1

General introduction and outline of the thesis
1.1 Pulmonary hypertension

Pulmonary arterial hypertension (PAH) is a rare, yet severe condition characterized by vascular proliferation and remodeling of the pulmonary arteries, resulting in an increase in pulmonary vascular resistance. Normally, the pulmonary circulation is a low pressure system with a mean pulmonary artery pressure (mPAP) of $\sim 14 \text{mmHg}$. When the mPAP exceeds 25 mmHg at rest, the diagnosis PAH is established. Several factors can induce PAH, for example left heart disease, congenital heart disease and chronic pulmonary embolism. However, in a large subgroup of patients no underlying cause can be found; this type of PAH is called idiopathic pulmonary arterial hypertension (IPAH) [34].

The right ventricle (RV) is part of the pulmonary circulation and pumps oxygen depleted blood to the lungs via the pulmonary arteries, and returns oxygenated blood back to the left ventricle (LV). The pulmonary circulation is a low pressure system and consequently, the RV is a thin walled structure [45]. However, in PAH the RV needs to adapt to the increased resistance and hypertrophies [44]. Despite this adaptation, the RV is not capable to sustain the long-term pressure overload and will eventually dilate and fail. Prognosis of PAH-patients is poor and survival is closely related to RV function [8, 119, 143].

Since patients present with non-specific symptoms such as shortness of breath and fatigue, PAH is often ignored by the patient and mistreated by the medical doctor. This results in a delay in the establishment of the diagnosis and patients can already be in class II/III of the WHO functional assessment classification (Table 1.1). Currently, the only curative treatment available in PAH-patients is lung transplantation. After lung transplantation RV afterload is normalized and RV function restores in most patients [107]. Partial reductions in RV afterload, as achieved by current therapies which mainly

<table>
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<th>Class</th>
<th>Description</th>
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<tr>
<td>I</td>
<td>Patients with PAH but without resulting limitation of physical activity. Ordinary physical activity does not cause undue dyspnea or fatigue, chest pain, or near syncope.</td>
</tr>
<tr>
<td>II</td>
<td>Patients with PAH resulting in slight limitations of physical activity. They are comfortable at rest. Ordinary physical activity causes undue dyspnea or fatigue, chest pain, or near syncope.</td>
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<tr>
<td>III</td>
<td>Patients with PAH resulting in marked limitation of physical activity. They are comfortable at rest. Less than ordinary activity causes undue dyspnea or fatigue, chest pain, or near syncope.</td>
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<tr>
<td>IV</td>
<td>Patients with PAH with inability to carry out any physical activity without symptoms. These patients manifest signs of right-heart failure. Dyspnea and/or fatigue may even be present at rest. Discomfort is increased by any physical activity.</td>
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have vasodilatory effects, are insufficient to prevent progression towards right heart failure in PAH-patients [33, 61]. Therefore, treatment strategies for PAH-patients, aimed to improve the everyday quality of life, have gained importance.

Exercise intolerance is the most disabling symptom in PAH-patients, which results from exertional fatigue and dyspnea. These exercise limitations cannot solely be ascribed to the decrease in cardiac function. As with other cardiac and pulmonary diseases, skeletal muscle dysfunction might contribute to exercise intolerance in PAH-patients [5, 22, 66, 82, 109]. A reduction in maximal volitional and non-volitional muscle strength was observed in PAH-patients which correlated with exercise related parameters such as VO\textsubscript{2max} and 6 minute walking distance [6, 66, 82]. In addition, exercise training improved exercise capacity in PAH-patients and altered peripheral muscle function. This suggests that reduced muscle strength might play a role in the reduction in exercise capacity.

1.2 Striated muscle function

To better understand the contribution of muscle dysfunction in the setting of PAH, first the general function of striated muscles is described. The distinct pattern of alternating light and dark bands perpendicular to the long axis is the most striking feature when viewing skeletal and cardiac muscles through a microscope and those muscles are therefore referred to as striated muscles.

Skeletal muscle

Skeletal muscles are build up by bundles of muscle fibers bound together by connective tissue. These muscle fibers are in turn composed of thin fibrils consisting of repeated units, called sarcomeres. The sarcomere consists of thick and thin filaments and is the smallest contractile unit in a muscle (Fig. 1.1). The thin filaments are composed of actin molecules, troponin complexes and tropomyosin strands which play an important role in the regulation of muscle contraction. The thick filaments are mainly composed of myosin molecules, with myosin heads extending out to the sides, forming cross-bridges with actin when the muscle is activated. In addition, for optimal active force generation, the structural integrity of the sarcomere is indispensable. This is at least in part provided by the passive elastic properties of the giant sarcomeric protein titin [77].

Muscle contraction

Skeletal muscle contraction results from the linked actions of electrophysiological, biochemical and mechanical processes, called excitation-contraction coupling. Skeletal muscles are innervated by motor neurons via the neuromuscular junctions. An activated motor neuron leads to depolarization of the muscle membrane at the neuromuscular junction. Depolarization induces an action potential that propagates along the muscle
Figure 1.1: Schematic representation of a skeletal muscle and the sarcomere. A muscle consists of a bundle of muscle fibers which run from tendon to tendon. A muscle fiber is build up by sarcomeres, the smallest contractile unit of a muscle. When an action potential reaches the T-tubulus, large quantities of Ca\(^{2+}\) are released from the sarcoplasmic reticulum via the ryanodine receptors. A sarcomere consists of thin filaments, composed of actin, troponin and tropomyosin, and thick filaments build up by myosin [151].

fiber length into its interior by way of the T-tubules. When the T-tubules depolarize, large quantities of calcium from the sarcoplasmic reticulum will be released into the cytoplasm via the ryanodine receptors (Fig. 1.1).

When calcium enters the sarcomeres, it binds to the troponin complex on the thin filament. This leads to a conformational change of tropomyosin thereby unblocking the actin binding sites (Fig. 1.2). Exposure of these sites allow myosin heads to bind to actin and to form a cross-bridge. A cross-bridge cycle can start and consists of four phases (Fig. 1.2): 1) myosin heads of the thick filament bind to actin, forming a cross-bridge; 2) movement of the cross-bridge, producing tension in the thin filament; 3) detachment of the cross-bridge (an ATP depending process); and 4) energizing the cross-bridge so it can attach again to actin and repeat the cycle. Each cross-bridge undergoes its own cycle of movement, independent of other cross-bridges. This means that at any instance only a portion of the cross-bridges are attached to actin, and are able to produce tension, while others are in the detached phase of the cross-bridge cycle. As long as calcium is present and there is enough ATP to detach the cross-bridges, cross-bridge cycling can
When Ca\textsuperscript{2+} enters the sarcomeres it binds to the troponin complex. This lead to a conformational change of tropomyosin, thereby unblocking the actin binding sites and cross-bridge cycling can start. Cross-bridge cycling consists of four phases and starts with 1) myosin head binds to actin - cross-bridge formation; 2) movement of the cross-bridge, producing tension in the thin filament; 3) cross-bridge detachment - an ATP depending process; 4) energizing the cross-bridge so it can attach again [151].

Muscle plasticity

Skeletal muscles are build up by different fiber types, based on the location and function of the muscle. Muscle fiber types are generally classified as fast-twitch (fast-glycolytic and fast-oxidative) or slow-twitch (oxidative) based on ATPase activity and energy substrate utilization.

As the names imply, slow-twitch muscle fibers primarily use oxidative phosphorylation to generate ATP and fast-twitch glycolytic muscle fibers mainly use anaerobic glycolysis to generate ATP. Consequently, slow-twitch muscle fibers are surrounded by more capillaries and contain more mitochondria than fast-twitch muscle fibers. As slow-twitch muscle fibers rely on oxidative phosphorylation they are more fatigue resistant. However, the ATPase activity is higher in fast-twitch muscle fibers, which determines the rate of cross-bridge cycling and thus shortening velocity. As a result fast-twitch muscle fibers can generate more power than slow-twitch muscle fibers. Human skeletal muscles are generally composed of both slow-twitch and fast-twitch muscle fibers [151].
The remodeling of skeletal muscles according to alterations in demand is called muscle plasticity. It is a well-recognized phenomenon in sports, where distinct adaptations of muscle tissue can be observed to maximize the performance of the muscles. This adaptation is very specific, and the degree of loading and number of muscle contractions is important. For example, high repetitive low-load exercise training can induce a fiber type shift towards more slow-twitch fatigue resistant muscle fibers [132]. While, exercise regimes with a high degree of loading will provoke muscle fiber hypertrophy [3, 59].

Inactivity or disuse, on the contrary, can induce muscle fiber atrophy and a fiber type shift towards more fast-twitch fatigable muscle fibers. This can, for example, be observed in astronauts during zero gravity or in leg and arm muscles after a cast is removed [3, 131]. Contradictorily, chronic over-stimulation of skeletal muscles can also lead to muscle atrophy and weakness. This weakness is not only caused by the reduction in muscle size, but also involves sarcomeric dysfunction [64].

**Cardiac muscle**

Cardiac muscle cells (i.e. cardiomyocytes) also contain sarcomeres, and the contractile function of these sarcomeres is similar to that of skeletal muscles. However, the mechanism by which depolarization of the plasma membrane leads to cardiomyocyte contraction is different from skeletal muscle. Depolarization of the plasma membrane leads to an influx of calcium through L-type calcium channels. This influx of calcium triggers the ryanodine receptors, which will again result in the release of large quantities of calcium from the sarcoplasmic reticulum into the cytoplasm. Calcium can bind to troponin and cross-bridge cycling and force generation can occur as described for skeletal muscle.

Similar to skeletal muscle, cardiac muscle can adapt to changes in demand. When the increase in demand is of relative short duration, the heart adapts by increasing heart rate and stroke volume. At the sarcomeric level this is modulated by post-translation modifications such as phosphorylation. For example, protein kinase A (PKA) is activated upon β-adrenergic receptor stimulation and is a key player in cardiac adaptation to increase cardiac demand as occurs during stress or exercise. However, a chronic increase in demand can induce cardiac hypertrophy, which can be a physiological adaptation, for example in endurance athletes or pregnant women [100, 148]. Long-term chronic overload leads to transcriptional changes, which can for example affect muscle function by alterations in myosin heavy chain isoform expression, the development of fibrosis and protein content [54]. Cardiac hypertrophy can also be pathological due to hypertension, cardiac muscle injury or valvular diseases, and can eventually lead to heart failure [69]. On the other hand, removal or reducing the load will lead to cardiac atrophy. Cardiac atrophy can also occur in healthy hearts for example during zero gravity, which reduces the load on the heart [108].
Permeabilized muscle fibers

To study the contractile function of striated muscles in PAH-patients, biopsies are indispensable. However, biopsies are small pieces obtained from the part of the muscle. Hence, the membrane is damage and fiber ends are not sealed by tendons, which disrupts normal excitation-contraction coupling. Therefore, we specifically study sarcomeric contractile function of permeabilized muscle fibers. In permeabilized muscle fibers, the membranous structures are made permeable, while leaving the sarcomeres intact. By exposing these fibers to exogenous calcium, sarcomeric function can be studied without the confounding effects of excitation-contraction coupling, sarcoplasmic reticulum and/or energy utilization.

The maximal active force ($F_{\text{abs}}$) developed by permeabilized muscle fibers depends on 1) the fraction of strongly bound cross-bridges ($\alpha_{fs}$); 2) the number of available cross-bridges ($n$); and 3) the force generated per cross-bridge ($F_{cb}$) (Eq. 1.2).

$$\alpha_{fs} = \frac{f_{\text{app}}}{f_{\text{app}} + g_{\text{app}}} \quad (1.1)$$

$$F_{\text{abs}} = \alpha_{fs} \cdot n \cdot F_{cb} \quad (1.2)$$

The fraction of strongly bound cross-bridges depends on the apparent rate of cross-bridge attachment ($f_{\text{app}}$) and cross-bridge detachment ($g_{\text{app}}$)(Eq. 1.1). For example, if cross-bridge detachment is slower, $g_{\text{app}}$ becomes smaller, which leads to an increase in $\alpha_{fs}$ and thus to an increase in force production. However, if both the attachment and detachment rates vary proportional, $\alpha_{fs}$ will not alter. The number of available cross-bridges depends on the amount of myosin and actin present. If less protein is present, less cross-bridges can be formed and less force can be generated. The force generated per cross-bridge is determined by the movement of the myosin head. Post translation modifications can alter the function of the myosin head and affect the force generated per cross-bridge. Sarcomeric dysfunction could thus result from dysfunction in one or more of these three determinants [121].

Equation 1.2 assumes that all actin binding site are exposed, which only occurs when the fiber is maximally activated. During maximal activation, enough calcium is present to saturate all troponin binding sites on the thin filaments and thus maximal force depends only on the above described factors. However, in vivo muscles are generally not maximally activated, but activated at submaximal firing rates, which generate submaximal cytosolic calcium concentrations ($[\text{Ca}^{2+}]$). The relation between the $[\text{Ca}^{2+}]$ and a steady state force ($F_{\text{ss}}$) follows a dose-response curve, described by the Hill equation (Eq. 1.3).

$$F_{\text{ss}} = \frac{F_{\text{max}}}{(1 + 10^{n(p\text{Ca}_{50} - p\text{Ca})})} \quad (1.3)$$

In which $F_{\text{max}}$ is the maximal force, $p\text{Ca}$ is $-\log [\text{Ca}^{2+}]$, and $p\text{Ca}_{50}$ is the $p\text{Ca}$ at which 50% of the maximal force is reached. The $p\text{Ca}_{50}$ provides a measure of calcium sensitivity.
of force generation. Changes in calcium sensitivity of the muscle can occur in both skeletal and cardiac muscles and can affect submaximal contractile function of the muscle [105, 137]. For example, a reduction in calcium sensitivity can result in a lower force generation at submaximal calcium levels.

1.3 Aim and guide through the thesis

Due to exertional fatigue and dyspnea the quality of life of PAH-patients is reduced. Previous studies have suggested that these symptoms are not only caused by RV dysfunction. The contractility of other muscles, such as the LV, respiratory and peripheral muscles could be affected as the demand placed on these muscles changes in PAH. The aim of the present thesis is to unravel the underlying pathophysiology of striated muscle dysfunction in PAH. This knowledge might provide new additional treatment targets to improve quality of life in PAH-patients.

As the heart consists of both a right and left ventricle, the function of these two ventricles is inextricably linked in both the healthy and diseased heart. The RV, which has to pump against an increased afterload in PAH, becomes hypertrophic and will eventually dilate and fail. However, cardiac dysfunction in PAH is not only limited to the RV. Also reductions LV function have been described. This may be caused by reduced filling of the LV, as a consequence of decreased RV output. In addition, septum bulging is a well-known cardiac characteristic in PAH which can further hamper LV filling. In chapter 2 we investigated whether atrophy and reduced contractility of LV cardiomyocytes contributes to the impaired LV function in PAH-patients.

Respiratory muscles of PAH-patients might be subjected to an increase in demand. PAH-patients hyperventilate during exercise, at rest and sometimes even during sleep, which places an increased burden on the inspiratory muscles. Recently, a reduction in maximal inspiratory pressure was reported in PAH-patients, suggesting inspiratory muscles weakness. This might contribute to the sensation of dyspnea, which results from an imbalance between the demand placed on the inspiratory muscles, and the capacity of the inspiratory muscle to generate pressure. The diaphragm is the main inspiratory muscle and in animal models of PAH, diaphragm contractile dysfunction was found. In chapter 3 we investigated the nature of this diaphragm weakness and the involvement of sarcomeric dysfunction of the diaphragm muscle in a rat model of PAH.

Encouraged by the finding that sarcomeric dysfunction contributes to diaphragm muscle weakness in PAH-rats, we investigated in chapter 4 whether this sarcomeric dysfunction was also present in PAH-patients. In addition, we measured in vivo inspiratory muscle function in these patients to determine whether diaphragm sarcomeric weakness contributes to inspiratory muscle weakness. As a potential therapeutic approach we tested the ability of a calcium sensitizer to improve diaphragm muscle contractility.

Due to cardiac dysfunction, physical activity of PAH-patients declines with disease progression. This reduced activity might affect the peripheral muscles of PAH-patients.
Maximal volitional and non-volitional muscle strength is reduced in PAH-patients and correlated with the reduction in VO$_2$max. This may indicate that peripheral muscle dysfunction contributes to exercise intolerance. However, the underlying cause of the reduction in muscle strength in PAH-patients remains unclear. In chapter 5 we investigated if sarcomeric dysfunction contributes to the reduction in peripheral muscle strength of PAH-patients.

The results of this thesis are discussed in chapter 6. A conclusion and future research perspective is provided in chapter 7 together with a summary in chapter 8.