Skeletal Muscle in Restless Legs Syndrome (RLS) and Obstructive Sleep Apnoea Syndrome (OSAS)
To family and friends

Jag lär mig livet mer och mer och jag förstår det allt mindre.

FOLKE ISAKSSON UR TERRA MAGICA
Britta Wåhlin Larsson

Skeletal Muscle in Restless Legs Syndrome (RLS) and Obstructive Sleep Apnoea Syndrome (OSAS)
Abstract

Restless legs syndrome (RLS) and Obstructive sleep apnoea syndrome (OSAS) are two sleep disorders that affect daily life with symptoms such as sleepiness and fatigue. It was therefore hypothesised that the skeletal muscle could be affected as symptoms from skeletal muscle are common. The overall aim of the thesis was to investigate aerobic capacity and structure of skeletal muscle in patients with OSAS and RLS and an age matched control group to provide information regarding the underlying mechanisms. The specific aims were to investigate muscle fibre composition, capillary network, capillary proliferation and sings of local inflammation in musculus tibialis anterior of RLS and OSAS.

OSAS and RLS patients had a significantly lower predicted VO$_2$ max expressed in ml/min/kg compared with the control group and in the OSAS group apnoes-hyponea index (AHI) was inversely correlated to maximal oxygen uptake Fibre type composition and muscle fibre cross sectional area in the tibialis anterior muscle was equal in all groups with a predominant proportion of slow type I fibres and a smaller fibre area in slow type I fibres compared to fast type II fibres. The distribution of fast fibres (I/IIA, IIA) did not differ except for the group IIX and IIA/IIX where OSAS and RLS had a significantly higher percentage. OSAS patients had a significantly higher number of capillaries per fibre (CAF) for slow type I fibres and CAF per fibre area (CAFA) for fast type II fibres. The CFPE-index (capillary to fibre perimeter exchange) and LC/PF-index (length of capillary/perimeter of fibre) were higher in both patient groups. Vascular endothelial growth factor (VEGF) and proliferating endothelial cells were analysed by double-immunofluorescence staining and were presented to a greater extent in the patient groups compared with the healthy controls. Based on normal amounts of T-cells and macrophages in the histological picture it was also demonstrated that local inflammation was not present in the tibialis anterior muscle of RLS and OSAS whish was also supported by the absence of expression of major histocompatibility complex class I molecules (MHC class I) on the surface of the tibialis anterior muscle fibres.

In conclusion, the low predicted VO$_2$ max together with higher percentage of type IIX and IIA/IIX muscle fibres indicates a low central capacity in the patient groups. The increased capillary network and the absence of inflammation indicate the occurrence of local hypoxia in tibialis anterior muscle in patients OSAS and RLS.

Key words: RLS, OSAS, aerobic capacity, skeletal muscle fibers, capillary, VEGF, proliferation, inflammation
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LIST OF ABBREVIATION

AHI    the number of apnoeas and hypopnoeas per hour
ATPase  adenosien triphosphatase
BMI    body mass index
CD3    a molecule associated with the T cell receptor. CD3 is expressed on all T cells.
CD31   monoclonal mouse anti-human, endothelial cell
CD31, Ki-67+  the percentage of capillaries expressing Ki-67
CD31, Ki-67-  the percentage of capillaries which do not express Ki-67
CD31, VEGF+  the percentage of capillaries expressing VEGF
CD31, VEGF-  the percentage of capillaries which do not express VEGF
CD68   is present in macrophages in many human tissues and is used an immunocytochemical marker for staining of monocytes/macrophages
C/Fi   sharing factor
CK    creatine kinase
Core zones a core zone is devoid of mitochondria and oxidative enzyme activity.
CFPE-index capillary-to-fibre perimeter exchange, an indicator of the interface between muscle fibre and capillary membrane.
COX    cytochrome c oxidase
FSS    fatigue severity scale
HIF    hypoxia-inducible factor
hsCRP  high sensitive C-reactive protein
Ki-67  proliferating endothelial cell marker
LC/PF-index length of capillary / perimeter of the fibre, an indicator of the degree of capillary tortuosity.
MyHC   myosin heavy chain
MHC classI major histocompatibility complex
NADH-TR nicotinamide-adenine-di-nucleotide tetrazolium reductase
OSAS   obstructive sleep apnoea syndrome
PF    perimeter
Ring-binden fibres the normal orientation of the myofibrils is distorted and is usually indicative of a disturbance in muscle tone
RLS    restless legs syndrome
SDH    succinate dehydrogenase
VEGF   vascular endothelial growth factor
VPF    vascular permeability factor
INTRODUCTION

Sleep disorders

When we have a good night sleep, we wake up feeling refreshed and alert and ready for our daily activities. Sleep is an important factor for recovery and has a significant role in human well-being and performance. A normal night sleep should be more than 6 hours per night [18] and if sleep is disturbed, the consequences include cognitive impairment and impaired quality of life [68]. When the length of sleep is reduced from 8 to 5 hours during 4 days daytime alertness will decrease [37] and it is known that sleep loss also affects for example physical performance in terms of decreased ability for endurance exercise [14]. Restless legs syndrome (RLS) and Obstructive sleep apnoea syndrome (OSAS) are two types of sleep disorders that affect daily life with symptoms such as fatigue and tiredness [6,24].

Restless legs syndrome (RLS)

Restlessness in the legs and its negative impact on sleep was first described in 1672 by Willis [116] and named Restless legs in 1945 by Ekbom [36] The prevalence of RLS varies considerably in the literature. Ulfberg and co-workers (2001) demonstrated a prevalence of 5.8% in a population of 4000 Swedish men aged between 18 and 64 years [109]. In two resent studies a prevalence of 11.5% in the general adult population in Norway and Denmark was reported [11] and Tison et al (2005) estimated the prevalence in a French population to be 8.5%, with a higher prevalence observed in women (10.8%) than in men (5.8%) [105].

Restless legs syndrome (RLS) is a sleep disorder with a wide range of sensory symptoms. The restless legs syndrome study group developed standardized criteria for the diagnosis of RLS, which was modified in 2003. The following four criteria are all required to make the diagnosis: a) desire to move limbs usually associated with para/dysesthesia. b) motor restlessness – feel forced to move to get relief from the symptoms. c) symptoms shall exclusively be present or worsen at rest and there shall be at least a partial and temporary relief with action. d) symptoms worsen in the evening or at night [5,111].

The cause of RLS is still largely unknown and there are studies indicating that RLS is partly an “inherited disorder” [4,13,106,118,119]. It is also known that a high percentage of relatives with RLS correlate with an early age of onset (before age 45) [4].

The relatively good effect of pharmacological treatment with dopaminergic drug indicate evidence for dopaminergic pathology in RLS but there are conflicting results whether the dopaminergic system is affected [3,106] or if the changes in the...
dopaminergic system are of primary or secondary nature [79]. Secondary causes of RLS may be associated with many causes and iron deficiency is assumed to be the most frequent cause.

The main symptom is the urge to move the legs and/or other extremities due to a very unpleasant sensation felt mostly deep inside the limbs that worsen during periods of rest or inactivity such as lying or sitting. These symptoms are partly or totally relieved by movement such as walking, bending, stretching, etc, at least for as long as the activity continues. The unpleasant sensation or the urge to move is worse in the evening or at night than during the day, or may only occur in the evening or at night [5,106].

RLS may cause the sufferer great difficulty in initiating sleep because of the urge to move the legs. When the symptoms cause the patient to wake up, getting back to sleep may be as difficult as trying to get to sleep in the first place. Severely affected RLS patients have the least sleep time of any sleep disorder and today it is known that sleep deprivation has both short-term and long-term consequences on health [6]. In the REST primary study [51] 60% of RLS sufferers reported that they lacked “energy” or felt fatigued. Complaints of headache on awakening and/or daytime headaches are frequent among RLS, as well as a high frequency of reported hypertension and heart problems which could be explained by the sleep loss reported by these subjects [109].

Treatment of RLS includes both non-pharmaceutical and pharmacological treatments, as well as treating the specific cause in the case of secondary RLS. There is no cure for primary RLS and the physical examination is usually normal [97,104]. Treatment with levodopa and dopamine agonists has a positive effect but augmentation can be a problem of dopaminergic treatment [97,106]. Iron is known to be a co-factor used by one of the enzymes in the production of dopamine and treatment with iron results in a reduction in RLS symptoms [3,106].

Before starting a pharmacological treatment other possibilities should be considered and implemented. Due to clinical observations it is recommended to avoid nicotine and alcohol although the effects were not systematically studied [6]. There are also findings demonstrating that aerobic exercise (walking 3 times a week during 12 weeks) was effective improving symptoms of RLS [10].

As symptoms of RLS are often located in the limb and typically occur during periods of inactivity, and are alleviated by movement including muscle contraction it would be of interest to examine the skeletal muscle in patients suffering from RLS to get a better understanding of the underlying mechanism of RLS.
Obstructive sleep apnoea syndrome (OSAS)

There is still no general agreement on how to define OSAS but it is often referred to as multiple episodes of breathing cessation (apnoeas or hypopnoeas) during sleep caused by collapse of the pharyngeal airway, associated with reduction in blood oxygen saturation. The diagnosis of OSAS also involves daytime impairment with mental and/or physical effects. OSAS was first defined in terms of the apnoea index (AI), i.e. the average frequency of apnoeas per hour of sleep. One common definition for OSAS was AI > 5. Now, the concept of the apnoea-hypnoea index (AHI), i.e. the number of apnoeas and hypopnoeas per hour, is more frequently used as a definition of OSAS. The cut off point of AHI is debated and varies between 5 and 20 [43,68].

Epidemiological studies over time differ not only in sampling but also in definition of OSAS. Estimated OSAS prevalence can vary from 0.7% to 4.7% in a population or some times even more (for review see [68]). The prevalence in men is two to three times that in women and the reasons are not entirely clear although hormonal factors clearly play a role [43].

Complaints about habitual snoring and daytime sleepiness are well established in patients with OSAS. They often describe their problems as lack of energy, fatigue or sleepiness [24]. Fatigue is sometimes defined as a decreased work capacity or diminished energy (physical fatigue) and it is also shown that OSAS patients have a decreased physical work capacity [2,112].

OSAS is confirmed with polysomnographic [7,31] or Embletta and Breas screening [30,78]. OSAS is under-diagnosed and under-treated and patients with OSAS are often heavy dependent on the health-care system [100]. The sleepiness impairs social functioning and work performance and accounts for a large socio-economic burden [43,108]. There is an increasing interest in OSAS due to its recognized associations with diseases such as cardiovascular and metabolic diseases. However there is an ongoing debate as to whether these factors are independent or could be due to confounding factors, especially obesity which is common among OSAS patients [43].

It is known that OSAS patients have a severe intermittent hypoxia during sleep [113,114] and blood oxygen saturation may be as low as 70% [101,110]. The role of intermittent hypoxia as a cause of the clinical symptoms of OSAS is largely unexplored but there is evolving literature suggesting an association between OSAS and a variety of adverse cardiovascular outcomes [115] such as hypertension coronary heart disease and stroke. There are also data indicating a dose-dependent activation of inflammatory pathway by intermittent hypoxia supporting a role in the pathophysiology of cardiovascular complications in OSAS [90]. It is generally unknown whether the cellular and functional properties of skeletal muscle are affected by OSAS. In muscles surrounding the upper airway (medium pharyngeal constrictor muscle and
genioglossus), a higher glycolytic activity and a higher percentage of fast type IIA muscle fibres have been reported in OSAS patients compared to normal controls [22,40,95]. However, these changes may result from increased mechanical influence on the muscle during snoring [99]. To our knowledge only one study has investigated limb skeletal muscle (quadriceps femoris) in patients with OSAS [91]. This study showed that the diameter of fast type II fibres was larger in OSAS compared to healthy controls. Moreover, there was an upregulation of cytochrome oxidase and phosphofructokinase enzyme activities. The authors suggested that these changes are indicative of the occurrence of intermittent hypoxia in skeletal muscle of patients with OSAS.

At first the only effective treatment of OSAS was tracheostomy [103] but is now used only very occasionally. Today continuous positive airway pressure (CPAP) is the standard treatment for OSAS and has been shown to have a major impact on symptoms [43,58], but there is evidence that CPAP treatment has very little impact on quality of life [58].

Smoking and alcohol are known to be risk factors for snoring and sleep apnoea and can increase the numbers of apnoeas as well as the duration [68]. Obesity is also a major risk factor and 70% of patients with OSAS are overweight [68]. Thus these factors should be considered in a treatment programme.

Another important risk factor is physical inactivity [43,68], a factor that is often ignored and therefore not debated. It has been shown that physical training together with CPAP treatment is beneficial for decreasing AHI but that physical training alone is not an adequate intervention for patients with OSAS [44,77]. However, different study designs and methods have been used which makes it difficult to draw clear conclusions and further studies are warrant.

Despite the fact that OSAS patients often explain their symptoms, beside sleepiness and tiredness, as fatigue or lack of energy, the question of whether the structural and functional properties of skeletal muscle are affected has not been fully examined.

Further studies of the skeletal muscle and aerobic capacity in patients with OSAS can provide important information as to the underlying mechanisms and treatments and thereby increase the opportunity to improve the quality of life for these patients.

**Muscle biology**

All movement depends on the correct functioning of skeletal muscle. The entire muscle is surrounded by the epimysium and the perimysium divides the skeletal muscle into a series of compartments. Within the perimysium the endomysium surrounds the individual skeletal muscle cells, or muscle fibres. The endomysium contains a capillary
network, satellite cells and nerve fibres (Fig. 1) Skeletal muscle is a very adaptive tissue and can adjust its metabolic and contractile characteristics in response to alteration in environmental demands [15,26,55,98].

Muscle fibre type distribution

It is well known that skeletal muscle is characterised by a marked heterogeneity in the muscle fibre population. Muscle fibres can be classified according to their morphologic and metabolic characteristics. Histochemical staining of myosin adenosine triphosphatase (ATPase) made it possible to delineate slow type I fibres and fast type II fibres [81]. When the staining techniques were developed an opportunity to reveal more fibre types by pre-incubation at different pH values were revealed, type I, IIA and IIB [19,20]. Immunohistochemical staining, based on the expression of myosin heavy chain isoforms, has also revealed "pure" and "hybrid" muscle fibre types. Pure fibre types contain a single Myosin heavy chain (MyHC) isoforms whereas hybrid fibres contain two or more MyHC isoforms [85]. Skeletal muscle fibres mainly present three MyHC isoforms (I, IIA, IIX) [62]. In addition to the main groups there are also "hybrid" fibres expressing I/IIA and IIA/IIX [8,84,85]. Slow type I fibres are the most oxidative form of fibres and fast type IIX fibres are the most glycolytic fibre whereas type IIA fibres are both glycolytic and oxidative (Table 1).

Muscle fibres are dynamic structures capable of changing their phenotype under various conditions, for example aging and altered neuromuscular activity [85]. The changes in MyHC isoforms tend to follow a scheme: MyHC type I ↔ MyHC type IIA

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Fig. 1 The organization of musculus tibialis anterior. Illustration from Fundamental of anatomy & physiology by Martini (2008)
MyHC type IIA ↔ MyHC type IIA/IIX ↔ MyHC type IIX. It has been shown that the proportion of fast type IIX and type IIA/IIX increases in response to inactivity and decreases following a period of intensive training [61,96].

Table 1 Characteristics of muscle fibre type I, IIA and IIX

<table>
<thead>
<tr>
<th>Fibre type</th>
<th>Type I</th>
<th>Type IIA</th>
<th>Type IIX</th>
</tr>
</thead>
<tbody>
<tr>
<td>Contraction time</td>
<td>Slow</td>
<td>Fast</td>
<td>Very fast</td>
</tr>
<tr>
<td>Activity</td>
<td>Aerobic</td>
<td>Long-term anaerobic</td>
<td>Short-term anaerobic</td>
</tr>
<tr>
<td>Force produced</td>
<td>Low</td>
<td>High</td>
<td>Very high</td>
</tr>
<tr>
<td>Oxidative capacity</td>
<td>High</td>
<td>High</td>
<td>Low</td>
</tr>
<tr>
<td>Glycolytic capacity</td>
<td>Low</td>
<td>High</td>
<td>High</td>
</tr>
</tbody>
</table>

Mitochondria and the respiratory chain

Mitochondria are present in the cytoplasm, and the total number varies depending on the amount of energy required by the cell. Mitochondria are localized where energy is required most and they tend to lie alongside the myofibrils and the sarcoplasmatic reticulum. Each mitochondrion has an inner membrane and an outer membrane, which are separated by an inter-membrane space. The citric acid cycle occurs in the mitochondrial matrix and the respiratory chain in the inner membrane [49].

NADH (the reduced form of nicotinamide adenine dinucleotide) and FADH$_2$ (the reduced form of flavin adenine dinucleotide), produced mostly by the citric cycle, provides electrons to the respiratory chain. The respiratory chain is composed of a five enzyme complex, which makes ATP production possible by oxidative phosphorylation. Succinate dehydrogenase (SDH) corresponds to enzyme complex II and cytochrome oxidase (COX) correspond to enzyme complex IV [59].

Pathological changes in the mitochondria structure, number, size and distribution can affect muscle function. Ischemia can induce a variety of mitochondrial changes which influence the function of aerobic respiration [50]. Accumulation of subsarcolemmal clumps of mitochondria and mitochondria devoid of COX are seen in various mitochondrial myopathies. These patients often suffer from muscle weakness [33,64]. A disorganised mitochondrial pattern is for example seen in trapezius muscle of subjects with work related myalgia [60,65]. These subjects had, among other
changes, fibres lacking COX and an increased occurrence of ragged-red fibres, a marker for mitochondrial dysfunction.

**Muscle capillarisation**

Capillaries can be identified by the size of their cross-section. Vessels in the endomysium with a diameter < 15 μm are mostly capillaries [23].

The histological structure of the capillaries permits a two-way exchange of gases and substances. It is known that muscle fibre types characterised by a high aerobic metabolism have a much denser capillary network than muscle fibre types characterised by a high glycolytic metabolism [55]. However, capillaries do not function as individual units but as part of an interconnected network, the capillary bed or capillary network (Fig 2). In this network the entrance to each capillary is guarded by a precapillary sphincter. Contraction of the sphincter leads to a stop or reduction in blood flow in the specific capillary and blood is diverted into other branches of the network. Relaxation of the precapillary sphincter results in increased blood flow in the capillary [74]. Skeletal muscle blood flow is closely coupled to metabolic demand and its regulation is mainly the result of the interplay of neural vaso activity and local vaso-active substances such as change in pH, PO₂ as well as adrenaline [27].

*Fig. 2 The organisation of a capillary bed.*

*Illustration from Fundamental of Anatomy & Physiology by Martini (2008)*
Angiogenesis

Angiogenesis is the development of new capillaries from an already established capillary network [21,89]. Endothelial cells form capillaries and are thus centrally involved in angiogenesis; they migrate and proliferate and then assemble into tubes with tight cell-cell connections to contain the blood [54].

The process of angiogenesis is controlled by both growth promoting factors and by growth inhibitory factors [45]. Although a variety of growth factors act as inducers of angiogenesis, one of the most specific factor for vascular endothelium is the vascular endothelial growth factor (VEGF) [12,63]. VEGF in turn can be regulated by several factors including growth factors, tumor suppressor factors and hypoxia through stimulation by hypoxia inducible factor (HIF) [17,41].

It is still controversial whether systemic hypoxia causes capillary growth in skeletal muscle. There are studies showing that systemic hypoxia induces angiogenesis [29,28,75] but there are also studies showing no change in capillary supply [71,86]. These results indicate that the degree of systemic hypoxia and the duration of exposure are important factors for angiogenesis in skeletal muscle.

Local hypoxia is known to cause capillary growth [48,89,98]. It is known that endurance exercise with restricted blood flow to working muscle enhances the expression of VEGF mRNA and that repetitive bouts of endurance exercise increase basal VEGF protein levels [47,46].

Angiogenesis could also be induced by metabolic or mechanical factors. AMP-activated protein kinase (AMPK) is activated when energy consumption exceeds energy production leading to an increase in VEGF expression [80]. It is also demonstrated that adenosine might be essential mediator for angiogenesis [1]. Growth of capillaries can also be initiated by mechanical factors related to increased blood flow (shear stress, capillary wall tension) [56].

Capillary parameters

The capillary supply can be assessed by counting the number of capillaries around each fibre (CAF) and CAF in relation to fibre area (CAFA). CFPE-index (capillary-to-fibre perimeter exchange) which represents the interface between the muscle fibre and the capillary will thereby be a more sensitive marker for change in the capillary bed compared to CAF and CAFA [23,53]. LC/PF-index, which represents the percentage of muscle fibre perimeter in contact with the capillary wall, is a marker that allows the evaluation of the tortuosity of capillaries around muscle fibres. LC/PF index takes into account three main morphological factors, i.e. number of microvessels, length of the
microvessels-to-fibre contact and the perimeter of the fibres [23]. Fig 3 shows the effects of microvessel number and microvessel length on microvessel indices.

Fig 3. Effects of microvessel number and microvessel length on microvessel indices. (A) represents the values of the indices before increase in capillary network. After angiogenesis CAF, C/F, and CFPE increase when the number of microvessels increases (B and D). When the length of the contact between microvessels and fibres increases, CAF, C/F, and CFPE remain unchanged, while LC/PF increases (C). If both microvessel number and microvessel length increase, CAF, C/F, and CFPE increase (D). This is due to the increase in microvessel number. Thus, the amplitude of CAF, C/F, and CFPE enhancement is the same as in B, while the amplitude of LC/PF enhancement is more important. If an increase in fibre perimeter occurs together with an increase in the number and the length of the microvessels, the enhancement of CFPE and LC/PF is less important (E). Abbreviations: d=length of a microvessel, LC=Σd, PF=perimeter of the fibre.

Illustration from Charifi et al (2003) J Physiol (Lond); 554 (2): 559-569.
Local inflammation in skeletal muscle

VEGF is also known as vascular permeability factor (VPF) and VEGF expression can be increased in relation to inflammation [17]. On the one hand VEGF stimulates endothelial cells to proliferate and to migrate, and on the other hand VEGF increase the permeability of microvessels [35].

Monocytes are part of the immune system and differentiate to macrophages entering the tissue from the blood. The macrophages are very powerful phagocytes and are necessary both for normal muscle function and tissue repair [67,72,73]. In resting healthy human vastus lateralis muscle 0-8 macrophages per 100 muscle fibres were found [72]. The numbers of macrophages are increased in different inflammatory myopathies [33,107]. T-lymphocytes belong to the adaptive immune system and are normally present in a low amount in healthy muscle [72] and will increase due to inflammation [69].

There are two types of major histocompatibility complex (MHC) molecules, MHC class I and MHC class II. The proteins encoded by the MHC are expressed on the surface of cells and present the antigen long enough for T cells to recognize it. MHC class I is generally absent or very weakly expressed in healthy skeletal muscle but are highly expressed in different inflammatory myopathies [69].
AIM OF THE THESIS

The overall aim of this thesis was to study the morphology of tibialis anterior muscle of patients with OSAS and RLS. It was hypothesised that the skeletal muscle could be affected in these patient groups as symptoms in skeletal muscle are common in RLS and OSAS.

The specific aims of this thesis were to investigate:

- aerobic capacity of patients with RLS and OSAS (study I and II).
- skeletal muscle morphology in Restless legs syndrome (RLS), a sleep disorder without systemic hypoxia (study I).
- skeletal muscle morphology in patients with Obstructive sleep apnoea syndrome (OSAS), a sleep disorder with severe intermittent hypoxia during the night (study II).
- the occurrence and localization of VEGF and proliferating endothelial cells in skeletal muscle of patients with RLS and OSAS (study III).
- signs of inflammation in skeletal muscle of RLS and OSAS patients (study IV).
MATERIAL AND METHODS

Subjects
All patients (OSAS and RLS) participating in the studies were diagnosed by one expert sleep physician at the Sleep Disorder Center, Avesta Hospital, Sweden. All patients were untreated and apart from RLS or OSAS the subjects were healthy. The control group was age-matched, healthy men and women. The subjects characteristics are presented in Table 2.

Table 2 Characteristics on subjects included in the study. Mean ± SD

<table>
<thead>
<tr>
<th>Age (years)</th>
<th>Subjects</th>
<th>n</th>
<th>Weight (kg)</th>
<th>Height (cm)</th>
<th>BMI</th>
</tr>
</thead>
<tbody>
<tr>
<td>OSAS 39 ± 7</td>
<td>Women</td>
<td>2</td>
<td>109 ± 26</td>
<td>180 ± 8</td>
<td>33.6 ± 6.5</td>
</tr>
<tr>
<td></td>
<td>Men</td>
<td>16</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RLS 40 ± 7</td>
<td>Women</td>
<td>12</td>
<td>78 ± 12</td>
<td>170 ± 7</td>
<td>27.0 ± 3.1</td>
</tr>
<tr>
<td></td>
<td>Men</td>
<td>8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Controls</td>
<td>Women</td>
<td>8</td>
<td>72 ± 10</td>
<td>173 ± 9</td>
<td>24.1 ± 2.2</td>
</tr>
<tr>
<td></td>
<td>Men</td>
<td>8</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

OSAS was confirmed with polysomnography [7,31] or Embletta and Breas screening [30,78]. Data on the severity of sleep apnoea in OSAS patients are presented in Table 3.

Table 3 Data on severity of the sleep apnoea from 18 OSAS patients.

<table>
<thead>
<tr>
<th>Measure</th>
<th>OSAS (n=18) mean (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lowest desaturation during sleep</td>
<td>79.6% (62-90)</td>
</tr>
<tr>
<td>Percent of sleep time with oxygen desaturation &lt; 90%</td>
<td>12.9% (0-84.1)</td>
</tr>
<tr>
<td>Mean apnoea-hypopnoea index AHI (the number of apnoeas and hypopnoeas per hour)</td>
<td>42.4 (16-90)</td>
</tr>
<tr>
<td>Duration of OSAS (years) (estimated by the patients together with the physician)</td>
<td>13.6 (5-25)</td>
</tr>
</tbody>
</table>

RLS patients were diagnosed according to the diagnosis criteria recommended by the International Restless Legs Syndrome Study Group together and with a ten-question RLS-severity scale [111]. None suffered from iron deficiency according to ferritin levels. Data on severity of symptoms in RLS are presented in Table 4.
Table 4 Data on the severity of symptoms in RLS patients.

<table>
<thead>
<tr>
<th></th>
<th>RLS (n=20) mean (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RLS severity scale</td>
<td>27 (12-40)</td>
</tr>
<tr>
<td>Ferritin (μg/L) Reference range</td>
<td>107 (15-391)</td>
</tr>
<tr>
<td>Onset of RLS (Age)</td>
<td>21 (5-38)</td>
</tr>
<tr>
<td>Duration of RLS (years)</td>
<td>19.6 (5-45)</td>
</tr>
</tbody>
</table>

Aerobic performance capacity and self reported physical activity

Aerobic performance capacity was obtained using a submaximal cycle ergometer test according to the procedure described by Åstrand and Rodahl [121].

Questions were designed to survey the participants daily physical activity. The questionnaire was based on the report, “LIV-90”, which is a survey report about the lifestyle of Swedish men and women and their performance and health [39].

Blood samples

Blood was collected in vacutainer tubes by venapuncture. The samples were centrifuged and stored in a freezer at –70°C before analysis. Patients with RLS and OSAS underwent routine blood testing, including assessment of creatine kinase (CK) and high sensitive CRP (hsCRP). Plasma levels of hsCRP were determined using Hitachi 911 and CK using Vitros 5.1 at the Department of Clinical Chemistry, Örebro University Hospital, Sweden.

Muscle sampling

Muscle biopsies were obtained from the thickest part of the tibialis anterior muscle belly using the semi-open biopsy technique described by Henriksson (1979) [52]. The tibialis anterior was chosen as it is an important postural muscle involved in balance control and in control of foot stability during normal walking and is therefore active for long periods daily [70].
Histological staining

Serial cross-sections, 8 and 10 μm thick, were cut in a cryostat microtome at -20ºC and mounted on glass slides. The sections were stained using routine histochemical methods for determination of nicotinamide adenine dinucleotide hydrogen dehydrogenase tetrazodium reductase (NADH-TR), succinate dehydrogenase (SDH), cytochrome oxidase (COX) and also a double staining for COX and SDH. NADH, SDH (complex II) and COX (complex IV) are enzymes of the mitochondrial respiratory chain [59]. These histochemical reactions essentially distinguish fibres with low mitochondrial content (non-staining) from those with high mitochondrial content (darkly stained). COX is stained brown, SHD is stained light blue and NADH a more intense blue [33]. The muscle biopsies were also stained for Gomori Trichrome where the mitochondrial accumulations, sarcoplasmatic reticulum and sarcolemma appear red, the myofibrils are green and the nuclei are reddish-purple [38].

All histological stainings were evaluated by two independent observers. The description of the abnormalities seen in tibialis anterior muscle is performed according to the nomenclature used in Dubowitz et al (2007) [33] (Table 5).

Table 5 Definitions of the terms used in study IV according to Dubowitz [33]

<table>
<thead>
<tr>
<th>Term of abnormality</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibre diameter variation</td>
<td>Differences in fibre size together with the distribution of large and small fibres</td>
</tr>
<tr>
<td>Accumulation of peripherally localized mitochondria</td>
<td>Intense staining along the cell membrane (Gomori)</td>
</tr>
<tr>
<td>Core zones (Irregular or central cores)</td>
<td>A core zone is devoided of mitochondria and oxidative enzyme activity</td>
</tr>
<tr>
<td>Ringbinden fibres</td>
<td>The normal orientation of the myofibrils is distorted usually indicative of disturbance in muscle tone</td>
</tr>
<tr>
<td>COX-negative fibres</td>
<td>Fibres devoided of cytochrome oxidase activity</td>
</tr>
<tr>
<td>SDH-negative fibres</td>
<td>Fibres devoided of succinate dehydrogenase activity</td>
</tr>
</tbody>
</table>
Immunohistochemistry

**Fibre type distribution**

Serial transverse sections, 5 µm thick, were cut using a microtome at −20°C (Leica CM1850) and mounted on glass slides. Muscle biopsies were air-dried at room temperature rinsed for 10 minutes in phosphate-buffers saline (PBS) and incubated for 20 minutes in goat serum. Sections were then incubated overnight with the primary antibody. The following primary monoclonal antibodies (mAb) against myosin heavy chains (MyHC) were used: mAb A4.840 against MyHC type I and mAb N2.261 against MyHC type I and IIA [60] (Table 6). Antibodies were purchased from the Developmental Studies Hybridoma Bank. Five fibre types were identified (type I, I/IIA, IIA, IIA/IIX, IIX). Type I fibres were strongly stained with mAb A4.840 and weakly stained with mAb N2.261. Type IIA fibres were strongly stained with mAb N2.261 and unstained with mAb A4.840. Type IIX fibres were unstained with both mAb N2.261 and mAb A4.840. Type I/IIA were weakly stained with mAb N2.261 and unstained with mAb A4.840. Type I/IIA fibres were strongly stained with both mAb N2.261 and mAb A4.840 (Fig. 4).

<table>
<thead>
<tr>
<th>fibre type</th>
<th>antibody</th>
<th>antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td><img src="null" alt="Gray" /></td>
<td><img src="null" alt="Black" /></td>
</tr>
<tr>
<td>IIA</td>
<td><img src="null" alt="Black" /></td>
<td><img src="null" alt="Gray" /></td>
</tr>
<tr>
<td>IIX</td>
<td><img src="null" alt="Gray" /></td>
<td><img src="null" alt="Gray" /></td>
</tr>
<tr>
<td>IIA/IIX</td>
<td><img src="null" alt="Gray" /></td>
<td><img src="null" alt="Gray" /></td>
</tr>
<tr>
<td>I-IIIA</td>
<td><img src="null" alt="Black" /></td>
<td><img src="null" alt="Black" /></td>
</tr>
</tbody>
</table>

Fig. 4 Classification of muscle fibres according to their staining for N2.261 and A4.840.
Following incubation with the primary antibody, the sections were rinsed with PBS buffer and incubated with a goat anti-mouse secondary antibody (Vector Laboratories). The slides were then washed in PBS for 10 minutes and incubated for 1 hour with Vectastain ABC reagent (avidin-biotin-complexregent). To visualize the primary antibody binding site, the diaminobenzidine (DAB) substrate kit for peroxidase (Vector, SK-4100) was used which results in a brown coloured end-product at the site of the target antigen. Analysis of fibre type distribution in all muscle fibres on the cross section was carried out and a mean of 233 ± 62 fibres were counted for each subject (Fig. 5).

Fig. 5 Showing 2 consecutive transverse sections stained for A4.840 and for N2.261. Type I fibres were strongly stained with A4.840 and unstained with N2.261. Type IIA fibres were strongly stained with N2.261 and unstained with A4.840. Type IIX fibres were unstained with both N2.261 and A4.840.
Table 6 Monoclonal and polyclonal primary antibodies used in all studies.

<table>
<thead>
<tr>
<th>Study</th>
<th>Type</th>
<th>Primary antibody</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>I and II</td>
<td>Monoclonal antibody</td>
<td>N2.261 MyHC type I and IIA</td>
<td>1:100</td>
</tr>
<tr>
<td>I and II</td>
<td>Monoclonal antibody</td>
<td>A4.840 MyHC type I</td>
<td>1:100</td>
</tr>
<tr>
<td>I and II</td>
<td>Monoclonal antibody</td>
<td>CD31 Endothelial cells</td>
<td>1:20</td>
</tr>
<tr>
<td>III</td>
<td>Monoclonal antibody</td>
<td>VEGF Vascular endothelial growth factor</td>
<td>1:50</td>
</tr>
<tr>
<td>III</td>
<td>Polyclonal antibody</td>
<td>Ki-67 Nuclear antigen in the cell cycle including the G1, S, G2 and M phases but not G0 phase</td>
<td>1:100</td>
</tr>
<tr>
<td>IV</td>
<td>Monoclonal antibody</td>
<td>CD3 A marker for mature T lymphocyte</td>
<td>1:50</td>
</tr>
<tr>
<td>IV</td>
<td>Monoclonal antibody</td>
<td>CD68 Detecting macrophages</td>
<td>1:100</td>
</tr>
<tr>
<td>IV</td>
<td>Monoclonal antibody</td>
<td>MHC classI Major histocompatibility complex molecule class I, essential for antigen presentation to cytotoxic T-cells</td>
<td>1:100</td>
</tr>
</tbody>
</table>

**Fibre area and perimeter**

Four to eight randomly selected areas were photographed at a magnification X 20 and used for determination of fibre area and fibre perimeter (Fig. 6). Obliquity in fibre sectioning was assessed using the form factor that represents: \((4\pi \times \text{fibre area}) / (\text{fibre perimeter})^2\) [23]. There were no differences between the form factors measured in biopsies taken in the control group \((0.80 \pm 0.04)\) compared with the OSAS patients \((0.78 \pm 0.03)\) and RLS patients \((0.79 \pm 0.04)\).
Randomly selected areas corresponding to a mean of 83 ± 23 fibres were photographed at a magnification X 20 and used for determination of the capillary network. Sequential estimation analysis has been done indicating that 50 fibres from one biopsy are sufficient to characterise capillary parameters [87]. The identification of capillaries was performed using the monoclonal antibody CD31 (Dako, Glostrup, Denmark; MO823), an antibody which primarily labels vascular endothelial cells (Table 6). Following washing in PBS, the slides were incubated over-night with the primary antibody and incubated in normal serum for 20 minutes. Peroxidase labelling was performed using a DAB substrate kit (Vector, SK-4100) that results in a brown coloured end-product at the site of the target antigen (endothelial cell).

The following capillary parameters were measured: (1) numbers of capillaries around a single fibre (CAF) and CAF relative to the area of the fibre (CAFA), (2) sharing factor (C/F), which was calculated first by determining the number of capillary contacts for the fibre in question followed by determination of the sharing factor for each of the capillaries around the fibre, (3) capillary to fibre perimeter exchange (CFPE-index) was calculated as the ratio between C/F and fibre perimeter and (4) microvessels tortuosity (LC/PF-index): in transversal sections, several microvessels are cut longitudinally due to their tortuous arrangement around the muscle fibre. The index of
tortuosity is expressed as a percent of muscle fibre perimeter in contact with the wall of
the microvessel, LC/PF-index (length of capillary/perimeter of fibre-index) (Fig. 7).

**Fig. 7** Muscle cross section stained with the monoclonal antibody CD31 for visualisation
of capillaries (1a).
In (1b), the capillary length in contact with the perimeter of the muscle fibre is shown for
four capillaries around one muscle fibre. Calculation of the tortuosity, expressed as a
percent of muscle fibre perimeter in contact with the capillary wall (LC/PF) was made.
In (1c) the sharing factor is first calculated by determining the number of capillary
contacts followed by determination of the sharing factor for each of the capillaries
around the fibre (1/4+1/2+1/2+1/3+1/3+1/3 = 2.25). CFPE-index was calculated as the
ratio between sharing factor and fibre perimeter.
Vascular endothelial growth factor (VEGF)

Double-immunofluorescence staining using two monoclonal antibodies (mAbs) was performed using a method described by Kadi et al (1999) [61]. Serial transverse sections, 5 μm thick, were cut using a microtome at −20°C (Leica CM1850) and mounted on glass slides. Muscle biopsies were air-dried at room temperature, rinsed for 10 minutes in phosphate-buffered saline (PBS), and incubated for 20 minutes in normal goat serum. Sections were then incubated overnight with the first primary antibody, monoclonal mouse anti-human CD31 (Dako, Glostrup, Denmark; MO823) (Table 6), at 4°C in a humid chamber. The sections were rinsed 3 x 10 min in PBS buffer and incubated with the first secondary antibody, goat anti-mouse antibody labeled with fluorescent Alexa fluor® 546, for 60 min at room temperature. The sections were washed for 3 x 10 min in PBS, followed by 20 min in normal serum and then incubated in FAB fragment (Jackson Immunoresearch laboratories) for 60 min. The sections were then incubated with the second primary antibody, purified mouse anti-human VEGF monoclonal antibody (A Becton Dickinson Co 555036, MO48417) (Table 6). Following incubation, the sections were washed for 3 x 10 min in PBS and incubated for 60 min with the second secondary antibody, goat anti-mouse antibody labeled with fluorescent Alexa fluor® 488, for 60 min at room temperature. A fluorescence microscope (Nikon Eclipse E400) connected to a computerized image system (SPOT Insight; Diagnostic Instrument, Sterling Heights, Michigan) was used. The percentage of capillaries expressing VEGF (CD31,VEGF+) and the percentage of capillaries which did not express VEGF (CD31, VEGF) were determined on the whole muscle cross-section (Fig. 8).
Double-immunofluorescence staining was performed using a monoclonal mouse anti-human CD31 (Dako, Glostrup, Denmark; MO823) (Table 6) as the first primary antibody, followed by the second primary antibody, polyclonal antibody, Ki-67 (Biocare Medical, CP249) (Table 6), using the same protocol as with VEGF. The percentage of capillaries expressing Ki-67 (CD31, Ki-67+) and the percentage of capillaries which did not express Ki-67 (CD31, Ki-67-) were determined on the whole muscle biopsy cross-section (Fig. 9).

**Fig. 8** Double-immunofluorescence staining for capillaries (red) and for VEGF (green) in human musculus tibialis anterior. (A) Same section double stained for capillaries (red) and for VEGF (green) showing capillaries with no VEGF expression. (B) Same section double stained for capillaries (red) and for VEGF (green) showing capillaries expressing VEGF.

**Proliferating endothelial cells (Ki-67)**

Double-immunofluorescence staining was performed using a monoclonal mouse anti-human CD31 (Dako, Glostrup, Denmark; MO823) (Table 6) as the first primary antibody, followed by the second primary antibody, polyclonal antibody, Ki-67 (Biocare Medical, CP249) (Table 6), using the same protocol as with VEGF. The percentage of capillaries expressing Ki-67 (CD31, Ki-67+) and the percentage of capillaries which did not express Ki-67 (CD31, Ki-67-) were determined on the whole muscle biopsy cross-section (Fig. 9).
Macrophages (CD68), T-cells (CD3) and MHC class I

Frozen specimens were cut in 6 μm thick sections using a microtome at −20°C (Leica CM1850). The following monoclonal antibodies were used: MHC class I (Dako, MO835, clone UCHT1) a marker for Major histocompatibility complex molecule class I essential for antigen presentation to cytoxic T-cells, CD3 (Dako, MO736, clone W6/32) a marker for mature T lymphocytes and CD68 (Dako, M0718, clone EBM11) a marker detecting macrophages (Table 6).

Cross sections were fixed on glass slides by acetone. After air drying, the sections were rinsed for 10 minutes in phosphate buffer. The primary monoclonal antibody, diluted in Dako REAL diluent (S2022 Dako, Denmark) was then added and incubated for 30 minutes at room temperature. After washing in phosphate buffer for 10 minutes the sections were incubated with Dako REAL Biotinylated secondary antibody for 25 minutes. The sections were washed for 10 minutes in phosphate buffer and incubated

Fig. 9 Double-immunofluorescence staining for capillaries (red) and for Ki-67 (green) in human musculus tibialis anterior. (A) Same section double stained for capillaries (red) and for Ki-67 (green) showing capillaries with no Ki-67 expression. (B) Same section double stained for capillaries (red) and for Ki-67 (green) showing capillaries expressing Ki-67.
with Dako REAL Streptavidin peroxidise for 25 minutes. The reaction was visualised with Dako REAL DAB/chromogen that results in a brown coloured end-product at the site of the target antigen. (Dako REAL Detection system Peroxidase/DAB, Rabbit/Mouse, Dako K5001)

All analyses (CD3 and CD68) were blinded and performed by a single observer. A test-retest was made to evaluate the reliability of the measurement, with no significant difference between the counts. The number of cells expressing CD3 (CD3⁺) and cells expressing CD68 (CD68⁺) were determined on the whole muscle biopsy cross-section and presented as number of CD3⁺ or CD68⁺ / 100 muscle fibres.

All immunohistochemical gradings for MHC class I were made by two observers. The grading was based on the pattern of expression on muscle fibre membranes as well as in the cytoplasm of the muscle fibre. Also, the distribution of the expression was considered [69]. The scale for grading is presented in Table 7.

*Table 7. Principal for grading expression of Major histocompatibility complex class I (MHC class I)*

<table>
<thead>
<tr>
<th>Grade</th>
<th>Criteria for MHC class I</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>No expression on muscle fibre membrane, capillaries stained (single, isolated, dark muscle fibres may be seen).</td>
</tr>
<tr>
<td>1</td>
<td>Expression on parts of the muscle fibre membrane in parts of the biopsy (single, isolated, dark muscle fibres may be seen).</td>
</tr>
<tr>
<td>2</td>
<td>Expression on parts of the muscle fibre membrane throughout the biopsy (single, isolated, dark muscle fibres may be seen).</td>
</tr>
<tr>
<td>3</td>
<td>Expression involving the total circumference of the muscle fibre membrane. Single isolated dark and/or granulated muscle fibres occur.</td>
</tr>
<tr>
<td>4</td>
<td>As 3, and in addition, groups of dark, granulated muscle fibres present.</td>
</tr>
<tr>
<td>5</td>
<td>Expression involving total circumference of the muscle fibre membrane with dark, granulated fibres present throughout the biopsy.</td>
</tr>
</tbody>
</table>
Statistics

Values are expressed as mean ± standard deviation in all studies. For comparisons between groups, ANOVA followed by Bonferroni was used. p ≤ 0.05 was considered as statistically significant. Calculation of the correlation coefficient was used to determine the relationship between two variables and this was used in study II III and IV. In study II, linear regression analysis was used to model the relationship between independent variable groups (OSAS or controls)) and dependent variables CFPE-index and LF/PF-index. Statistical analyses were performed using Statistix®8 (Analytical Software, Tallahassee, USA)
RESULTS

Study I

The structural characteristics of musculus tibialis anterior and the aerobic capacity in patients with RLS was investigated and compared with healthy age matched controls. RLS patients had a significantly lower predicted maximal oxygen uptake expressed in ml/min/kg compared with the control group (33.8 ± 8.1 vs 40.2 ± 5.7 ml·min⁻¹·kg⁻¹, p = 0.012).

Muscle fibre cross sectional area was equal in RLS and controls with a smaller fibre area in slow type I fibres (5288 ± 1222 µm² for RLS and 4926 ± 691 µm² for controls) compared to fast type II fibres (6389 ± 2273 µm² for RLS and 7217 ± 1582 µm² for controls). Muscle fibre type composition was similar in both the RLS group and in the control group with a predominant proportion of slow type I fibres (67.5 ± 8.9 vs 71.5 ± 9.2%). The distribution within fast fibres (IIA, IIA) was also similar except for the group IIX and IIA/IIX where RLS had a significantly higher percentage compared with controls (3.0 ± 4.6 vs 0.3 ± 0.4%, p=0.017).

The index for microvessel tortuosity, LC/PF-index, was significantly higher for RLS compared with controls, both for slow type I fibres (p=0.002) and fast type II fibres (p=0.008). The numbers of CAF, CAFA or CFPE for slow and fast fibres did not differ significantly between the two groups. All microvessel parameters are presented in Table 8.

Table 8 Microvessel indices for type I and type II muscle fibres in tibialis anterior muscle from 18 OSAS patients, 20 RLS patients and 16 controls.

<table>
<thead>
<tr>
<th></th>
<th>OSAS (n=18)</th>
<th>RLS (n=20)</th>
<th>Control (n=16)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mean</td>
<td>SD</td>
<td>mean</td>
</tr>
<tr>
<td>CAF type I</td>
<td>5.7</td>
<td>1.0</td>
<td>4.8</td>
</tr>
<tr>
<td>CAF type II</td>
<td>5.6</td>
<td>1.0</td>
<td>4.5</td>
</tr>
<tr>
<td>CAFA type I</td>
<td>1.1</td>
<td>0.2</td>
<td>1.0</td>
</tr>
<tr>
<td>CAFA type II</td>
<td>0.8</td>
<td>0.1</td>
<td>0.8</td>
</tr>
<tr>
<td>CFPE type I</td>
<td>7.2</td>
<td>1.0</td>
<td>6.1</td>
</tr>
<tr>
<td>CFPE type II</td>
<td>6.2</td>
<td>0.9</td>
<td>5.1</td>
</tr>
<tr>
<td>LC/PF type I</td>
<td>23.4</td>
<td>4.9</td>
<td>22.3</td>
</tr>
<tr>
<td>LC/PF type II</td>
<td>18.7</td>
<td>4.7</td>
<td>19.3</td>
</tr>
</tbody>
</table>

Number of capillaries around a single fibre (CAF), CAF relative to the area of the fibre (CAFA), capillary to fibre perimeter exchange (CFPE), microvessel tortuosity (LC/PF).
Study II

Predicted maximal oxygen uptake (maxVO₂), clinical parameters (duration of OSAS, desaturation during sleep and AHI) and muscular parameters (muscle phenotype, muscle fibres cross sectional area and capillary network) in 18 OSAS patients and 16 age-matched controls were examined. The relationships between the muscular parameters, predicted maximal oxygen uptake, and the clinical symptoms were also assessed.

The OSAS group had significantly lower predicted relative maxVO₂ (31.3 ± 10.5 ml·min⁻¹·kg⁻¹ body weight) compared with the control group (40.2 ± 5.7 ml·min⁻¹·kg⁻¹ body weight), (p = 0.0047) and predicted relative maxVO₂ in OSAS patients was inversely correlated to AHI (r = -0.6, p = 0.017).

A questionnaire including questions regarding physical activity level was used. Fourteen patients with OSAS and 10 controls had very low- or low physical activity level. Two patients with OSAS and 4 controls had moderate physical activity level. Two from each group had high level of physical activity and none had very high level. There was no significant difference between the two groups regarding physical activity level.

Muscle fibre cross sectional area was similar in OSAS and controls with a smaller fibre area in slow type I fibres (5586 ± 1586 µm² for OSAS and 4926 ± 691 µm² for controls) compared to fast type II fibres (6815 ± 1447 µm² for OSAS and 7217 ± 1582 µm² for controls). Analysis of muscle fibre type distribution showed a predominant proportion of slow type I fibres in tibialis anterior both in OSAS (70.6 ± 6.8%) and in controls (71.5 ± 9.2%). There were no significant differences in the proportions of type I and type IIA between OSAS and controls. Fast type IIX and IIA/IIX fibres were rarely detected in both groups but there was a significantly higher percentage of type IIX and IIA/IIX fibres in OSAS (1.5 ± 2.3%) compared to controls (0.3 ± 0.4%) (p = 0.040).

Patients with OSAS have a significantly higher number of capillaries per fibre (CAF) for slow type I fibres compared with controls (p = 0.0031). CAF per fibre area (CAFA) was significantly higher in the OSAS group for fast type II fibres compared with the control group (p = 0.0163). CFPE-index was significantly higher in OSAS both in slow and fast fibre types compared with controls (p = 0.0007 for slow type I fibres and p = 0.0126 for fast type II fibres). LC/PF-index was significantly higher in type I and type II fibres from OSAS compared with controls (p = 0.0003 for slow type I fibres and p = 0.0285 for fast type II fibres). Microvessel indices for type I and type II muscle fibres are presented in Table 8.

The dependent variable group (OSAS or control) was significantly related to both LC/PF-index (r² = 0.28, p = 0.0008) and CFPE-index (r² = 0.24, p = 0.0019)
Study III

The occurrence and location of VEGF and proliferating endothelial cells in musculus tibialis anterior of patients with RLS and OSAS compared to healthy age matched controls was investigated. Furthermore, data from two earlier studies were used to compare LC/PF-index and CFPE-index between RLS and OSAS.

Double immunofluorescence staining for capillaries (anti-CD31) and VEGF (anti-VEGF) showed that capillaries expressed VEGF in all three groups. However, the number of capillaries expressing VEGF (CD31, VEGF+) varies between the groups. Both OSAS (69.9 ± 26.6%) and RLS (60.9 ± 21.9%) have a significantly higher percentage of CD31,VEGF+ capillaries compared to the controls (34.1 ± 22.7%) (Fig. 10).

Double immunofluorescence staining for capillaries (anti-CD31) and proliferating cells (Ki-67) showed few capillaries expressing Ki-67 in all three groups. OSAS had significantly higher percentage of CD31,Ki-67+ capillaries compared to RLS and controls (7.6 ± 4.9% for OSAS, 3.9 ± 2.0% for RLS and 2.1 ± 1.7% for control) (Fig. 10). The differences in CD31,Ki-67+ between RLS and controls did not reach the level of significance. There was a correlation between CD31,VEGF+ capillaries and CD31,Ki-67+capillaries (r = 0.46, p = 0.0056).

When comparing OSAS and RLS regarding capillary geometry, a significantly higher CFPE-index of type I (p = 0.0027) and type II (p = 0.0037) muscle fibres was found in...
OSAS compared to RLS but there was no significant difference in LC/PF-index between the two groups. BMI was significantly higher in OSAS (33.7 ± 5.6) compared with RLS (27.2 ± 3.4) and controls (24.4 ± 1.9).

**Study IV**

In order to examine whether local inflammation in skeletal muscle occur in patients with OSAS and RLS, different inflammatory markers were investigated in the tibialis anterior muscle of patients with OSAS and RLS and compared to healthy controls. Furthermore, a second aim was to elucidate if changes also appear in mitochondrial function and structure.

Plasma samples were analysed for hsCRP and CK to evaluate systemic inflammation (Table 9). There were no differences in hsCRP and CK between the groups but a positive correlation between hsCRP and BMI was identified ($r=0.6350$, $p<0.001$). Using hsCRP as an adjunct to major risk marker for cardiovascular disease a value $>3$ mg/L is considered as high risk [82].

**Table 9** *Creatine kinase (CK) and high sensitive C-reactive protein (hsCRP) in patients with RLS and OSAS*

<table>
<thead>
<tr>
<th></th>
<th>CK Ukat/L mean ± sd</th>
<th>hsCRP mg/L mean ± sd</th>
</tr>
</thead>
<tbody>
<tr>
<td>OSAS</td>
<td>2.54 ± 1.28</td>
<td>3.03 ± 2.81</td>
</tr>
<tr>
<td>RLS</td>
<td>1.69 ± 1.06</td>
<td>2.23 ± 2.75</td>
</tr>
<tr>
<td>reference range</td>
<td>0.8 - 6.7</td>
<td>&lt; 2</td>
</tr>
</tbody>
</table>

The muscle biopsy findings staining for NADH-TR, SDH, COX and Gomori Trichrome were normal except for minimal changes of low diagnostic significance, i.e. the occurrence of a few atrophic fibres, a few fibres with centrally placed nuclei, or a non-significant fibre diameter variation (Fig. 11). Accumulation of peripherally localized mitochondria in some fibres was seen, together with irregular cores in some fibres (Fig. 12). No COX- or SDH-negative fibres were seen in any of the subjects. In 4 OSAS patients and 1 RLS patient ringbinden fibres were seen (Fig. 13), a phenomenon usually indicative of a disturbance in muscle tone.
Fig. 11 Section stained for NADH-TR showing non-significant fibre diameter variation

Fig. 12 Section stained for NADH-TR showing core zone

Fig. 13 Section stained NADH-TR showing a ringbinden fibre.

Fig. 14 Section stained for MHC class I.

Fig. 15 Section stained for T-cells

Fig. 16 Section stained for macrophages
None of the subjects showed any expression of MHC class I according to the criteria for grading the expression of MHC class I. All patients with RLS and OSAS as well as the control group were graded as normal (0-1) (Fig 14).

Staining for T-cells (anti-CD3) (Fig. 15) and macrophages (anti-CD68) (Fig. 16) showed presence of both T-cells and macrophages in all three groups but there were no significant difference between the groups according to CD3⁺ cells and CD68⁺ cells (Table 10).

**Table 10.** Percentage of T-cells (CD3⁺) and percentage of macrophages (CD68⁺) in patients with OSAS and RLS and in controls.

<table>
<thead>
<tr>
<th></th>
<th>OSAS mean ± sd</th>
<th>RLS mean ± sd</th>
<th>Control mean ± sd</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CD3⁺ T-cells (%)</strong></td>
<td>1.6 ± 1.6</td>
<td>1.9 ± 1.3</td>
<td>1.8 ± 0.9</td>
<td>ns</td>
</tr>
<tr>
<td><strong>CD68⁺ Macrophages (%)</strong></td>
<td>1.4 ± 0.8</td>
<td>2.1 ± 1.2</td>
<td>1.6 ± 1.0</td>
<td>ns</td>
</tr>
</tbody>
</table>
DISCUSSION

Subjects

Eighteen patients with OSAS and 20 patients with RLS were recruited at the Avesta Sleep Research Centre and diagnosed by a sleep physician. RLS is diagnosed by only four diagnostic criteria but it is important to emphasize that the criteria are well standardized and used world-wide [5]. OSAS was confirmed with the gold standard polysomnography or with Embletta and Breas screening [30,78]. Polysomnography is a more complicated method and a more complete examination can be achieved compared to Breas and Embletta. All three methods measure saturation and thereby AHI and the basis for the diagnosis of OSAS is the number of AHI. There are still discussions as to where the cut-off point for the numbers of AHI should be [68]. AHI > 5 is still common but cut-off points of AHI > 10, 15, and even 20 have been proposed. In our study a high cut-off point was used and AHI was 42.4 with the range between 16-90.

The healthy controls were recruited by advertising at Örebro University and were age matched with the patient groups. The age interval chosen was 20 to 50 years of age, to avoid influence of the accelerating muscle growth in youth or the age-related decline in muscle mass [117].

The higher BMI seen in OSAS patients compared to controls might influence the results of a higher capillary network but Gavin et al (2005) showed that BMI does not account for differences in capillarisation [42].

OSAS is most common in men [34] and RLS is most common in women [11,105]. However in our study, the subjects were consecutively recruited and the distribution between men and women is therefore not equal. Porter et al (2002) showed that microvascularisation of tibialis anterior muscle is similar in men and women [88] which implies that the larger capillary network seen in RLS and OSAS compared with controls is not due to gender differences.

Aerobic capacity level

Aerobic capacity was determined as predicted relative maximal oxygen uptake. The submaximal test procedure instead of a maximal test was used because of the extreme demand during a maximal exercise test and the unwillingness of the patients to perform this test. When comparing the 3 groups (OSAS, RLS and controls), the OSAS was the group with the lowest predicted value while the control group had the highest. The low aerobic capacity seen in RLS and OSAS might explain the symptoms such as perceived fatigue to some extent. In the OSAS group AHI was inversely correlated to maximal
oxygen uptake indicating that the severity of the syndrome affects the aerobic capacity or that low aerobic capacity increase AHI.

Women generally have lower maximal oxygen uptake compared to men [117]. Despite the fact that the OSAS group was comprised of more men than women, the relative predicted maximal oxygen uptake was higher in controls compared to OSAS, thus speaking in favour of a low physical capacity level in OSAS.

**Muscle fibre distribution and fibre area**

The similar distribution of slow type I and fast type II fibre in the tibialis muscle, with a predominate proportion of slow type I fibres in the three groups are in accordance with earlier studies [88]. The percentage of fast type IIX and IIA/IIX fibres was significantly higher in RLS and OSAS compared to controls. As the proportion of fast type IIX is increased in skeletal muscle in response to inactivity or decreased in response to physical activity, this indicates that OSAS and RLS patients are more sedentary compared to controls [61,96].

**Muscle fibre capillarisation**

The capillary network was larger in both OSAS and RLS compared to controls and was largest in the OSAS group. Increased capillary network is in agreement with earlier reported findings after endurance training [23,57]. The cause for this adaptation is proposed to be the occurrence of local hypoxia within the muscle during exercise. VEGF and HIF are also enhanced during exercise, most probably due to local hypoxia in the muscle, thus stimulating angiogenesis [47,46].

OSAS is a sleep disorder with known intermittent arterial hypoxia during sleep [114], likely causing local hypoxia in the skeletal muscle. To compensate for the hypoxia during sleep, angiogenesis may occur by the same underlying mechanisms as after endurance training i.e. enhanced expression of VEGF. In the present study only the OSAS patients had a significantly higher BMI compared to controls whereas VEGF expression was located in the tibialis anterior muscle both in RLS and OSAS, indicating that the elevated VEGF is associated with a larger capillary network.

RLS patients do not show arterial hypoxia, however local hypoxia due to vasoconstriction might exist in these patients. This hypothesis is strengthened by the relief of symptoms by limb-movement which cause muscle contraction that are known to increase blood flow and oxygen supply [9,27]. The fact that RLS patients often move their legs to temporarily relieve the symptoms could therefore be a strategy to increase the blood flow.
The two groups of patients also have a low aerobic capacity level. This might indicate poor levels of central (stoke volume, cardiac output) as well as peripheral (aerobic enzyme activity, mitochondria function) factors involved in the regulation of the maximal oxygen uptake [15]. Therefore patient with RLS and OSAS may benefit from physical training to provide better delivery of oxygen to the muscle.

**VEGF and Ki-67**

To verify if an ongoing process of endothelial cell proliferation occurs, a double-immunofluorescence staining with CD31/VEGF and also CD31/Ki-67 was performed. VEGF and Ki-67 were expressed in capillaries in human skeletal muscle but were more highly expressed in RLS and even more pronounced in OSAS compared to controls. This strengthened the hypothesis that hypoxia is a factor involved in the angiogenesis seen in patients with RLS and OSAS.

**Inflammation in skeletal muscle**

Besides stimulating angiogenesis, VEGF is also known to act as a vascular permeability factor (VPF), is involved in inflammation [17,35,76], and VEGF expression is induced during inflammation [17,120]. Could it therefore be possible that the higher expression of VEGF seen in tibialis anterior muscle in RLS and OSAS is not due to local hypoxia but to systemic or local inflammation?

To assess if there were signs of inflammation in tibialis anterior muscle in RLS, OSAS and controls, well known immunohistochemical markers for inflammation (MCH class I, CD3 and CD68) were used and the expression of MHC class I, CD3+ cells or CD68+ cells was not increased. Normally both T-cells and macrophages do exist in a small number in skeletal muscle [72] which is in accordance with the present data indicating absence of local inflammation.

There are studies reporting that OSAS patients have elevated plasma levels of inflammatory markers such as TNF-α and IL-6 interpreted as systemic inflammation [102,120]. High levels of plasma VEGF have also been reported in patients with OSAS suggesting the existence of systemic inflammation leading to cardiovascular disease [66,92]. However plasma VEGF was not analysed in the present study. Another indicator of inflammation is C-reactive protein (CRP) and elevated levels of CRP have been reported in patients with OSAS [93]. In our study mean hsCRP levels in OSAS patients was 3.03 mg/L (reference range <2) which is slightly increased above normal reference range. In addition a positive correlation between hsCRP and BMI (r=0.6350,
p< 0.001) was noted in the present study however it has earlier been shown that higher CRP is more likely associated with higher BMI than with OSAS [25].

Boyd et al (2004) performed morphometric analysis on upper airway tissue from OSAS patients. These data suggested that inflammatory cell infiltration and denervation changes affect not only the mucosa but also the upper airway muscle [16]. If the inflammatory state seen in the upper airway region is due to systemic inflammation this should also be present in the muscle examined in our study but this was not the case.

Whether RLS patients have an elevated level of plasma VEGF is unknown. However, it has been reported that patients with RLS have normal levels regarding inflammatory cytokines in plasma [94]. In the present study hsCRP in the RLS patients was 2.23 mg/L (reference range <2), which is considered to be normal. Taken together no signs of inflammation are present in muscle from RLS.

In conclusion, tibialis anterior muscle in OSAS and RLS is not affected by local inflammation (study IV), which supports the hypothesis of local hypoxia causing the upregulation of VEGF. The high expression of VEGF may be beneficial as angiogenesis will contribute to an increased perfusion of potentially ischemic tissues, thus limiting the symptoms from the muscle.

**Histopathological findings**

In clinical investigations it is common to examine sections of muscle fibres, using routine histochemical methods for determination of NADH, SDH, COX and also a double staining for COX and SDH, together with Gomori Tricrome staining. Small amounts of ring-binden fibres, irregular cores fibres or COX- and SDH-negative fibres are common and seldom indicative of a particular disease. These type of stainings could also give an indication of structural and architectural changes in the skeletal muscle [33].

The consolidated evaluation of the stained sections was made by two independent observers and the results showed good inter-individual correlation. Both RLS- and OSAS patients had minimal changes such as core zone fibres and ring-binden fibres (or ring fibres). The core zone is devoid of mitochondria and oxidative enzyme activity, and can be found in patients with different myopathies [32]. Ring-binden fibres are a phenomenon usually indicative of disturbance in muscle tone [33,83]. There were very few occurrences of all findings and those assessed were not of any diagnostic significance but it may be an indication that tibialis anterior muscle in RLS and OSAS is in some way affected and therefore further investigations are required.
CONCLUSIONS

Restless legs syndrome (RLS) and Obstructive sleep apnoea (OSAS) syndrome are two types of sleep disorders that affect daily life with symptoms such as fatigue and tiredness [24,106]. These two syndromes are accompanied by changes in skeletal muscle and physical fitness level.

- The predicted relative maxVO$_2$ was lower in both RLS and OSAS patients compared to healthy controls.
- Muscle fibre type composition was similar in all three groups with a predominant proportion of slow type I fibres compared to fast type II fibres.
- Muscle fibre cross sectional area was equal in the groups with a smaller fibre area in slow type I fibres compared to fast type II fibres.
- Fast type IIIX and IIA/IIIX fibres were rarely detected in all three groups but there was a significantly higher percentage of type IIIX and IIA/IIIX fibres in OSAS and in RLS. As the proportion of fast type IIIX fibres is increased in skeletal muscle in response to inactivity and decreased in response to physical activity this further indicates low physical fitness level in the patient groups.
- Musculus tibialis anterior in RLS and OSAS patients have a larger capillary network compared to healthy age matched controls. CFPE- index (capillary to fibre perimeter exchange) and LC/PF-index (length of capillary/perimeter of fibre) was elevated in both RLS and OSAS.
- Double immunofluorescence staining for capillaries (anti-CD31) and VEGF (anti-VEGF) showed that capillaries express VEGF in the tibialis anterior muscle in all three groups with a significantly higher expression in RLS and OSAS compared to controls.
- Double immunofluorescence staining for capillaries (anti-CD31) and proliferating endothelial cells (Ki-67) showed that there are occurrence of proliferating endothelial cells in musculus tibialis anterior in all three groups with a more significant expression in OSAS compared to controls.
Increased capillarisation was not due to inflammation as no expression of MHC class I was detected and the number of T-cells (CD3) and macrophages (CD68) were equally present in all three groups.
FUTURE PERSPECTIVES

In this thesis local hypoxia in the muscle was suggested to cause the larger capillary network and a higher percentage of capillaries expressing VEGF both in OSAS and RLS. OSAS do have a well known arterial intermittent hypoxia during sleep. RLS patients do not show arterial hypoxia, however local hypoxia due to vasoconstriction might exist. Occurrence of impaired capillary blood flow in RLS is strengthened by relief of symptoms by limb movement as muscle contraction is known to increase blood flow and oxygen supply. Whether blood flow is influenced in resting skeletal muscle in RLS patients is unknown and need further investigation.

OSAS and RLS patients have a lower aerobic capacity compared to healthy controls that indicates poor levels of central factors involved in maximal oxygen uptake. Therefore patient with RLS and OSAS may benefit from physical training aimed to provide better capacity to increase delivery of oxygen to the muscle. There are only a few studies (two in OSAS patients and one in RLS patients) evaluating the effect of physical training, indicating that physical training benefit the symptoms of RLS and OSAS. However, the RLS study did not measure any physiological parameters during the study which made it more difficult to conclude if the training did influence factors involved in oxygen uptake. The investigations on OSAS patients showed conflicting results. One study showed no improvement while the other study showed increased physical capacity. However in both studies symptom relief was noted. These diverging results are probably due to differences in training protocol and methods for assessment of training effects. On the basis of our results it would be of interest to propose an exercise training protocol that ensures an increase in central (stoke volume, cardiac output) as well as peripheral (aerobic enzyme activity, mitochondria function) factors involved in the regulation of the maximal oxygen uptake and to assess the effect on clinical symptoms.
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