myogenic factors, such as MyoD and Myogenin, affecting skeletal muscle mass and function [99]. Post-burn, release of Transforming Growth Factor beta (TGF-β) from the bone matrix is increased, promoting myotube atrophy and muscle weakness by increasing SMAD3 phosphorylation [100, 101]. Blocking bone resorption, by e.g. pamidronate, can limit the release of pro-apoptotic factors, decreasing effects on skeletal muscle [100]. Further research is needed to elaborate the mechanisms of TGF-β in the muscle after burns and to investigate other bone myogenic factors affecting skeletal muscle post-burn.

4 Potential mechanisms of exercise on post-burn muscle wasting

Apart from systemic effects of the burn wound(s) itself, disuse aggravates skeletal muscle wasting post-burn, especially during the hospitalization period. But even after discharge, most burn patients remain physical inactive for a prolonged time, worsening negative effects on skeletal muscle mass and function. Both pharmacological and nutritional aids are used to minimize muscle mass loss in cachexia and sarcopenia, although with limited efficacy [102]. Since physical activity has been shown to exert beneficial effects on muscle mass preservation in cachexia, sarcopenia and disuse, the hypothesis arises that increasing the level of physical activity during the post-burn period may have similar positive effects on skeletal muscle mass preservation.

However in post-burn, clinical care is focused on overall stabilization of general health parameters and wound care. Current treatment protocols include: nutritional support, prevention of sepsis, and physical interventions to restore functionality, prevent hypertrophic scars and maintain a good range of motion of the limbs [103]. Despite their use in other diseases (critical ill, cancer, heart failure etc.) to prevent skeletal muscle wasting, physical exercise intervention programs are not commonly used in the rehabilitation of major burns, although they are considered to be safe and efficacious to restore physical function [104, 105]. Physical exercise starting immediately post-hospitalization is associated with improved quality of live, reduced secondary hospitalizations and prolonged survival in metabolic diseases [14]. The underuse of physical exercise programs post-burn is mainly due to a poor fund of knowledge leading to fear of worsening the burn wounds, possible additional pain, aggravating hypermetabolism and, above all, a limited amount of evidence [17, 106]. Hence, to raise awareness among health care providers about the necessity of implementing exercise in the standard of care of early burn rehabilitation, it is of a high importance to prove the beneficial effects of exercise on the different pathways involved in skeletal muscle wasting and to refute the supposed negative consequences[17, 106].

4.1 Exercise effects in cachectic conditions

A hypothesis of the molecular mechanisms of the beneficial effects of exercise on muscle mass losses post-burn can be constructed based on other cachectic conditions. For example, cancer, renal failure and rheumatoid arthritis show similar pathological mechanisms, including inflammatory responses and disuse. Muscle mass losses are counteracted by exercise in these systemic diseases, and possibly also in burns, by lowering inflammation and oxidative stress, increasing insulin sensitivity and limiting the activity of proteolytic pathways [107-111].

In cancer cachexia and heart failure patients, exercise stimulates the PI3K-AKT pathway by increasing levels of anabolic hormones insulin and IGF-1, which in turn increase mTORC1

levels, induce protein synthesis and inhibit protein breakdown [110, 112]. An upregulation of key regulators involved in protein translation and synthesis, mTORC1, S6K1 and GSK-3β has been noted during immediate post-exercise recovery periods in cancer cachexia [110]. Activation of the PI3K-AKT pathway also leads to the inhibition of FOXOs, decreasing the activity of the atrogene transcription program[112]. Hence, physical exercise is a potent stimulant for protein synthesis, even in wasting conditions, despite an acute suppression of protein synthesis [110].

Exercise reduces the number of inflammatory cytokines (like TNF-α and IL-6) and glucocorticoids, further limiting the activation of FOXOs and NFκB [113]. These anti-inflammatory effects of exercise may also block the effects of TNF-α in mediating insulin sensitivity, contributing to an increased insulin sensitivity. This leads to an additional increase in activation of PI3K-AKT pathway, further restoring protein balance, as seen in cancer cachexia [110].

Myostatin signaling seems to be reduced after exercise as well, leading to decreases in SMAD2/3 levels, as seen in heart failure patients [114]. Another important exercise-induced transcription factor is PGC1-α. Exercise has been shown to prevent losses of muscle mass by increased signaling of PGC1-α, preventing sarcopenia [115]. PGC1-α exerts its effects by inhibiting FOXOs and NF-kB signaling, and is associated with lower oxidative stress, inflammation, apoptosis, autophagy, UPS activation and increases in mitochondrial biogenesis[116].

Based on these findings we can conclude that exercise stimulates and/or inhibits several molecular pathways including PI3K-AKT, FOXOs, NFκB and myostatin in several cachectic conditions, leading to an increased protein synthesis and decreased activation of the atrogene transcription program (inducing autophagy and the UPS and thus protein breakdown), stimulating muscle growth and attenuating muscle atrophy [14].

4.2 Exercise studies post-burn

The above-mentioned findings are mainly based on studies with healthy subjects or cachectic patients other than burns. However, since the exercise effects are conserved mostly in these molecular pathways, they represent a promising intervention to attenuate or reverse the muscle wasting process in burn patients as well. A recent meta-analysis by Yang et al. (2021), including children with severe burns, has shown that physical activity in the form of resistance training leads to significant increases in muscle strength, lean body mass and cardiopulmonary function (VO2 peak) without increasing energy expenditure [117]. The results demonstrate that exercise programs can be effectively used to prevent muscle catabolism in burn patients. This study also shows that further research is necessary, since only a limited number of 12 studies could be identified and small sample study biases could have influenced the overall conclusions [117]. Besides, the focus of the studies is mainly limited to the clinical effects of exercise, focusing on body composition and muscle function, and lack information concerning fundamental processes such as the (molecular) mechanisms underlying the effects of exercise on skeletal muscle.

Our hypothesis that exercise alters signaling pathways associated with skeletal muscle function improvement after burns has been confirmed in an interesting animal study by Song et al.[118]. In this study, the protein synthesis pathway PI3K-AKT was activated by exercise, with simultaneous significant increases in mTOR and eEF2 expression. Also, decreases in the E3 ligase MURF-1 were found, but no change could be observed in Atrogin-1 expression after a physical exercise program. It should be noted that in this study a burn model with hindlimb unloading was used[118]. Other animal studies show increases in protein synthesis rates and decreases in protein breakdown after insulin or IGF-1 treatment [38, 119].

Although our knowledge concerning the effects of exercise on the molecular mechanisms of muscle wasting post burn is limited, the few studies published in burns in combination

with the findings in other cachectic conditions confirm the hypothesis that exercise is a promising intervention to prevent muscle wasting in burns.

5 Summary

After a severe burn, skeletal muscle wasting is observed among other symptoms, affecting overall health and quality-of-life of burn patients. The fundamental processes that underlie the process of skeletal muscle wasting in burns are incompletely understood, as well as the counteracting effects of physical rehabilitation in preservation of muscle mass.

Skeletal muscle wasting after burns is the result of a skeletal muscle protein imbalance. Decreases in the anabolic hormones such as insulin and IGF-1 lead to decreased activation of the PI3K-AKT-mTOR signaling pathway. In addition, an increased overall energy expenditure leads to excessive activation of AMPK, contributing to a decreased protein translation by inhibition of eEF2. Accelerated proteolysis post-burn increases the availability of free intracellular amino acids, activating mTORC1 independent of PI3K-AKT and increasing protein synthesis. The UPS is the main mechanism associated with the accelerated breakdown of myofibrils during post-burn muscle wasting, through an upregulation of the atrogene transcription factors MURF-1 and Atrogin-1 by, among others, FOXO proteins. Besides, post-burn an upregulation of autophagy-related genes can be found, partly via these FOXOs. Hence, although protein synthesis is slightly elevated, the increased proteolysis leads to an overall negative protein balance post-burn. However, studies on the pathways involved are scarce and inconclusive.

Apart from the systemic effects of the burn wound itself, disuse worsens the undesirable effects on skeletal muscle mass. Physical activity has been shown to exert beneficial effects on muscle mass preservation in cachexia, sarcopenia and disuse. The current limited use of physical exercise programs in the post-burn situation is mainly due to a lack of evidence in this population. It can by hypothesized that increasing physical activity after burns may have similar positive effects on skeletal muscle mass. The few studies that are available

have shown that exercise programs can be effectively used in burn patients. Experimental studies of burns in rodents show that exercise has multiple beneficial effects i.e. 1) stimulation of the PI3K-AKT-mTOR pathway; 2) increases of protein translation pathways; 3) decreases MURF-1 but not Atrogin-1 and 4) increases in protein synthesis and decreases proteolysis after insulin and/or IGF-1 treatment.

Our knowledge concerning the effects of burns and the possible beneficial effects of exercise on the molecular mechanisms of muscle wasting is still in its infancy. Therefore, a continued research effort is needed to further unravel the different mechanisms and to fully elaborate the contribution of the different pathways leading to protein synthesis and skeletal muscle proteolytic rates post-burn.

6 References

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Chapter 4

Molecular pathways of postburn skeletal muscle wasting in the early flow phase

Dombrecht D, Van Daele U, Van Asbroeck B, Schieffelers DR, Guns PJ, van Breda E. Skeletal muscle wasting after burn is regulated by a decrease in anabolic signaling in the early flow phase. Burns. 2023;49(7):1574-84.

1 Abstract

Following burns a sustained catabolic stress response is activated, resulting in skeletal muscle wasting. A better understanding of the underlying mechanisms of postburn skeletal muscle wasting is essential for the development of preventive and/or therapeutic strategies. Six weeks old female rats underwent a sham, 10% or 40% total body surface area scald burn. Ten days post-injury, severely burned animals gained significantly less weight compared to sham treated and minor burned animals, reflected in a significantly lower ratio of muscle to total body weight for Soleus (SOL) and Extensor Digitorum Longus (EDL) in the severely burned group. Postburn, total fiber number was significantly lower in EDL, while in SOL the amount of type1 fibers significantly increased and type2 fibers significantly decreased. No signs of mitochondrial dysfunction (COX/SDH) or apoptosis (caspase-3) were found. In SOL and EDL, eEF2 and pAKT expression was significantly lower after severe burn. MURF1,2,3 and Atrogin-1 was significantly higher in SOL, whilst in EDL MURF1,2,3 was significantly lower postburn. In both muscles, FOXO3A was significantly lower postburn. This study identified postburn changes in muscle anthropomorphology and proteins involved in pathways regulating protein synthesis and breakdown, with more pronounced catabolic effects in SOL.

Keywords

Burns, Muscle wasting, Skeletal muscle, Signaling pathways, Protein turnover

Highlights

- Muscle wasting characteristics are dependent of fiber type 10 days postburn
- In the early flow phase postburn anabolic signaling is diminished in SOL and EDL
- Catabolic signaling is more pronounced in SOL 10 days postburn
- No sign of apoptosis and mitochondrial dysfunction was found 10 days postburn

2 Introduction

Skeletal muscle wasting is a prominent hallmark of the systemic stress response after a burn injury [1]. To improve functionality and quality of life of burn survivors, counteracting muscle wasting is highly important [2]. Although many facts of the metabolic consequences of burns are revealed, filling the gaps regarding underlying molecular mechanisms is crucial in the development of evidence based therapeutic strategies in burn rehabilitation [1, 3].

Evidence supports that following a severe burn a sustained pathophysiological stress response is activated, characterized by persistent inflammatory and humoral stress responses, inducing a state of whole-body hypermetabolism, insulin resistance and muscle wasting up to nine months postburn [4-9]. However, to date, experimental studies are mainly focused on the acute effects of burns (one to five days postburn) [10, 11]. Despite its systemic nature, skeletal muscle wasting is dependent on muscle fiber type [12]. Previous research shows that after burns predominantly type 2 muscles are more susceptible to muscle wasting compared with predominantly type 1 muscles [13, 14].

Muscle wasting during the catabolic flow phase is, at the cellular level, a result of changes in intracellular signaling pathways adjusting to metabolic stimuli, resulting in a protein imbalance [15]. These pathways have been partly unraveled under various pathological conditions, such as in cancer and critical ill patients, but remain relatively unknown in burn patients [16, 17]. Although it has been shown that during the catabolic phase postburn an increase in protein breakdown is one of the main causes of skeletal muscle wasting, literature concerning postburn protein synthesis is rather inconclusive [18, 19]. Whereas some studies show a postburn increase in protein synthesis due to increased levels of free amino acids, other studies show a decrease due to different activation of intracellular pathways [20-22]. From other wasting conditions, it is known that one of the most important pathways determining the anabolic or catabolic state of a muscle is the PI3K-AKT pathway (‘phosphoinositide-3-kinase’-‘Protein kinase B’ (PKB)), stimulated by anabolic hormones (Figure 4.1) [23]. Downstream of PI3K, AKT phosphorylates a set of substrates,

including mTORC1 (‘mammalian target of rapamycin complex 1’) with downstream p70S6K (‘Ribosomal protein S6 kinase’), blocking apoptosis and inducing protein synthesis, gene transcription and cell proliferation [24]. Key downstream regulators of translation initiation signaling are eEF2 (‘Eukaryotic elongation factor 2’), stimulating RNA translation, and eIF2α (‘Eukaryotic translation initiation factor 2α’), recognizing stress conditions [15, 25, 26].

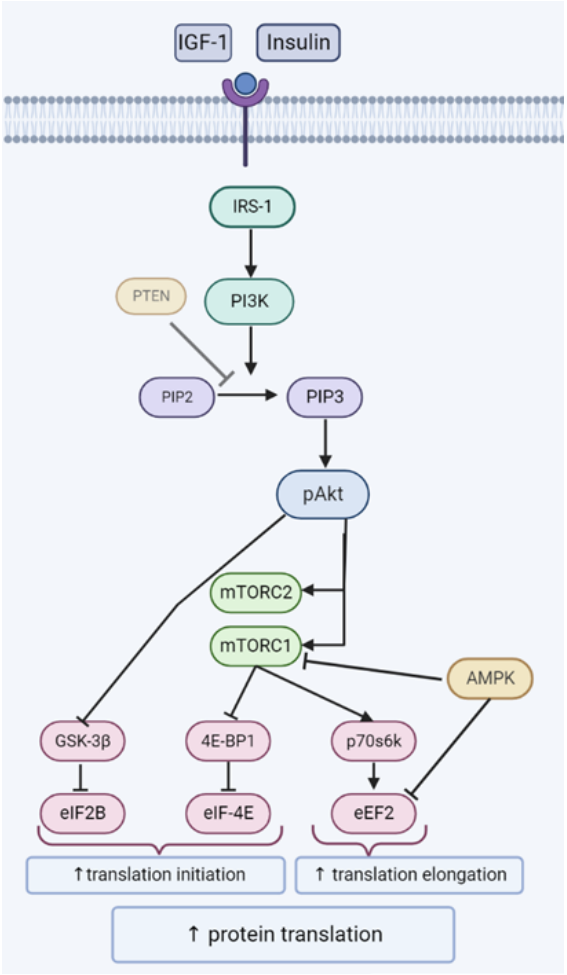


Figure 4.1: PI3K-AKT (‘phosphoinositide-3-kinase’-‘AKT’) signaling pathway of protein synthesis in skeletal muscle. Upon Insulin or IGF-1 (‘Insulin like growth factor-1) binding to its receptor, IRS-1 (‘insulin receptor substrate-1’) activates PI3K, generates PIP3 (‘phosphatidylinositol (3,4,5)-trisphosphate’) at the plasma membrane, facilitating phosphorylation of AKT. Subsequently, downstream GSK-3β is inhibited (consequently inhibiting eIF2B) and parallel downstream mTORC1/2 (‘mammalian target of rapamycin complex 1/2’) are activated, of which mTORC1 inhibits 4EBP1 (‘eukaryotic initiation factor 4E binding protein’), consequently inhibiting eIF-4E (‘Eukaryotic translation initiation factor 4E’), and stimulates p70s6k (‘70kDa ribosomal protein S6 kinase’) (consequently inhibiting eEF2k which inhibits eEF2 (‘translation initiation factor 2B’)), resulting in the initiation of protein translation.

Protein breakdown in skeletal muscle wasting is regulated by the Ubiquitin Proteasome System (UPS) and autophagy [17, 27]. Key enzymes in proteasomal degradation are atrogenes MURF-1 (‘muscle RING finger-1’) and Atrogin-1 (‘muscle atrophy F-box’ (MAFbx)), strongly upregulated in skeletal muscle wasting conditions [28, 29]. Atrogene expression is regulated by various intracellular signaling pathways, including the PI3K-AKT pathway (Figure 4.2) [24]. Also FOXOs (‘Forkhead box protein O transcription factors’),

stimulated by decreases in insulin/IGF-1 and increases in inflammatory cytokines and glucocorticoids, play an important role in regulating the UPS and a number of essential autophagy related genes [15, 30]. In many catabolic diseases apoptosis often precedes protein degradation in skeletal muscle [31].

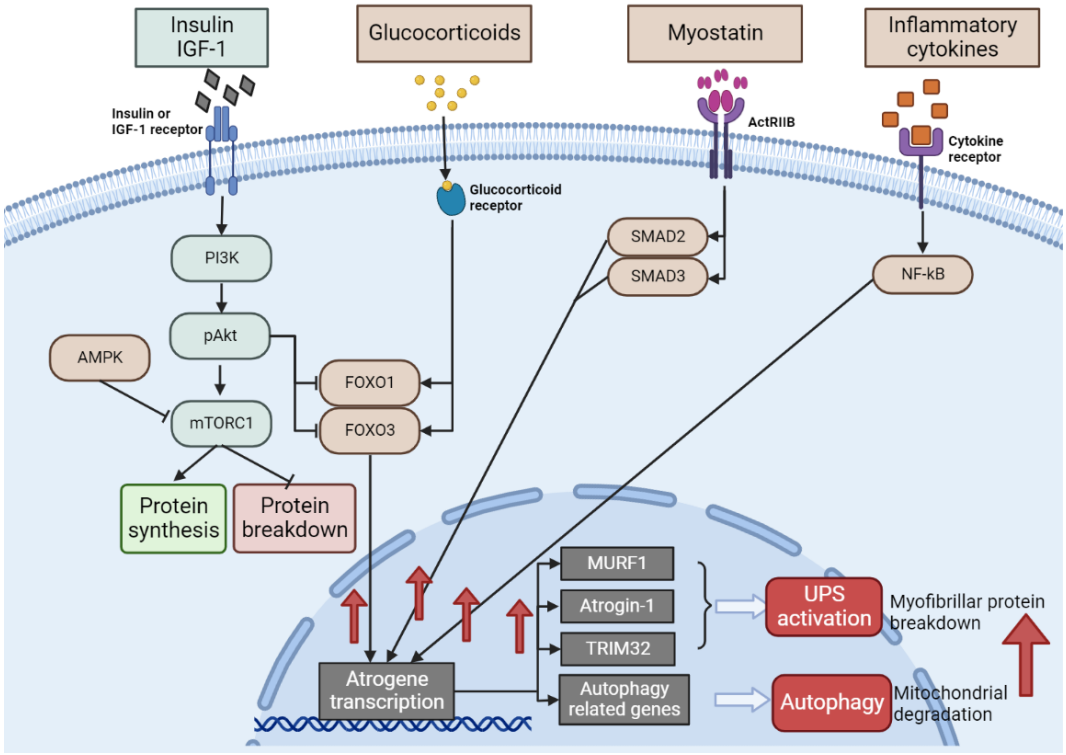


Figure 4.2 Main intracellular signaling pathways of skeletal muscle activating the UPS and Autophagy as found during other atrophy conditions. The atrogene transcription program is activated by 1) decreases in insulin or IGF-1 signaling, decreasing the activity of the PI3K-Akt-mTOR pathway, increasing expression of transcription factors FOXO1 and FOXO3; 2) increases in glucocorticoid release resulting in increases of FOXO1/3; 3) increased amounts of inflammatory cytokines leading to higher NF-κB expression; 4) transcription factors SMAD2 and SMAD3, stimulated by myostatin. The atrogene transcription program consequently leads to proteolysis via increased activity of MURF-1, atrogin-1, TRIM32 and autophagy related genes, stimulating the Ubiquitin Proteasome System (UPS) and Autophagy. IGF-1, insulin like growth factor 1; PI3K-Akt-mTOR, phosphoinositide 3-kinase-Akt-mammalian target of rapamycin; FOXO, forkhead box protein O; NF-κB, nuclear factor-κB; MURF-1, muscle-specific RING-finger 1; TRIM32, Tripartitie Motif Containing 32.

Studies investigating the role of above-mentioned pathways in postburn muscle wasting are scarce [1, 16]. We investigated Soleus muscle (SOL), with predominantly type 1 muscle fibers, and Extensor Digitorum Longus muscle (EDL), with predominantly type 2 muscle fibers, separately in a female rat burn model. To obtain a better understanding of affected signaling pathways, we analyzed pAKT, p70S6k, eIF2α and eEF2 for protein synthesis and FOXO3A, MURF-1 and Atrogin-1 for protein breakdown [15, 27, 30, 32]. We hypothesize that after severe burns skeletal muscle wasting is caused by a protein imbalance resulting from altered expressions of protein synthesis regulating proteins and/or activation of atrogene transcription factors MURF-1 and Atrogin-1, induced by the inflammatory response in the postburn flow phase [33]. Besides, we hypothesize that postburn muscle wasting is regulated differently in type 1 and type 2 muscle fibers.

3 Material and methods

3.1 Animal model

All procedures were reviewed and approved by the Ethical Committee of laboratory animals of the University of Antwerp under number 2020-52. 30 female Sprague-Dawley rats (Charles River) of six weeks old were acclimated individually in specialized ventilation cabinets with a temperature of 22°C ten days prior to burn injury. Rats were randomly assigned to one of three groups: (1) sham treatment (S; n=10), (2) minor burn submitted to a 10% total body surface area (TBSA) burn (MB; n=10) or (3) severe burn submitted to a 40% TBSA burn (SB; n=10). Three animals were excluded from analysis for different reasons (death during anesthesia n=1, hindlimb infections n=1, burn size not according to protocol n=1).

3.2 Burn model

Burns were inflicted according to the modified Walker-Mason model inflicting a deep dermal burn and inducing metabolic changes [34-36]. Animals were administered

buprenorphine (0,05mg/kg) (Temgesic, Schering-Plough, Belgium) and anaesthetized with 1-3% isoflurane (Zoetis, UK) in 100% oxygen. Next, rats were shaved with a clipper, placed in a mold exposing 10% (minor burn group) or 30% (severe burn group) TBSA of their back and submerged in 100°C water for ten seconds [37]. An intraperitoneal injection of Ringers lactate was administered, rats were subsequently placed in a mold exposing 10% TBSA along their ventral side and submerged for three seconds in 100°C water for the severe burn group and in room temperature water for the minor burn group. Sham treated rats underwent the same procedures but all in room temperature water. Rats were administered Ringers lactate 30min, four hours and eight hours postburn (resulting in a total of 4ml/kg/%TBSA) and buprenorphine 12 hours postburn. Animals stayed in individual cages in specialized ventilation cabinets for the remainder of the experiment, received food and water ad libitum (Ssniff 'complete feed for rats & mice', consisting of 9% Fat, 24% Protein and 67% Carbohydrates) and were monitored daily using the Functional Observational Battery adjusted to the specific features of burns [38]. Animals showed normal ambulatory patterns without signals of pain.

3.3 Tissue collection

Ten days after the burn procedure, animals were euthanized by an intraperitoneally injection of 250mg/kg pentobarbital (Kela, Belgium). Animals were weighed, blood was immediately drawn by cardiac punction and hindlimb muscles (SOL and EDL) of both hindlimbs were harvested, weighed and one of each stored in a cryotube in liquid nitrogen. SOL and EDL from the other hindlimb of each animal were prepared for immunohistochemical analysis, placed on a cork, covered in Neg-50 Frozen Section Medium (Thermo Scientific, USA) and frozen in liquid nitrogen-cooled isopentane (2-methylbutane) (Honeywell, US). All samples were stored in a -80°C freezer until further analysis.

3.4 Immunohistochemistry

Fiber type distribution (total fiber count, amount of type 1, type 2a and type 2b muscle fibers) was determined using a MyHC staining. Since this is a semi-qualitative technique it was performed on 6 (randomly chosen) muscles for each group. Unfixed transversal muscle sections (10µm) were air-dried at room temperature and fixated with Paraformaldehyde (10 minutes). Slides were pre-incubated with TBS-Tx, washed with TBS-T, blocked with horse serum (1:10) for 30min and incubated overnight (at room temperature) with specific primary antibodies: anti-laminin (mouse; Bio-Techne NB300-144; 1:500) and isoform-specific myosin heavy chain (MyHC) antibodies: MyHC type 1 (BA-D5 mouse MIgG2b 1:5), MyHC type 2a (SC-71 mouse MIgG1 1:5) from Developmental Studies Hybridoma Bank (DSHB; Iowa City, IA, USA). Slides were washed in TBS-T and incubated for one hour with corresponding immunoglobulin-specific secondary antibodies were used: Alexa fluor 405 (anti-rabbit; Invitrogen A31553) IgG, Alexa fluor 555 (anti-mouse; Invitrogen A21147) IgG2b and Alexa fluor 633 (anti-mouse; Invitrogen A21126) IgG1. Afterwards, slides were washed in TBS-T, covered with 'Vectashield with DAPI' (Vector Laboratories) and air-dried. Images were taken at x10 magnification with a NIKON Eclipse Ti series high throughput fluorescent microscope and ImageJ software was used for further analysis.

To quantify apoptosis, slides were incubated with cleaved caspase-3 (cell signaling #9661, 1:300). To investigate mitochondrial dysfunction, the activity of the respiratory enzymes Cytochrome c oxidase (COX) and succinate dehydrogenase (SDH) was measured using the VitroView COX/SDH Double Histochemistry Stain kit (VB-3022) following manufacturer's instructions. Images were captured at x10 magnification with an Olympus BX43 microscope and analysed with ImageJ software.

Investigators were blinded to burn group for all histological analyses.

3.5 Western blotting

We determined, using Western Blotting, the expression of several key proteins 10 days after a severe burn injury, known to be involved in skeletal muscle wasting. Snap-frozen SOL and EDL were collected in RIPA lysis buffer (Cell-Signaling, #9806) supplemented with phosphatase and protease inhibitor Tablets (Roche, 4906845001 and 11836170001: 1 Tablet of each/10 mL) in specially designed CK-28R tubes with 2.8mm ceramic beads (Bertin Technologies P000916-LYSK0-A). A Precellys 24 (Bertin Technologies P000669-PR240-A) was used for tissue homogenization (6800rpm for 3x30sec) and after centrifugation of the lysate (10min at 14000rpm at 4°C) protein suspension was extracted. Total protein concentration was quantitated using Pierce BCA Protein Assay kit (ThermoFisher #23225) following manufacturer's instructions. Diluted samples (1µg/µl) were heat-denatured for five minutes at 100°C.

Based on linear range analysis, 15 µg protein was loaded on Bolt 4-12% Bis-Tris gels (life technologies, NW04125BOX). Proteins were separated, transferred to a PVDF membrane. A total protein staining was performed using Revert 700 Total Protein Stain Kit (Li-COR biosciences, 926-11010). Membranes were scanned with an IR scanner (Odyssey Imaging System) and de-stained. Membranes were blocked in Intercept blocking buffer (Li-Cor Biosciences, 927-60001) and incubated overnight at 4°C with the following rabbit IgG primary antibodies: anti-Akt1 (phospho S473; 1:1000; ab81283; Abcam)), anti-EEF2 (1:10000; ab75748; Abcam), anti-p70S6kinase (1:1000; 9202 Cell signaling), anti-eIF2α (1:1000; 9722 Cell signaling), Anti-MURF1+MURF3+MURF2 (1:1000; ab172479; Abcam), anti-Fbx32 (1:1000; ab168372; Abcam) and anti-FOXO3A (1:1000; ab23683; Abcam). After washing, a secondary anti-rabbit IgG IR-labeled secondary antibody (Li-COR LI 926-32211; 1:20000 dilution in Li-Cor blocking buffer supplemented with 0.01% SDS) was added for one hour and washed again. Membranes were imaged with an IR scanner (Odyssey imaging system). Analysis was completed with Empiria Studio software, and the signal from the protein bands was normalized to the Total Protein Stain signal.

3.6 Statistical analysis

Statistical analysis was performed using JMP-16. Experimental data for each burn group were expressed as means ±SEM. Differences between groups were analyzed using the Kruskal-Wallis test for general analysis and for immunohistological stainings. For western blots, linear mixed models was used, with the number of blots as a random effect to exclude technical variation between blots. Differences were considered to be statistically significant at $p<0.05$.

4 Results

4.1 Total body mass and muscle weight

Severely burned animals gained significantly less body weight compared to sham treated (12±3.2g vs 27.8±3.4g; p=0.003) and compared to minor burned animals (12±3.2g vs 22.7±1.5g; p=0.03). Minor burned animals also gained less body weight compared to sham treated animals, although this difference was not significant (p=0.45) (Figure 4.3).

Muscle wet weights of both SOL and EDL were not significantly different between groups (p=0.09 and p=0.47 respectively) (Table 4.1). To account for differences in total body weight between the different groups at baseline, ratio of muscle wet weight-to-total body weight(baseline) was used. This ratio was significantly lower in severely burned animals compared with sham treated animals for both SOL (0.45±0.01 vs 0.50±0.01; p=0.03) and EDL (0.47±0.01 vs 0.54±0.01; p=0.008). In EDL, this ratio was also significantly lower in the severe burn group compared to the minor burn group (0.47±0.01 vs 0.53±0.01; p=0.02) (Figure 4.3).

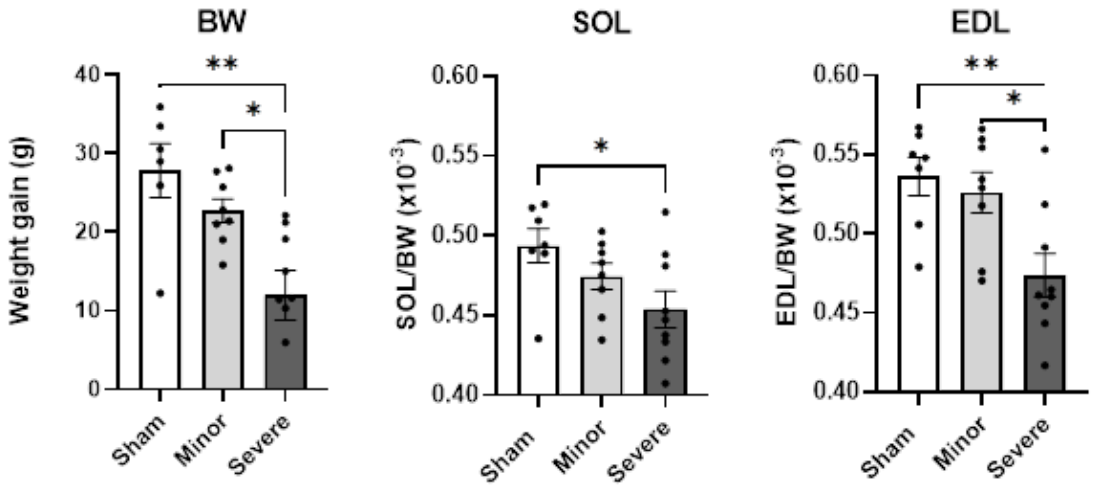


Figure 4.3: Difference in total body weight (BW) gain (weight day 10 minus weight day 0 postburn), ratio of m. Soleus (SOL) and m. Extensor Digitorum Longus (EDL) wet weight (mg) at 10 days postburn to total body weight at baseline (BW) (g) for sham, minor and severe burned animals. N=9 per group, error bars represent SEM, significant difference *p<0.05, **p<0.01

	Body weight baseline (g)	Body weight end (g)	Body weight gain (g)	SOL (mg)	EDL (mg)
Sham	200.7±8.7	228.5±6.4	27.8±3.4	96.1±4.3	104±4.7
Minor burn	194.5±2.6	217.2±2.2	22.7±1.5	89.5±3.3	99.4±4
Severe burn	227±7.2#*	239±7.5#	12±3.2#*	103.4±5.3	107.8±5.5

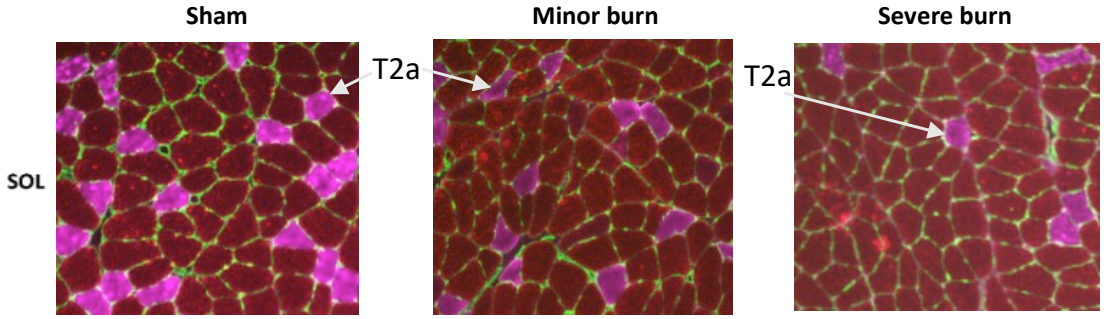
Table 4.1 Total body weight(g) at baseline and 10 days postburn (end) and wet weights (mg) of m. Soleus (SOL) and m. Extensor Digitorum Longus (EDL) 10 days postburn. Values are expressed as mean ± SEM. N=9 per group. *significant difference vs sham; #significant difference vs minor burn group; Significance if p<0.05

4.2 Muscle morphology

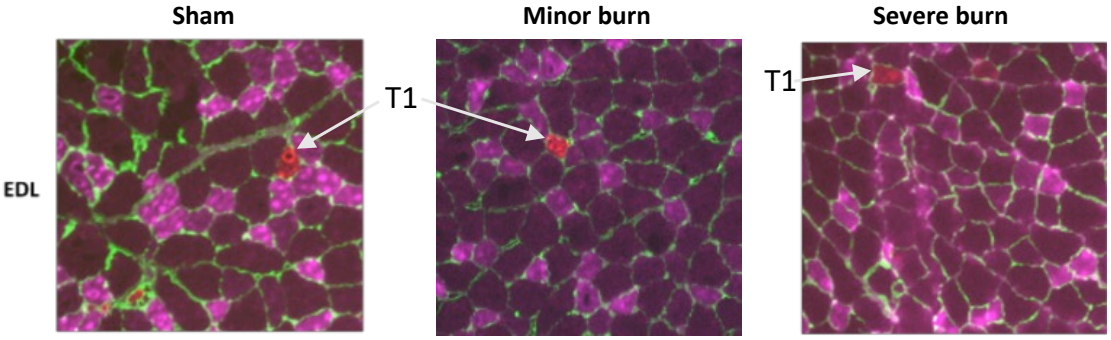
Muscle fiber typing (Figure 4) of SOL and EDL is shown in figure 5. In EDL total number of fibers is significantly lower in the severe burn compared to the sham treated group (177±14 vs 237±21; p=0.03). In SOL a lower total fiber count was present in the severely burned animals compared to sham treated animals, but this was not significant (p>0.05).

In SOL but not in EDL significant changes were found in type 1 and type 2 fiber distribution when comparing burn and sham treated groups. Number of type 1 muscle fibers was

significantly higher in SOL of severely burned animals compared to both minor burned (87±2 vs 77±2; p=0.02) and sham treated animals (87±2 vs 76±2; p=0.005). Likewise, type 2 muscle fiber count was significantly lower in SOL of severely burned compared to minor burned animals (14±2 vs 24±2; p=0.005), whereas the difference between severely burned and sham treated animals did not reach significance (14±2 vs 22±2; p=0.07). Looking more specifically at type 2a and type 2b muscle fiber subdivision, number of type 2b fibers was significantly lower in severely burned compared to sham treated animals (0±0 vs 1±0; p=0.003), whereas for the number of type 2a fibers no significant difference was found for severe vs sham treated animals (significance for SOL; p=0.0547).



No black T2b fibers visible. The majority are red T1 fibers, alternated with some purple T2a fibers (1 indicated with an arrow in each section).



All red T1 fibers are indicated with an arrow. Purple T2a fibers are alternated by black T2b fibers in each section.

Figure 4.2 Example of the used immunohistology MyHC staining on transversal muscle sections (10µm) of sham treated, minor and severely burned Soleus (SOL) muscle samples and ~~on~~ of sham treated, minor and severely burned Extensor Digitorum (EDL) muscle samples captured at 10x magnification. Green indicates the basal membrane, red type 1 fibers, purple type 2a fibers and black type 2b fibers

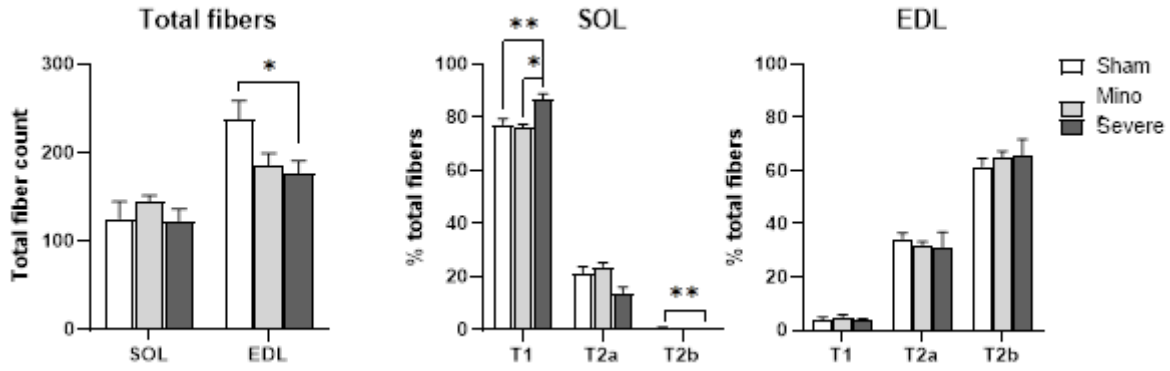


Figure 4.3: Muscle fiber type distribution: total fiber count, % of type 1 (T1), type 2a (T2a) and type 2b (T2b) fibers (specific fiber type count/total fiber count) for Soleus (SOL) and Extensor Digitorum Longus (EDL) transversal muscle sections (10µm) muscles from sham treated, minor and severe burned animals after 10 days. N=6 per group, error bars represent SEM, Significant difference *p<0.05, **p<0.01

4.3 Mitochondrial oxidative activity and apoptosis

A COX/SDH staining was performed to evaluate mitochondrial oxidative capacity, but no COX negative fibers or red ragged fibers were found in SOL or EDL sections of sham treated, minor or severe burn animals. Further, a cleaved Caspase-3 staining showed no signs of apoptosis ten days post-injury.

4.4 Skeletal muscle protein synthesis: pAKT, p70S6K, eEF2 and eIF2α

eEF2 was significantly lower in SOL of severely burned animals compared to sham treated animals ($p<0.0001$) (Figure 4.5B). Also, the expression of pAKT and p70S6K in SOL was significantly lower in severely burned compared to sham treated animals ($p=0.003$ and $p=0.044$, respectively) (Figure 4.5C and E). In EDL eEF2, pAKT, p70S6K and eIF2α were significantly lower in severely burned animals compared to sham treated animals ($p<0.0001$, $p=0.005$, $p=0.015$ and $p=0.044$ respectively) (Figure 4.5F-I).

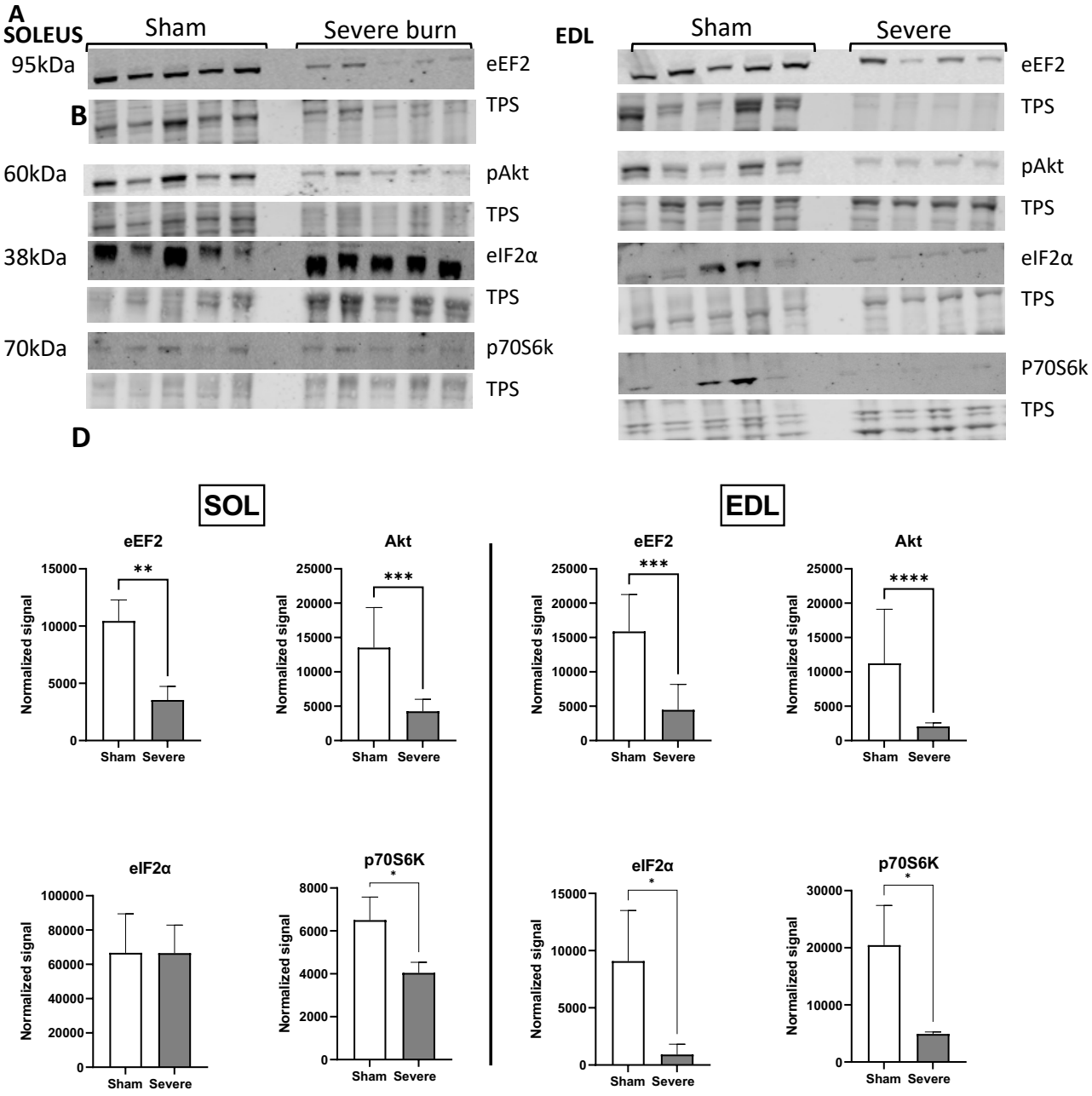
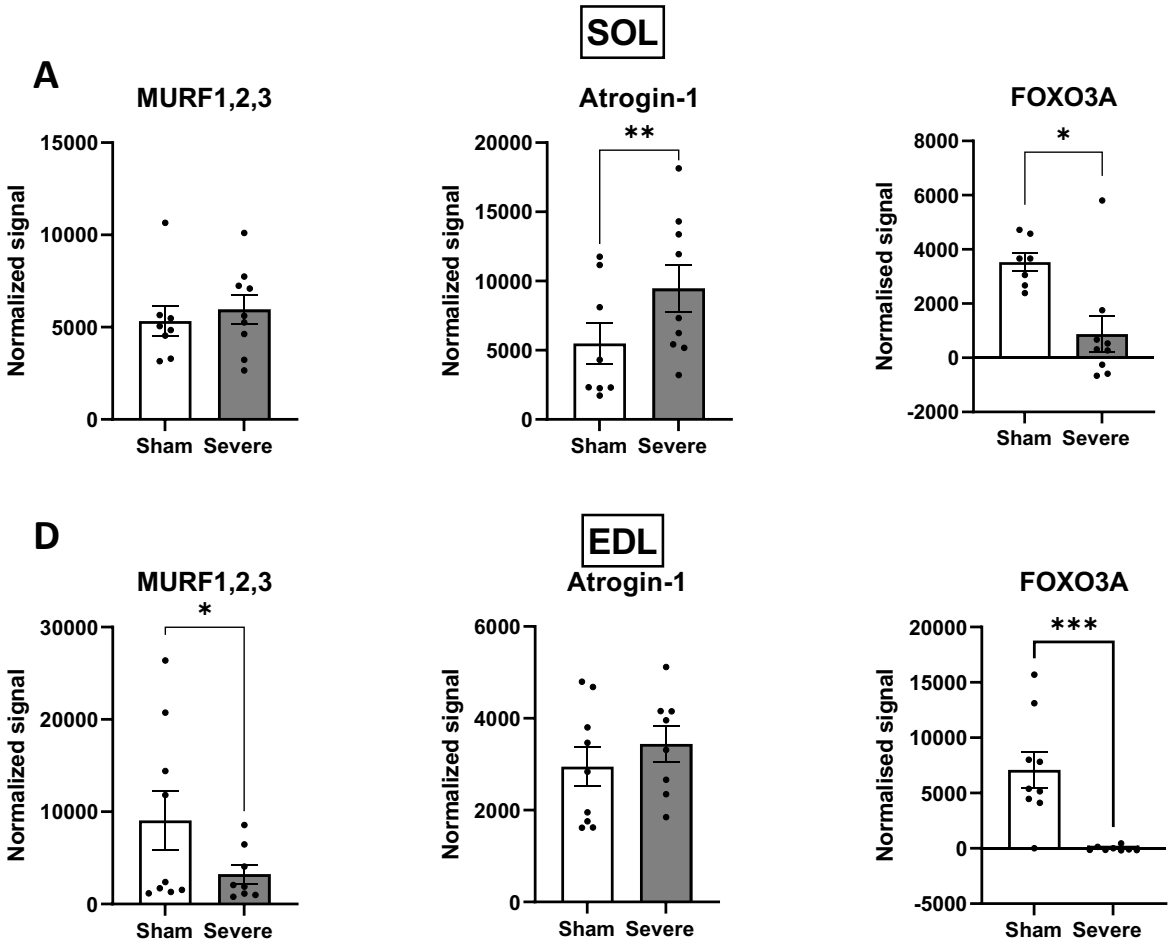


Figure 4.4 A: Western blots for eEF2, Akt, eIF2α and p70S6K of Soleus (SOL) and Extensor Digitorum Longus (EDL) muscle. The immunoblots depicted are representative of samples of sham treated and severely burned animals 10 days post injury. For every protein, the Total Protein Stain (TPS) at the corresponding molecular height is depicted. B-I: Protein signals of Western blots normalized to Total protein stains in sham treated versus severe burn groups 10 days postburn. Bars represent SEM. A-E: Normalized protein signals in SOL F-I: Normalized protein signals in EDL. N=9 per group, error bars represent SEM, significant difference **p<0.01, ****p<0.0001

4.5 Skeletal muscle protein breakdown: MURF-1, Atrogin-1 and FOXO3A

In SOL, both MURF-1 and Atrogin-1 were higher 10 days after the severe burn, however only the difference in Atrogin-1 was significant (p=0.002) (Figure 4.6A-B). FOXO3A showed a significant lower expression in the severely burned group compared to the sham treated group (p=0.01) (Figure 4.6C). In EDL of severely burned animals, we found a significant lower expression of both MURF-1 (p=0.02) and FOXO3A (p=0.0004) compared to sham treated animals (Figure 4.6D and F). No significant difference was found for Atrogin-1 in EDL of severely burned animals compared to sham treated animals (p=0.41) (Figure 4.6E).



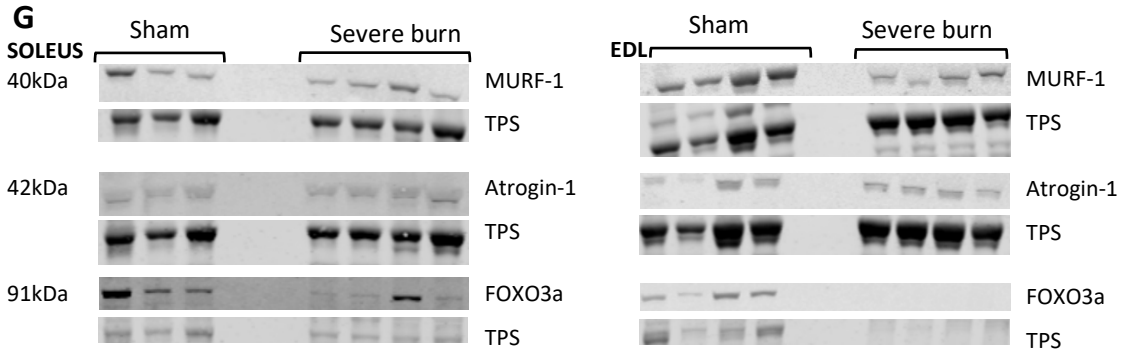


Figure 4.5 Protein signals of Western blots normalized to Total protein stains in sham versus severe burn groups 10 days postburn. Bars represent SEM. A. Normalized MURF1,2,3 protein signal in Soleus muscle. B. Normalized Atrogin-1 protein signal in Soleus muscle. C. Normalized FOXO3A protein signal in Soleus muscle D. Normalized MURF1,2,3 protein signal in EDL muscle. E. Normalized Atrogin-1 protein signal in EDL muscle. F. Normalized FOXO3A protein signal in EDL muscle. G. : Western blots for MURF1,2,3, Atrogin-1 and FOXO3A of Soleus and Extensor Digitorum Longus muscle. The immunoblots depicted are representative of samples of sham and severely burned animals 10 days post injury. For every protein, the Total Protein Stain (TPS) at the corresponding molecular height is depicted. N=9 per group, error bars represent SEM, significant difference *p<0.05 **p<0.01, ***p<0.001

5 Discussion

The purpose of this study was to investigate the effects of burns on skeletal muscle, including pathways affecting protein synthesis and breakdown. We found changes in body weight, muscle weight and muscle morphology 10 days postburn, consistent with previous observations both in rats [11, 12, 39-41] and humans [5, 42, 43]. Besides, we demonstrated that the expression of different proteins involved in signaling pathways that regulate skeletal muscle protein balance was altered postburn, but interestingly not all key signaling proteins appear affected. To date, experimental studies are mainly focused on the acute effects of burns on clinical parameters like (lean) body mass, muscle force and overall protein content [11-13, 40, 44]. The Walker-Mason burn model for rats was used, which induces a hypermetabolic response similar as in humans [45-47]. Davenport et al (2019) confirmed that using this model, full thickness burns were created with a predefined surface area, making this the most suitable model for in-depth burn research [48].

5.1 Body weight and skeletal muscle morphology

Ten days postburn we found a significant lower body weight gain compared to sham treated animals, which is consistent with observations in other studies [12, 45, 46]. This difference correlates to the severity of the burn injury, where a higher %TBSA led to a lower body weight gain. We also observed, in line with other investigations, a lower ratio of muscle wet weight to body weight, suggesting that lower body weight gain is most likely due to less lean body mass, and thus skeletal muscle mass [10, 39, 40].

We found a decrease in total number of fibers in EDL after severe burn, supporting the hypothesis that after a burn injury type 2 (fast-twitch) muscles are more prone to muscle wasting after burns than type 1 (slow-twitch) muscles. In SOL, muscle fibers shifted towards more type 1 (slow-twitch) fibers. These findings are in line with other catabolic conditions such as sepsis, starvation, cachexia and aging [13, 49], but are in contrast with conditions such as microgravity and hindlimb unloading, where previous research has shown a switch from type 1 to type 2 fibers [50, 51]. Although we have no clear explanation for these differences, other studies have suggested that the difference in metabolic response between type 1 and type 2 muscle fibers is caused by a more pronounced upregulation of the ubiquitin proteolytic pathway in fast-twitch muscles, causing muscle catabolism [13, 14]. Because of these discrepancies, we investigated the effect of burns on skeletal muscle protein regulation in predominantly type 1, EDL, and type 2, SOL, muscles separately.

5.2 Regulation of skeletal muscle protein metabolism after burns

Since no clear anthropomorphic changes were found 10 days after minor burns , we decided not to perform Western Blots for the muscles of minor burned animals. We found a lower expression of both pAKT, p70S6K and eEF2 in both SOL and EDL in comparison to sham treated animals. These findings are similar to distorted pathways in other cachectic diseases such as cancer [16, 17]. The lower pAKT after burns is indicative for a decreased activation of the PI3/AKT pathway, presumably due to endocrine disturbances [52]. Lower p70S6K indicates lower mTOR signaling postburn [43]. These findings are of major

importance since the PI3K/AKT pathway is one of the most important pathways determining the anabolic or catabolic state of skeletal muscle [24]. In addition, protein synthesis can be affected independent of the PI3K/AKT pathway by downstream proteins, such as eEF2. In contrast to our results, Song et al. (2012) found that total eEF2 was not altered in pediatric burn patients (at 10 and 49 days postburn) suggesting a non-canonical role for eEF2 [42]. eIF2α was found only to be lower in EDL postburn, but not in SOL. Although it is not easy to comprehend the reason for different expression of eIF2α in EDL and SOL postburn, our findings confirm the speculations of Rios-Fuller et al. (2020) that the pathological regulation of eIF2α is muscle type specific [53].

Surprisingly, we did not find a higher expression of MURF-1, Atrogin-1 and FOXO3A in muscle samples of severely burned animals. Only Atrogin-1 in SOL showed a significant higher expression 10 days after the burn injury. Previous studies have shown that energy-ubiquitin-dependent proteolysis, regulated by MURF-1 and Atrogin-1, is stimulated after burn injuries [10]. For instance, Quintana et al (2021) have shown a higher MURF-1 and Atrogin-1 expression in Gastrocnemius muscle [41], whereas Lang et al (2007) have showed that also at mRNA level both E3 ligases are upregulated after burns [11]. One factor that might explain the discrepancies between our findings and these studies are the different measurement timepoints, i.e. 10 days postburn, well into the flow-phase, compared to 48 hours until four days postburn in the above mentioned studies. Therefore, based on these findings, we hypothesize that both E3 ligases are upregulated postburn but in type 1 or mixed muscles the increases are sustained over a longer period of time.

5.3 Mitochondrial oxidative activity and apoptosis

There is no agreement as to the role of apoptosis in postburn skeletal muscle wasting. Previous studies reported an increase in apoptosis in skeletal muscle postburn, while our results showed no increase in caspase-3 activity either in SOL or EDL [40, 54]. However, our results are in agreement with the studies by Duan et al (2016), reporting that caspase-3 levels decreased to basal levels at day 10 postburn, and by Merrit et al (2012), reporting no

significant increases in activated caspases during the postburn flow phase [40, 43]. We assume that the contribution of apoptosis to muscle wasting after burns is important in the first few days after the burn injury, as Duan et al. (2016) found marked increases in caspase-3 levels on one and four days postburn [40]. It should be noted that apoptosis has been shown to be upregulated in immobilization an sedentary behavior, which also contributes to muscle wasting after burns, but was not taken into account in the present study [55, 56].

On muscle sections of the burned animals, COX/SDH staining revealed no positive fibers. Positive complexes II (SDH) and IV (COX) would indicate mitochondrial impairments encoded by both nuclear and mitochondrial DNA (mtDNA)[57]. Our results indicate that at 10 days postburn the nuclear DNA, encoding SDH, is not impaired and that there are no mutations of the mitochondrial DNA, encoding subunits of COX. This is in contrast to previous studies in cancer cachexia, where loss of oxidative phenotype and mitochondrial content was found concomitant with muscle wasting [58]. Even after sepsis impaired mitochondrial complex enzymes have been observed long after the sepsis has resolved, shown by lower complex II and complex IV activity [59].

6 Conclusion

In conclusion, we have shown that at 10 days postburn changes in muscle anthropometry are present and that proteins related to signaling pathways regulating protein synthesis and protein breakdown are altered, with more pronounced catabolic effects in SOL. In both SOL and EDL anabolic signaling was clearly reduced postburn. Overall, our findings support the idea that severe burns lead to a protein imbalance, induced by metabolic changes altering intracellular signaling pathways. This understanding may help to guide future research to elucidate the relative contribution of pathways and molecules leading to postburn muscle wasting in the postburn flow phase.

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Chapter 5

Molecular pathways of postburn skeletal muscle wasting in the chronic flow phase

Dombrecht D, Van Daele U, Van Asbroeck B, Schieffelers DR, Guns PJ, van Breda E; Burn injury dysregulates protein signaling in type 1 and type 2 muscles in rats. *Submitted to 'Burns'*

Background: Skeletal muscle wasting is one of the systemic hallmarks of severe burn injuries. The underlying mechanisms of muscle wasting post-burn, and in particular the differences between muscle fiber types, are not well understood. This study aimed to investigate the protein signaling pathways involved in skeletal muscle atrophy following severe burn injury.

Methods: 11 rats were submitted to sham injury and 11 rats to a severe burn (40% total body surface area burn) using the Walker-Mason burn model. Muscle samples from predominantly type II m. extensor digitorum longus (EDL) and predominantly type I m. soleus (SOL) were collected 40 days post-burn. Body and muscle weights, immunohistochemistry and Western blotting were used to assess anthropometric differences and the expression of key proteins involved in protein imbalance causing muscle wasting.

Results: Rats with severe burn injury showed less body weight gain (39.73±10.97g vs 77.12±4.83g; $p<0.01$) and lower muscle wet weights of both SOL (123.1±3.63mg vs 102.7±3.67mg; $p<0.001$) and EDL (127.9±2.95mg vs 110.3±2.80mg; $p<0.001$) compared to sham-treated rats. Immunohistochemical analysis revealed no significant differences in fiber type distribution between the groups. Western blotting analysis showed altered expression of proteins involved in protein synthesis and proteolysis pathways, with differences between SOL and EDL muscles. In SOL, severe burns induce inhibition of anabolic pAkt ($p<0.0001$) and eEF2 signaling ($p<0.05$), while in EDL increased levels of pAkt ($p<0.01$) were found. Catabolic E3 ligases MURF1,2,3 and Atrogin-1 show higher activation in SOL after severe burn injury ($p<0.0001$; both) but not in EDL, were decreases were found ($p<0.0001$; $p<0.001$; respectively). Analysis of myokines post-burn show in SOL lower expression of Decorin ($p<0.0001$) and higher of myostatin ($p<0.0001$), but in EDL both myostatin and irisin expressions were lower ($p<0.001$; $p<0.05$; respectively).

Conclusions: Severe burn injury resulted in skeletal muscle wasting, as evidenced by decreased body weight gain in growing rats and reduced muscle weights. Protein signaling pathways related to protein synthesis and proteolysis are dysregulated in muscles of burned rats, with significant differences between slow- and fast-twitch muscles. Further research is needed to understand the long-term effects of postburn metabolism on skeletal muscle protein balance and develop targeted therapies to prevent muscle wasting in burn survivors.

Key words

Burns, Skeletal muscle, Signaling pathways, Muscle atrophy, Myokines

1 Background

Skeletal muscle wasting is, among sustained inflammation, insulin resistance and hypermetabolism, one of the hallmarks of the systemic hypercatabolic response after a severe burn injury [1, 2]. Following the acute 'ebb phase', 24-48 h after the injury, the catabolic 'flow phase' persists for months or even years, resulting in, among others, a decrease in lean body mass and muscle strength, adversely affecting morbidity and quality of life of burn survivors [3]. In response to the increased metabolic demands and to provide nutrients for essential organ function and wound healing post-burn, skeletal muscle protein breakdown increases [4, 5]. A redistribution of amino acids is crucial to survive, but muscle catabolism sustains long after wound healing, indicating involvement of additional mechanisms [3, 6].

Under normal physiological conditions, skeletal muscle mass is maintained by a dynamic protein balance [7, 8]. After a severe burn injury, skeletal muscle protein balance is disturbed not only on the site of the burn, but in the whole body. Protein synthesis cannot counterbalance increased rates of protein breakdown, resulting in skeletal muscle atrophy [9, 10]. Both protein breakdown, mainly executed by the ubiquitin proteasome system

(UPS) and autophagy, and protein synthesis are intracellularly regulated by several signaling pathways [8, 11] (for a detailed overview of processes involved post-burn, see [12]). Previous studies suggest that in catabolic conditions the regulation of protein synthesis and proteolysis is different in type I and type II muscle fibers, with more pronounced protein breakdown in type II muscle fibers [13]. In burn studies, similar differences between muscle types have been suggested as well [14]. However despite present knowledge of fundamental muscle protein regulation processes in health and different disease states, little is known about the processes in postburn muscle fibers.

Protein synthesis is mainly regulated by the PI3K-AKT ('phosphoinositide-3-kinase-AKT/PKB(Protein Kinase B)') pathway downstream of insulin and insulin-like growth factor-1. AKT serves as a key protein phosphorylating a set of downstream substrates, among which eEF2 ('Eukaryotic elongation factor2'), blocking apoptosis and inducing protein synthesis [15]. eEF2 activity is also affected by AMPK ('AMP-activated protein kinase'), a key regulator of energy homeostasis [16]. On the other hand, proteolysis is mainly activated by two key atrogenes, MURF-1 ('muscle RING-finger protein-1') and Atrogin-1 ('Muscle Atrophy F-box'). Atrogene transcription is upregulated by FOXOs ('forkhead box protein O transcription factors'), downstream of glucocorticoids. FOXOs can be phosphorylated and deactivated by Akt [17]. A burn injury activates an inflammatory stress response, causing the release of pro-inflammatory cytokines, and a humoral response through upregulating stress hormones, affecting the anabolic and catabolic pathways. In addition, also postburn insulin resistance and immobilization contribute to changes in protein signaling [18].

Mechanical factors such as contraction of skeletal muscle affect proteins involved in these pathways as well by secreting specific cytokines, called myokines, involved in the autocrine and paracrine regulation of metabolism in the muscle itself, as well as other metabolic organs [19]. A well-known catabolic myokine is myostatin, decreasing muscle mass by promoting proteolysis and inhibiting protein synthesis [20, 21]. Two important anabolic myokines released by physical activity are decorin, promoting muscle fiber hypertrophy by

inhibiting myostatin, and irisin, inducing expression of pro-myogenic response genes enhancing protein synthesis [20, 22, 23].

To date, our knowledge of the long-term effects of postburn metabolism on the intracellular pathways affecting net skeletal muscle protein balance is lacking. Understanding the underlying processes of muscle wasting can have major implications for future treatment options, like preventive exercise therapy targeting specific pathways potentially leading to positive systemic effects [24]. We hypothesize that severe burns continue to induce skeletal muscle atrophy 40 days post-burn, resulting from a net negative protein balance with increased levels of catabolic signaling proteins and decreased levels of anabolic signaling proteins. For that purpose, we evaluated, in a severe (40% of total body surface area) rat burn model, protein signaling pathways in predominantly type II m. extensor digitorum longus (EDL) and predominantly type I m. soleus (SOL) in response to severe burn injury.

2 Methods

2.1 Animal model

All experimental protocols were approved by the Ethical Committee of laboratory animals of the University of Antwerp (number 2020-52) and performed in accordance with relevant regulations. Twenty-two female Sprague-Dawley rats (Charles River) of six weeks old were acclimated individually seven days prior to the injury. Rats were randomly assigned to one of two groups: (1) sham treatment (S; n=11) or (2) severe burn (SB; n=11), submitted to a 40% total body surface area (TBSA) burn. One animal was excluded from analysis due to an infection at the hindlimb.

2.2 Burn model

Burns were inflicted according to the modified Walker-Mason model inflicting a deep dermal burn and inducing metabolic changes [25-27]. Animals were administered

buprenorphine (0,05mg/kg) (Temgesic, Schering-Plough, Belgium) and anaesthetized with 1-3% isoflurane (Zoetis, UK) in 100% oxygen. Next, rats were shaved with a clipper and placed in a pre-made mold exposing 30% TBSA of their back and submerged in 100°C water for ten seconds [28]. An intraperitoneal injection of Ringers lactate was administered, rats were subsequently placed in a mold exposing 10% TBSA along their ventral side and submerged for three seconds in 100°C water. Sham treated rats underwent the same procedures but all in room temperature water. Rats were administered Ringers lactate 30min, four hours and eight hours post-burn (resulting in a total of 4ml/kg/%TBSA) and buprenorphine 12 hours post-burn. Animals stayed in individual cages in specialized ventilation cabinets for the remainder of the experiment, received food and water ad libitum (Ssniff 'complete feed for rats & mice', consisting of 9% Fat, 24% Protein and 67% Carbohydrates) and were monitored daily using the Functional Observational Battery adjusted to the specific features of burns [29]. After 48 hours, animals showed normal ambulatory patterns without signals of pain.

2.3 Tissue collection

Forty days after the burn procedure, animals were euthanized by an intraperitoneally injection of 250mg/kg pentobarbital (Kela, Belgium). Rats were weighed, blood was immediately drawn by cardiac punction and hindlimb muscles (SOL and EDL) of both hindlimbs were harvested, weighed and one of each stored in a cryotube in liquid nitrogen. SOL and EDL from the other hindlimb of each animal were prepared for immunohistochemical analysis, placed on a cork, covered in Neg-50 Frozen Section Medium (Thermo Scientific, USA) and frozen in liquid nitrogen-cooled isopentane (2-methylbutane) (Honeywell, US). All samples were stored in a -80°C freezer until further analysis.

2.4 Immunohistochemistry

Fiber type distribution (total fiber count, amount of type I, type IIA and type IIB/X muscle fibers) was determined using a MyHC staining. Since this is a semi-qualitative technique it

was performed on 6 (randomly chosen) muscles for each group. Unfixed transversal muscle sections (10µm) were air-dried at room temperature and fixated with Paraformaldehyde (10 minutes). Slides were pre-incubated with TBS-Tx, washed with TBS-T, blocked with horse serum (1:10) for 30min and incubated overnight (at room temperature) with specific primary antibodies: anti-laminin (mouse; Bio-Techne NB300-144; 1:500) and isoform-specific myosin heavy chain (MyHC) antibodies: MyHC type I (BA-D5 mouse MIgG2b 1:5), MyHC type IIa (SC-71 mouse MIgG1 1:5) from Developmental Studies Hybridoma Bank (DSHB; Iowa City, IA, USA). Slides were washed in TBS-T and incubated for one hour with corresponding immunoglobulin-specific secondary antibodies were used: Alexa fluor 405 (anti-rabbit; Invitrogen A31553) IgG, Alexa fluor 555 (anti-mouse; Invitrogen A21147) IgG2b and Alexa fluor 633 (anti-mouse; Invitrogen A21126) IgG1. Afterwards, slides were washed in TBS-T, covered with 'Vectashield with DAPI' (Vector Laboratories) and air-dried. Images were taken at x10 magnification with a NIKON Eclipse Ti series high throughput fluorescent microscope and ImageJ software was used for further analysis.

Investigators were blinded to burn group for all histological analyses.

2.5 Western blotting

To assess the expression of key proteins involved in skeletal muscle wasting 40 days after a severe burn injury, Western blotting was performed. Snap-frozen SOL and EDL were collected in RIPA lysis buffer (Cell-Signaling, #9806) supplemented with phosphatase and protease inhibitor Tablets (Roche, 4906845001 and 11836170001: 1 Tablet of each/10 mL) in specially designed CK-28R tubes with 2.8mm ceramic beads (Bertin Corp. P000916-LYSK0-A). A Precellys 24 was used (6800rpm for 3x30sec) and after centrifugation (10min at 14000rpm at 4°C) protein suspension was extracted and transferred to new ice-cold tubes. Total protein concentration was quantitated using Pierce BCA Protein Assay kit (ThermoFisher #23225) following manufacturer's instructions. Diluted samples (1µg/µl) were heat-denatured for five minutes at 100°C.

Based on linear range analysis, 15 µg protein was loaded on Bolt 4-12% Bis-Tris gels (life technologies, NW04125BOX). Proteins were separated, transferred to a PVDF membrane. A total protein staining was performed using Revert 700 Total Protein Stain Kit (Li-COR biosciences, 926-11010). Membranes were scanned with an IR scanner (Odyssey Imaging System) and de-stained. Membranes were blocked in Intercept blocking buffer (Li-Cor Biosciences, 927-60001) and incubated overnight at 4°C with the following rabbit IgG primary antibodies: anti-Akt1 (phospho S473; 1:10000; ab81283; Abcam)), anti-EEF2 (1:10000; ab75748; Abcam), Anti-MURF1+MURF3+MURF2 (1:1000; ab172479; Abcam), anti-Fbx32 (1:1000; ab168372; Abcam), anti-FOXO3A (1:800; ab23683; Abcam), anti-FOXO3A (phosphoS253) (1:800; ab47285; Abcam), anti-irisin (anti-FNDC5, 1:1000, ab174833, Abcam), anti-decorin (1:1000; ab175404; Abcam) and anti-GDF8/myostatin (1:300; ab203076; Abcam). After washing, a secondary anti-rabbit IgG IR-labeled secondary antibody (Li-COR LI 926-32211; 1:20000 dilution in Li-Cor blocking buffer supplemented with 0.01% SDS) was added for one hour, followed by another round of washing. Membranes were imaged with an IR scanner (Odyssey imaging system). Analysis was completed with Empiria Studio software, and the signal from the protein bands was normalized to the total protein stain bands.

2.6 Statistical analysis

Statistical analysis was performed using JMP-16. Experimental data for each burn group were expressed as means ±SEM. Differences between groups were analyzed using the Kruskal-Wallis test for general analysis and for immunohistological stainings. For western blots, linear mixed models was used, with the number of blot as a random effect to exclude technical variation between blots. Statistical difference was determined at $p<0.05$.

3 Results

3.1 Total body weight and muscle weight

Rats with severe burn injury had less body weight gain in compared to sham treated rats (39,73±10,97g vs 77,12±4,83g; p=0.0045) (Figure 5.1). Both SOL and EDL wet weight was lower in burned rats (Table 5.1) and accordingly, the ratio of muscle wet weight-to-total body weight (baseline) was lower in severely burned animals compared to sham treated animals for both SOL (0.53±0.02mg vs 0.62±0.02mg; p=0.0013) and EDL (0.57±0.02mg vs 0.65±0.2mg; p=0.0105) (Figure 5.1).

	Body weight baseline (g)	Body weight end (g)	Sol (mg)	EDL (mg)
Sham	197,9±5.284	275±4,696	123.1±3.625	127.9±2.948
Burn	193±4,289	232,7±9,572	102.7±3.671	110.3±2.802
Significance	P=0.4853	P=0.0006	P=0.0009	P=0.0004

***Table 5.1**: Total body weight (g) at baseline and 40 days postburn (end) and wet weights (mg) of m. Soleus (SOL) and m. Extensor Digitorum Longus (EDL) 40 days postburn. Values are expressed as mean ± SEM. N=11 per group. Kruskall Wallis test for significance; *significant difference if p<0.05*

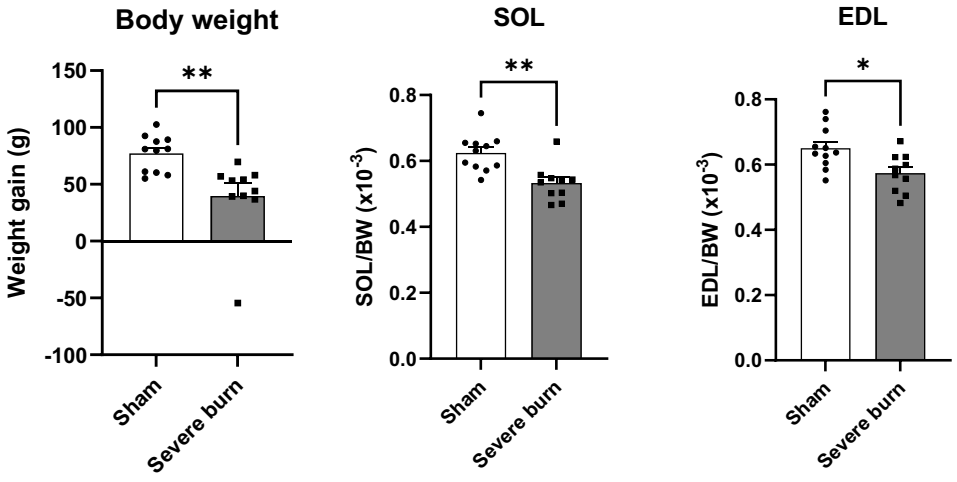

***Figure 5.1**: Difference in total body weight (BW) gain (weight day 40 postburn minus weight right before burn injury), ratio of m. Soleus (SOL) and m. Extensor Digitorum Longus (EDL) wet weight (mg) at 40 days postburn to total body weight at baseline (BW) (g) for sham and severe burned animals. N=11 per group, error bars represent SEM, significant difference *p<0.05, **p<0.01*

3.2 Muscle morphology

The mean value of total muscle fibers counted in a section of a MyHC stained muscle was higher in SOL of burned animals compared to sham animals (648±13 vs 591± 20, respectively; p=0.0303). In EDL muscle, this difference was not significant (772±59 vs 715±34, respectively; p=0.4302). In both muscles, no significant differences were found in type I and type II fiber distribution when comparing burn and sham treated groups. (Figure 5.2 A-C)

Looking at cross-sectional area (CSA) of SOL and EDL muscle fibers, significant differences were found between burn and sham groups in SOL but not in EDL muscle. Mean CSA of type I muscle fibers is lower in SOL of severely burned animals compared to sham (1734.29±84.42µm² vs 2003.90±81.98µm² respectively; p=0.0403). For type II muscle fibers, the CSA was not significantly different in SOL. In EDL, no significant differences were found concerning CSA. (Figure 2 D-E)

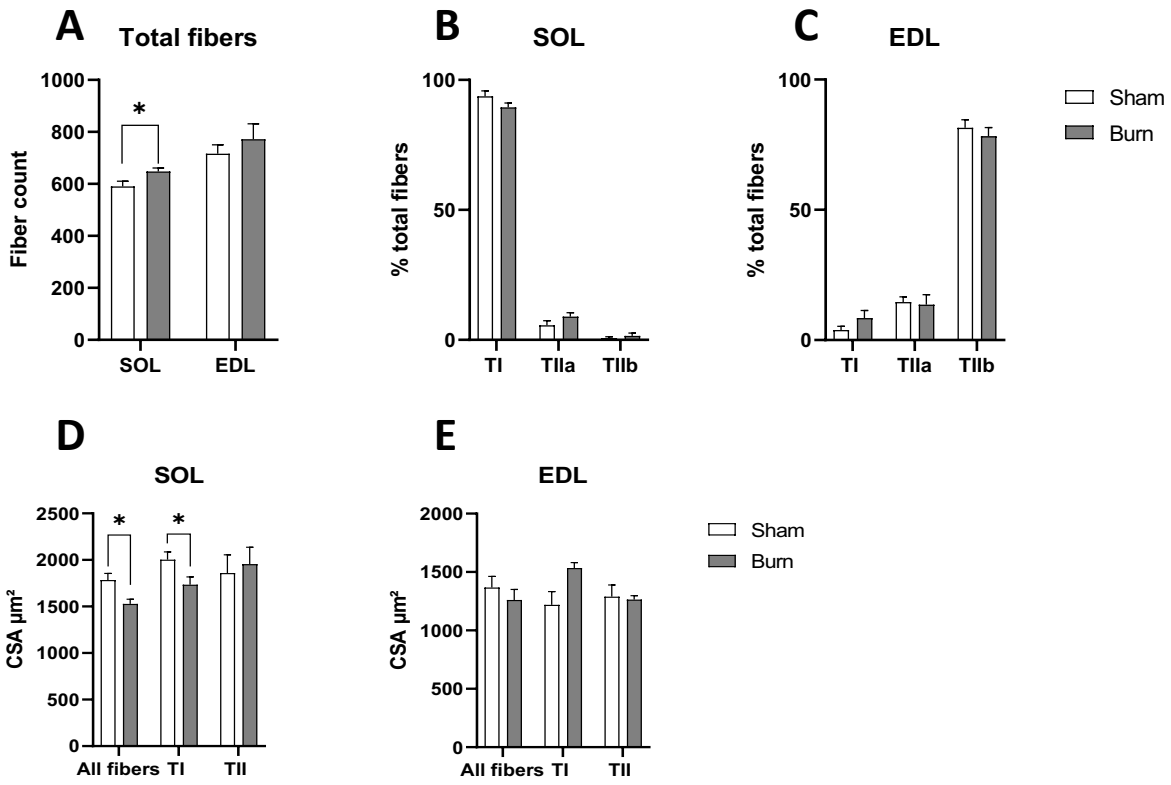

***Figure 5.2**: Muscle fiber type distribution for both Soleus (SOL) and Extensor Digitorum Longus (EDL) muscle sections (10µm) from sham treated and burned animals after 40 days: total fiber count (A), % of type I (TI), type IIa (TIIa) and type IIb (TIIb) fibers (specific fiber type count/total fiber count) (B,C) and cross-sectional area (CSA) of all muscle fibers and type I (TI) and type II (TII) muscle fibers separately (D,E). N=9 per group (only qualitative stained sections were used), error bars represent SEM, significant difference *p<0.05*

3.3 Anabolic pAkt and eEF2 signaling is inhibited in SOL but not in EDL muscle after severe burn injury

Among the signaling pathways affecting protein synthesis and therefore controlling muscle size, the PI3K-AKT pathway plays a critical role [18]. We investigated the effect of severe burns on the PI3K-AKT pathway in SOL and EDL by focusing on two key proteins, AKT and eEF2. Significant lower expressions of AKT and eEF2 were found in SOL ($p<0.0001$ and $p=0.0188$ respectively) after severe burns compared to sham treated animals (Figure 5.3). In EDL an increase in AKT was found in severely burned rats ($p=0.0011$), but no significant difference was found for eEF2 protein expression ($p=0.0997$) (Figure 5.3).

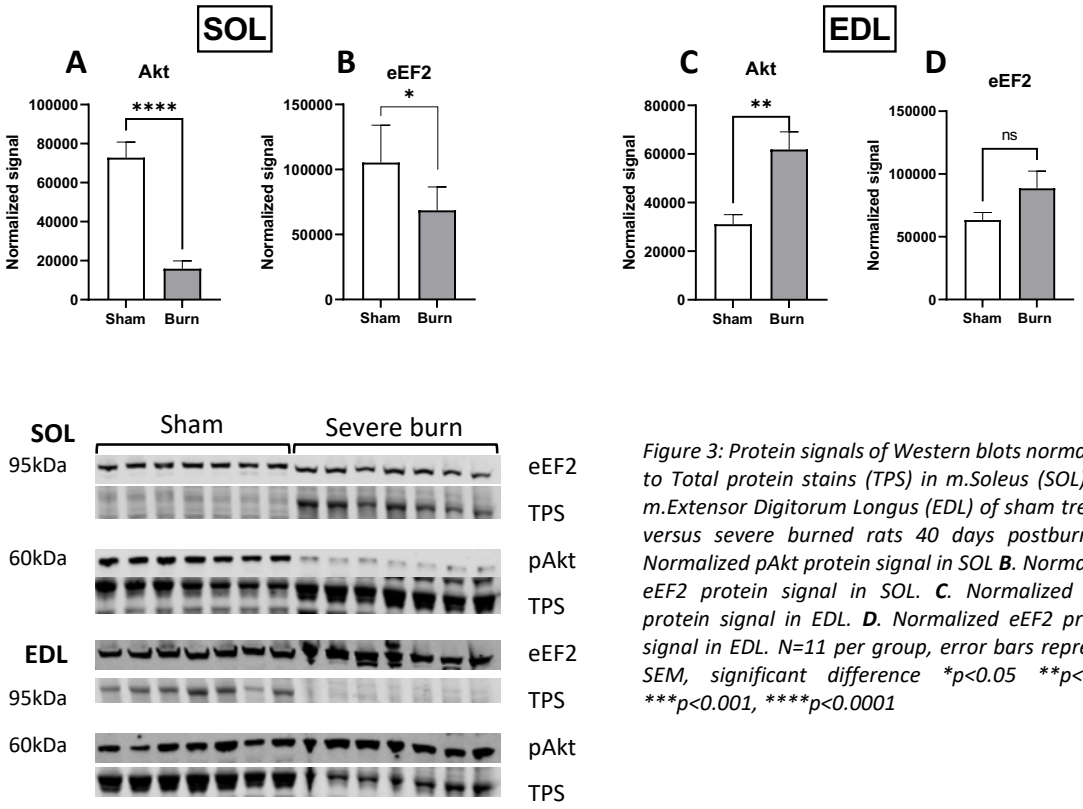

*Figure 3: Protein signals of Western blots normalized to Total protein stains (TPS) in m.Soleus (SOL) and m.Extensor Digitorum Longus (EDL) of sham treated versus severe burned rats 40 days postburn. **A**. Normalized pAkt protein signal in SOL **B**. Normalized eEF2 protein signal in SOL. **C**. Normalized pAkt protein signal in EDL. **D**. Normalized eEF2 protein signal in EDL. N=11 per group, error bars represent SEM, significant difference *p<0.05 **p<0.01, ***p<0.001, ****p<0.0001*

3.4 Catabolic E3 ligases MURF1,2,3 and Atrogin-1 are activated in SOL but not in EDL muscle after severe burn injury

To determine whether burn-induced muscle wasting is mediated by atrogenes and the activation of the proteolytic system, we looked into protein expression of MURF1,2,3 and Atrogin-1 in SOL and EDL (Figure 5.4). In response to severe burn injury, western blot analysis of MURF1,2,3 and Atrogin-1 showed higher expressions in SOL ($p<0.0001$). In EDL however, a lower expression of both atrogenes was found ($p<0.0001$ for MURF1,2,3 and $p=0.0001$ for Atrogin-1). To examine the effect of severe burn injury on signaling pathways activating the proteolytic system, we examined the expression of FOXO3a and pFOXO3a in both SOL and EDL (Figure 5.4). FOXO3a expression was lower in SOL of severely burned rats ($p=0.0034$), and also the phosphorylated form, pFOXO3a, was lower in these animals, but not significantly. In EDL, no significant differences were found for either FOXO3a of pFOXO3a protein expression when comparing burned and sham treated animals.

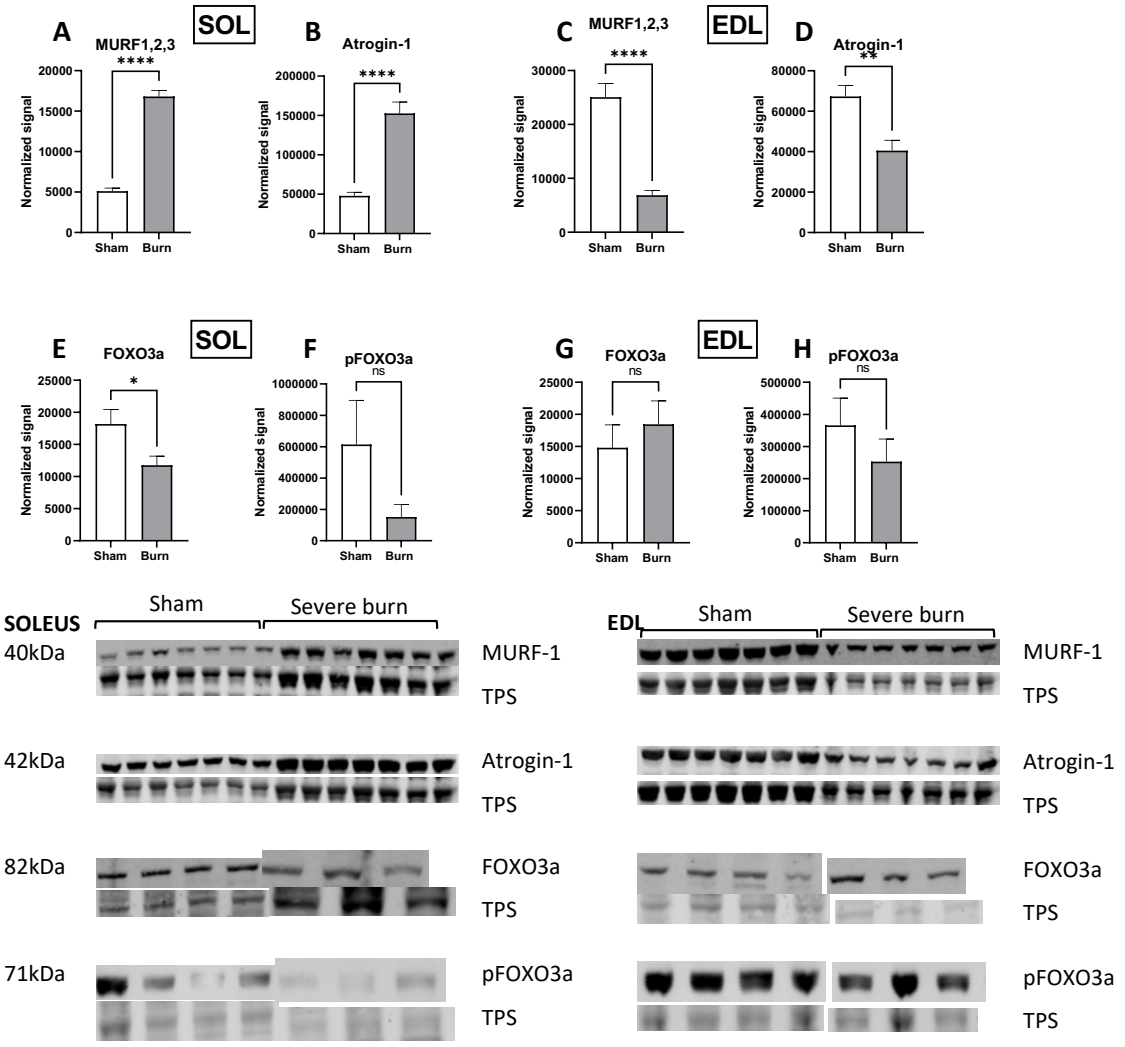

***Figure 5.4**: Protein signals of Western blots normalized to Total protein stains in m.Soleus (SOL) and m.Extensor Digitorum Longus (EDL) of sham treated versus severe burned rats 40 days postburn. A. Normalized MURF1,2,3 protein signal in Soleus muscle. B. Normalized Atrogin-1 protein signal in Soleus muscle. C. Normalized FOXO3A protein signal in Soleus muscle D. Normalized FOXO3A protein signal in Soleus muscle E. Normalized MURF1,2,3 protein signal in EDL muscle. F. Normalized Atrogin-1 protein signal in EDL muscle. G. Normalized FOXO3A protein signal in EDL muscle. H. Normalized pFOXO3A protein signal in EDL muscle. N=11 per group, error bars represent SEM, significant difference *p<0.05 **p<0.01, ***p<0.001, ****p<0.0001*

3.5 Myokines myostatin and decorin in SOL and irisin in EDL are involved in postburn muscle wasting

To evaluate whether post-burn muscle atrophy of SOL and EDL is affected by myokines, we examined the expression of anabolic myokines Irisin and Decorin and catabolic myokine Myostatin (Figure 5.5). Whilst Decorin revealed a lower expression in SOL of severely

burned rats (p<0.0001), no significant difference was found for Irisin expression (p=0.2094). In EDL, anabolic Irisin showed a lower expression (p=0.0126), but no significance difference was found for Decorin (p=0.3984). Myostatin showed a higher expression in SOL of severely burned rats (p<0.0001), whilst a reversed effect was found in EDL, with a lower myostatin expression compared to sham treated rats (p=0.0001).

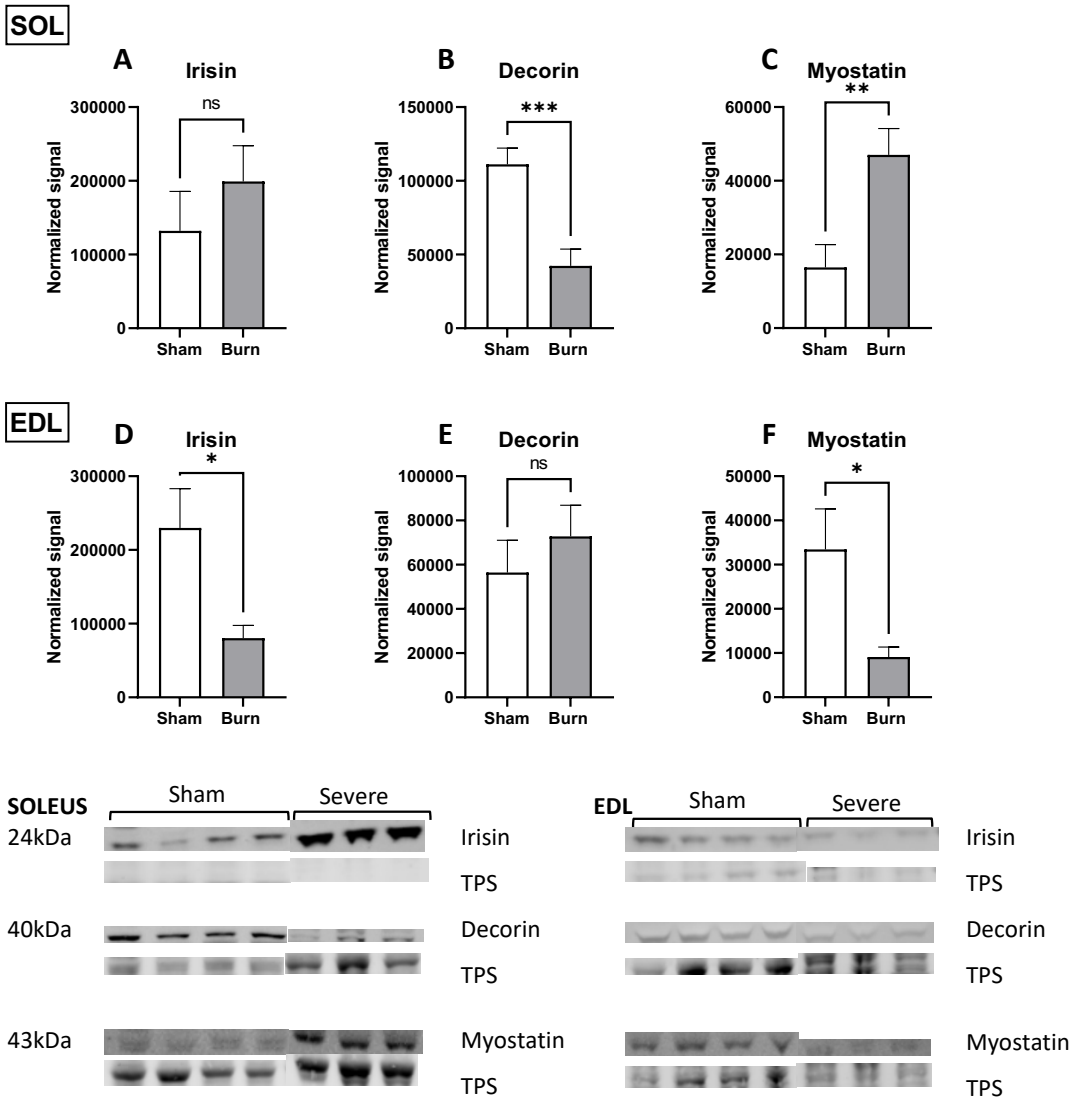

Figure 5.5*: Protein signals of Western blots normalized to Total protein stains in m.Soleus (SOL) and m.Extensor Digitorum Longus (EDL) of sham treated versus severe burned rats 40 days postburn. A. Normalized Irisin protein signal in Soleus muscle. B. Normalized Decorin protein signal in Soleus muscle. C. Normalized Myostatin protein signal in Soleus muscle D. Normalized Irisin protein signal in EDL muscle E. Normalized Decorin protein signal in EDL muscle. F. Normalized Myostatin protein signal in EDL muscle. N=11 per group, error bars represent SEM, significant difference *p<0.05 **p<0.01, ***p<0.001, ****p<0.0001; ns=no significance*

4 Discussion

The current study confirms previous studies and adds new information regarding prolonged skeletal muscle atrophy after severe burn injury [30, 31]. The most important findings of the present study are the difference in type 1 and type 2 skeletal muscle protein metabolism post-burn. In addition, we - report a different role of myokines in different muscle fibers.

Postburn hypermetabolism has been shown to continue up to 2 years post-burn, leading to catabolic effects in skeletal muscle [2]. However, evidence of processes leading to muscle mass loss post-burn is limited as most research is limited to the early flow phase (until 5 days post-burn). Studies focused on a longer observational period by Quintana et al (2015) and Fry et al (2016) found prolonged muscle wasting at 14 and 17 days post-burn in both rats and humans respectively [30, 31]. Our findings are in line with the findings of these studies, showing lower ratios of muscle-to-total body weight. Interestingly, we also found differences between muscle types by investigating predominantly type I (m. Soleus) and type II (m. Extensor Digitorum Longus) skeletal muscles. Accordingly, at 40 days postburn muscle atrophy processes in different muscle fiber types are, in parallel with the persistent hypermetabolic state, still unresolved.

Muscle mass is directly affected by protein balance, post-burn a net negative protein balance in skeletal muscle have been reported, with increases in protein breakdown but with inconsistent results in the rate of protein synthesis [32, 33]. Fang et al (1997) showed in rats that different muscle types respond differently to a burn injury, with differences in molecular pathways [14]. Such differences have also been reported in other diseases with a major catabolic component.

Evidence suggests a higher rate of protein breakdown in type II compared to type I muscle fibers [34]. Also on the level of mRNA, 24 hours after a severe burn higher ubiquitin mRNA

levels were found in EDL compared to SOL [14]. In contrast to these findings, we demonstrated a higher expression of atrogenes MURF-1 and atrogin-1 in SOL, whilst in EDL expression of these proteins was lower compared to sham animals. Analyses of FOXO3a and pFOXO3a, involved in atrogene transcription, are less consistent. In SOL a lower expression of FOXO3a was found post-burn, whilst in EDL no significant differences could be detected. This is surprising since MURF-1 and Atrogin-1 expressions are partially activated by FOXO3a. Yu et al (2018), showed higher expression of FOXO3a and lower expression of pFOXO3a in tibialis anterior at day 7 post-burn [35]. Although an explanation for the different findings is not easy to provide, we hypothesize that molecular regulation of protein breakdown post-burn is no only dependent of muscle fiber type but to time as well.

In SOL more but smaller muscle fibers were found post-burn, reflecting a shift from slow-twitch muscle towards a faster phenotype. At the level of protein synthesis, a similar pattern was found. In SOL of burned animals both anabolic signaling proteins pAkt and downstream eEF2 protein expressions were lower. Surprisingly, in EDL pAkt showed a higher expression post-burn, while for eEF2 no difference could be detected. The latter findings are partly in line with findings of Song et al (2012), who showed that eEF2 activation in severely burned patients persists for 49 days post-burn in skeletal muscle, however they did not distinguish between different muscle fiber types [32]. Recently, we showed decreased phosphorylation of Akt (pAkt expression) in EDL 10 days post-burn [36]. This is in line with the findings of Yu et al. (2016 and 2018) who reported lower pAkt expression in tibialis anterior muscle 7 days post-burn [37]. Altogether, these results indicate that 40 days after a burn injury type I muscles fibers have a higher activation of catabolic pathways and a lower activation of anabolic pathways, contributing to a catabolic protein imbalance whereas type II muscles on the other hand show a downregulation of catabolic signaling and a small increase in anabolic signaling, possibly counteracting further detrimental effects. Such hypothesis, however, requires further investigation.

Myokines are relatively newly discovered proteins that are released from contracting muscle cells and play an important role in maintaining homeostatic processes in an autocrine, paracrine and endocrine manner [38]. The myokines myostatin, irisin and decorin are emerging in literature as targets to counteract muscle wasting in various conditions in response to exercise [19]. A previous study using serum of chronic burn patients showed higher circulating myostatin (a negative regulator of muscle mass) concentrations [39]. This is in line with our results, showing that myostatin protein levels increase in SOL whereas the concentration of decorin (inhibiting myostatin) decreases post-burn [22]. In the predominant type II EDL, opposite results were seen. Myostatin and decorin expressions are in line with our other observed results, showing a compensatory/recovering mechanism of EDL at this point whilst SOL remains in a catabolic stage. For irisin, a significant lower expression was found in EDL but not in SOL of postburn animals. Since irisin expression is highly correlated to muscle mass, the lower expression in EDL could be due to lower total muscle mass [22]. Since it has shown that both irisin and decorin are upregulated through exercise, they can be regarded as potential physical therapeutic targets to counteract muscle atrophy during postburn recovery [22, 23]. Recently, we showed in a multicenter clinical trial with severe burned patients that an exercise program, consisting of resistance and aerobic training, started during the acute phase of burns increased muscle size and strength parameters [40].

The current study has some limitations. Firstly, food and water intake were not measured during the time course of the study, whereas differences in food intake could influence nutritional status and weight of the animals. Secondly, activity of the animals in the cages was not monitored. Sedentary behavior after the injury could have detrimental effects on body mass and/or muscle weight. Although more research is necessary we believe that the effect of both the acute food intake and physical activity level is only limited. No signs of discomfort were observed after 24 hours post-burn, leading to the believe that the effects of these factors after 40 days will be limited.

5 Conclusions

To the best of our knowledge, the current study is the first to report decreased catabolic signaling in type 2 m. Extensor Digitorum Longus and increased catabolic signaling in type 1 m. Soleus 40 days postburn, which extends beyond the time frames examined in most previous studies. When we combine the results of the present study with our results at 10 days postburn, we hypothesize that in the early flow phase type II muscle fibers are more prone to muscle wasting as type I fibers. Interestingly, during the chronic flow phase the phenomena seem to reverse. Further studies should aim to unravel the molecular pathways involved in these long-term mechanisms in more detail and investigate possible treatment strategies by e.g. exercise. A better understanding of the molecular regulation of postburn muscle wasting in the long term and the involvement of myokines can lead to the development of physical therapeutic approaches.

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Chapter 6

The effects of early exercise on molecular mechanisms of postburn muscle wasting

Dombrecht D, Van Daele U, Van Asbroeck B, Schieffelers DR, Guns PJ, van Breda E.; The impact of exercise on postburn muscle wasting: unraveling molecular pathways and anabolic potential in a rat burns model. *Almost ready to submit in 'Journal of Applied Physiology'.*

1 Introduction

Burn injuries are accompanied by a sustained and complex pathophysiological response. After the hypometabolic 'ebb phase', the first 24-48hours postburn, the body goes into a prolonged systemic hypermetabolic 'flow phase' [1]. An abundance of inflammatory and stress signals, continuing even after wound healing, gives rise to a catabolic state with a range of consequences, including muscle wasting [2, 3]. Postburn muscle atrophy is aggravated by hospital-associated disuse and is associated with adverse outcomes, such as longer hospitalizations, a significant decline in functional capacity and overall quality of life [4, 5].

Unraveling the mechanisms underlying skeletal muscle wasting after burns is crucial for developing effective interventions to mitigate these debilitating consequences. Despite great efforts in other catabolic populations such as cancer cachexia and critical ill patients, research in burn patients is still scarce [6, 7]. Recent research has shed light on molecular processes, providing crucial insights in postburn muscle wasting [8]. A disrupted protein balance with changes in both anabolic and catabolic signaling proteins leads to muscle atrophy and decreased total body weights after burns [Preliminary data]. Besides, differences in molecular processes were found dependent on muscle type, with acutely type II muscle fibers being more susceptible to muscle atrophy, whilst chronically type II muscle fibers show more upregulation of catabolic proteins [Preliminary data][9].

Exercise can counteract negative effects of prolonged disuse during hospitalization and is a known systemic anabolic factor affecting various molecular signaling pathways [10, 11]. Previous research has shown positive effects of exercise on skeletal muscle in burn patients, but the specific molecular and cellular mechanisms underlying these benefits remain largely unknown [12-15]. Unraveling these pathways, provides crucial insights into potential therapeutic exercise interventions but can also identify novel therapeutic targets to further block muscle wasting and improve morbidity postburn.

We hypothesize that a progressive aerobic training protocol starting in the initial flow phase can mitigate muscle pathophysiological changes associated with post burn muscle wasting. The purpose of the current study was to investigate the effects of a long-term treadmill training program initiated immediately after thermal injury on protein expressions of anabolic and catabolic signaling pathways in both fast-twitch dominant m. Extensor Digitorum Longus and slow-twitch dominant m. Soleus in a rat model.

2 Methods

2.1 Animal model

All experimental protocols were approved by the Ethical Committee of laboratory animals of the University of Antwerp (number 2020-52) and performed in accordance with relevant regulations. Forty-four female Sprague-Dawley rats (Charles River) of six weeks old were acclimated individually seven days prior to the injury. Rats were randomly assigned to one of four groups: (1) sham treatment (S; n=11), (2) sham treatment – exercise (SE; n=11), (3) severe burn (B; n=11) or (4) severe burn – exercise (BE; n=11). Severe burns encompass a 40% total body surface area (TBSA) burn. One animal was excluded from analysis due to an infection at the hindlimb after the burn procedure.

2.2 Burn model

Burns were inflicted according to the modified Walker-Mason model inflicting a deep dermal burn and inducing metabolic changes [16-18]. Animals were administered buprenorphine (0,05mg/kg) (Temgesic, Schering-Plough, Belgium) and anaesthetized with 1-3% isoflurane (Zoetis, UK) in 100% oxygen. Next, rats were shaved with a clipper and placed in a pre-made mold exposing 30% TBSA of their back and submerged in 100°C water for ten seconds [19]. An intraperitoneal injection of Ringers lactate was administered, rats were subsequently placed in a mold exposing 10% TBSA along their ventral side and submerged for three seconds in 100°C water. Sham treated rats underwent the same

procedures but all in room temperature water. Rats were administered Ringers lactate 30min, four hours and eight hours postburn (resulting in a total of 4ml/kg/%TBSA) and buprenorphine 12 hours postburn. Animals stayed in individual cages in specialized ventilation cabinets for the remainder of the experiment, received food and water ad libitum (Ssniff 'complete feed for rats & mice', consisting of 9% Fat, 24% Protein and 67% Carbohydrates) and were monitored daily using the Functional Observational Battery adjusted to the specific features of burns [20]. Animals showed normal ambulatory patterns without signals of pain.

2.3 Exercise model

All animals underwent treadmill acclimatization 3 days prior to the experiment (10min/day, progressive speed increase from 10cm/sec for 3min to 40 cm/sec for 40sec). Burns were inflicted 24 hours after the last acclimatization session. After 48 hours of recovery, rats of exercise groups (SE and BE) were submitted to a progressive running protocol on a treadmill (..) for 1 hour 4 days/week with a slope of 15°. The protocol consisted of a warm-up phase, followed by multiple intervals of increased intensity (35-40cm/sec in training one to 40-45cm/sec in training 20) alternated with periods of relative recovery (25cm/sec) and a cooling-down phase. Total time in high intensity intervals increased from 35 minutes in training 1 to 50 minutes in training 20. Notes were taken if rats could not sustain the total running period or got a large amount of shocks.

2.4 Exhaustion test

An exhaustion test was conducted on rats belonging to the exercise groups on the 5th day after burn injury and 5 days prior to sacrifice. On the running treadmill at 15° inclination rats started running at 20cm/sec, increasing speed every minute until 60cm/sec. At 60cm/sec, rats ran until 60min or exhaustion (when rats stayed 5sec on the electric grid or within 3 cm of the grid). Total running time, total running distance and max speed were documented for each rat.

2.5 Tissue collection

Forty days after burn procedure, animals were euthanized by an intraperitoneally injection of 250mg/kg pentobarbital (Kela, Belgium). Rats were weighed, blood was immediately drawn by cardiac punction and hindlimb muscles (SOL and EDL) of both hindlimbs were harvested, weighed and one of each stored in a cryotube in liquid nitrogen. SOL and EDL from the other hindlimb of each animal were prepared for immunohistochemical analysis, placed on a cork, covered in Neg-50 Frozen Section Medium (Thermo Scientific, USA) and frozen in liquid nitrogen-cooled isopentane (2-methylbutane) (Honeywell, US). All samples were stored in a -80°C freezer until further analysis.

2.6 Immunohistochemistry

Fiber type distribution (total fiber count, amount of type 1, type 2a and type 2b muscle fibers) was determined using a MyHC staining. Unfixed transversal muscle sections (10µm) were air-dried at room temperature and fixated with Paraformaldehyde (10 minutes). Slides were pre-incubated with TBS-Tx, washed with TBS-T, blocked with horse serum (1:10) for 30min and incubated overnight (at room temperature) with specific primary antibodies: anti-laminin (mouse; Bio-Techne NB300-144; 1:500) and isoform-specific myosin heavy chain (MyHC) antibodies: MyHC type 1 (BA-D5 mouse MIgG2b 1:5), MyHC type 2a (SC-71 mouse MIgG1 1:5) from Developmental Studies Hybridoma Bank (DSHB; Iowa City, IA, USA). Slides were washed in TBS-T and incubated for one hour with corresponding immunoglobulin-specific secondary antibodies were used: Alexa fluor 405 (anti-rabbit; Invitrogen A31553) IgG, Alexa fluor 555 (anti-mouse; Invitrogen A21147) IgG2b and Alexa fluor 633 (anti-mouse; Invitrogen A21126) IgG1. Afterwards, slides were washed in TBS-T, covered with 'Vectashield with DAPI' (Vector Laboratories) and air-dried. Images were taken at x10 magnification with a NIKON Eclipse Ti series high throughput fluorescent microscope and ImageJ software was used for further analysis.

Investigators were blinded to different groups for all histological analyses.

2.7 Western blotting

We determined, using Western Blotting, the expression of several key proteins 40 days after a severe burn injury, known to be involved in skeletal muscle wasting. Snap-frozen SOL and EDL were collected in RIPA lysis buffer (Cell-Signaling, #9806) supplemented with phosphatase and protease inhibitor Tablets (Roche, 4906845001 and 11836170001: 1 Tablet of each/10 mL) in specially designed CK-28R tubes with 2.8mm ceramic beads (Bertin Corp. P000916-LYSK0-A). A Precellys 24 was used (6800rpm for 3x30sec) and after centrifugation (10min at 14000rpm at 4°C) protein suspension was extracted and transferred to new ice-cold tubes. Total protein concentration was quantitated using Pierce BCA Protein Assay kit (ThermoFisher #23225) following manufacturer's instructions. Diluted samples (1µg/µl) were heat-denatured for five minutes at 100°C.

Based on linear range analysis, 15 µg protein was loaded on Bolt 4-12% Bis-Tris gels (life technologies, NW04125BOX). Proteins were separated, transferred to a PVDF membrane. A total protein staining was performed using Revert 700 Total Protein Stain Kit (Li-COR biosciences, 926-11010). Membranes were scanned with an IR scanner (Odyssey Imaging System) and de-stained. Membranes were blocked in Intercept blocking buffer (Li-Cor Biosciences, 927-60001) and incubated overnight at 4°C with the following rabbit IgG primary antibodies: anti-Akt1 (phospho S473; 1:10000; ab81283; Abcam)), anti-EEF2 (1:10000; ab75748; Abcam), Anti-MURF1+MURF3+MURF2 (1:1000; ab172479; Abcam), anti-Fbx32 (1:1000; ab168372; Abcam), anti-FOXO3A (1:800; ab23683; Abcam), anti-FOXO3A (phosphoS253) (1:800; ab47285; Abcam), anti-irisin (anti-FNDC5, 1:1000, ab174833, Abcam), anti-decorin (1:1000; ab175404; Abcam) and anti-GDF8/myostatin (1:300; ab203076; Abcam). After washing, a secondary anti-rabbit IgG IR-labeled secondary antibody (Li-COR LI 926-32211; 1:20000 dilution in Li-Cor blocking buffer supplemented with 0.01% SDS) was added for one hour and washed again. Membranes were imaged with an IR scanner (Odyssey imaging system). Analysis was completed with Empiria Studio software, and the signal from the protein.

2.8 Statistical analysis

Statistical analysis was performed using JMP-16. Experimental data for each burn group were expressed as means ±SEM. Differences between groups were analyzed using the Kruskal-Wallis test for general analysis and for immunohistological stainings. For western blots, linear mixed models was used, with the number of blot as a random effect to exclude technical variation between blots. For exhaustion test data within groups, Wilcoxon rank test was used. Differences were considered to be statistically significant at $p<0.05$.

3 Results

3.1 Total body and muscle weights

Mean body weights and muscle wet weights are shown in table 6.1. Mean body weight gain and the ratios of muscle wet weight over total body weight are shown in figure 6.1. Body weight gain of animals in the exercise groups was significantly higher compared to non-exercise groups. Wet weights of m. Soleus and m. Extensor Digitorum Longus was higher post-exercise in the sham group, but not in the burned group. When normalizing for total body weight, the ratio of muscle weight/body weight was significantly higher in exercise groups compared to non-exercise groups for both m. Soleus (SE 0.749±0.108 vs S 0.624±0.056; p=0.0030 and BE 0.577±0.054 vs B 0.533±0.055; p=0.0030) and m. Extensor Digitorum Longus (SE 0.783±0.106 vs S 0.650±0.064; p=0.0078 and BE 0.650±0.134 vs B 0.521±0.182; p=0.0078).

Table 1 - Total body and muscle weights					
	Sham (S) (n=11)	Sham-exercise (SE) (n=11)	Burn (B) (n=10)	Burn-exercise (BE) (n=11)	p-value exercise effect
Body weight baseline (g)	197.92±17.53	183.18±12.68	192.92±12.87	181±14.79	**Sham: p=0.0018** Burn: p=0.0537
Body weight end (g)	275.04±15.58	301.73±22.7	232.74±30.27	253.32±29.04	**Sham: p=0.0070** Burn: p=0.1101
Body weight gain (g)	77.12±16.03	128.27±26.61	39.73±34.68	81±16.89	**Sham: p<0.0001 Burn: p<0.0001**
m. SOL weight (mg)	123.10±12.02	136.18±13.65	102.7±11.61	102.40±12.22	**Sham: p=0.0324** Burn: p=0.9556
m. EDL weight (mg)	127.91±9.77	142.73±15.69	110.25±8.86	118.64±30	**Sham: p=0.0134** Burn: p=0.1906

Table 6.1 Total body weights at baseline (day 0) and at 40 days postburn (end), body weight gain during the experiment (weight day 40 minus weight day 0 postburn) and wet weights of m. Soleus (SOL) and m. Extensor Digitorum Longus (EDL) in each experimental group. Values expressed as mean±SEM.

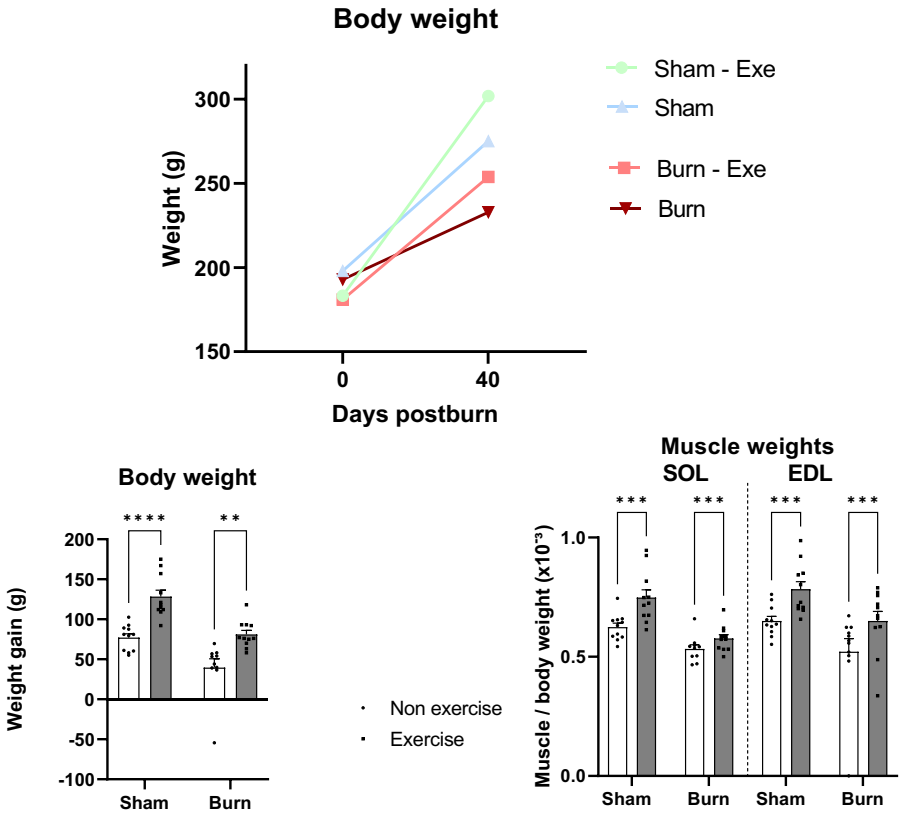

Figure 6.1 (A) Evolution of total body weight, (B) effect of exercise on body weight gain in sham and burned animals, (C) effect of exercise on muscle wet weights (ratio of muscle wet weight at 40 days postburn to total body weight at baseline) of m. Soleus (SOL) and m. Extensor Digitorum Longus (EDL) in different experimental groups. N=11 per group, error bars represent SEM, significant difference **p<0.01, ***p<0.001, ****p<0.0001

3.2 Exercise effect

To evaluate efficiency of the exercise protocol, an exhaustion test was used to compare total running distance at the start and the end of the experiment. Both sham and burned groups showed a significant increase in total running distance (SE 1037±157.1; $p<0.0001$ and BE 297.7±173; $p=0.0273$) at the end of the experiment. The increase was significantly greater in the sham group compared to the burned group ($p=0.0001$).

3.3 Muscle morphology

No differences were found in SOL and EDL for the distribution of type I, type IIa or type IIb muscle fibers when comparing sedentary and exercised animals with either sham or burn treatment. Also when looking at cross-sectional area of TI and TII muscle fibers in both SOL and EDL muscles, no differences were found.

3.4 Protein signaling pathways

To evaluate signaling pathways affecting protein balance, we evaluated protein expressions of Atrogin-1, MURF1,2,3, FOXO3a and pFOXO3a for protein breakdown, and pAkt and eEF2 for protein synthesis, as shown in figure 6.2. In exercised animals from the sham (SE) and burned (BE) group significant lower expressions were found for both SOL and EDL muscles concerning Atrogin-1 ($p<0.0001$ for S vs SE and B vs BE in both SOL and EDL), MURF1,2,3 (SOL: S vs SE $p=0.0004$; B vs BE $p<0.0001$; EDL: S vs SE and B vs BE $p<0.0001$) and FOXO3a ($p<0.0001$ for S vs SE and B vs BE in both SOL and EDL) in comparison with sedentary animals of the same group (S and B). For pFOXO3a only a significant higher expression was found in EDL of exercised animals from the sham group when compared to the sedentary group ($p=0.0007$). pAkt showed an increased expression in SOL of both exercised sham and burned animals ($p=0.0042$ and $p=0.0063$ respectively), whilst in EDL pAkt expression was significantly lower after exercise in the burn group ($p=0.0002$). In both SOL and EDL of sham and burned groups eEF2 expression was significantly lower in exercised animals ($p<0.0001$ for S vs SE and B vs BE in both SOL and EDL).

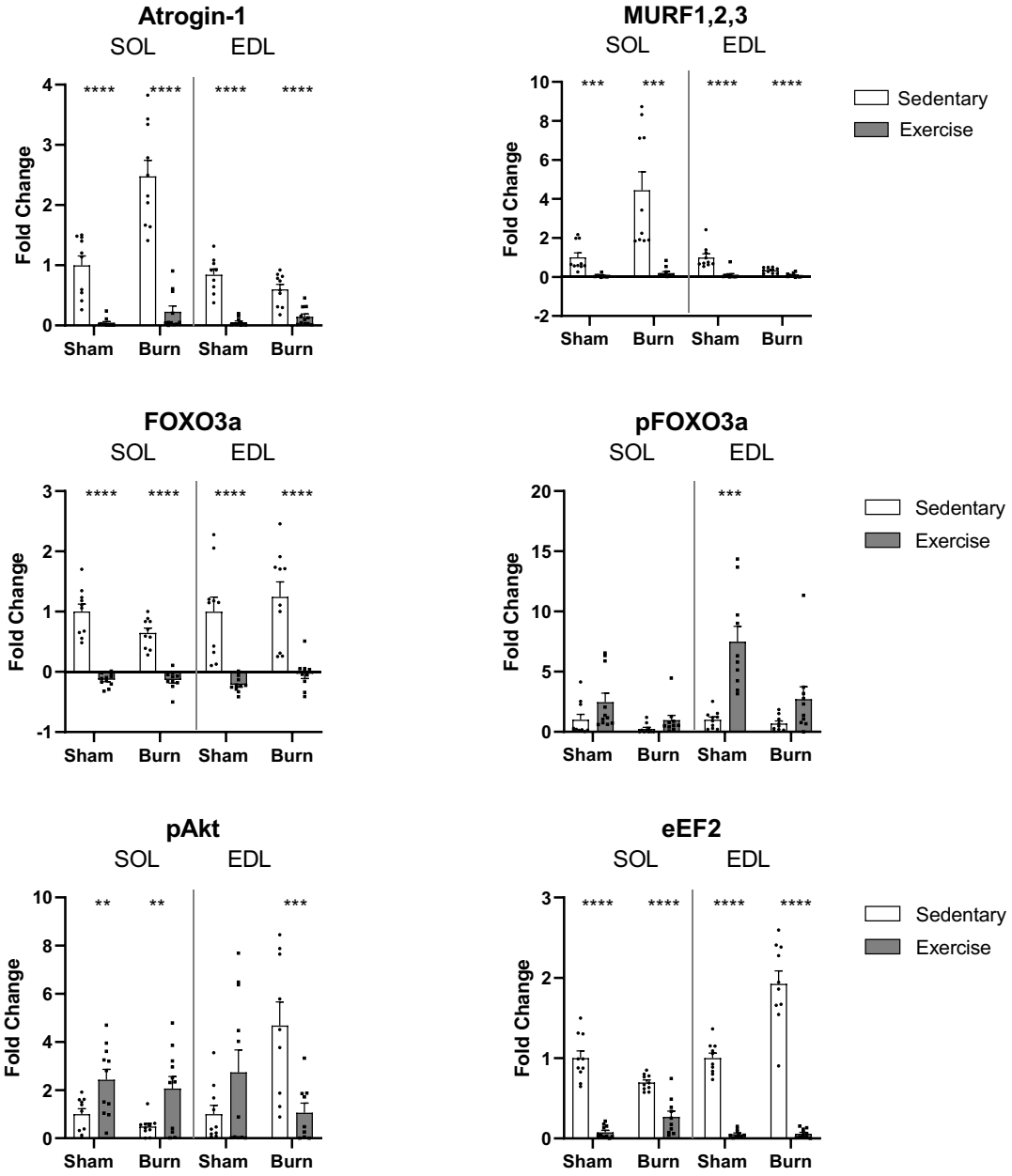

Figure 6.2 Protein signals of Western blots normalized to Total protein stains in m.Soleus (SOL) and m.Extensor Digitorum Longus (EDL) of sedentary versus exercised sham treated and severe burned rats 40 days postburn. A. Proteins involved in catabolic signaling pathways Atrogin-1, MURF1,2,3, FOXO3a and pFOXO3a; B. Proteins involved in anabolic signaling pathways; N=11 per group, error bars represent SEM, significant difference **p<0.01, ***p<0.001, ****p<0.0001

3.5 Myokines

Protein expression of catabolic myokine Myostatin and anabolic myokines Decorin and Irisin were measured to evaluate changes after exercise postburn, as shown in figure 6.3. Myostatin shows significant lower expressions in exercised animals in both Sham and burned groups (SOL: S vs SE $p<0.0001$; B vs BE $p=0.0004$; EDL: both $p<0.0001$). For Decorin significant higher expressions were found in exercised animals of both groups in SOL (S vs SE $p=0.0024$; B vs BE $p=0.0019$) whilst in EDL only a higher expression was found in sham animals with exercise compared to sedentary ($p=0.0315$). For both muscles, significant higher expressions of Irisin were found after exercise in sham and burned groups (SOL: S vs SE $p=0.0031$; B vs BE $p=0.0219$; EDL: (S vs SE and B vs BE $p<0.0001$).

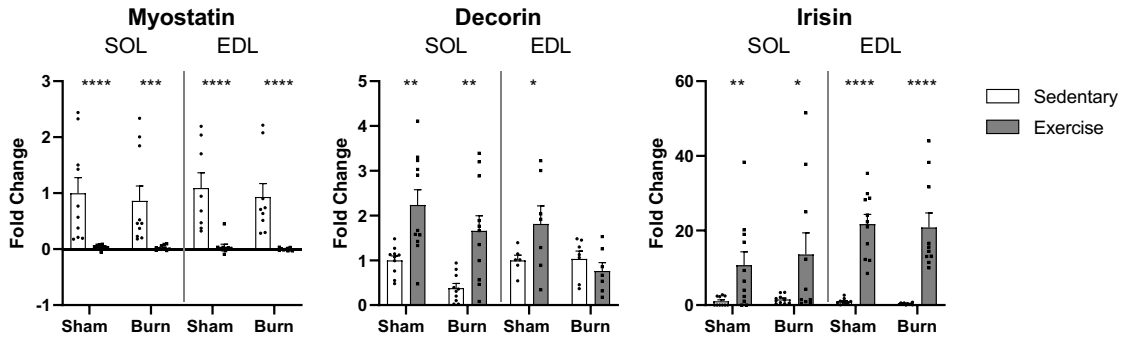

Figure 6.3 Protein signals of Western blots normalized to Total protein stains in m.Soleus (SOL) and m.Extensor Digitorum Longus (EDL) of sedentary versus exercised sham treated and severe burned rats 40 days postburn. Western blots of myokines involved in muscle wasting pathways, Myostatin, Decorin and Irisin; N=11 per group, error bars represent SEM, significant difference **p<0.01, ***p<0.001, ****p<0.0001

4 Discussion

We investigated in a rat model the effects of long term treadmill training on postburn skeletal muscle wasting, myokine expression and intracellular protein synthetic and proteolytic signaling. Our main findings demonstrate a decrease in catabolic signaling and a positive effect on myokine expression in postburn skeletal muscle by exercise. These molecular changes led to an increase in muscle and total body weights compared with the non-exercise group. This is the first study to report positive effects of a long-term aerobic exercise protocol on postburn catabolic signaling pathways in predominantly type I (m. Soleus) and type II (m. Extensor Digitorum Longus) skeletal muscles.

It is well established that as a result of both the persistent hypermetabolic response, activated by severe burn injury, and disuse, as a result of postburn adverse effect and hospitalization, skeletal muscle mass loss occurs [8, 21]. Previous studies in cancer cachexia and critical illness have demonstrated that physical exercise is protective against disease-induced muscle wasting [11, 22]. Although there have been a few studies showing positive effects of exercise postburn in rodents [23-25] and humans [12-15], most of these studies have focused on the effects of exercise during the early postburn period. Besides, it is well-known that skeletal muscle wasting leads to delays in rehabilitation and major functional impairments, playing an important role in the quality of life of burn survivors[13, 15]. To be able to implement exercise programs in burn rehabilitation, insights in both the clinical and fundamental effects of these programs is crucial. Knowledge regarding the underlying molecular processes involved in exercise-mediated protection against postburn muscle wasting is lacking.

The present study shows that exercise was effective in increasing body weight gain in both control and burn groups, indicating that exercise positively impacts overall body weight. Exercise preserved muscle mass during the postburn period, shown by higher relative muscle weights after exercise (Figure 6.1). Saemen et al. (2015) reported increases in SOL

muscle mass after a resistance exercise protocol post-burn as well, but they found no increase plantaris muscle mass, while our study shows positive effects in both muscle types [24]. A meta-analysis of human studies confirms postburn increases in lean body and leg lean mass by resistance exercise [26]. Besides, a recent multicenter trial showed significant increases in quadriceps muscle layer thickness by both aerobic and anaerobic exercise [27].

In order to understand the molecular factors that underly the observed changes in skeletal muscle, we investigated protein expressions of key regulatory proteins related to catabolic and anabolic signaling pathways. In exercised animals from both sham and burn groups, lower expressions of catabolic factors Atrogin-1, MURF1,2,3 and FOXO3a were found in both SOL and EDL muscles, indicating a potential reduction in protein breakdown. Similar results were found by Song et al (2015), who shows in a burn-disuse model a decrease in MURF-1 after a combination of exercise and insulin therapy, but no changes in Atrogin-1 [23]. In contrast to our findings, they also found increases in p-Akt, mTOR and eEF2 after exercise post-burn [23]. At present we don't have a clear explanation for the different findings but it is plausible to assume that the combination with the hindlimb unloading model, as used in the study of Song et al. (2019), played an important role [23]. Disuse leads to decreases in anabolic signaling, hence effects of exercise on anabolic proteins in a model including hindlimb unloading are possibly larger [8]. Another explanation for the different findings can be the type of exercise. Song et al. (2019) used an anaerobic resistance training protocol, whilst we used aerobic exercise [23]. Anaerobic exercise is known to stimulate myofibrillar protein synthesis through activating the PI3K/Akt pathway [28]. Future research should explore complementary effects of aerobic and anaerobic training protocols.

Further analysis led to interesting findings concerning effects of exercise on myokine expression. Myokines affect downstream signaling pathways and could play a crucial role in exercise-mediated protection against muscle wasting [29]. First of all, we found that exercise resulted in lower expressions of the catabolic myokine myostatin (a negative

regulator of muscle mass) in both sham and burned groups. These results are in line with previous findings in sarcopenia, cancer and heart failure, where the anti-inflammatory effects caused by a decrease in myostatin by exercise counteracts muscle wasting [30-32]. Secondly, higher expressions of the myokines irisin and decorin were found in the exercise groups. Irisin is a pro myogenic factor, inducing skeletal muscle hypertrophy and shown to attenuate denervation induced atrophy [33]. Decorin acts as a pro-myogenic factor as well, by reducing specific ubiquitin ligases Atrogin-1 and MURF-1 [33]. Accordingly, in the present study we found higher decorin concentrations, together with lower Atrogin-1 and MURF-1 expressions after exercise post-burn.

Although changes are found between the different muscle types in pathways inducing muscle atrophy post-burn, exercise did not have specific effects on the different muscle types [34]. Positive effects were found for all catabolic proteins in both SOL and EDL muscle. To our knowledge, this study is the first to report changes in myokines by exercise post-burn. Our findings suggest that exercise may modulate myokine expression, further supporting the beneficial effects of exercise in mitigating muscle wasting after burn injuries. Further research on the molecular pathways influenced by myokines and their potential interactions with anabolic factors would be valuable for developing targeted therapeutic intervention. Certainly in burn patients, with difficulties to exercise, myokine-based drugs could be an interesting therapeutic strategy.

While our study provides valuable insights, it is essential to acknowledge its limitations. Only female rats were used in this investigations, as they tend to increase their food intake to compensate for the increased energy expenditure caused by running exercise. This allowed for a comparison of muscle masses between freely eating exercised rats and sedentary rats with similar body weights. However, future research could also investigate the effects of exercise in male rats and compare the results between genders [35]. Additionally, while the study evaluated several key proteins and myokines, there may be other molecular pathways involved in postburn muscle wasting that were not explored in

this investigation. Further research in human populations and more extensive molecular analysis could provide a more comprehensive understanding of the effects of exercise on postburn muscle wasting.

5 Conclusion

Overall, the results of this study provide valuable insights into the molecular and cellular mechanisms underlying postburn muscle wasting. By demonstrating the positive effects of a progressive aerobic training protocol initiated during the initial flow phase of burns, the study suggests that exercise could be a potential therapeutic intervention for burn patients to preserve muscle mass and improve recovery outcomes. The findings also highlight the importance of considering muscle type (fast-twitch vs. slow-twitch) when studying the effects of exercise on muscle atrophy in this population.

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Chapter 7

General discussion and future perspectives

This research project was initiated to shed light on the fundamental effects of exercise on post-burn muscle wasting. By elucidating the underlying mechanisms, we can lay the foundation for the implementation of early exercise as a viable treatment strategy for burn survivors, addressing several metabolic sequelae. To unravel this complex interplay, specific sub aims were formulated, collectively forming the core of this thesis.

In **Chapter 3,** we have provided an overview of the current literature concerning the fundamental processes that underlie skeletal muscle wasting in burns, as well as the effects of physical rehabilitation in the perseveration of skeletal muscle mass. In **Chapter 4** we unraveled the molecular mechanisms of skeletal wasting in the early flow-phase in a rat burns model. In **Chapter 5**, our study investigating the molecular mechanisms of skeletal wasting in the late flow-phase in a rat burns model is presented. Overall, the results from both studies provide evidence that muscle wasting is present in the early flow phase and persists during the later stages post-burn, with differences in fast- and slow-twitch muscle fibers. In **Chapter 6** we investigated whether an early exercise program in severely burned rats could counteract skeletal muscle wasting post-burn. We showed that early exercise has protective effects on post-burn muscle wasting through altering the expression of anabolic and catabolic signaling proteins. *(Figure 7.1)*

We confirm our main hypothesis that the implementation of an early physical exercise protocol can effectively mitigate skeletal muscle wasting within a severe burns rat model by attenuating catabolic signaling and positively affecting myokine expression.

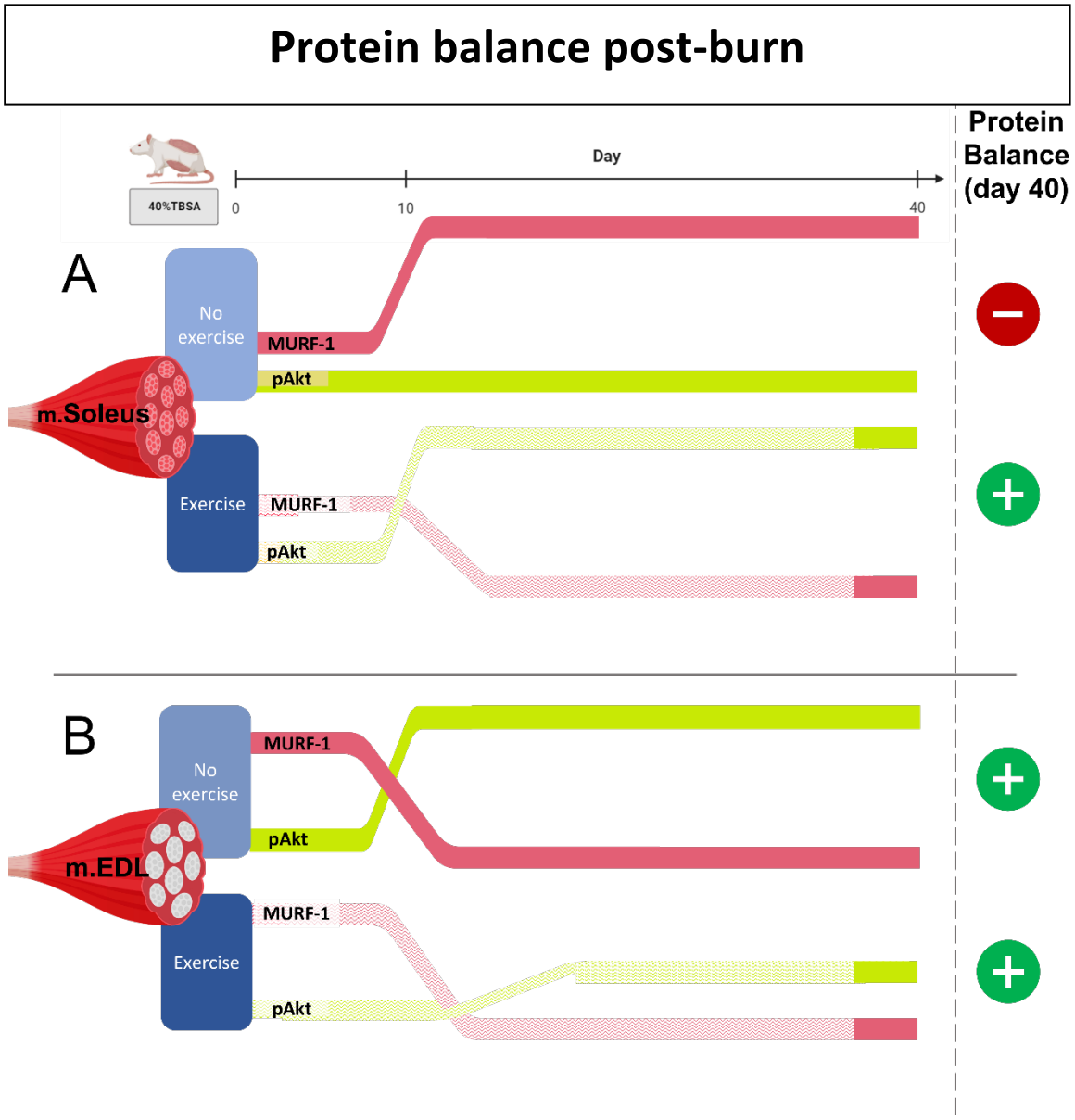

Figure 7.1 Graphical presentation of protein balance post-burn based on the results of the different chapters. Presented are the evolution of anabolic protein pAkt and catabolic protein MURF-1 both with and without exercise in type 1 dominant musculus Soleus (A) and type 2 dominant musculus Extensor Digitorum Longus (EDL) (B) after a severe burn injury (40%TBSA). The curves are semi-quantitative and based on both previous literature and our own study results on day 10 (no exercise) and day 40 (no exercise and exercise) post-burn, with a hypothesized evolution in between. Dashed lines represent hypothesized results during the exercise period (no measurements and no previous literature available). In the last column, the hypothesized protein balance, purely based on these proteins, is shown.

1 General discussion

Prior to the start of this doctoral research, colleague David Schieffelers had started his research focusing on the clinical implications of early exercise in mitigating postburn skeletal muscle wasting. He conducted a comprehensive survey of burn clinicians to gain an overview of the current practice of inpatient burn rehabilitation across Europe. The study revealed that healthcare professionals have a limited understanding of metabolic burn pathophysiology, leading to inadequate use of early exercise in burn management [1]. Insufficient scientific research contributes to an important lack of understanding. The studies presented in this doctoral thesis were designed to bridge this critical knowledge gap. We created a comprehensive overview of the state-of-the-art knowledge of intricate mechanisms driving skeletal muscle wasting postburn and effects of exercise, as presented in **Chapter 3,** on which we based the experimental studies. Integrating physical exercise into the therapeutic regimen could contribute to a more holistic approach to manage and mitigate muscle loss in burn survivors and ultimately to enhance their overall recovery and quality of life.

1.1 Characterization of the experimental model

In vitro models are limited in replicating all aspects of the burn pathophysiology and all the intricate systemic features. Animal models for in-depth burn research are still essential to unravel the post-burn pathological mechanisms [2]. The Walker-Mason burn model for rats was used, which induces a hypermetabolic response similar as in humans [3,4,5]. Using rats also allowed us to create a standardized study cohort [6]. All animals were inflicted a burn encompassing 40% TBSA, had an identical age, similar medication regimens and anesthesia protocols. In human burns studies, these factors are often variable within the study population, making it more difficult to draw concrete conclusions [7]. Rats were specifically selected as preferred animal model since their metabolic characteristics are the most closely approximate to those of humans, with the exclusion of larger animal models such as pigs and sheep, which are more difficult to take care of and exercise [2].

1.2 Postburn muscle wasting mechanisms

In **Chapter 4 and 5** we contributed to the understanding of the mechanisms and their time-course in skeletal muscle wasting following a burn injury. Our experimental studies were built upon three primary research questions, being: (1) What is the time course of postburn muscle wasting? (2) What is the role of different protein signaling pathways in the development of postburn muscle wasting? (3) Do alterations in myokines impact postburn muscle wasting? Throughout all research questions, we have analyzed muscle-specific mechanisms, with emphasis on the dissimilarities between type 1 (represented by Soleus, SOL) and type 2 (represented by Extensor Digitorum Longus, EDL) muscles.

1.2.1 *Time course of muscle atrophy*

We confirmed the existence and prolonged nature of skeletal muscle wasting after severe burn injury, by lower SOL and EDL muscle weights in combination with lower total body masses 10 and 40 days post-burn. Ten days post-injury, burned animals exhibited only half the weight gain compared to sham animals. This trend continued at 40 days post-burn, highlighting the harmful consequences of burns beyond the acute phase, in line with prolonged hypermetabolism [8,9]. In human burn patients, catabolism of lean body mass persists for up to 9 months beyond full wound healing [10]. Reduced food intake, higher energy expenditure and associated difficulties in determining the adequate nutrient intake lead to reductions in overall body mass but also to the over-activation of lipid signaling pathways [11,12,13]. A remodeling of fat storages, with increases in lipid accumulation in vital organs and central fat accumulation, unfolds [14,15]. These changes result in an unfavorable body composition, detrimentally affecting overall health and increasing risks of complications, such as organ dysfunction [13].

1.2.2 *The role of different protein signaling pathways*

We hypothesized that postburn skeletal muscle wasting is characterized by elevated catabolic signaling proteins and diminished levels of anabolic signaling proteins, with distinct regulation in type 1 and type 2 muscle fibers. We found lower expressions of anabolic proteins Akt and eEF2 and higher expressions of catabolic proteins Atrogin-1 and

MURF-1 in SOL 10 days post-burn, even more pronounced at 40 days post-burn. In contrast to the clear findings in SOL, results in the predominantly type 2 fiber type EDL were more complex and yielded conflicting results. In line with our hypothesis, anabolic signaling was lower 10 days post-burn but this decrease had stabilized 40 days post-burn, Akt expressions were even higher compared to control. Catabolic proteins in EDL showed, surprisingly, at both timepoints lower expressions in severely burned compared to sham animals. This is remarkable since EDL muscle weights decreased, indicating significant muscle atrophy. The most plausible explanation for this inconsistency is that EDL have a higher sensitivity to acute breakdown, yet may recover faster than the SOL. Studies conducted in the acute (24-72 hours) post-burn phase have shown a more pronounced upregulation of proteasome activities in EDL, indicating a higher sensitivity to myofibril breakdown at this timepoint compared to SOL muscle [16,17]. Additionally, in acute sepsis, a preferential atrophy of fast glycolytic muscle fibers is seen as well [18]. Presumably type 2 muscles, which are prone to rapid inflammatory muscle atrophy, undergo acute breakdown initially, but may subsequently reach a plateau or even trigger compensatory mechanisms [16,17,18]. Another explanation could be the movement of the animals in their cages. Although we haven't analyzed moving behavior it is possible that normal physical activity prevents muscle wasting processes in EDL. Type 2 muscles not only experience faster degradation, they also have a greater recovery capacity compared to type 1 muscles [19]. Low-intensity exercises or even minimal loading could be sufficient to mitigate the effects of burns on type 2 muscle fibers [19]. Nevertheless, these compensatory mechanisms do not seem to be able to completely prevent the negative effects of burns, since we still found lower EDL muscle weights at 40 days post-burn.

1.2.3 *Impact of myokines*

Finally, we investigated the correlation between myokine expression and postburn changes in muscle mass and fiber type-specific protein expression. We found increased myostatin expression in SOL and decreased expression in EDL. Myostatin is known to stimulate genes associated with protein degradation pathways, such as the ubiquitin-proteasome system, and simultaneously inhibiting protein synthesis by suppressing the Akt-mediated signaling

pathway (see chapter 3) [20]. The myostatin expressions found in our study seem to follow this evidence, inducing similar alterations of downstream proteins. More evidence comes from the results of Brightwell et al. (2020), who reported increased myostatin protein signaling in mice spinotrapezius muscle, a mixed fiber type muscle, 21 days post-burn, eliciting a fibrotic muscle phenotype hindering regenerative capacity [21]. In addition, Lang et al. (2001) observed increased myostatin expressions in the fast-twitch part of the Gastrocnemius muscle 24 hours post-injury [22]. This aligns with our previous hypothesis suggesting a more acute involvement of fast-twitch muscle fibers in postburn muscle atrophy, restoring over time.

In summary, these studies have shed light on the dynamic nature of postburn muscle wasting, emphasizing the importance to carefully consider muscle type, timing, and myokine expression in burn rehabilitation strategies. These findings highlight the need for tailored approaches in burn rehabilitation.

1.3 Therapeutic impact of exercise on postburn muscle wasting mechanisms

The study presented in **Chapter 6** aimed to contribute to filling the knowledge gap on efficacy and mechanisms of exercise in postburn muscle wasting. The exercise intervention study aimed to (1) demonstrate the efficacy of physical exercise to mitigate postburn muscle wasting (2) evaluate the effects of exercise on protein signaling pathways in postburn muscle wasting and (3) quantify exercise effects on (dysregulated) myokine expression post-burn.

1.3.1 *Protective effects on muscle wasting*

Severely burned animals in the exercise group exhibited higher muscle weights and a twofold increase in mean body weight gain compared to non-exercised animals. Remarkable is the exercise-induced restoration of postburn body weight to levels typical for animals of corresponding age. Such normalization underscores the significant

importance of exercise, considering that low body weight is a defining factor contributing to the adverse effects of burns and subsequent negative outcomes. Notably, even in the absence of adjusted nutrition, exercise demonstrates the capacity to restore body weight in rats. The effect of exercise on muscle weight post-burn was more pronounced in EDL compared to SOL. Since exercise has more atrophy protective effects on fast-twitch muscle fibers compared to slow-twitch, exercise was expected to further enhance a positive protein balance and restore muscle weights in EDL post-burn [23,24]. Our muscle weight data confirm this hypothesis. In contrast, the weight of the SOL muscle lags behind and does not match that of the non-exercise sham group. Accordingly, SOL did not exhibit recovery in protein signaling at 40 days post-burn in our previously discussed studies. This suggests that exercise is unlikely to have significant effects on slow-twitch fibers and is thus unable to fully counteract the catabolic effects caused by burns.

1.3.2 *Muscle specific impact on protein signaling pathways*

Post-burn exercise reduces catabolic signaling in both SOL and EDL, shown by reduced levels of FOXO3a and E3 ubiquitin ligases MURF-1 and Atrogin-1. Anabolic protein signaling was higher in SOL post-exercise, as hypothesized. However, in EDL both Akt and eEF2 protein expressions were lower compared to the non-exercise group. Several explanations could explain the discrepancies between type 1 and type 2 muscle types, when relating them to our previous results. Anabolic protein signaling did not show a decrease at 40 days post-burn in EDL, making it more difficult for exercise to induce a significant increase. Aerobic exercise is known to primarily affect protein catabolism in type 2 muscles. Our results indeed show lower FOXO3 levels, by impacting downstream expressions of MURF-1 and Atrogin-1 [23]. While our protocol involved aerobic endurance exercise, the addition of resistance exercise could additionally enhance anabolic signaling in type 2 muscles. Anaerobic exercise activates the PI3/Akt pathway, stimulating myofibrillar protein synthesis [25,26].

1.3.3 *Exercise and myokine expression*

Irisin and Decorin, two anabolic myokines known to be upregulated by exercise, emerged as key players in post-burn exercise effects [27,28]. Exercise counteracted the postburn

higher expression of myostatin in SOL, the lower expression of Decorin in SOL and of Irisin in EDL. Exercise reversed the myokine expressions negatively affected by the burn, but also caused positive changes in myokines that were initially unchanged. A similar beneficial impact is observed in cancer cachexia [29]. Downstream signaling pathways are affected by these changes [30]. Decorin inhibits MURF-1 and Atrogin-1 expression, also our study showed a concurrent increase in Decorin and a decrease in MURF-1 and Atrogin-1 in SOL [28]. In EDL, where Decorin did not exhibit a significant increase following exercise, Irisin may play a more substantial role [31]. In both muscle types, exercise induced lower myostatin expression contributes to inhibiting catabolic processes, which is also seen in patients with heart failure [32]. Additionally, myokines are recognized for their systemic benefits, making them an interesting target for exercise therapy in burns. In cancer research, myokines are increasingly acknowledged as intriguing targets not only for addressing cancer cachexia, but also for potentially impeding cancer progression through the anti-inflammatory effects induced by exercise [33,34].

In summary, the intervention study has shed light on the potential of exercise training to inhibit burn-induced muscle atrophy in both fast- and slow twitch muscles, but just as the progression of burn atrophy, also the exercise effects are different in both muscle types. Overall, the cumulative impact of exercise yields positive outcomes on both muscle types, contributing to an overall positive effect, as shown by successfully restoring total body weights following a severe burn injury. Future research will need to further unravel both the advantageous systemic effects and the specific (molecular) mechanisms in both muscle types, in combination with the exploration of the effects of anaerobic exercises.

2 Methodological implications

A major strength of the studies investigating the underlying mechanisms of muscle wasting in the late flow phase and the intervention study (**Chapter 5 and 6**), is the extended follow-up period. Although it is widely recognized that the negative effects of burns persist beyond wound healing, studies investigating the underlying mechanisms at later timepoints were lacking. To our knowledge, these are the first studies investigating fundamental processes with a follow-up period longer than 14 days. For the purpose of interpreting the results in relating to clinical implications, a notable strength lies in the researcher's participation in clinical studies, more specifically in the study of DR Schieffelers investigating the effects of exercise training on muscle wasting in adults with acute burn injury in a clinical setting [35]. Besides, interesting discussions concerning exercise therapy emerged out of the hospital visits, involving the primary researcher, clinical doctors, physicians or the burn patients themselves.

Several pathways involved in regulating protein balance in skeletal muscle were studied, but not all possible protein pathways were taken into account. We evaluated several key proteins and myokines found to be important in other burn studies, as well as in research investigating muscles in other cachectic conditions such as cancer, but other molecular pathways may be involved in postburn muscle wasting as well. Besides, some parameters were not measured in our studies which could provide useful information. Differences in food and water intake could influence nutritional status and weight of the animals. Also sedentary behavior or increased activity of specific animals could affect body mass and/or muscle weights. We believe that the effect of both the acute food intake and physical activity level is limited, since no signs of discomfort were observed from 24 hours postburn. Lastly, our plan to measure Resting Energy Expenditure (REE) was hindered due to technical complications with the equipment. Within post-burn rehabilitation, therapists are often concerned about exacerbating hypermetabolism by the integration of exercise [36]. To alleviate such concerns, measuring REE can offer valuable insights.

3 Future perspectives

In the clinical setting, burn patients show reduced physical activity levels during hospitalization until long after burn center stay, aggravating skeletal muscle wasting. Being bed-bound compared to physically active in the acute period post-burn can affect muscle outcomes. Therefore, in future studies it is important to take sedentary behavior in the post-burn period into account by using e.g., activity tracking in the cages. Exploring the effects of post-burn sedentary, e.g. by including a reliable disuse group, compared to active behavior can broaden our knowledge of skeletal muscle outcomes in the short and long term and consequently affect rehabilitation and treatment strategies in and outside the hospital.

We demonstrated the positive effects of an aerobic exercise program on muscle wasting, using a progressive treadmill protocol for 5 weeks. Despite current efforts of unravelling differential effects of various training types, the effects of resistance training in burn patients are not known yet. Burn patients are often bed-bound, making them able to perform strength exercises more easily compared to aerobic exercise protocols. Future research should focus on further unraveling the fundamental effects of aerobic exercise training, the effects of resistance training, and the, possibly cumulative, effect of combination training on skeletal muscle post-burn.

Given the limitations of both pharmacological and nutritional approaches to combat skeletal muscle wasting post-burn, the potential of exercise shown in this thesis hold significant importance [37]. The restoration of total body and muscle weights by exercise post-burn presents a possibility to mitigate complications arising from burns both on the short- and long- term post injury. Integrating exercise programs with personalized nutritional strategies hold immense promise within the clinical burn setting. Remarkable effects from combining early exercise interventions with nutrition have been evident in other populations, such as critically ill patients and those with cancer cachexia [38,39,40]. Future studies should explore this potential in burn patients as well. Besides, establishing

robust guidelines for the implementation of exercise programs within burn centers can substantially enhance their application and augment the knowledge base among healthcare practitioners. In this manner, restoring muscle mass, and simultaneously diminishing correlated side effects, offers the potential to enhance functionality and quality of life of burn patients.

Being included in the clinical study of Colleague David Schieffelers advocating clinical benefits of targeting postburn muscle wasting by early exercise training [35], we were confronted with difficulties in the application of exercise in human burn patients. Patients are often demotivated to exercise by e.g. pain, upcoming operations, medication,… Besides, we faced difficulties in motivating the staff to execute the training protocols. Hence, it can be useful to identify biomarkers of the population the most at risk for severe muscle wasting. Research in other muscle wasting diseases, such as sarcopenia, is currently evolving to the use of biomarkers as a tool to facilitate and accelerate the diagnostic process as well [41]. The results of our myokine analysis has shown the possibility of using myostatin as a marker reflecting alterations in skeletal muscle signaling pathways post-burn. Besides, myostatin is considered a promising target molecule for the treatment of muscle wasting. Blocking myostatin activity has shown significantly increased muscle mass, however clinically sufficient treatments have not been found yet [20]. Hence, further research should look into the possibility of biomarkers in burn patients as well.

4 Clinical implications

In general, clinical therapists treat muscles primarily as a musculoskeletal organ with the primary objective of maintaining functionality, especially following burn injuries. A critical knowledge gap remains concerning burn injuries as internal disorders with systemic effects that profoundly affect skeletal muscles as metabolic organs and the underlying physiological mechanisms. This thesis contributes in bridging this knowledge gap. As stated in our future perspectives, clinicians are often scared to worsen complications. By further unraveling underlying molecular pathways of postburn muscle wasting we contribute to a

better understanding of postburn systemic sequelae. The demonstration of physical exercise as a tool to prevent and reverse postburn muscle wasting, is, including exploration of the intricate molecular processes that drive these beneficial outcomes, can pave the way for more effective treatment strategies. Exercise can mitigate the adverse consequences of disuse beyond the isolated effects of a physical exercise program itself. This thesis has successfully highlighted the importance and effectiveness of a physical exercise program to counteract skeletal muscle wasting post-burn by disclosing some of the molecular mechanisms of skeletal muscle protein metabolism. To indicate the clinical significance and promote the inclusion of (early)exercise within burn rehabilitation standards, we integrate the study's findings with a clinical investigation by David Schieffelers [35]. In this study, the feasibility and safety of early exercise in severe burn patients during burn center stays is proven with minimal health risks. This study underscores that exercise is an easy applicable tool even in burn patients, with, in contrary to pharmacological substances, mostly no side effects. We hereby aim for a greater motivation among burn clinicians to monitor, prevent and treat systemic complications such as muscle wasting.

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Summary

Severe burns induce several acute and chronic metabolic perturbations. A prominent hallmark persisting up to two years post-burn is skeletal muscle wasting, profoundly impacting Quality of Life of the burn survivor. Insights from studies in other cachectic populations show the promising potential of exercise as a preventive and therapeutic approach to counteract muscle wasting. After a burn injury, the specific molecular pathways orchestrating muscle wasting and the precise impact of exercise remain elusive. This research project was initiated to shed light on the fundamental effects of exercise on post-burn muscle wasting. By elucidating the underlying mechanisms, we can lay the foundation for the implementation of early exercise as a viable treatment strategy for burn survivors, addressing several metabolic sequelae.

In **Chapter 3,** we provided an overview of the current literature, emphasizing that the existing knowledge concerning the effects of burns and the possible beneficial effects of exercise on the molecular mechanisms of muscle wasting is still in its infancy. This limited understanding of metabolic pathophysiology of post-burn muscle wasting might explain the limited use of early exercise as a therapeutic tool in burn patients.

In **Chapter 4** we demonstrated that 10 days post-burn muscle wasting is present, as shown by lower total body weight gain and muscle weights for both SOL and EDL in severely burned rats. The majority of analyzed proteins involved in protein synthesis pathways showed significant lower expressions in both muscles post-burn (eEF2, pAkt and p70S6K). However, for protein breakdown pathways, no higher expressions were found for the majority of analyzed proteins in muscles from severely burned animals compared to sham (MURF-1, FOXO3A). Only Atrogin-1 was significantly higher in SOL post-burn. At 40 days post-burn severely burned animals still show lower total body and SOL and EDL muscle weights, as demonstrated in **Chapter 5.** Morphological analysis showed that in SOL more but smaller muscle fibers were present post-burn, and a shift towards a faster phenotype took place. Likewise, molecular analysis showed significant lower expressions of anabolic

pAkt and eEF2 and higher expressions of catabolic MURF1,2,3 and Atrogin-1 in SOL of severely burned animals. Surprisingly, in EDL the opposite result was found, with a significant higher expression of pAkt and lower expressions of MURF-1,2,3 and Atrogin-1. We also looked into the expression of several myokines to evaluate whether these affect post-burn muscle atrophy. Anabolic myokine Decorin showed a significant lower expression in SOL of the severely burned animals, similar to Irisin in EDL. Catabolic myokine Myostatin showed a higher expression in SOL, but not in EDL 40 days post-burn. Overall, these results provide evidence that muscle wasting is present in the early flow phase and persists during the later stages post-burn, but the specific mechanisms are time and muscle dependent. During the early flow phase protein imbalances are present, with type 2 muscles being more vulnerable compared to type 1 muscles. During the chronic flow phase, this phenomenon seems to be reversed.

In **Chapter 6** we showed that early exercise, a progressive treadmill training protocol, has protective effects on post-burn muscle wasting. Anthropomorphic analysis shows an increase in total body and skeletal muscle weights of SOL and EDL in exercised animals after burn. Accordingly, catabolic signaling pathways show significant lower expressions of Atrogin-1, MURF-1 and FOXO3a after exercise in both SOL and EDL. Anabolic signaling was not observed to be significantly increased by exercise post-burn, only pAkt showed a significant higher expression in SOL whilst eEF2 even decreased significantly in both muscles by exercise. Several myokines involved in exercise-mediated protection against muscle wasting by affecting downstream signaling pathways were analyzed as well. Exercise resulted in lower expressions of the catabolic myokine Myostatin and higher expressions of the anabolic myokine Irisin after burns in both muscles. Additionally in SOL, higher Decorin expressions were found after exercise. These findings suggest that exercise may modulate myokine expression, further supporting the beneficial effects of exercise in mitigating muscle wasting after burn injuries.

In conclusion, we can confirm that an early physical exercise protocol can effectively mitigate skeletal muscle wasting within a severe burns rat model by attenuating catabolic signaling and positively affecting myokine expression.

Samenvatting

Ernstige brandwonden veroorzaken verschillende acute en chronische metabole verstoringen. Een van deze gevolgen is spieratrofie, dat aanhoudt tot twee jaar na het oplopen van de brandwonde en een grote invloed heeft op de levenskwaliteit van de brandwondenpatiënt. Studies in andere populaties die onderhevig zijn aan spierafbraak tonen het veelbelovende potentieel aan van lichaamsbeweging als preventieve en therapeutische tool. Momenteel zijn de specifieke moleculaire paden die spieratrofie veroorzaken na een brandwondeletsel en het precieze effect van lichaamsbeweging nog onduidelijk. Dit onderzoeksproject is daarom opgericht om de fundamentele effecten van lichaamsbeweging op spieratrofie na brandwonden te onderzoeken. Door de onderliggende mechanismen te ontrafelen, kan de basis worden gelegd voor de implementatie van vroegtijdige lichaamsbeweging als een behandelingsstrategie, waarbij verschillende metabole verschijnselen kunnen worden aangepakt.

In **hoofdstuk 3** gaven we een overzicht van de huidige literatuur, waarin we benadrukten dat de bestaande kennis over de effecten van brandwonden en de mogelijke gunstige effecten van lichaamsbeweging op de moleculaire mechanismen van spieratrofie nog in de kinderschoenen staat. Dit beperkte begrip van de metabole pathofysiologie van spieratrofie na brandwonden zou het beperkte gebruik van vroegtijdige lichaamsbeweging als therapeutisch middel bij brandwondenpatiënten mede kunnen verklaren.

In **hoofdstuk 4** toonden we aan dat 10 dagen na verbranding spieratrofie aanwezig is, gekenmerkt door minder gewichtstoename en lagere spiergewichten van zowel SOL als EDL. De geanalyseerde eiwitten betrokken bij eiwitsynthese vertoonden significant lagere expressies in beide spieren na de verbranding (eEF2, pAkt en p70S6K). Voor eiwitafbraakroutes werden echter geen hogere expressies gevonden voor de geanalyseerde eiwitten in spieren van ernstig verbrande dieren (MURF-1, FOXO3A). Enkel Atrogin-1 was significant hoger in SOL na verbranding. Ook op 40 dagen na de verbranding vertoonden ernstig verbrande dieren een lager lichaamsgewicht en spiergewicht in SOL en EDL, zoals aangetoond in **hoofdstuk 5**. Morfologische analyses toonden aan dat er in SOL meer maar kleinere spiervezels aanwezig waren na de verbranding en dat er een

verschuiving plaatsvond naar een sneller fenotype. Ook toonde moleculaire analyses significant lagere expressies van anabole proteïnen pAkt en eEF2 en hogere expressies van catabole proteïnen MURF1,2,3 en Atrogin-1 in SOL van ernstig verbrande dieren. Verrassend genoeg werd in EDL het tegenovergestelde resultaat gevonden, met een significant hogere expressie van pAkt en lagere expressies van MURF-1,2,3 en Atrogin-1. Hiernaast keken we ook naar de expressie van verschillende myokines om te evalueren of deze spieratrofie na verbranding beïnvloeden. Anabool myokine Decorin toonde een significant lagere expressie in SOL van de ernstig verbrande dieren, vergelijkbaar met Irisin in EDL. Catabool myokine Myostatin toonde een hogere expressie in SOL, maar niet in EDL 40 dagen na de verbranding. Samengevat leveren deze resultaten bewijs dat spieratrofie na verbranding aanwezig is in de vroege flow fase, en aanhoudt in de latere stadia, maar dat de specifieke mechanismen afhankelijk zijn van het tijdspunt en spiertype. Tijdens de vroege flow fase is er een onevenwicht in eiwit-aanmaak en -afbraak, waarbij type 2 spieren kwetsbaarder zijn dan type 1 spieren. In de chronische flow fase keert dit fenomeen om.

In **hoofdstuk 6** werd aangetoond dat vroege fysieke training, in de vorm van een progressief trainingsprotocol op de loopband, beschermende effecten heeft tegen spieratrofie na verbranding. Antropomorfische analyses toonde na verbranding een toename aan van het totale lichaamsgewicht en het skeletspiergewicht van SOL en EDL bij getrainde dieren. Gelijkaardig vertonen catabole proteïnen significant lagere expressies na training in zowel SOL als EDL (Atrogin-1, MURF-1 en FOXO3a). Anabole proteïnen waren niet significant verhoogd door training na verbranding, alleen pAkt vertoonde een significant hogere expressie in SOL, terwijl eEF2 zelfs significant afnam in beide spieren door training. Verschillende myokines die betrokken zijn bij inspanning gerelateerde bescherming tegen spieratrofie, door het beïnvloeden van lagere signalen, werden ook geanalyseerd. Training resulteerde in lagere expressies van het catabole myokine Myostatin en hogere expressies van het anabole myokine Irisin na verbranding in beide spieren. Daarnaast werd in SOL een hogere expressie van Decorin gevonden na training. Deze bevindingen suggereren dat lichaamsbeweging de myokine-expressie kan moduleren,

wat de gunstige effecten van lichaamsbeweging op spieratrofie na brandwonden verder ondersteunt.

Samenvattend kunnen we bevestigen dat een vroegtijdig fysiek trainingsprotocol effectief spieratrofie kan verminderen in een ernstig brandwonden rat model door de vermindering van catabole proteïnen en het positief beïnvloeden van myokine-expressies.

Acknowledgments

Om mijn boekje, en mijn doctoraat in het algemeen, af te sluiten, ben ik een welgemeende dankjewel verschuldigd aan een heel aantal personen. Daarom als afsluiter nog een uitgebreid dankwoord aan iedereen die mij, op welke manier dan ook, gesteund of geholpen heeft tijdens de voorbije jaren om mijn werk mee tot een goed einde te brengen.

Om bij het begin te starten, natuurlijk bedankt aan mijn promotoren **prof. Dr. Van Daele** en **prof. Dr. Van Breda** om mij de kans te geven dit doctoraat aan te gaan en mij door het hele traject te ondersteunen. Bedankt Ulrike, om mee op de fundamentele boot te springen, en door jouw passie voor brandwonden een grondige onderbouw te geven aan ons onderzoek. Ook bedankt om ons telkens als we weer grootse plannen hadden, met onze voetjes op de grond te houden en te doen herinneren aan het belangrijkste aspect, de klinische transleerbaarheid. Bedankt Eric, voor jouw onuitputtelijke energie, ideeën en passie voor het onderzoek. Bedankt om alles in goeie banen te helpen leiden en, op elk moment van de dag, open te staan voor grote en kleine vragen. Jij ziet altijd alles positief in, en kon op de juiste momenten aanvoelen wanneer een extra duwtje in de rug nodig was, met de nodige humor en fiets-babbels! Ook **prof. Dr. Pieter-Jan Guns** werd toegevoegd aan mijn promotorenteam. Bedankt Pieter-Jan, om jullie labo open te stellen aan 'die van kine'. Als vreemde eend in de bijt op T2 heb je mij steeds welkom doen voelen en laten kennis maken met deze, voor mij op vele vlakken nieuwe, wereld. Jouw feedback heeft mijn werk steeds beter gemaakt, al was het soms na wat gezucht.

Naast de steun van mijn promotoren, had ik het geluk om in 2 mooie groepen terecht te komen, MOVANT en Fysiofarmacologie, of 'T2'. Bedankt MOVANT om mij vanaf dag 1 welkom te doen voelen. Voornamelijk in de periode voor en tijdens corona heb ik veel aan jullie gehad. In het speciaal wil ik hier toch **Ante** en **Kato** bedanken, samen starten geeft toch direct een verbondenheidsgevoel. Vanaf het begin konden we op de bureau onze onzekerheden, frustraties, maar ook lichtpuntjes delen. Verder zijn Lies en Lies ook van onschatbare waarde voor MOVANT. Bedankt, **Lies D.**, voor jouw feedback waar nodig. Hoe jij alles aanpakt en het dan ook nog eens er zo 'makkelijk' doet uitzien zijn een inspiratie

voor velen! **Lies P**., jij bedankt om altijd zo snel alle verzoekjes/bestellingen/.. uit te voeren! Altijd paraat voor een babbeltje, ook vroeg in de ochtend.

In het bijzonder moet ik dan nog, intussen Dr., **David** bedanken. Jij hebt me laten kennis maken met werkelijk elk aspect van de brandwonden zorg. Vanaf dag 1 vond ik je een inspirerend persoon, en dit is doorheen onze samenwerking enkel meer en meer gebleken. Alle uren die we in Stuivenberg en Neder-Over-Heembeek hebben gespedeerd, hebben me doen inzien hoe zorgend en tegelijk hoe een enorme doorzetter je bent. Jouw passie voor brandwonden heeft me heel veel bijgebracht, en samen waren we een goed team om de gulden middenweg te vinden tussen het perfectionisme en 'zo zal het ook wel goed zijn'. Bedankt om me op dit PhD pad te begeleiden!

Verder bestaat MOVANT nog uit heel veel mooie mensen en gepassioneerde onderzoekers. Spijtig genoeg heb ik niet iedereen even goed leren kennen door eerst corona, en nadien meer en meer tijd door te brengen in het labo, maar jullie zijn allemaal toppers en ik ben heel blij dat ik jullie op dit pad ben tegengekomen!

Voor ik overga naar de collega's van T2, moet ik eerst nog **Birgit** heel hard bedanken. Na een jaar kreeg ik er een 'maatje' bij, een 2^{e} kine van het MOVANT-team die zich kwam verdiepen in het fundamentele labo onderzoek. Samen alles moeten leren schept een band, om het klein uit te drukken. De uren, of beter dagen, die wij gespendeerd hebben om de kleinste dingen uit te zoeken, om technieken te optimaliseren, om protocollen uit te schrijven,… zijn ontelbaar. Maar ik ben zo dankbaar dat we dat met 2 konden doen. Frustraties, maar ook goede momenten en successen ervaren waren veel aangenamer samen met jou! Je bent echt een fantastische onderzoekster, jouw werk mentaliteit is opmerkelijk, en ook al zie je het nu nog niet altijd, je doctoraat gaat echt helemaal goed komen! Hiernaast ben je ook een prachtige persoon en goeie vriendin geworden. Onze trip naar Capri, en miljoen stappen in Napels, ga ik niet snel vergeten! Op naar een volgende trip!

T2, waar moet ik beginnen.. Ik kwam terecht in een labo waar ik niet begreep wat de toestellen waren, waar geregeld muizen door de gang kwamen gereden, en waar dan ook nog eens verschillende groepen samen zaten. Gelukkig werken er enkel goeie onderzoekers die me warm ontvangen hebben en steeds open stonde voor elke (stomme) vraag.

First my own desk, with Birgit, but where also **Callan** was sitting from the moment I started at the lab. Thank you Callan for being the easy-going person as you are, for being there when we needed to complain, for always bringing peace to our desk. Few months to go for you, your PhD was some with ups and downs as well, but it will end in a good way! **Sander,** toen jij startte dacht ik eerst dat het gedaan ging zijn met de rust op onze bureau, dat was misschien ook deels wel zo, maar op een goede manier. Ondanks je rommel was ik blij dat jij dat plekje hebt ingenomen, ik moet je zeker bedanken voor het uitwisselen van goede ideeën, maar ook de vele babbels, waar we soms plots doorhadden dat we een uur verder waren.. Ik ben nog steeds jaloers op je schrijftalent, je gaat hier ver mee komen! We gaan zeker nog verdere sportplannen maken samen! Then we have the 'Erasmus-student' desk, where a lot of nice students have passed, but the most important for me was **Alessandra.** Ale, thank you actually for just being who you are! You are really such a warm, nice, really hard working person. I am so happy you ended up at our desk, and we got to know you well. From just mentioning during a talk that Capri was so beautiful to inviting us to your home (I can never forget your moms food) and spending the week in Salerno, Capri and Naples. Next one a trip to Turin?

Buiten onze bureau zijn er nog vele hardwerkende onderzoekers die zeker een benoeming verdienen. **Matthias,** jij leerde me kennis maken met 'hét badjes lab', mijn eerste dagen op T2 bestonden uit pipetteren en grafieken lezen waar ik niks van begreep. Je bleef nadien ook telkens open staan voor vragen, waarvoor bedankt! **Cedric,** jouw inspiratie en onderzoeksbrein is onuitputtelijk, bedankt voor jouw ideeën en interessante gesprekken. Jullie gaan beide een mooie carrière tegemoet, veel post-doc succes! **Michelle,** altijd klaar voor een babbeltje over fitness, voeding, je hond of 'het hoge Noorden'. Je doet dat daar geweldig en gaat het nog ver brengen! **Freke**, ondanks tegenslagen vind je altijd een weg

om door te gaan, bedankt voor onze fijne babbeltjes! **Melissa**, met Western blot-problemen, en dat waren er veel, konden we altijd bij jou ten rade. Bedankt om samen naar oplossingen te zoeken. **Kasper**, de sportieve dokter, wat jij allemaal gedaan krijgt op een dag, ik snap het niet. Bedankt voor de loopjes, toffe triathlon gesprekken, maar ook de interessante discussies over exercise studies. **Benji**, de muizen-king, voor ideeën om een nieuwe studie op te zetten 1 adres. Jij blijft gaan, en zal je doctoraat met een hele hoop data kunnen eindigen! **Dustin**, one of the warmest persons from T2, you were always there for us, thank you for being there we needed you. **Celine,** the Italian fury of T2. You have so much energy and from being an Erasmus student to working at T2, you always had the right mentality and just keep going. Thank you for the talks over lunch, and the runs now.

Dan hebben nog de Fysiologie bureau, waar ik toch meer dan eens per dag binnen viel. **Julie**, bedankt voor alles. Doorheen de jaren onze gedeelde loop-passie ontdekt en mee naar de bureau verplaatst. Onze loopjes waren vaak een welkome onderbreking van de dag. Ondanks dat het onze planning niet altijd vergemakkelijkte (‘die blot zal wel een kwartiertje langer kunnen blijven zitten’), zorgden de loopjes altijd voor frisse energie, zeker wanneer het minder goed ging. Bedankt ook voor de babbels aan’t koffiemachine, onze 2^{e} gedeelde ‘passie’… Nog effe doorzetten en je doctoraat komt ook helemaal goed! En zolang blijf ik ’s middags bereid voor loopjes. **Siel**, ik ken niemand meer hardwerkend dan jou. Bedankt om mijn mede-vroege vogel te zijn (al is dat misschien niet goed uitgedrukt, want vroeg, laat, nacht, jij bent er gewoon altijd) en te tonen dat alles kan, zolang je het maar wil. Je stond steeds klaar om elke vraag te beantwoorden, of om effe ons hart te luchten, hoe druk je het zelf ook had. En euh, naar de vogeltjesmarkt, da komt er nog van he!! Dan **Jens,** we zullen ongeveer gelijk afleggen uiteindelijk, dus ook voor u een goed einde! Bedankt om de rust zelve te zijn de voorbije jaren. Hopelijk tot snel! **Bo** en **Michiel,** merci voor de varkentjes-introductie. Nog veel succes ook voor jullie!

Een van de grootste bedankjes gaat nog naar **Mandy**. Bedankt Mandy, voor je geduld met ons. Bedankt om alles uit te leggen vanaf werkelijk 0, en je te proberen in onze schoenen te zetten. Bedankt ook om open te staan voor onze ideeën, als we weer met iets ongewoon

afkwamen. Je stond steeds open om nieuwe, dingen te proberen, en samen met ons problemen uit te zoeken en op te lossen. Je bent heel goed in je job, hebt me enorm veel bijgeleerd en zonder jou zou dit niet gelukt zijn en ik nu niet staan waar ik nu sta. Veel geluk de komende jaren met je huisje en gezinnetje! Wie zeker ook niet vergeten mag worden is **Griet.** Bedankt voor je geduld, hulp en tijd. Je hebt ons steeds met open armen ontvangen en altijd geholpen met alle problemen. Zonder jou zou T2 niet zijn wat het is (en zou je er gewoon niet binnen kunnen van de rommel). Bedankt ook aan alle andere professoren van T2 voor de ideeën en feedback, en bedankt **Guido**, om het labo recht te houden.

Verder wil ik ook nog mijn persoonlijke omgeving bedanken. Het leven bestaat uiteraard niet alleen uit werken, en ik wil toch wel enkele personen bedanken om mij daaraan te doen herinneren.

Annelore, Lena, Marthe, Paulien, Sammeke en **Stephanie**, merci om er altijd te zijn voor mij! Bedankt om mij op te vangen of net af te leiden als ik het nodig had, om interesse te hebben, en er gewoon voor mij te zijn eender wanneer. Sorry als ik jullie soms verwaarloos(d heb), maar hierbij dus nog bedankt voor alle etentjes, weekendjes, drankjes, of gewoon hang momentjes. Jullie zijn de beste vriendinnen die ik me kan wensen.

Carmen, Marijke, Kiara, Stivie, en eigenlijk ook nog heel ACSS en den Hamburg, merci voor de sportieve afleiding. Na het werk met jullie gaan lopen/fietsen/zwemmen, heeft ervoor gezorgd dat ik alles op een rijtje heb kunnen houden. Naast het afreageren hebben ook de vele babbels tussenin, waarbij we het dan vaak net niet over mijn doctoraat hadden, mij altijd goed gedaan. Bedankt daarvoor, en dat gaan we zeker blijven doen!!

Hannah, Dario, Lynn en Kira, dat laatste jaar pendelen voor de inwendige kine heeft ons allemaal dichter bij elkaar gebracht. Bedankt aan jullie om samen dat laatste jaar door te komen, zonder dat zou ik ook nooit aan mijn doctoraat begonnen zijn. Op nog vele etentjes en brunchkes! Ook **Eline, Manon, Milan, Tine, Nick** en **Bram,** merci om die 5 jaren kine wat aangenamer te maken. Ookal is maar de helft van ons in de kine gaan werken, de

studentenjaren waren alleszins wel plezant met jullie! En Bram, ookal snapte je waarschijnlijk niet waarom ik dat nog 4 jaar zou willen doen, toch bedankt om samen die masterproef tot een goed einde te brengen! Topteam!

Bedankt ook aan team Poupou en team Oude brouwerij. Ik weet nog dat ik tijdens het werken in de Oude Brouwerij telefoon kreeg dat ik aan mijn doctoraat mocht starten, en we dat ineens goed gevierd hebben. Dus vooral **Stijn, Syd, Liesse** en **Drets,** jullie mogen toch ni ontbreken in mijn bedanking na intussen toch al zo lang met jullie samen te werken. Bedankt voor de nodige afleiding 's avonds en in het weekend.

Bedankt ook aan mijn beide families, **Dombrechtjes** en **Hendrickxen**. Ik weet dat jullie, ondanks dat jullie waarschijnlijk niet helemaal begrepen waar ik nu net mee bezig was, toch trots zijn. Hopelijk heeft de verdediging hier een beetje duidelijkheid over kunnen scheppen. **Bomma**, bedankt om me nog geregeld van eten te voorzien, telkens met de vraag 'maar wat doet ge nu eigenlijk'. Dan wel steeds gevolgd door 'ik zou het ni kunnen ze'. Bedankt om me te steunen, eender wat ik doe. Hetzelfde geld voor **Oma en Opa**, als ik over mijn doctoraat vertel zie ik aan oma haar blik dat ze enorm trots is, bedankt daarvoor!

Margot, Maarten en **Anneke,** bedankt voor jullie onvoorwaardelijke steun! Bedankt Margot, voor de ontelbare autoritjes samen naar de unief, de ene keer zwijgend omdat we nog half sliepen, de andere keer zagend en klagend omdat er weer iets was mislukt, en de andere keer ik die jouw uitleg vroeg over een of ander proces waar ik niets van snapte. Het heeft die 4 jaar toch veel aangenamer gemaakt. Ik weet dat mama en papa hierboven trots zijn op ons alle drie.

Bedankt ook **Dart, Hilde, Lise, Emilie en Thomas**. In mijn laatste masterjaar ben ik nog bij Emilie gaan aankloppen om uitleg te vragen wat een doctoraat nu net is, en nu staan we hier. Bedankt om me op te nemen in jullie warm gezinnetje, om interesse te hebben in wat ik doe en klaar te staan als ik jullie nodig heb. Ook bedankt aan **Louis** voor de knuffels het laatste jaar!

En als laatste, maar zeker niet te vergeten, bedankt **Joppe.** Maar misschien ook een beetje sorry Joppe. Sorry dat het voorbije jaar voor misschien 95% bestond uit schrijven en sporten. Maar bedankt om me eraan te herinneren dat het leven meer is dan dat. Bedankt om me uit ons kot te sleuren als het nodig was, en bedankt om me ook net te laten doen als dát nodig was. Bedankt om voor mij klaar te staan als ik het nodig had, maar ook om zaken op jou te nemen als ik er geen tijd voor had. Al was ik slechtgezind, verliep mijn dag niet hoe ik het wou, of was er weer eens iets mislukt, jij slaagde er altijd weer in om mij op een of andere (stomme) manier aan het lachen te krijgen. Bedankt om te zijn wie je bent en mij graag te zien hoe ik ben.

Dorien