Molecular mechanisms mediating development of pulmonary cachexia in COPD
Dedicated to my family:
Jelisaveta, Toma, Kaća, Jaša, Jela, Vanja and Maria

Actiones nostras, quaesumus Domine, aspirando praeveni et adiuvando prose-quere: ut cuncta nostra oratio et operatio a te semper incipiat et per ta coepta finiatur. Per Christum Dominum nostrum. Amen.
Molecular mechanisms mediating development of pulmonary cachexia in COPD
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Title: Molecular mechanisms mediating development of pulmonary cachexia in COPD
Publisher: ÖrebroUniversity 2014
www.oru.se/publikationer-avhandlingar

Print: Örebro University, Repro 08/2014

ISSN 1652-4063
Abstract


Cigarette smoking (CS) represents the main causative agent underlying development and progress of COPD. Recently, involvement of CS in the pathogenesis of COPD-associated muscle abnormalities is becoming increasingly evident. Nevertheless, involved triggers and underlying mechanisms remain largely unknown. This study was conceived in order to examine effects of cigarette smoke exposure on skeletal muscle morphology, vascular supply and function. For this purpose, we have specifically designed murine COPD/emphysema model and gastrocnemius muscle was examined, while in-vitro experiments were conducted using murine C2C12 skeletal muscle myocytes.

In addition to the mild emphysematous changes present in the lungs of CS-exposed mice, our results demonstrated evident signs of muscle atrophy reflected by decreased fiber cross-sectional area, profound fiber size variation and reduced body mass. Furthermore, we have observed impairment in terminal myogenesis and lower number of myonuclei in skeletal muscles of CS-exposed animals despite evident activation of muscle repair process. Additionally, our results demonstrate capillary rarefaction in skeletal muscles of CS-exposed animals which was associated with deregulation of hypoxia-angiogenesis signaling, reduced levels of angiogenic factors such as HIF1-α and VEGF and enhanced expression of VHL and its partner proteins PHD2 and Ube2D1. The results of our in-vitro experiments demonstrated that VHL and its ubiquitination machinery can be synergistically regulated by TNF and hypoxia consequentially impairing angiogenic potential of skeletal muscle myocytes. Finally, we have shown that CS elicits chronic ER stress in murine skeletal muscles which is associated with activation of ERAD and apoptotic pathways as mirrored by elevated expression of Usp19, caspase 12 and caspase 3 in skeletal muscles of CS-exposed animals. Moreover, molecular and morphological alterations in CS-exposed mice resulted in impairment of muscle function as reflected by their impaired exercise capacity.

Taken together, from our results it is evident that cigarette smoke exposure elicits set of morphological, vascular and functional changes highly resembling those observed in COPD. Additionally, CS induces wide range of molecular alterations and signaling pathway deregulations suggesting profound effects of cigarette smoke exposure on skeletal muscle cell homeostasis.

Keywords: COPD, cachexia, atrophy, cigarette smoke, myogenesis, angiogenesis

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List of papers

This thesis is based on the following papers and manuscripts:


Basic VT, Jacobsen A, Sirsjö A, Samy AH. Chronic cigarette smoke exposure impairs skeletal muscle regenerative capacity in murine COPD/emphysema model. Manuscript


Other publications not included in this thesis:

Zhang B, Elmabsout AA, Khalaf H, Basic VT, Jayprakash K, Kruse R, Bengtsson T, Sirsjö A. The periodontal pathogen Porphyromonas gingivalis changes the gene expression in vascular smooth muscle cells involving the TGFbeta/Notch signalling pathway and increased cell proliferation. BMC Genomics. 2013 9;14:770
Abbreviations

COPD            Chronic obstructive pulmonary disease
PC             Pulmonary cachexia
CS                  Cigarette smoke
HIF1-α           Hypoxia inducible factor one alpha
VEGF           Vascular endothelial growth factor
VHL           von Hippel Lindau tumor suppressor
PHD2           Prolyl hydroxylase two
Ube2D1 Ubiquitin conjugating enzyme two D one
Ube1           Ubiquitin activating enzyme one
Usp19          Ubiquitin specific protease 19
TNF           Tumor necrosis factor
TA               Tunicamycin
ER                  Endoplasmic reticulum
GLUT1       Glucose transporter 1
FEV1          Forced expiratory volume 1
GOLD       The global initiative for chronic obstructive lung disease
ATP              Adenosine three phosphate
BMI            Body mass index
FFM           Fat free mass
IGF-1          Insulin growth factor one
AKT           V- Akt Murine Thymoma Viral Oncogene Homolog
UPS          Ubiquitin proteolytic system
MAPK           Mitogen activated protein kinase
JNK                c-JUN NH2 terminal kinase
IRE1         Inositol required kinase one
ERK            Extracellular signal-regulated kinase
XBP1           X-box binding protein 1
MHC            Myosin heavy chain
Ub                 Ubiquitin
NFkB            Nuclear factor kappa be
TNFR          Tumor necrosis factor receptor
IL1, 6,8,18    Interleukine one, six, eight, eightin
ZNF496      Zinc finger protein four hundred ninety six
Notch1         Notch1
Myh3           Embryonic myosin heavy chain
FGF1           Fibroblast growth factor
ROS/RNS      Reactive oxygen/nitrogen species
SC               Satellite cell
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1. COPD, A LUNG DISEASE WITH SYSTEMIC FEATURES

Tobacco smoke is a complex, dynamic and reactive mixture containing more than 5000 chemicals [1]. This toxic and carcinogenic mixture represents the most common source of toxic chemical exposure and chemical mediator of disease in humans [1,2]. Tobacco-related diseases, including chronic obstructive pulmonary disease (COPD), account for 3.7% of the world burden of disability-adjusted life-years (DALYs), a measure of lost years of healthy life [3]. These diseases are projected to impose a worldwide burden of $47 trillion health dollars by 2030 [3].

COPD is a lung disorder characterized by progressive airflow obstruction and remodeling of the airways (chronic bronchitis) and the destruction of lung parenchyma tissue (emphysema) [4]. These pathological changes originate from an abnormal inflammatory response in the lungs which represents the innate and adaptive immune response to long term exposure to cigarette smoke [4]. In addition to pulmonary features, it is now well known that COPD is associated with significant systemic manifestations, such as renal and hormonal abnormalities, cardiovascular diseases, anemia, osteoporosis, and cachexia [5].

Cachexia or wasting syndrome is a complex, debilitating metabolic syndrome associated with a wide variety of chronic illness such as cancer, AIDS, congestive heart failure, tuberculosis, and chronic obstructive pulmonary disease (COPD) [6]. Clinically, cachexia manifests with excessive weight loss in the settings of an ongoing disease, with involuntary and progressive loss of skeletal muscle mass, with a variable loss of fat mass [7]. The prevalence of cachexia is high in COPD (20-40% depending on definition and disease stage) and appears more prevalent in the emphysematous phenotype of the disease [8,9]. Pulmonary cachexia increases mortality and is associated with poor quality of life and loss of peripheral and respiratory muscle function [10,11].

1.1. Cigarette smoking and COPD pathogenesis: a vascular story

Cigarette smoking (CS) is the most commonly encountered risk factor for the development of COPD [4,12-15]. CS toxicity causes an inflammatory process in the central airways, peripheral airways and lung parenchyma.
This is associated with their structural remodeling caused by repeated injury and repair process [4,16,17]. Consequential respiratory function decline due to irreversible airflow limitation represents the most important clinical feature of COPD.

COPD encompasses two major clinical phenotypes: 1) chronic bronchitis and 2) emphysema. Examination of lung tissue in patients with chronic bronchitis shows thickened bronchial walls with luminal narrowing, as well as mucous and mucopurulent debris accumulation within the airways [4,16,17]. Furthermore, cell hyperplasia, thickening of the subepithelial basement membrane, bronchial wall fibrosis, hyperplasia of the subepithelial seromucinous glands and a chronic inflammatory infiltrate are present within the respiratory epithelium [4,16,17].

Small airway obstruction is an important pathologic feature of COPD. The histomorphology includes infiltration and accumulation of macrophages containing smoker’s pigment within respiratory bronchiole lumina, alveolar ducts, and alveoli [16,17]. Disease severity classification has been done according The Global Initiative for Chronic Obstructive Lung Disease (GOLD) and includes stages I–IV, based primarily on pulmonary function tests (forced vital capacity [FVC] and forced expiratory volume in 1 second [FEV1]) [4,16,17].

There are two distinct types of emphysema that share the common pattern of destruction of alveolar walls and result in enlargement of airspaces distal to terminal bronchioles and lung function decline. Thus, centrilobular emphysema which is most commonly caused by cigarette smoking involves destruction of alveoli centered on the respiratory bronchiole and the proximal acinus [4,16,17]. Microscopic examinations of this emphysema subtype show punctuate areas of small airspace destruction, often associated with the deposition of pigment [4,16,17]. Panlobular emphysema is the other subtype of emphysema which is caused by α1-antitrypsin deficiency and represents an inherited disorder involving mutations within chromosome 14 [4,16,17]. Panlobular emphysema involves destruction of alveolar tissue with dilation of small airspaces throughout the lungs. Furthermore this destruction is pronounced at the lung bases, while it is not so severe in the upper lobes [4,16,17]. Misbalance between protease/anti-protease activity in response to augmented inflammatory response has been suggested as the potential mechanism underlying emphysema development [17]; however several studies question this hypothesis and associate inflammatory response with more advanced stages of emphysema [18-20]. Moreover, most of the individuals with the genetic α1-antitrypsin
deficiency do not develop emphysema-unless they start smoking cigarettes despite misbalance in protease/anti-protease activity [21].

Recent studies have highlighted toxic effect of CS per se and concordant destruction of lung tissue architecture, specifically pneumocytes, endothelial, and myofibroblastic cells [21-24]. CS appears to elicit prototypic insult and injurious response of the alveolar cells leaving simplified septa, with a progressive decrease in capillary exchange area [22,24]. Furthermore, an early onset of lung vasculature alterations suggests etiological role of vascular insufficiency in emphysema development. Indeed, destruction of lung parenchyma capillary bed and disruption of hypoxia-angiogenic signaling in animal models reveals significance of HIF1-α/VEGF pathway in the maintenance of lung structural integrity [22,24-26]. Thus disruption in VEGF signaling results in arrested lung development, simplification of alveolar structure in the neonate and emphysema in the adult models [25,26]. More importantly, there is growing evidence that human emphysema is associated with decreased HIF1-α/VEGF gene expression [27]. Moreover in-vitro studies directly link CS with impaired VEGF signaling and apoptosis of alveolar epithelial cells [28]. Molecular aspects and regulatory pathways underlying HIF1-α/VEGF signaling deregulation in emphysematous lungs however remain elusive.
1.2. COPD: a multifactorial systemic disease. Skeletal muscle dysfunction as an independent risk factor in COPD

Skeletal muscle dysfunction is considered to be one of the key systemic co-morbidities in COPD [5,29-32]. Highly debilitating in nature, it is shown to significantly impacts life quality and mortality rate in patients [31,33]. Initially, skeletal muscle dysfunction is manifested in the exercise intolerance and dyspnea on exertion [33,34]. As the disease progresses, these symptoms develop to inability to perform normal every-day routines and severe disability [31]. Moreover, skeletal muscle dysfunction and consequential exercise limitation reduce capacity for pulmonary rehabilitation which has been established as one of the most effective therapeutical means in COPD [33-40]. Intriguingly, depressed muscle strength and reduction in muscle endurance observed in COPD patients cannot be ex-
plained entirely on the basis of the decline in lung function and impaired gas exchange [41-43]. Evidence in favor of a poor relationship between lung function and exercise performance were suggested in studies conducted in patients who had undergone lung transplantation as well as those using bronchodilatators where despite marked increase in the lung function exercise limitation persists [42,43]. Reduced exercise capacity and limb muscle weakness rather than degree of dyspnea leads to COPD patient disability and correlate with poor prognosis and higher utilization of health care resources [44].

Reduction in muscle strength and endurance was shown to predominantly affect lower limbs of patients with COPD [33,45-49]. As an example, 20-30% reduction in quadriceps femoris strength and 30% in quadriceps muscle endurance has been reported in COPD patients relative to control subjects [46,48]. The degree of reduction in limb muscle strength and endurance was demonstrated to correlate with the severity of the disease process, poor exercise performance, and increased dyspnea and worsening of quality of life [46]. Furthermore, this reduction represents powerful predictor of increased mortality in severe COPD patients [50].

In upper limbs, in opposite to the lower limbs overall muscle strength appears to be preserved despite reduced exercise capacity. Reduced muscle endurance might be consequence of the work load difference between different muscle groups [45], what is further evident in respiratory muscles (diaphragm). Diaphragm muscle in COPD patients demonstrates set of adaptive changes associated with intensively trained muscle [51]. These adaptive alterations include increased mitochondrial density, capillarization, and increased oxidative capacity and enhanced myosin ATPase activity [51,52]. Moreover, shortening of diaphragm sarcomeres designed to reverse the negative influence of hyperinflation has been demonstrated [51]. Reduction of diaphragm strength is believed to be a consequence of hyperinflation-induced shortening of diaphragmatic length, which has a negative influence on the pressure-length relationship.
1.3. Pulmonary cachexia as an underlying factor in the development of skeletal muscle dysfunction in COPD. Characteristics of peripheral musculature in COPD

Weight loss and particularly the fat-free mass loss (FFM) is a frequent complication in patients with COPD [8,9]. This disproportionate loss of FFM in COPD is often referred to as pulmonary cachexia (PCS). The clinical diagnosis of cachexia is traditionally based on determination of body weight or body mass index (BMI). However, this traditional approach warrants several disadvantages. Firstly, muscle wasting was demonstrated to be present in COPD patients with normal weight [45]. Additionally, BMI index was shown to correlate poorly with the exercise capacity and mortality rate in COPD [163]. Thus, assessment of fat-free mass represents more precise determinant of muscle wasting shown to strongly correlate with the mortality rate and exercise capacity in COPD [163].
It has been well established that limb muscles of COPD patients develop significant reduction in muscle mass and cross-sectional area (muscle atrophy) [10,53]. The extent of the lower limb muscle loss was shown to be greater than that of the whole body weight, indicating a preferential loss of muscle tissue over other body tissues [10,46,54]. However, it is still not clear whether decrease in muscle mass reflects in proportional strength reduction. Thus, normalized to cross-sectional area difference between muscle strength in COPD and control subjects was not evident, suggesting that factors other than simple atrophy (i.e. mass-independent mechanisms) underlay the COPD-related skeletal muscle dysfunction [55,56]. In support to this notion muscle fiber atrophy appears to be specific to fiber types IIA/IIX and IIX which exhibit disturbed oxidative capacity, mitochondria rarefaction and reduced mitochondrial biogenesis what might in turn impair muscle capacity to produce energy and generate force [57-59]. Reduction in the proportion of types I (slow-twitch oxidative) fibers and increase in the proportion of type IIb (fast twitch glycolytic) fibers in lower limb muscles of COPD patients might be an important factor underlying increased leg muscle fatigability and reduced endurance due the fact that type II fibers are more fatigue-prone [60-63]. In addition to the impairment in skeletal muscle oxidative capacity due to the fiber type shift towards glycolitic phenotype, reduction in vascular density might adversely affect oxidative capacity in peripheral musculature of COPD patients [62]. Severe COPD patients exhibit drastic reduction in capillary number in vastus lateralis, with 47% decrease in the capillary-to-fiber ratio [64]. Decreased capillarization is observed already in skeletal muscles of mild COPD patients and it is shown to worsen with the disease progression [62,65,66]. Intact vascular network is essential to fiber nourishment and maintenance of muscle homeostasis, thus decrease in the capillary density in skeletal muscles of COPD patients is associated with an increase in serum and muscle lactic acid levels during exercise and an early onset of contractile fatigue [67,68].

1.4. Factors associated with the maintenance of muscle mass in COPD.
The molecular perspective

Muscle mass is determined by the net balance of two major factors including protein turn-over and myonuclei turn-over. Increased protein turn-over has been reported in COPD, both due decreased protein synthesis as well as accelerated protein degradation [69-71]. Interestingly, reduction in
protein synthesis is not limited to underweight COPD patients but occurs even in long-term smokers in the absence of compromised lung function [72]. When it comes to catabolic processes, accelerated degradation of muscle fibril proteins as well as intermediaries of muscle catabolism such as methyl-histidine and pseudouridine have been reported in serum and urine of COPD patients [73-75].

1.4.1. Molecular pathways associated with muscle anabolism are suppressed in skeletal muscles of COPD patients

The essential pathway regulating muscle anabolism and hypertrophy involves the insulin-like growth factor-1 (IGF-1)-Akt signaling cascade [76,77]. In skeletal muscles of cachectic COPD patients decreased levels of IGF-1 protein have been reported [37]. In contrast, circulatory levels of IGF-1 appear not to be altered in patients with pulmonary cachexia, though significant depletion was reported during acute exacerbations [78-81]. IGF-1 is the main and the most potent activator of Akt signaling pathway in the cell [77]. Akt signaling pathway favors anabolic processes by stimulating cell growth and proliferation as well as by suppressing programmed cell death [76,77]. Interestingly, Akt pathway activation has been observed in skeletal muscles of cachectic COPD patients despite reduction in protein synthesis and depletion of IGF-1 protein [37,82]. This might come as a futile attempt of myocytes to restore muscle mass and cellular homeostasis. In cachectic patients with pronounced fiber atrophy, however Akt activation is completely absent though IGF-1 mRNA has been elevated [83,84].

1.4.2. Activation of ubiquitin proteolytic system (UPS) and enhanced protein degradation in skeletal muscles of COPD patients

The ubiquitin-proteasome system (UPS) is known to degrade the major contractile skeletal muscle proteins and play a major role in muscle wasting [85,86]. Diverse factors and cellular events can activate UPS and subsequent muscle wasting [79,85-91]. These include pro-inflammatory cytokines, activation of various caspases and cell apoptosis as well as different types of cell insults such as endoplasmic stress and hypoxia [79,83,86,91,92]. They evoke degradation of cellular proteins via two discrete and successive steps. In the first step, the protein substrate is tagged by covalent attachment of multiple ubiquitin molecules to generate
the polyubiquitin chain which is further recognized and degraded by the downstream 26S proteasome complex [93]. This process is conducted via three different enzyme families. The ubiquitin-activating enzyme, E1, initiates ubiquitin ligation by adenylating ubiquitin [93]. One ATP molecule is expended for each E1-ubiquitin linkage [93]. The ubiquitin molecule is transferred to the ubiquitin-conjugating enzyme E2, which transiently carries ubiquitin [93]. E2 works in conjunction with the ubiquitin ligase E3, which is responsible for conferring substrate specificity on the reaction [93]. E3 mediates the transfer of ubiquitin to an internal lysine of the target protein [93]. The second step involves recycling of ubiquitin molecules which is mediated by ubiquitin recycling enzymes such as isopeptidases, also known as ubiquitin-specific proteases (USPs) and deubiquitinating enzymes (DUBs) [94,95].

Figure 3. Ubiquitin proteolytic system

UPS-dependent protein degradation of muscle contractile proteins is demonstrated to occur via activation of the muscle-specific E3 ligases such as atrogin-1 and muscle-specific RING finger protein 1 (MuRF1) which are regulated by the fork-head transcription factors FOXO-1 and 3 [83,85]. Furthermore, other UPS members such as Nedd4, Usp19, E3q and E2(14k) are reported to be involved in skeletal muscle atrophy during
different catabolic conditions [79,82,85,96-98]. In muscles of COPD patients, activation of UPS is reflected by increased protein ubiquitination and proteosomal activity [79]. Increased expression of muscle specific E3 ligases such as Atrogin-1 and Murf-1 as well as activation of FOXO-1,3 signaling pathways are reported in skeletal muscles of patients with pulmonary cachexia [79,82,99]. Increase in the Atrogin-1 expression within skeletal muscles of cachectic COPD patients appears to involve activation of mitogen-activated protein kinase (MAPK; p38, JNK ERK) signaling pathway [100]. Interestingly, in response to chronic cigarette smoke exposure MAPK signaling gets activated in skeletal muscles of rodents promoting UPS activation and muscle atrophy [101]. In addition other E3 ligases such as Nedd4, regulator of Notch1 signaling and VHL, regulator of HIF1-α/VEGF signaling pathway are reported to be elevated in the muscles of cachectic COPD patients and animal models further evidencing involvement of UPS in the development of pulmonary cachexia [65,79,83].

1.4.3. Muscle regeneration capacity and myonuclei turn-over

Adult skeletal muscle fibers are terminally differentiated, their nuclei are post mitotic and are thus, not able to replicate [102]. Each myonuclei is responsible for gene expression in its surrounding cytoplasm. Muscle regeneration after injury or during recovery from atrophy requires activation of local muscle stem cells (Satellite cells, SC), their subsequent proliferation followed by cell cycle exit and the execution of myogenic program [102-104]. Fusion of terminally differentiated muscle cells (myocytes) to damaged myofibers (myonuclear accretion) plays an essential role in the process of muscle repair [102-105]. The region of cytoplasm effectively controlled with an individual myonuclei is termed as myonuclear domain [77,105,106]. Loss of the myonuclei due apoptosis and alteration in myonuclear domain represent frequent feature of atrophied muscle [77,106]. Alteration in myonuclear domain and impaired skeletal muscle regenerative capacity are increasingly recognized as important factors involved in the development of muscle dysfunction and wasting in patients with COPD [37,83,107-111]. Skeletal muscles of cachectic COPD patients demonstrate evident signs of DNA fragmentation and myocytes apoptosis [112,113]. This is believed to adversely affect myonuclei accretion and increase the overall myonuclear domain contributing in turn towards fiber atrophy [92,111,114]. Though, immunohistochemical assessment of levels of active caspase-3, which is a reliable indicator of muscle cell apoptosis,
did not demonstrate differential expression or reliable positive staining COPD patients or matching controls [115], quantification of active caspase-3 using other methods such as western blot or ELISA has not been conducted yet. Moreover, levels of myogenic differentiation regulators such as MyoD and myogenin, which are essential for muscle regenerative capacity, are reported to be significantly decreased in patients with pulmonary cachexia [34,37,81,83,99,114]. In addition to this, evident impairment in regenerative capacity as well as satellite cell activation and ongoing repair process has been observed in diaphragm of COPD patients [52]. Impaired satellite cell function as a result of interference with their ability to proliferate, differentiate, or fuse ultimately results in the loss of skeletal muscle tissue; however mechanisms leading towards this event remain uninvestigated.

1.4.4. The role of myostatin in the regulation of muscle mass in COPD

Myostatin is a transforming growth factor-β family member that acts as a negative regulator of skeletal muscle mass [77,116]. It is expressed predominantly by skeletal muscle cells and released to the circulation wherefrom it acts on muscle tissue, by binding a cell-bound receptor called the activin type II receptor [116]. Myostatin was shown to induce muscle atrophy by inhibiting myogenesis, suppressing IGF-1/Akt pathway and activating UPS [77]. Increased circulatory levels of myostatin as well as myostatin transcript levels in skeletal muscles have been reported in cachectic COPD patients [83,108]. The mechanism by which myostatin induces skeletal muscle atrophy in COPD is still unknown, however increased myostatin levels correlate strongly with the increase in the UPS activity in skeletal muscles of patients with pulmonary cachexia [83].

1.4.5. Skeletal muscle capillarization is impaired in COPD. Molecular insight

Skeletal muscle tissue is characterized by tremendous plasticity [117]. In response to the increase in the workload such as during exercise training, muscle tissue undergoes vast morphological alterations as well as metabolic reprogramming favoring muscle hypertrophy [81,117-119]. These alterations involve increase in the fiber area, capillary number and oxidative capacity. In contrast, during muscle disuse, reverse chain of events is present [119]. The capillary supply is vital for nutrient and oxygen nourish-
ment of muscle fibers, waste removal, regulation of oxidative status and muscle mass maintenance [117]. In COPD, decrease in skeletal muscle capillarization as well as additional vascular impairments such as decrease in capillary to fiber contact and diffusion area are reported early in the disease onset and they worsen as the disease progresses [62,64-66]. Early vascular impairments appear to activate futile compensatory response and overexpression of pivotal promoters of muscle angiogenesis including hypoxia inducible factor 1-alpha (HIF1-α) and vascular endothelial growth factor (VEGF) on mRNA level which is absent in severe stages of the disease, with even significant depletion in VEGF protein levels. [65,120] In addition, von Hippel Lindau protein (VHL), negative regulator of HIF1-α/VEGF signaling is increased in skeletal muscles of COPD patients [65]. Interestingly, muscles of COPD patients exhibit impaired adaptive response to exercise hypoxia which is manifested by impaired VEGF accumulation and de novo capillarization [35]. This observation is extended to peripheral blood mononuclear cells of patients with COPD, reported to mount inadequate adaptive response to hypoxia challenge via still not elucidated mechanism [121].
HIF1-α acts as the main cellular oxygen sensor, and regulator of tissue angiogenesis [122,123]. Its regulation is kept under tight control and represents an oxygen dependant process [122]. In normoxic conditions, HIF1-alpha is maintained at low levels by hydroxylation at the proline residues 402 and/or 564 by the family of prolyl hydroxylases (PHD1-4) and subsequent ubiquitination by the von Hippel Lindau tumor suppressor protein (VHL) which facilitates its proteasomal degradation [124-128]. VHL, E3 ligase is part of a larger ubiquitination complex that includes Elongin-B, Elongin-C, Cul2, RBX1 (Ring-Box 1) and a ubiquitin-conjugating enzyme.
E2 (Ube2D1) which all play indispensable role in the HIF1-α regulation [128-130]. In contrast, hypoxia inactivates oxygen-dependent prolyl hydroxylases and VHL allowing HIF1-α to stabilize, dimerize with β subunit, translocate to nucleus and becomes transcriptionally active [122]. In the nucleus, HIF1 transcriptional complex interacts with cofactors such as CREB Binding Protein/p300 (CBP/p300) and the DNA polymerase II complex to bind to HREs (Hypoxia-Responsive Element) and activate transcription of over 300 target genes [122]. HIF1-target genes such as vascular endothelial growth factor (VEGF), glucose transporter-1 (Glut1), lactate dehydrogenase (LDH), Nitric Oxide Synthase (NOS) and erythropoietin (Epo) promote angiogenesis, glycolysis and cell survival [123,126,130].

1.5. Triggers and mechanisms of pulmonary cachexia. The story so far

Several local and systemic factors have been implicated in the etiopathology of pulmonary cachexia. These include chronic muscle disuse, cigarette smoking per se, increased oxidative/nitrosative stress, hypoxia, and systemic inflammation [32]. Additional factors such as nutrition, age and medication (glucocorticoids) might additionally contribute towards development of pulmonary cachexia, however they will not be discussed in this review [32].

1.5.1. Chronic disuse

Exercise limitation as well as generally inactive life style is a common feature in COPD. Chronic disuse elicits muscle remodeling having features somewhat similar to those observed in patients with COPD [81,119]. Thus, reduced proportions of type I fibers, attenuation of oxidative enzyme capacity and mitochondrial biogenesis, fiber atrophy, reduction of antioxidant enzyme levels and lower capillary density result are feature of both, COPD and muco-skeletal de-conditioning due sedentary life style [57,58,81,119,131,132]. These morphological, structural and biochemical abnormalities result in a significant reduction in the skeletal muscle strength and endurance. However, chronic disuse appears to affect predominantly type I muscle fibers, while in COPD atrophy of type II fibers type reflects pathological mechanisms additional to fiber type I atrophy and physical inactivity further affecting muscle performance [57,61,131].

1.5.2. Oxidative/nitrosative stress
Reactive oxygen species (ROS) arise as a byproduct during mitochondrial respiration, or from other oxidant producing systems in the muscle such as the xanthine oxidase system [133,134]. In reaction with the nitric oxide (NO), ROS produce reactive nitrogen species (RNS) [133,134]. Impairment or overwhelming tissue capacity for the clearance of reactive species leads towards development of oxidative/nitrosative stress [133,134]. In COPD, cachectic COPD patients exhibit greater muscle and systemic oxidative/nitrosative stress at rest and after exercise compared to non-cachectic patients and controls [99,135,136]. Furthermore, systemic as well as local oxidative stress is negatively correlated with FFM and muscle strength in cachectic patients [135,137]. In addition, increase in the mitochondrial ROS production is associated with activation of UPS and fiber atrophy in skeletal muscles of cachectic COPD patients [99]. Presence of the oxidative/nitrosative stress in the skeletal muscles of COPD patients is further evidenced by the reduction of basal anti-oxidant levels, attenuation of exercise-induced expression of anti-oxidant enzymes as well as increased H2O2 production by the muscle mitochondria [136,138]. Oxidative/nitrosative stress promotes muscle catabolism via myocytes apoptosis, inhibition of protein synthesis, suppression of myogenesis and activation of UPS [54,139,140], however direct mechanistic link between muscle wasting in COPD and presence of the oxidative/nitrosative stress remains to be established.

1.5.3. Systemic and/or local inflammation

Systemic and/or local inflammation is a common feature of COPD [5,19,141]. Hence, increased number of circulating inflammatory cells and elevated serum levels of C-reactive protein, fibrinogen, circulating leukocytes and pro-inflammatory cytokines, including tumor necrosis factor (TNF), interleukin-8 (IL8), interleukin-6 (IL6), interleukin-18 (IL18), soluble TNF receptors 55 (sTNF-R55) and 72 (sTNF-R75) have been reported in patients with COPD [7,19,37,72,78,81,142-152]. In addition, inflammatory responses were significantly augmented during episodes of acute exacerbations [146-148].

Inflammatory mediators have long been implicated in muscle atrophy and cachexia [118]. In particular, TNF was originally designated as ‘cachectin’ in the recognition of its catabolic actions [77,153]. However, potential involvement of TNF in the pathogenesis of pulmonary cachexia remains debatable [154]. While some studies report elevated circulatory as
well as skeletal muscle TNF levels (up to 5 fold increase), others fail to reproduce this, or even report opposite results [7,19,81,113,155]. More recent studies, our group found no evidence for inflammatory process in skeletal muscles from patients with COPD, despite presence of systemic inflammation [78]. The reason for this discrepancy is unknown; however, it was speculated that the difference in the specificity of ELISA kits previously used to measure TNF levels was questionable [154]. Nevertheless, recent results and usage of the newest generation of ELISA kits do not seem to support this hypothesis [7,19,37,59,150,151,156]. Interestingly, elevated circulatory TNF has been reported even in healthy long-term smokers, and the increase in the TNF appears to be positively correlated with the duration of the CS exposure [157-159]. More likely, the reasons behind these contradictory results are arising due to factors such as the use of relatively low numbers of patients, the presence or absence of hypoxemia, severe weight loss, exacerbation of pulmonary manifestation. Recent results appear to support presence of inflammatory process in skeletal muscles, reflected by inflammatory cell infiltration, increased binding activity of nuclear factor kappa B (NFkB) in severe COPD as well as elevated IL-18 levels in moderate to severe COPD patients [113,144,160]. The involvement of inflammatory mediators in skeletal muscle dysfunction is also suggested by the observation that systemic inflammation markers correlate with poor muscle contractile performance in COPD patients [146,147]. Thus, serum IL8 levels in COPD patients during acute exacerbation and serum IL6 and TNF in aged COPD patients correlate negatively with quadriceps muscle strength [146,147]. Moreover, low FEV1 values correlate with increased plasma levels of C-reactive protein and IL6 in severe COPD which appears to be associated with diminished limb muscle strength, reduced exercise endurance, poor health status and quality of life, independent of other factors such as age, sex, and smoking history [148,149].

Inflammatory cytokines, and TNF in particular are shown to promote muscle atrophy via activation of several proteolytic pathways [153]. These include ubiquitin proteolytic system (UPS), ER stress response and caspases activation as well as myocytes apoptosis [86,153,161,162]. Furthermore, TNF is reported to suppresses mitochondrial biogenesis and impairs muscle oxidative capacity [59,163]. Indeed, activation of these pathways and oxidative impairment in skeletal muscles of COPD patients has been demonstrated in several studies [58,67]. Though, clear mechanistic connection between TNF and muscle abnormalities in COPD is still lacking.
The cellular origins of pro-inflammatory cytokines in the serum of stable COPD patients remain unclear. According one hypothesis, their source might be the “spill-over” from the lungs, where intense inflammatory processes develop in the vasculature, parenchyma and airways. However, no direct correlations have been found between sputum and plasma concentrations of diverse pro-inflammatory cytokines have been found [145]. This suggests that organs other than the lungs such as peripheral muscles as well as, diaphragm and intercostal muscles might contribute to elevated levels of systemic inflammatory mediators. This is supported by the fact that strenuous resistive breathing and whole body exercise in healthy humans induce significant elevations of plasma pro-inflammatory cytokine levels, including IL6, IL-1β, and TNFα [164,165]. The fact that the ventilatory muscles could be a source of systemic inflammation is further supported in an animal model of inspiratory resistive loading, where increased work of breathing significantly upregulates IL6, IL1β and TNFα expressions within the diaphragm and a recent study which has confirmed that TNFα and IL6 levels are significantly elevated in the intercostal muscles of COPD patients [166,167].

1.5.4. Hypoxia/Hypoxaemia

As the COPD severity increases, mismatch between ventilatory function and perfusion causes significant alveolar hypoxia and consequential decrease in the blood oxygen saturation (hypoxaemia) [168,169]. This may be exacerbated by sleep or exercise [169]. Uncorrected chronic hypoxemia is associated with the development of systemic features of COPD, including pulmonary hypertension, secondary polycythemia, systemic inflammation, and skeletal muscle dysfunction [169]. In healthy subjects, chronic hypoxia was shown to decrease muscle strength and endurance, promote muscle atrophy and attenuate mitochondrial Krebs cycle enzyme activity [170,171]. Additionally, the proportions of type I fibers in quadriceps muscles of hypoxemic COPD patients vs non-hypoxemic COPD patients were significantly lower [60]. Furthermore, a positive correlation between the arterial partial pressure of oxygen (PaO2) and percentage of type I fibers in the vastus lateralis muscle was reported in patients with COPD [47,172]. Hypoxaemia was speculated to additionally contribute to the muscle wasting in COPD by decreasing anabolic hormone levels and increasing pro-inflammatory cytokine levels as well as via generation of ROS, and oxidative stress [173-175].
Figure 5. Hypothetical triggers and mechanisms mediating pathogenesis of pulmonary cachexia

1.5.5. Cigarette smoking per se

Chronic smoking is the main risk factor underlying COPD pathogenesis [12,176]; however, the potential effects of smoking on skeletal muscle function remain unclear. Chronic cigarette smoking per se appears to elicit skeletal muscle abnormalities associated with COPD such as muscle fiber atrophy (type I and II), fiber type re-composition as well as reduction in peripheral muscle strength and endurance even prior to COPD development [72,177,178]. Furthermore, long-term cigarette smoking was shown to impair muscle metabolism, inhibit protein synthesis and reduce skeletal muscle oxidative capacity [72]. In addition, elevated expression of atrogin-1 and myostatin has been reported in long-term smokers [72].
1.6. Chronic cigarette smoking per se elicits skeletal muscle abnormalities associated with COPD. Lessons learned from CS-exposed animal models.

In animal models of COPD, adverse effects of cigarette smoke exposure on skeletal muscle morphology and function appear to be more evident [163,179-181]. In addition to the development of emphysema, chronic exposure of rodents to CS results in the systemic features that closely resemble the early signs of the extra-pulmonary manifestations observed in patients with COPD [163,179-181]. Thus, exposures of C57BL/6 mice to CS during 6 months lead to the marked increase in the circulatory levels of the pro-inflammatory cytokine TNF and a chemokine eotaxin [163,179,180,182]. Levels of the pro-inflammatory markers, IL-1β, -3, -17, and RANTES were however, elevated to the lesser extent [179]. Moreover, significant role of the TNF in the development of emphysema as well loss of FFM has been demonstrated in TNF receptor 2 (TNF-R75) KO mice after cigarette smoke exposure [162]. In addition, elevated circulatory TNF has been associated with muscle wasting and dysfunction in CS-exposed mice [163], while disturbance in muscle cell homeostasis has been attributed at least partially to the increased local muscle TNF levels [180]. Chronic exposure of rodents to cigarette smoke attenuated muscle anabolism via suppression of Akt signaling pathway, promoted muscle catabolism via activation of UPS and caused fiber atrophy (type I and type IIa) as well as fiber type re-composition shifting muscles towards glycolytic profile [163,179,180,183]. In analogy to COPD patients, activation of UPS in skeletal muscles of animal models was reported to involve activation of MAPK signaling and increased p38, JNK and ERK phosphorylation [101,180]. Furthermore, CS elicited systemic inflammatory response as well as systemic and local oxidative/nitrosative stress in AKR/J mice, as well as an oxidative damage to vital muscle proteins, fiber atrophy and muscle dysfunction [181]. Chronic cigarette smoke exposure further demonstrated to suppress angiogenesis and cause capillary rarefaction in murine models of COPD, which affects more robustly oxidative muscles, and in the lesser extent glycolytic muscles [163,184]. Interestingly, smoking cessation appears to activate a pro-angiogenic state in the skeletal muscles of rodents and elevates muscle VEGF levels, further implying that CS represents potent inhibitor of muscle angiogenesis as suggested in COPD [180]. In addition to this, activity of mitochondrial citrate synthase and beta-hydroxyacyl CoA dehydrogenase in skeletal muscles was report-
ed to be adversely affected by the CS suggesting that CS suppresses mito-
chondrial biogenesis as reflected by lower values of PGC-1 [163,179].

Despite tremendous advantages given by utilization of rodent models in
studies investigating CS role in the pathogenesis of primary disease as well
as systemic manifestations such as cachexia and muscle dysfunction cer-
tain warrants need to be taken into consideration. First of all there is sig-
nificant difference between upper respiratory tract of rodents and humans
which might reflect into different pathological features of the primary
disease as well as systemic manifestations [185]. In difference to patients,
rodents exposed to CS do not develop chronic bronchitis but exclusively
emphysema [185,186]. Furthermore, rodent models develop relatively
mild form of the primary disease which could be closest to the type I of
the primary disease according the GOLD classification [185,186]. Moreo-
ver, the CS dosage/body mass and treatment duration utilized in published
studies is still questionable regarding the proximity to the reality in human
patients. Additionally, genetic susceptibility to CS-induced skeletal muscle
abnormalities in different mice strain has not been studied up to date. The
same is valid when it comes to studying effects of emphysema severity and
muscle alterations.
2. METHODOLOGY

2.1. Cell lines and cell culturing

Murine skeletal muscle myoblasts (Figure 5), C2C12 (Sigma-Aldrich, Germany) were cultured in Dulbecco’s modified Eagle medium (PAA, Austria) supplemented with 10% fetal bovine serum (PAA, Austria), 2mM L-glutamine (Life Technologies, Sweden), and 0.1% PEST (50UI/ml penicillin and 50µg/ml streptomycin, Life Technologies, Sweden) and incubated at 37°C (5% CO2, 21% O2 and 74% N2) in the CO2 incubator (Binder, Germany). In all experiments prior to treatment, C2C12 myoblasts were seeded into collagen coated plates and induced to differentiate when reached 80%-90% confluence by shifting C2C12 to DMEM containing 2% horse serum, 1mM L-glutamine (Life Technologies, Sweden), and 0.1% PEST (Life Technologies, Sweden). C2C12 myocytes were considered fully differentiated 96h after induction of differentiation.

2.2. Hypoxia exposure, TNF and tunicamycin stimulation of C2C12

Fully differentiated C2C12 were treated with different concentrations of TNF (Peprotech, Israel) and maintained at normal oxygen conditions or deprived for oxygen (5% CO2, 1% O2 and 94% N2) at specific time points. In Study IV C2C12 were treated with 5µg/ml tunicamycin in order to induce endoplasmic reticulum stress response. Hypoxia experiments have been performed using hypoxia incubator (Binder, Germany) capable to finely regulate oxygen levels in the range from 0.2%±0.2% to 98%±0.2%. Controls have been maintained in the redundant CO2 incubator (Binder, Germany) under identical conditions. No significant cell death was observed in response to TNF and tunicamycin treatment or hypoxia exposure.
2.3. Transfection with siRNA

Prior to transfection, fully differentiated myocytes were subjected to a 6 hr pre-starvation in DMEM supplemented with 0.1% horse serum. Four different siRNA duplexes were used, targeting different regions of the USP19 mRNA (SI01463315, SI01463322, SI01463329, SI01463336; Qiagen, Hamburg, Germany). All four duplexes were used in all assays at equal concentrations. Transfection was conducted in an antibiotic-free medium with total siRNA concentrations of 10-200nM, using Lipofectamine 2000 reagent (Life Technologies-Invitrogen, Stockholm, Sweden) as per manufacturers instruction. After 5 hrs, supplemented DMEM was added, to give a total concentration of 2% horse serum and 1mM L-glutamine. Treatment was terminated after 48 hours.
2.4. 129 SvJ mice

The 129/SvJ mice (Jackson Laboratory, Bar Harbor, ME) were bred and maintained under specific, pathogen free conditions involving 12 hours dark/light cycles inside adequate vivarium facilities at the University of Rochester, USA. 3R4F research cigarettes (University of Kentucky, Lexington, KY, USA) were used to generate a mixture of sidestream smoke (89%) and mainstream smoke (11%) by a Teague smoking machine (Model TE-10, Teague Enterprises, Woodland, CA) at a concentration of ~100 mg TPM/m3 to avoid the possible toxicity to mice at a high concentration of CS (n=6) [187,188]. The level of carbon monoxide in the chamber was 350 ppm. The 129/SvJ mice (8-10 weeks old, 22-25 g body weight) received 5 h exposures per day, 5 days/week for 6 months, and were sacrificed 24 h after the last CS exposure. Control mice (n=8) were exposed to filtered air in an identical chamber according to the same protocol described for CS exposure. All animal procedures described in this study were approved by the Animal Research Committee of the University of Rochester, USA.

2.5. Muscle excision and post-excision handling

Animals were killed 24 h after the last CS exposure. Mice were anesthetized using 100 mg/kg pentobarbital sodium (Abbot Laboratories, Abbot Park, Illinois). Gastrocnemius muscle specimens (~150 mg) in both legs were dissected, cleaned for fat and connective tissue, placed into sealed vials, snap frozen in liquid nitrogen, and stored at −80°C until analysis.

2.6. Total RNA extraction and cDNA synthesis

Total RNA from C2C12 myocytes was extracted using the Total RNA Kit I (Omega Bio-tek, Norcross, UK). RNA was quantified using a Nanodrop Spectrophotometer (ND-1000; Thermo Fisher Scientific, Sweden). 1 µg RNA was reversed transcribed with a High-Capacity cDNA Reverse Transcription Kit (Life Technologies, Sweden). The reaction was performed according to the manufacturer’s instructions using Uno Thermoblock Thermal Cycler (Biometra, Germany).

Muscle specimens cut in pieces, snap frozen in the liquid nitrogen and disrupted using Micro-Dismembrator II (B Braun, Melsungen, Germany). Total RNA was isolated from the obtained homogenized material with the
RNEasy Fibrous Tissue Mini Kit (Qiagen, Valencia, California, USA) according to the instructions specified by the manufacturer. RNA was quantified by the Nanovue Plus spectrophotometer (GE Healthcare, UK). Total RNA (0.75 μg) was reverse-transcribed to cDNA by using SuperScript II first-strand synthesis kit (Invitrogen, Carlsbad, California, USA) following detailed instructions described by the manufacturer. The reaction was performed according to the manufacturer’s instructions using Uno Thermoblock Thermal Cycler (Biometra, Germany).

2.7. Polymerase chain reaction (PCR) analysis

cDNA synthesis from total RNA was performed as described above. To amplify XBP1 mRNA, PCR was performed for 35 cycles (heating DNA at 95 for 2 min, 95°C for 15 s; 62.5°C for 30 s and 68°C for 1 min) using the PCR primers 5’- ACACGCTTGGAATGGACAC-3’ and 5’- CCATGGGAAGATGTTCTGGG’ and Kod Hot DNA polymerase master mix (Novagen, UK) using RT-PCR Detection System 2700 (Applied Biosystems). The fragments representing spliced and unspliced XBP1 were visualized on 2.5% agarose gels with ethidium bromide staining.

2.8. Quantitative RT PCR analysis

Quantitative RT-PCR gene expression analysis was performed on ABI Prism Sequence Detection System 7900HT (PE Applied Biosystems, Foster City, California, USA). Genes targeted in the expression analysis were provided as Assay-on-demand by Applied Biosystems (Foster City, California, USA). The probes were labeled using FAM as the reporter dye and TAMRA as the quencher dye. Each sample was analyzed in duplicate under the following conditions: 2 min at 50 °C, 10 min at 95 °C, 15s at 95 °C and 1 min at 60 °C. PCR amplifications were correlated against a standard curve. Reactions were performed in the MicroAmp optical 96-well reaction plates (PE Applied Biosystems, Foster City, California, USA).

2.9. Western blot analysis

The excised muscles were homogenized in the ice cold lysis buffer (Santa Cruz Biotechnology, CA, USA) containing a protease inhibitor cocktail (Sigma Aldrich, Germany). Whole cell lysates from C2C12 were prepared using radioimmunoprecipitation (RIPA) buffer (150mM NaCl, 1% NP-
40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), and 50nM Tris, pH 8.0) with a cocktail of protease inhibitors (Sigma-Aldrich, Germany). In the Study IV, formalin fixed paraffin embedded tissue blocks were deparaffinized in xylene and rehydrated in gradient ethanol and antigen retrieval was performed in tris-based buffer (20 mM Tris-HCl buffer (pH 9) containing 2% SDS) for 20 min in 100°C and for 2 hours on 80°C. After retrieval process 50μl of RIPA (150mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), and 50nM Tris, pH 8.0) buffer was added and centrifuged for 10 minutes at 10000 g on 4°C in order to extract total protein. Total protein concentration was either measured using the Micro Bicinchoninic Acid (BCA) Protein Assay Kit (Thermo Fisher Scientific, Sweden) - microplate procedure or Bradford assay (Sigma Aldrich, Germany). Equal amounts of total protein (20 µg-100 μg) were separated under reducing conditions using 7.5%, 10% and 12% SDS page and transferred onto PVDF membrane (Amersham, UK) in a transblot electrophoretic transfer cell (Bio-Rad Laboratories, USA). Membranes were probed overnight at 4°C using:

Study I: rabbit polyclonal anti-HIF-1α (Novus Biologicals, UK) in 1:1,000 dilution, rabbit polyclonal anti-VHL (Cell Signaling Technology, Beverly, MA, USA) in 1:1,000 dilution, rabbit polyclonal anti-PHD2 in 1:1,000 dilution (Santa Cruz Biotechnology), rabbit polyclonal anti-VEGF in 1:1,000 dilution (Santa Cruz Biotechnology), rabbit polyclonal anti-UBE2D1 in 1:1,000 dilution (Abnova, Taiwan), and rabbit polyclonal anti-α-tubulin in 1:10,000 (Abnova) used as loading control. Presence of nuclear protein yield was assessed and confirmed using rabbit polyclonal anti-TBP 1:1,000 (Abnova) used as nucleolar loading control (unpublished results).

Study II: rabbit polyclonal anti-Jarid2 (Abnova, Taiwan) in 1:1000 dilution, mouse monoclonal anti ZNF496 in 1:1000 dilution (Abnova, Taiwan), rabbit polyclonal anti-Notch1 (Abnova, Taiwan) in 1:2000 dilution, mouse polyclonal anti-myogenin in 1:2000 dilution (Santa Cruz Biotechnology, Santa Cruz, CA, USA), mouse monoclonal anti-Pax7 in 1:50 dilution (Supernatant, DSHB), and rabbit polyclonal anti-TBP in 1:1000 (Abnova, Taiwan) used as loading control.

Study III: rabbit polyclonal anti-HIF1-α in 1:1000 dilution,(Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit polyclonal anti-VHL diluted 1:1000 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit polyclonal anti-VEGFA diluted 1:1000 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit polyclonal anti-prolyl hydroxylase 2 (PHD2)
diluted 1:2000 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit polyclonal anti-Ube2D1 diluted 1:1000 (Abnova, Taiwan), rabbit polyclonal anti-α-tubulin diluted 1:10000 (Abnova, Taiwan), rabbit polyclonal anti-Ube1 diluted 1:1000 (Abnova, Taiwan) and rabbit polyclonal anti-ubiquitin (Sigma Aldrich, Germany).

Study IV: rabbit polyclonal anti-Usp19 (Novus Biologicals, UK) in 1:1000 dilution, rabbit monoclonal anti-pPERK in 1:1000 dilution (Sigma Aldrich, Germany), rabbit polyclonal anti-Caspase12 in 1:2000 dilution (Santa Cruz Biotechnology, Santa Cruz, CA, USA) rabbit monoclonal anti-cleaved-Caspase 3 (Cell Signaling, MA, USA) and rabbit polyclonal anti-tubulin in 1:10000 (Abnova, Taiwan).

Membranes were developed using an enhanced chemiluminescence system (Amersham, UK) and exposed to Hyperfilm enhanced chemiluminescence (Amersham, UK). Densitometric analysis was performed using the NIH software package ImageJ (ImageJ 1.46j, NIH, Bethesda, MD, USA)

2.10. Immunostainings

Serial, 5 µm thick, gastrocnemius muscle transverse sections, were cut at -22°C using Leica CM1850 cryostat attached to positively charged glass slides (Superfrost, Menzel Gläser, Braunschweig, Germany) or purchased (10µm thick , AMSBio, UK). Immunostaining was performed as follows.

Study I: Muscle cross-sections were briefly submerged in 1% BSA blocking solution and incubated overnight with 1:100 anti-CD31 (MO823; Dako, Glostrup, Denmark). CD31 signal was revealed with DAB. In addition, H&E staining was performed.

Study II: Muscle cross-sections were immunostained with anti-Pax7 (1:5, supernatant, Developmental Studies Hybridoma Bank, DSHB, University of Iowa, IA, USA), anti-Laminin (Sigma Aldrich, Germany) and anti-Jarid2 (1:100, Abnova, Taiwan). Incubation was performed overnight at 4 °C after antigen retrieval with 100 mM sodium citrate and blocking first with a solution containing 10% goat serum diluted in 1% BSA, 0.3% Triton X-100 in PBS and then with anti-mouse AffiniPure Fab fragment (Jackson Immunoresearch, 1:10) to avoid unspecific binding. TRITC-conjugated anti-rabbit (Jackson Immunoresearch, USA) and FITC-conjugated anti-mouse (Jackson Immunoresearch) secondary antibodies were used to reveal Jarid2, Laminin and Pax7 expression signal. Nuclei were visualized by counterstaining with DAPI.
Study III: Immunohistochemical slides containing FFPE tibialis anterior cross-sections were purchased from (AMSBio, UK). Sections were deparaffinized in xylene and rehydrated in the serial dilutions of ethanol after what the antigen retrieval procedure in Tris-EDTA (1M Tris-HCL, 0.1M EDTA, pH9) buffer has been performed using microwave for the period of 20 min. Following this procedure sections were incubated in the 0.3% Triton X-100 (Applichem, UK) solution in PBS for 30 minutes and then incubated for 1h in the blocking solution containing 1% BSA and 10% goat serum. Thereafter sections were incubated with primary antibody raised against VHL, PHD2 (1:100 dilution, Santa Cruz Biotechnology, CA, USA), Ube2D1, Ube1 (1:100 dilution, rabbit polyclonal, Abnova, Taiwan) and Ub (1:100 dilution, rabbit polyclonal, Sigma Aldrich, Germany), overnight at 4oC. Primary antibody was detected after incubation with FITC-labeled secondary antibody raised in goat and directed against rabbit IgG1 (H+L) for 1h on room temperature. DAPI was used to visualize the nuclei in the muscle specimens. As the negative control, primary antibody was omitted in the reaction mix.

Immunocytochemical analysis of VHL expression in C2C12 myocytes was performed in 8 chambers immunocytochemistry slides (Sarstedt, Sweden). Differentiated C2C12 have been fixed with 4% paraformaldehyde for 15 min and permobilized with 0.3% Triton X-100 for 30 min. After 1h blocking with 1% BSA, anti-VHL (Santa Cruz, CA, USA) was added in 1:100 dilution and sections were incubated overnight at 4oC. VHL signal was revealed using secondary, FITC-labeled anti-rabbit antibody (Jackson Immunoresearch, PA, USA) after 1h incubation on the room temperature. Nuclei were visualized with DAPI.

Study IV: Immunostaining with anti-Usp19 (1:100, Novus Biologicals, UK), was performed overnight at 4 °C after antigen retrieval with Tris-EDTA buffer (1M Tris-HCL, 0.1M EDTA, pH9) and blocking first with a solution containing 10% goat serum (diluted in 1% BSA, 0.3% Triton X-100 in PBS). FITC-conjugated goat anti-rabbit (1:300; Jackson Immunoresearch, PA, USA) secondary antibody was used to reveal Usp19 expression signal. Nuclei were visualized by counterstaining with DAPI (VectorLab, UK). As a negative control we have omitted primary antibody and incubated directly with secondary antibody in the 1/300 dilution. No false positive signal was detected.

In all studies, images were acquired with a light microscope (Olympus BX60) connected to a computerized image system (Cell Images) and edited
using the Photoshop CS.5.1. Reported figures are representatives of all examined fields.

2.11. Assessment of muscle capillarity

Capillaries were identified by staining with monoclonal antibody detecting CD31 antigen (1:100 dilution, MO823; Dako, Glostrup, Denmark) and diaminobenzidine. Capillary analysis was performed using light microscope (Olympus BX60) connected to a computerized image system (Cell Images). Capillary-to-fiber ratio parameter was determined by analyzing number of capillaries surrounding more than 200 individual fibers per section. Briefly, digital images of 4-6 randomly chosen cross-sectional areas were taken at ×40 magnification, area perimeter outlined using free-hand selection tool, and total number of fibers and surrounding capillaries was determined. Quality of cross-sections was assessed by measuring roundness index of individual fibers with the aid of an image morphometry program (ImageJ 1.32j). Only fibers entirely included in the outlined area and with the roundness index ≥0.8 have been included in the analysis.

2.12. Assessment of muscle morphology and mean fiber cross-sectional area

Mean fiber cross-sectional area (CSA) has been determined in digital photographs taken from each H&E section at x10 magnification using a Magnafire digital camera with 30-ms exposure and software (Optronics Inc., Galena, CA, USA). With the aid of an image morphometry program (ImageJ 1.46j, NIH, Bethesda, MD, USA), the outline of individual fibers were traced and parameters such as fiber area were expressed in μm². The smallest and the biggest fiber diameters in addition to the fiber cross-sectional roundness index were determined for each individual fiber. The roundness index (recorded as a value between 0 and 1) is the ratio of the cell area relative to the area of a circle that fully enclosed that cell. Circular cells have a value approaching 1 while non-circular cells have smaller values. The cut-point for roundness index that was accepted for inclusion in CSA evaluation was ≥0.8. More than 300 individual fibers per cross-section have been included in the CSA evaluation.

The number of fibers with centralized nuclei and number of myonuclei per individual fiber were determined in slides stained with anti-Laminin which is used to determine fiber border (lamina). DAPI staining was used
to visualize peripheral and central nuclei. A series of images were taken at 20x magnification from a minimum of 5 fields within the cross section in each slide (more than 300 fibers per slide). The images were loaded into the ImageJ 1.46 software and enlarged. A cell counter plugin was utilized to count the number of fibers, myonuclei and fibers with central nuclei. The same parameters were also assessed using fluorescent microscopy at 100x magnification (Olympus BX60). Double-blind analysis was performed and the mean of two measurements were included in the manuscript.

2.13. Determination of exercise capacity in 129 SvJ mice

Exercise endurance in mice was measured using a motorized rodent treadmill with an electric grid at the rear of the treadmill (Columbus Instruments, Columbus, OH). Exercise tolerance was determined by the run duration (min) and run distance in meters (calculated from the run time and speed of the treadmill). The mice were placed on the treadmill and allowed to adapt to the surroundings for 3–5 min before starting the exercise. The treadmill was started at a speed of 8.5 m/min with a 0° incline. After 9 min, the speed and incline were raised to 10 m/min and 5°, respectively. The speed was increased by 2.5 m/min every 3 min to a maximum of 40 m/min, and the incline slope was increased by 5° every 9 min to a maximum of 15°. The exercise test continued until the mouse was exhausted (defined as the inability of the animal to maintain the running exercise despite repeated contact with the electric grid).

2.14. In-silico promoter analysis

In the absence of experimentally validated promoter sequence for murine Usp19 gene, the most probable promoter sequence was determined using Proscan, version 1.7, Promoter Prediction 2.0 and MatInspector 2.3. Potential transcription factor binding sites have been determined using TFSEARCH 1.3. software package. Threshold was set to 0.9.

2.15. Statistical Analysis

Normality of the data distribution was assessed using Anders-Darling equation. Where data was normally distributed, statistical significance was assessed using independent student T test and the results were represented
as mean ± SD. Where testing statistical difference between more than 2 means, ANOVA was implemented. In the case where data was not normally distributed statistical significance was tested using Mann-Whitney test and data represented as median with IQR. Spearman’s rank correlation coefficient (r) was calculated to determine the relationship among the variables. Differences were considered significant at P< 0.05. Statistical analysis was performed with SPSS v.16 Statistics Software.
3. AIMS OF THE THESIS

Pulmonary cachexia is characterized by an unknown pathogenesis and therapeutic strategies with limited or no success. Thus, understanding chain of events leading towards muscle wasting and dysfunction in this condition, especially from the molecular perspective, could help identify novel therapeutic targets capable to halt and reverse progression of this highly debilitating syndrome.

The specific aims of this thesis were:

1) Examining effects of chronic CS exposure on skeletal muscle morphology, capillarization and function.
2) Further understanding molecular triggers, mediators and mechanisms underlying morphological, vascular and functional abnormalities of peripheral musculature in response to CS.
3) Investigate the role of UPS in the development of CS-induced muscle abnormalities

4. RESULTS AND DISCUSSION


Chronic cigarette smoke (CS) exposure elicits skeletal muscle abnormalities in rodents [163,179-181]. These abnormalities highly resemble those observed in COPD patients with cachexia complication and include metabolic alterations such as reduction in oxidative capacity as well as morphological changes including fiber atrophy and decreased capillarization [163,179,180]. Molecular mechanisms mediating decrease in skeletal muscle capillarization in response to CS remain largely unknown. The objective of the present investigation was to assess effects of chronic CS exposure on the transduction of the hypoxia-angiogenic signal and capillarization in the skeletal muscle tissue. For this purpose, we have exposed 129/Sv] mice to CS for 6 months. This strain was previously demonstrated to exhibit higher intrinsic resistance to CS-induced lung emphysema com-
pared with other susceptible strains such as C57BL/6J and DBA/2 [188-190].

As the result of 6 months of CS exposure a significant decrease in skeletal muscle capillarization was observed in our murine model. Capillary-per-fiber parameter was chosen to be determined since it represents one of the most reliable parameters assessing muscle capillarity [191]. We have chosen to focus on glycolytic, gastrocnemius muscle rather than previously investigated soleus muscle [163], due several reasons. First of all, murine gastrocnemius is the most widely studied muscle in the terms of assessing muscle capillarization as well as molecular mechanisms underlying maintenance of muscle capillary network [192-195]. Moreover, hypoxia-angiogenesis signaling cascade was studied in depth in the gastrocnemius muscle specific knock-down models for VEGF gene as well as HIF1-α gene and in relation to the maintenance of muscle mass and muscle performance [192,194,196]. Second, in COPD, reduced capillarization has been reported in glycolytic muscles such as quadriceps femoris, vastus lateralis and tibialis anterior [62,64,65, 66]. In difference to the previously published report demonstrating decrease in capillarization confined predominantly to the oxidative muscles such as soleus [163], we have shown that CS exposure also decreases skeletal muscle capillarization in glycolytic muscles such as gastrocnemius. However, it is important to emphasize significantly longer time of exposure to CS in our experimental model (24 weeks vs 8 weeks) [163].

In addition to the significant decrease in skeletal muscle capillarization, crucial molecular mediators of muscle angiogenesis were differentially expressed in CS-exposed mice. Thus, the most important finding represents up-regulation of von Hippel Lindau tumor suppressor protein (VHL) as well as partner E2 ubiquitin conjugating enzyme (Ube2D1) and prolyl hydroxylase 2 (PHD2) which extended our report of VHL overexpression in the muscles of COPD patients [65]. These partner proteins are involved in the negative regulation of HIF/VEGF signaling and inhibition of tissue angiogenesis [125,128,130]. Underlying mechanism involves hydroxylation of HIF1-α protein in the position Lys564 and Lys402 which facilitates HIF1-α recognition by the VHL E3 ligase complex, its polyubiquitination and subsequent proteosomal degradation [125,128,130]. Indeed, we have observed decreased HIF1-α protein expression levels which were further reflected in the reduction in VEGF expression on the protein level and associated capillary rarefaction. Intriguingly, even though trend could be observed we have not detected statistically significant difference in the
VEGF transcript levels as expected due the role of HIF1-α in the transcriptional regulation of VEGF. The explanation for this apparent discrepancy might be presence of systemic or local hypoxia which is known to potently stabilize VEGF transcript levels, thus they rarely mirror transcription rate in the tissue [163,197,198]. Presence of hypoxia is further suggested by the mild, but statistically significant over-expression of HIF1-α transcript in the muscles of the CS-exposed animals. This is in line with the reports of HIF1-α transcript over-expression in hypoxic muscle associated with chronic ischemia and physical exercise [199,200]. More robust decrease in the VEGF protein expression relative to the mRNA levels has been previously reported and might be a consequence of the established role of VHL in the regulation of VEGF expression on the protein level as well [198]. Moreover, similar alterations in HIF1-α/VEGF signaling in skeletal muscles of cachectic COPD patients have been reported by our group [65]. Thus, VHL overexpression has been accompanied with decreased capillarization in tibialis anterior of mild to moderate COPD patients despite mild overexpression of HIF1-α and VEGF transcripts [65]. This overexpression of pivotal promoters of muscle angiogenesis including HIF1-α and VEGF on mRNA level might arise as a futile response of ischemic muscle tissue to hypoxia insult which is blunted by the VHL overexpression.

Decrease in the skeletal muscle capillarization and alterations in HIF1-α/VEGF signaling in our model are associated with the significant decrease in gastrocnemius cross-sectional area and fiber atrophy. This is not surprising having in mind importance of vasculature network in the maintenance of muscle mass and fiber nourishing, oxygen supply and metabolic waste removal [117]. Furthermore, depletion of HIF1-α levels might cause a significant metabolic reprogramming of myocytes due to crucial role of HIF1-α in the transcriptional regulation of numerous metabolic genes [122]. This in turn might contribute to the energetic impairment and depression of muscle strength and endurance, such as observed in this study. In agreement with gastrocnemius-specific HIF1-α and VEGF knockout murine models [192,196] our model demonstrated significant impairment in exercise capacity as manifested as shorter run time and distance in the treadmill exercise. It is important to emphasize rather mild development of emphysema in our model suggesting mild decrease in lung function and contribution of ventilatory insufficiency to the exercise intolerance observed in this study [187].

Potentially, significant drawback of this study was not measuring systemic effects of CS exposure such as presence of systemic inflammation
and levels of pro-inflammatory cytokines such as TNF, IL6 and IL1-β which have been implicated in pathogenesis of skeletal muscle abnormalities in COPD and animal models [32,179,180]. Furthermore presence of carboxyhemoglobin as well as partial pO2 and oxygen saturation might provide evidence of presence of hypoxaemia in our model as well as possible connections to muscle alterations.

4.2. Chronic cigarette smoke exposure impairs skeletal muscle regenerative capacity in murine COPD/emphysema model

The preservation of skeletal muscle mass is central to maintaining mobility and quality of life and also impacts on our capacity to recover from illness [201]. Skeletal muscle tissue has a remarkable capacity to regenerate what plays a crucial role in the maintenance of muscle mass and the ability to elicit adaptive changes in response to increased functional demands [102,201]. Skeletal muscle regeneration reconstitutes most aspects of the developmental myogenesis and represents highly regulated process in which the central role play muscle satellite cells (SC), the core of muscle regenerative potential [103,202]. In response to diverse stimuli such as mechanical injury as well as during recovery from fiber atrophy satellite cell gets activated, re-enters the cell cycle and execute terminal myogenic program in order to fuse to damaged fiber and repair it [102]. Impairment in skeletal muscle regenerative capacity has been observed in patients with PCS and animal models of the disease, and might play an important role in the pathogenesis of pulmonary cachexia [81,83,99,107,111,203]. Decreased expression of putative regulators of myogenesis such as myogenic regulatory factor 5 (MYF5), MyoD and myogenin have been demonstrated in the muscles of cachectic COPD patients [81,83,99,114]. However, molecular mechanisms underlying deterioration of muscle regenerative capacity as well as potential triggers of this event have not been previously studied. This study was designed in order to investigate whether long-term cigarette smoke exposure induces alterations in the expression of the pivotal regulators of myogenesis and affects skeletal muscle regenerative capacity. For this purpose 129 SvJ mice have been exposed to cigarette smoke (CS) during 6 months.

In line with previous report by Nakatani et al. which demonstrated decrease in the fiber type I and II CSA in CS-exposed rats [183], the results of this study demonstrated distinct morphological abnormalities in skeletal muscles of CS-exposed animals including significant decrease in the gas-
trocnemius cross-sectional area and prominent fiber size variation, suggesting atrophy of specific fiber types. However, assessment of the fiber type distribution and association atrophic changes with the particular fiber type was not conducted due solely availability of FFPE sections which are incompatible with mATPase immunohistochemical method or availability of adequate antibodies. In addition, we have detected increased number of fibers with central nuclei as well as reduced number of myonuclei in muscles of CS-exposed mice. These pathological findings suggested the presence of muscle damage as well as ongoing repair process in response to CS exposure. Similar morphological changes were recently reported in the diaphragm and muscles of COPD patients [107,111,204]. In order to assess whether decreased myonuclear accretion in CS-exposed animals is associated with the suppression of myogenesis we have measured expression of crucial regulators of myogenesis in our animal model. Myonuclear accretion is a part of terminal myogenesis and fusion of myogenic precursor cells to damaged fibers is a process mainly regulated by myogenin protein [102]. In agreement with previous reports [81, 83, 9, 205], reduced in myonuclear accretion was associated with reduction in myogenin levels in the muscles of CS-exposed mice. Exact mechanisms leading towards this impairment remain elusive. One likely explanation is that myogenin protein is additionally destabilized in the skeletal muscles of the current model by activated VHL ubiquitination machinery, as previously reported [206]. Moreover, reduced HIF1-α signaling is known to adversely affect terminal myogenesis [244].

In addition to the impairment in terminal myogenesis, our data demonstrates evidence of activation of satellite cells and an ongoing repair process in skeletal muscles of CS-exposed animals. This is reflected by an increased expression of activated Notch1, pivotal regulator of developmental and regenerative myogenesis [103,207,208]. Increase in Notch1 signaling due damage stimuli activates SC and stimulates their proliferation via enhanced transcription of Pax7 regulatory factor [208]. In line with this, we have observed elevation of Pax7 transcript levels in CS exposed animals. Thus, chronic cigarette smoke exposure induced muscle damage as well as concurrent repair process through activation of SC and muscle regeneration pathways, which is in agreement with observations in intercostals and limb muscles of COPD patients [107, 111]. Furthermore, our data suggests the possibility that chronic exposure to CS can lead towards exhauster of limb muscle SC pool and regenerative potential as
observed in severe COPD [111]. In support to the existence of an ongoing repair process in gastrocnemius of CS exposed mice we have found elevated levels of embryonic myosin heavy chain Myh3 which is transiently expressed exclusively in regenerating fibers [102,103,209,210]. Presence of concurrent damage and repair process in the gastrocnemius muscles was further evidenced by the presence of inflammatory cell infiltration in the muscles of CS-exposed animals. Of special interest was detection of allograft inflammatory factor (AIF1) expressing macrophages exclusively in the muscles of CS-exposed mice. In accordance with the findings in the Study I, presence of AIF1 positive macrophages has been previously associated with the vascular impairments in the skeletal muscle [211].

Activation of satellite cell and execution of myogenesis is a highly regulated, spatio-temporally controlled process in which epigenetic regulation is heavily implicated [202,212-216]. This prompted us to investigate whether CS exposure interferes with epigenetic regulation of myogenesis in our model. As a suitable target we have decided to focus on Jarid2, potent regulator of developmental gene expression as well as myogenesis [213-220]. There are several reasons behind this decision. First of all, Jarid2 is a direct transcriptional repressor of Notch1 [214,215], regulator of SC activation, cell fate decision and muscle repair [103,207]. Furthermore, Jarid2 is an important part of polycomb repressive complex 2 (PCR2), major epigenetic regulator of regenerative myogenesis [202,216,219,221,222]. Polycomb signaling regulates SC activation via control of Notch1 signaling pathway, SC proliferation via Pax7 and terminal myogenesis via transcriptional regulation of myogenin expression [202,208,212,221]. Interestingly, Jarid2 appears to de-repress myogenin promoter what is in line with its reported role in terminal differentiation [217,220]. In support, we have localized Jarid2 expression to SC in the gastrocnemius muscles of our model as well as to the surrounding capillaries. Moreover expression of Jarid2 is altered in skeletal muscles of COPD patients after exercise and Jarid2 expression was reduced in atrophied muscles suggesting important role of Jarid2 in the regulation of the muscle mass during anabolic and catabolic stimuli [98,223]. Decrease in the muscle Jarid2 expression levels could potentially bring several favorable consequences for the muscle repair process. Jarid2 depletion might be involved in the activation and proliferation of SC via de-repression of Notch1 and Pax7 as well as in the attempt to restore myogenin levels and fusion competence of regenerating fibers.
4.3. TNF stimulation induces VHL overexpression and impairs angiogenic potential in skeletal muscle myocytes

In the Study I, we have shown that chronic cigarette smoke exposure (CS) causes alterations in hypoxia-angiogenesis signaling which were associated with the significant decrease in capillary density and fiber atrophy in gastrocnemius muscles of 129 SvJ mice. However, it was still not clear whether observed alterations arise as a response to direct effects of CS exposure or systemic effects such as hypoxia (hypoxaemia) or systemic inflammation might mediate development of observed abnormalities. This study was designed in order to examine effects of pro-inflammatory cytokine TNF on hypoxia angiogenesis signaling and skeletal muscles capillarization. For that purpose we have chosen C2C12 myocytes as a previously well established experimental model [163, 206, 226, 227].

Tumor necrosis factor (TNF) is a potent pro-inflammatory cytokine that has been widely implicated in the muscle catabolism in many chronic diseases [153,224]. The role of TNF in the pathogenesis of PCS is still debatable; however the question gained new attention in the recent literature [7,151]. In addition to the elevated circulatory TNF, increased levels of TNF receptors as well as augmented NFkB and MAPK signaling have been recently reported in skeletal muscle of COPD patients [37,100,143]. Elevated circulatory TNF was indisputably detected in unstable COPD, during acute exacerbations [143]. Moreover, in animal models, cigarette smoke exposure elicits substantial increase in the circulatory TNF and muscle TNF levels [163,180]. In the Study II, we have observed presence of inflammatory cells and increased expression of CD68 and AIF1 protein suggesting ongoing inflammatory process in the skeletal muscles of CS-exposed mice. Furthermore, in-vitro, TNF appears to reproduce many of the molecular alterations observed in the muscles of PCS patients such as suppression of oxidative metabolism, inhibition of protein synthesis and above all promotion of muscle degradation via activation of ubiquitin proteolytic system (UPS) [59,87,139]. TNF promotes muscle catabolism via overexpression and activation of UPS, especially E3 ligases such as Atrogin-1 and Murf-1 which potently degrade muscle contractile proteins [86,87]. In addition, in the current study we have shown that TNF can modulate expression of von Hyppel Lindau tumor suppressor (VHL), E3 ligase which is involved in the regulation of crucial cellular processes such as adaptation to hypoxia, cell cycle and cytoskeleton maintenance. [225]. Interestingly, TNF has significantly increased VHL protein expression,
while it has down-regulated VHL mRNA levels. The reason for this discrepancy is unknown; however it suggests existence of a feedback mechanism and auto-regulation of VHL expression in response to TNF stimulation. In support, expression of VHL protein was significantly elevated after 24h while the decline in the mRNA levels started approximately 32h after stimulation. It is tempting to speculate that down-regulation of VHL transcript might arise as an attempt of myocytes to limit potentially hazardous affects of augmented VHL signaling and restore cellular homeostasis. In agreement with this hypothesis, we have observed the same effect of long-term TNF stimulation on Atrogin-1, Murf-1 and Usp19 expression, which have all be attributed as potent promoters of muscle catabolism [85,96]. Furthermore, mRNA levels of ubiquitin molecule have also been depleted in the same time point suggesting silencing transcription of UPS genes in response to the long-term TNF stimulation [226]. How TNF increases VHL expression in C2C12 myocytes in this study has not been assessed. TNF increases muscle expression of other E3 ligases via NFkB signaling pathway and enhanced transcription [86,227] however TNF can utilize NFkB signal increase protein expression by protein stabilization without enhancing gene expression levels [228]. More likely, TNF stabilizes VHL protein via MAPK signaling pathway (Basic et al, unpublished observations).

VHL is a part of a large E3 ligase complex which requires interaction with partner proteins such as prolyl hydroxylase family member PHD2 and ubiquitin conjugating enzyme E2D1 (Ube2D1) in order to ubiquitinate its target proteins [128,130]. In line with this, the current study demonstrates increased expression of PHD2 and Ube2D1in response to TNF stimulation. This clearly suggests that TNF stimulation could promote catabolic environment in C2C12 myocytes and adversely affect expression and function of VHL target proteins even in normoxic conditions. Indeed, HIF1-α is reported to be potently ubiquitinated in response to TNF stimulation [229]. In consent, our study demonstrates mild reduction in HIF1-α protein levels after TNF stimulation which was associated with significant reduction in the expression of HIF1-α regulated-genes including VEGF and Glut1. A drawback of this study was a lack to assess levels of HIF1-α ubiquitination in response to TNF treatment, however fully differentially C2C12 are notoriously difficult to transfect what reasoned us against performing these type of experiments.

Expressions of VHL as well as associated UPS members have predominant nuclear localization in murine tibialis anterior. Nuclear localization of
VHL was additionally confirmed in C2C12 myocytes. This is certainly in collision with predominant cytoplasmic localization reported in other tissues [230]. This might be the consequence of nuclear sequestration, as suggested earlier [231], which could limit their activity in the cytoplasmic compartment and additionally stabilize HIF1-α. In support, skeletal muscles express higher HIF1-α levels in normoxic conditions relative to other tissues [232]. However, recent reports demonstrate nuclear localization of ubiquitinated HIF1-α suggesting high VHL activity in the nuclear compartment [229]. Furthermore, nucleus appears to be the place with the strongest UPS activity in the muscle tissue as suggested by strong nuclear signal for ubiquitin protein detected in this study.

The pathological picture of COPD is mainly dominated by the presence of intermittent hypoxia and systemic inflammation. The interaction of these two factors and the effects they exert on developing complications in COPD are unclear. We have tried to mimick this situation in the design of the current study and in view of our original observation of HIF and VHL overexpression in muscles from COPD patients [65]. The results of the current study demonstrated increase in the VHL protein expression in response to TNF stimulation, with significant down-regulation of VHL mRNA levels. This prompted us to investigate whether other systemic factors associated with COPD, such as hypoxia, can alter VHL gene expression in skeletal muscle myocytes. In agreement with previously published data [233,234], introduction of hypoxia elevated expression levels of VHL mRNA and restored basal levels of VHL mRNA after TNF treatment. This suggests that elevated mRNA VHL levels in CS exposed mice might be a result of muscle adaptation to hypoxia stimuli. However, it is less likely that hypoxia itself would elicits vascular abnormalities observed in Study I since VHL overexpression is reported to be the rate limiting step and mere compensatory mechanism in response to excessive HIF1-α stabilization and prolonged VEGF signaling [234]. These findings indicate the presence of a complex interaction between inflammatory markers and the available O2 levels, which enhanced HIF1-α ubiquitination and disturbed transduction of hypoxia-angiogenic signaling.
4.4. Cigarette smoke exposure up-regulates Ubiquitin specific protease 19 in murine skeletal muscles as an adaptive response to prolonged ER stress

Cigarette smoke (CS) exposure has been previously associated with increased endoplasmic reticulum (ER) stress and development of emphysema in COPD patients and animal models [236,237]. Furthermore CS elicits ER stress in various in-vitro models and promotes catabolism via UPS activation [238,239]. Whether CS elicits ER stress and activates ERAD in skeletal muscles has not been previously investigated. This study aimed to investigate whether chronic CS exposure induces ER stress and activates ERAD in skeletal muscles of COPD/emphysema model.

For this purpose we have chosen to study expression of Ubiquitin specific protease 19 (Usp19), UPS and ERAD member, recently associated with skeletal muscle atrophy in different catabolic conditions [96]. In addition, Usp19 regulates cellular response to diverse stress stimuli such as ER stress and hypoxia, as well as vital cellular processes such as cell cycle progression, differentiation, apoptosis and angiogenesis [240-242]. The results of the current study demonstrated elevated expression of Usp19 in skeletal muscles of CS-exposed 129 SvJ mice. In the Study I and II we have shown that skeletal muscles from CS-exposed 129 SvJ strain have lower mean fiber CSA and exhibit other signs of atrophy such as fiber size variation and myonuclei loss. All together these results are in agreement with recent report by Liu et al. [101] demonstrating Usp19 overexpression in skeletal muscles of CS-exposed rats which was associated with MAPK signaling activation and fiber atrophy. In the current study, we have localized Usp19 expression to the peri-nuclear area of gastrocnemius fibers suggesting ER localization and possible involvement in the muscle ER stress response and ERAD signaling. This hypothesis was evaluated and supported by in-silico analysis of Usp19 promoter, demonstrating stress-response elements and further evidenced by in-vitro experiments where tunicamycin, pharmacological inducer of ER stress increased Usp19 mRNA expression in C2C12 myocytes in a dose and time dependent manner. Moreover, elevated Usp19 expression in skeletal muscles of CS-exposed mice was accompanied by increased expression of activated caspase -12 and caspase -3, markers of chronic ER stress response, myocyte apoptosis and muscle proteolysis [89, 90, 92]. Presence of myocyte apoptosis in CS-exposed animals is in accordance with impaired regenera-
tive capacity of the skeletal muscle reported in the Study II as well as findings in patients with COPD [37,83,107-111].

Additionally, we aimed to investigate whether factors previously associated with the pathogenesis of PCS and COPD [131] can modulate Usp19 expression in our in-vitro model. Even though being a hypoxia-regulating gene, through stabilizing effects on HIF1-α protein [242], Usp19 expression was not modulated by hypoxia in skeletal muscle myocytes. In agreement, hypoxia activates PERK/eIF4 as well as Ire/XBP branch of UPR [243], which were not found to be activated in the muscles of CS-exposed animals.

Stimulation by TNF did not elevate Usp19 mRNA expression in C2C12 myocytes suggesting that systemic inflammation and TNF in particular is probably not a mediator of the CS-induced Usp19 overexpression in the current study and neither in the study by Liu et al [101]. Moreover, our results favor the idea that the initial stimuli for the Usp19 mRNA overexpression in skeletal muscles of CS-exposed mice is prolonged ER stress and activation of Ire1/MAPK signaling as reflected by activation of caspase -12 and caspase -3, downstream targets of this pathway [89,90].

Finally, this study was the first to demonstrate that ER stress suppresses myogenesis via transcription of muscle myofibrilar proteins, likely due to Usp19 overexpression.

5. CONCLUSIONS AND FUTURE PERSPECTIVES

The rationale for the investigations included in this thesis is a series of our reports in patients with COPD [30, 65, 66, 78]. Of the special relevance is the demonstration of VHL overexpression as well as disturbance in HIF1-α/VEGF signaling in skeletal muscles from patients with COPD [65]. That was the first study to investigate HIF1-α/VEGF axis in a disease long-characterized by the presence of intermittent hypoxia [131, 168, 169, 170].

From the results of the current studies, it is evident that cigarette smoke exposure elicits many of the skeletal muscle abnormalities in the murine model, previously observed in the human disease [131]. Thus, CS exposure results in an impaired vascular supply of the skeletal musculature which is associated with VHL overexpression and consequential deregulation in the hypoxia-angiogenesis signaling.
In addition, CS elicits skeletal muscle damage and could further contribute to the development of muscle abnormalities by impairing muscle regenerative capacity.

Cigarette smoke can possibly elicit muscle morphological and vascular abnormalities through, or in synergism with systemic factors such as hypoxia/hypoxaemia and systemic inflammation, and via activation of UPS. CS can possibly impose an ER stress to myocytes which overwhelms their resolving capacity and activates pathways associated with programmed death, ubiquitin recycling and suppression of myogenesis on the transcriptional level.

Taken together, CS has potency to elicit vast molecular alterations in skeletal musculature and cause deregulation in diverse signaling pathways in myocytes. UPS appears to be at the intersection and have an important contributive role to the development of these alterations. The triggers and mechanisms underlying activation of UPS in response to CS remain to be elucidated; however UPS seems to be legitimate target for the future investigations and drug development.

6. ACKNOWLEDGMENTS

My supervisors-Thank you for admitting me to your research group and giving me the chance to undertake this thesis. I have learned a lot from you, how the research life works, and especially how life in general works. Co-authors- Thank you for the fruitful and extremely pleasant cooperation.

My friends- Thank you, without you and your help, especially in the last months of my work, I would never succeed.

My family- Thank you, without your endless love and help, especially financial, this thesis would never been done.
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