Applying proteomics and metabolomics for studying human skeletal muscle with a focus on chronic trapezius myalgia

Jenny Hadrévi
“An essential aspect of creativity is not being afraid to fail”

- Edwin Land

Till min familj
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Abstract

Work related musculoskeletal disorders are the dominating causes of reported ill-health in industrialized countries. These chronic pain conditions are one of the most costly public health problems in Europe and North America. When work related musculoskeletal disorders are considered to be of muscular origin and the trapezius muscle is affected, the common appellation is trapezius myalgia. Since little is known about the genesis or how it is maintained, it is of great importance to better understand the pathophysiology of trapezius myalgia; doing so will better enable recommendations for prevention, treatment and rehabilitation. Several hypotheses have been presented based on biochemical alterations in the muscle, suggesting increased signaling of inflammatory substances and altered metabolism. Previous research has not been able to present the comprehensive picture of the muscle in pain. Thus there is a demand for more comprehensive research regarding the biochemical milieu of the chronic trapezius muscle.

Proteomic and metabolomic methods allow non-targeted simultaneous analyses of a large number of proteins and metabolites. The main emphasis in this thesis is on a proteomic method, two-dimensional differential gel electrophoresis (2D-DIGE). The method is validated to human skeletal muscle biopsy research with laboratory specific settings. In the baseline study, there were 14 metabolic, contractile, structural and regulatory proteins that differed significantly in abundance when trapezius and vastus lateralis muscles were compared. Using the validated 2D-DIGE method and the baseline study, a comparison between healthy and myalgic muscles was made. Biopsies from female cleaners with and without myalgia were compared to obtain results from women with the same type of work exposure. In the multivariate model, 28 identified unique proteins separated healthy and myalgic muscle and were grouped according to function: metabolic (n=10), contractile (n=9), regulatory (n=3), structural (n=4), and other (n=2). Finally, a second screening method, metabolomics, was introduced to analyze differences in metabolite content as a complement to and verification of the proteomic results. Gas chromatography-mass spectrometry (GC-MS) was performed on muscle interstitial fluid samples obtained with microdialysis, and differences in the abundance of extracellular metabolites were revealed.

The 2D-DIGE method is a reliable method to analyze human skeletal muscle. The outcomes of the proteomic analyses were dependant on the statistical approach. Systematic differences in protein and metabolite
content were detected using a multivariate approach. Univariate analyses were used to analyze individual proteins for their significance. The significant proteins in the baseline study were predominately related to muscle fiber type which correlated with the differences in fiber type content between trapezius and vastus lateralis. The proteomic and metabolomics studies where myalgic and healthy muscles were compared provide us with new clues and new aspects regarding the pathophysiology of the myalgic muscle.

Technically advanced methods employed in the thesis enabled an explorative screening of proteins of relevance for the pathophysiology of the myalgic muscle. The results of these analyses may contribute to the formulation of future hypothesis that need to be further evaluated.
## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>2D-DIGE</td>
<td>two dimensional-difference in gel electrophoresis</td>
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<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
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<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
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<td>BVA</td>
<td>biological variation analysis</td>
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<td>CRP</td>
<td>C-reactive protein</td>
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<td>DIA</td>
<td>differential in-gel analysis</td>
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<tr>
<td>FADH$_2$</td>
<td>flavin adenine dinucleotide-hydrogen</td>
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<td>FDR</td>
<td>false discovery rate</td>
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<tr>
<td>GC-MS</td>
<td>gas chromatography-mass spectrometry</td>
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<td>HSP</td>
<td>heat shock protein</td>
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<td>IHC</td>
<td>immunohistochemistry</td>
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<td>IL</td>
<td>interleukin</td>
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<td>IPI-human</td>
<td>international protein index - human</td>
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<td>LC-MS</td>
<td>liquid chromatography-mass spectrometry</td>
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<tr>
<td>LDH</td>
<td>lactate dehydrogenase</td>
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<td>MALDI-TOF</td>
<td>matrix assisted laser desorption/ionization-time of flight</td>
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<td>MS/MS</td>
<td>mass spectrometry</td>
</tr>
<tr>
<td>MyLC</td>
<td>myosin light chain</td>
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<td>MyHC</td>
<td>myosin heavy chain</td>
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<tr>
<td>NADH</td>
<td>nicotinamide adenine dinucleotidenicotine-hydrogen</td>
</tr>
<tr>
<td>NIST</td>
<td>National Institute of Standards and Technology</td>
</tr>
<tr>
<td>OPLS-DA</td>
<td>orthogonal partial least square – discriminant analysis</td>
</tr>
<tr>
<td>PEA</td>
<td>N-palmitoylethanolamide</td>
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<tr>
<td>PLS-DA</td>
<td>partial least square - discriminant analysis</td>
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<tr>
<td>Abbreviation</td>
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<tr>
<td>PSD</td>
<td>post source decay</td>
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<td>PVDF</td>
<td>polyvinylidene difluoride</td>
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<td>SDS-PAGE</td>
<td>sodium dodecyl sulphate-polyacrylamide gel electrophoresis</td>
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<tr>
<td>SNS</td>
<td>sympathetic nervous system</td>
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<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
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<tr>
<td>TnT/C/I</td>
<td>troponin T/C/I</td>
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<tr>
<td>TSST</td>
<td>Trier social stress test</td>
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<tr>
<td>VCWS</td>
<td>Valpar Component Work Stations</td>
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<tr>
<td>VIP</td>
<td>variable of importance</td>
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Sammanfattning på svenska


Proteiner renades fram och analyserades med så kallad tvådimensionell gelelektrofores. Genom att tillföra en elektrisk spänning och kemikalier, fördelas de enskilda proteinerna efter isoelektrisk laddning (i första dimensionen) och efter molekylvikt (i andra dimensionen). När analysen är klar erhålls en gel med små prickar. Dessa prickar skärs ut för att därefter proteinbestämmas med hjälp av masspektrometriska metoder. Metoderna bygger på att enskilda proteiner klyvs till fragment (peptider) och identifieras med hjälp av en så kallad masspektrometer.

grund för vidare analyser av trapezius muskeln då skillnader i proteinförekomst mellan frisk och smärtande trapezius muskel jämförs.

Tredje delarbetet (paper III) är en studie där muskelbiopsier tagna från trapeziusmuskeln hos personer med kronisk värk i nack-skulderregionen samt besvärssfria personer jämförs med avseende på proteininnehåll. Skillnader i proteinerna relaterade till ämnesomsättning, muskelkontraktion och cellulär stress hittades.


Sammanfattningsvis utvärderas i denna avhandling en proteinkemiskt avancerad metod i avseendet att få en så övergripande och tillförlitlig analys av skelettmuskel som möjligt. Vilket utfall som ges av den övergripande analysen påverkas av vilken typ av statistisk analysmetod man väljer. Metoden tillämpas vidare för att analysera muskelbiopsier i avseendet att jämföra proteinuttrycket mellan frisk och smärtande trapezius muskel. En ytterligare metod används för att komplettera resultaten från proteinanalysen med avseende på metabolitinnehåll. Resultaten visar skillnader mellan frisk och smärtande trapeziusmuskeln med avseende på en trolig förändrad ämnesomsättning, förändring av muskels kontrakionsproteiner samt cellulär stressrespons i den sjuka muskeln. Även om en heltäckande förklaring till mekanismerna bakom trapezius myalgi inte kan formuleras med dessa resultat, så utgör de viktiga ledtrådar till fortsatta studier rörande kroniska smärtstillstånd i musklerna.
Original papers

This thesis is based on the following papers:

**Paper I**


**Paper II**


**Paper III**


**Paper IV**


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Introduction

General background

Work related musculoskeletal disorders are the dominating causes of reported ill-health and one of the most costly public health problems in Europe and North America [7; 105]. When work related musculoskeletal disorders are hypothesized to be of muscular origin and affecting the trapezius muscle, the common appellation is trapezius myalgia. The main risk factors to develop trapezius myalgia are suggested to be intrinsic properties of the muscle in combination with external risk factors such as work load, work posture [68; 142; 143; 15] mental stress [88; 119] and combinations of these [32; 89; 29; 136]. Since little is known about the genesis or how it is maintained, it is of great importance to better understand the pathophysiology of trapezius myalgia. Understanding the pathophysiology of the myalgic muscle can better enable prevention, treatment and rehabilitation. Many different hypothetical models have been proposed [57; 54; 71]. Within these models, biochemical research data have been sparse although assumptions of biochemical alterations in the muscle are often proposed. Several research projects have been launched with the assumption of for example, inflammation and metabolic alterations in the muscle (reviewed by Visser and van Dien [146]). Thus, there is a demand for more comprehensive human research in order to provide a better picture of the maintenance and genesis of myalgia.

Proteomics and metabolomics together with multivariate analysis allow a simultaneous analysis of a large number of proteins, and are applied in the search for new possible aspects of the disorder. The development of these technically advanced methodologies enables explorative screening of proteins of importance to the pathophysiology of the myalgic muscle. The main emphasis in this thesis is on a proteomic method, two-dimensional differential gel electrophoresis (2D-DIGE). The method was validated to human skeletal muscle research with laboratory specific settings. A baseline study was made where the protein pattern of trapezius was compared to our most well studied muscle, the vastus lateralis. Using the validated 2D-DIGE method and the baseline study, a comparison between healthy and myalgic muscle was made. Biopsies from female cleaners with and without myalgia were compared, providing us with results from women with the same type of work exposure. Finally a second screening method was introduced, analyzing differences in metabolite content to complement and verify the proteomic results. This metabolomic study was made on muscle interstitial fluid obtained using microdialysis. Results from these explorative screening
methods provide us with new data and new aspects on the pathophysiology of myalgia.

**Human skeletal muscle**

More than 600 skeletal muscles, accounting for approximately 40% of our body weight, are the effector organs that produce body movement, maintain posture and tonus, produce heat and contribute to energy balance. The muscles in the human body show great variety of sizes, shapes and fiber architecture that reflect their anatomical locations and functional specialization. In spite of these differences, the vast majority of the trunk and limb muscles share a similar tissue organization.

Skeletal muscle consists of long, multinucleate myofibers surrounded by capillaries and embedded in connective tissue. Bundles of muscle fibers, separated by connective tissue, where blood vessels and nerves are found, form the muscle fascicles. The muscle fibers contain myofibrils built up of repeating units called sarcomeres. These give skeletal muscle its typical striated appearance under the microscope. Adjacent sarcomeres in a myofibril are connected by the z-discs. The major contractile proteins in the sarcomere are actin, building the thin filaments, and myosin, building the thick filaments. During muscle contraction, myosin functions as a motor making the thin filaments physically slide along the thin filaments, thereby shortening the sarcomere [23]. A number of proteins, including troponin and tropomyosin, bridge the thick and thin filaments.

**Proteins of muscle contraction**

Proteins involved in muscle contraction dictate the muscle specific characteristics, e.g. contractility, contraction velocity and fatigue resistance. Thick and thin filaments, connected via the myosin head, slide to shorten the distance between the z-discs during muscle contraction. Troponin and tropomyosin are, together with actin, the major components of the thin filaments [114; 23]. Tropomyosin has a coiled coil rod that extends the length of the actin filaments and, together with the troponins, regulates the interaction between actin (thin filaments) and myosin (thick filaments). The troponin complex consists of three subunits troponin C (TnC), troponin T (TnT), and troponin I (TnI). In the non-contracting state TnI is bound to actin, and tropomyosin covers the myosin-actin binding sites inhibiting the interaction between the myosin head and actin. As the motor-neurons signal the muscle to contract, Ca$^{2+}$ is released into the sarcomere. Subsequently, tropomyosin changes position and allows weak myosin-actin binding. In turn this leads to activation of the myosin ATPase, which consumes an ATP,
leading to further translocation of the tropomyosin along the actin filaments and finally, a strong stroke between actin and myosin as the filaments slide toward each other and the muscle contracts.

The myosin molecule is a hexamer composed of two identical myosin heavy chains (MyHCs), two alkali (essential) light chains (MyLC) and two regulatory (phosphorylatable) MyLCs [100; 101]. Each MyHC consists of a head (or motor) domain containing the ATPase activity site and the actin binding site, and a long rod (figure 1). The rods of the two MyHCs are intertwined forming an alpha helical coiled coil. A thin neck, where the essential and regulatory MyLCs are situated, connects the rod and head domains of the MyHC molecule.

The major MyHC isoforms present in human skeletal muscle are the fast isoforms MyHC2A and MyHC2X, typically present in fast-contracting type 2 fibers, and the MHCI/β present in type I, i.e. slow contracting muscle fibers [101]. MyHC embryonic and MyHC neonatal/fetal are expressed during fetal development but are later down regulated, with rare exceptions such as the masticatory and extraocular muscles [114]. Myosin light chains (MyLCs) are also expressed in different isoforms. In human skeletal muscle, different essential (or alkali) MyLC isoforms have been identified [10; 101]. Fast skeletal muscle fibers typically contain MyLC-1fast and MyLC-3fast. Slow skeletal muscle fibers also contain MyLC-1slow/ventricular (also called MyLC-1slow-b). MyLC-emb/atrial is expressed in developing skeletal muscle and atrial myocardium. Regulatory (or phosphorylatable) MyLCs are phosphorylated by MyLC kinase during muscle contraction. In human skeletal muscle fibers, two different regulatory MyLC isoforms are expressed, MyLC-2 fast and MyLC-2 slow. The regulatory MyLCs play a modulatory role.

Figure 1. The myosin molecule
by increasing force production at low levels of calcium activation in human skeletal muscle [126]. Slow and fast isoforms of myosin light chains are predominately expressed with the same type of MyHC isoform. The myosin composition correlates to the contractile properties of the muscle and for a long time has been considered the best marker of muscle function. As the contribution of other sarcomeric proteins to muscle contraction is gradually better elucidated; it becomes clear that the contractile properties of a muscle fiber are the result of the total composition of sarcomeric proteins [114].

**Muscle fiber types**

Muscle fibers of different types build up muscle tissue. Muscle fibers are traditionally divided into different fiber types (1, 2a, 2b) depending on the action of the ATPase situated on the myosin head. The ATPase activity may be revealed histochemically at different pH values [30]. In comparison to type 2 fibers, type 1 fibers have a higher oxygen demand due to their higher abundance of mitochondria [5; 86; 125]. Type 1 muscle fibers also have more capillaries [5]. Type 1 fibers are slow contracting with low glycolytic ability and use energy stored as triglycerides in the blood. Type 1 fibers have high levels of oxidative metabolism since mitochondria and myoglobin occur in larger amounts than in the type 2 fibers. Type 2 fibers are fast contracting (type 2b are faster than type 2a) and have a high glycolytic ability as they utilize most of their energy from glycogen stored in muscle. Muscles with different function are build of different proportions of type 1 and type 2 fibers. The traits of each muscle in the body are partially dictated by muscle performance. The proportion of type 1 and type 2 fibers can be altered depending on how the muscle is used; this is called the training effect [6]. Overall, limb muscle has a higher content of type 2 fibers correlating with motile movement; whereas stabilizing muscles, such as the trapezius, have a higher content of type 1 fibers. This type of traditional classification relies entirely on the activity of the ATPase situated on the MyHC. More comprehensive information about the contractile characteristics of the muscle can be obtained using different biochemical techniques that detects more proteins simultaneously. Comprehensive proteomic studies comparing different types of skeletal limb muscle have been preformed [20; 98]. These previous studies show that the distribution of different isoforms of contractile proteins is practically in agreement with fiber type content.

**Trapezius**

The human trapezius muscle is a large and powerful muscle located superficially over the posterior aspect of the neck, the shoulder and the upper part of the back (figure 2). The muscle is divided into three areas: the
ascending, descending and the transverse part, depending on the direction of the muscle fibers. The most studied part is the descending part, with fibers going from the cervical part to the shoulder [85; 86]. The trapezius muscle rotates the shoulder blade and extends or laterally flexes the neck backwards and forwards and to the sides; it is also involved in the movements of the arms, facilitating the lifting of the arms over the shoulder.

The healthy trapezius muscle has been thoroughly characterized [85; 86] and compared to the vastus lateralis [61] in previous morphological studies. The vastus lateralis muscle has approximately 50% type 1 fibers, whereas the descending part of the trapezius muscle has approximately 65% type 1 fibers [58; 84-86]. The trapezius muscle has higher androgen receptor content in comparison to vastus lateralis [61] and also, in correlation with the higher abundance of type 1 fibers, a denser capillary network [85; 61].

Figure 2. The trapezius muscle (left) and the vastus lateralis muscle (right).

**Trapezius myalgia**

The most palpable characteristic of trapezius myalgia is by definition sustained pain in the upper shoulder region. More subjective symptoms such as constant muscle fatigue and stiffness, radiating pain combined with increased muscle tone during passive movements, painful locations and palpable discrete hyperirritable spots, so-called trigger points contribute to the diagnosis [97].
Pain is an unpleasant sensory experience derived from actual or potential tissue damage. The pain sensation is transmitted by specialized nerve receptors (nociceptors) that serve as injury (noxious stimuli) receptors. Nociceptors are sensitive to chemical substances released from damaged cells, and the pain transmission has a discrete set of neuronal pathways. Chronic pain, defined by recurrent pain sensations over a prolonged period of time [132], may be induced from sensitization, as nociceptor threshold is lowered and the response to same stimuli is increased. Sensitization often leads to radiating pain and an increase of the sensitive area [90]. Sensitization can either be peripheral or central relating to where the chemical imbalance in the cells occurs, i.e. the peripheral or central nervous system.

In accordance with the name myalgia, the pain is believed to be muscular. Many studies regarding the underlying pathophysiological conditions of the myalgic trapezius have been performed. Several of the hypotheses related to the pathophysiology of myalgia are based on biochemical alterations in the muscle, suggesting increased signaling of inflammatory substances and altered metabolism (reviewed by Visser and Van Dien 2006 [146]). Thus for myalgia a comprehensive understanding of the muscle pain has not been attained.

**Previous pathophysiological research**

**Microcirculation**

In a study regarding work related musculoskeletal disorders, Knardal et al. [71] hypothesizes that neck shoulder pain originates from the blood vessels rather than the muscle cells. This explanatory model presents a mechanism similar to that of migraine, where arterial vasodilation stretches the blood vessel wall causing mechanical activation and releases pain producing substances. These substances further activate and sensitize nociceptors. Knardal’s model conflicts with previous experimental results showing decreased microcirculation in the myalgic trapezius [81; 79; 120] and impaired vasodilation response in the sore forearm [116; 104]; this impaired microcirculation is believed to be an effect of sympathetic dysregulation which occurs in stress-situations. In other studies myalgic muscle has during intervention shown a similar increase in blood flow in response to low force repetitive work as healthy muscle. However, after low force repetitive work (during the recovery period) the local blood flow in patients with myalgia was still increased compared to healthy controls [111; 120]. In another study [39], where subjects were exposed to a less intensive low force exercise than in the above mentioned experiments, there was no prolonged effect of the
low force exercise and blood flow returned to baseline during the recovery period. A local limitation in blood flow due to heterogenous muscle activity and thus increased intramuscular pressure, would require prolonged hyperaemia in the recovery period [111]. Heterogenous muscle activation has been demonstrated as increased neuronal firing rates activate type 2 fibers to a larger extent in chronic pain cases [62].

**Fiber types**

Fiber type content explains the contractile characteristics of a muscle and reflects its innervations. The Cinderella hypothesis [54] is an explanatory model based on earlier work by Henneman [49], who introduced the Hennemans size principle. The Hennemans size principle explains the innervation pattern of the muscle as there is an orderly recruitment of the motor units, which are the smallest functional units of the muscle. Small, low threshold motor units (type 1 fibers) are always recruited first, ahead of larger type 2 fiber motor units. Motor units with higher thresholds are activated at higher force levels and are inactive as soon as there is a decreased force level. In the Cinderella hypothesis [54], the low threshold units are assumed to remain constantly active until complete relaxation of the muscle. At low intensity work the low threshold motor units are constantly active, suggesting a lack of complete muscle relaxation [49], which is relevant for developing and perpetuating trapezius myalgia. As a further effect of the constant muscle activation, type 1 fibers are hypothesized to eventually get worn out. A complementary model by Gissel et al. [44] created on the basis of animal experiments, suggests Ca$^{2+}$ accumulation in the sarcomere and subsequent degradation of membrane proteins [35; 44].

Biopsies from patients experiencing trapezius myalgia have been investigated for fiber type content and fiber area in order to provide evidence for the Cinderella hypothesis. Women suffering from trapezius myalgia, a significantly increased percentage of type 1 fibers was demonstrated [81; 60], when comparing relatively pain free myalgic patients to patients experiencing severe pain. In another study [59], male forest machine workers were examined. The two groups in this study were matched according to work exposure. Results were contradictory since no differences in fiber type content were observed between pain free healthy men and myalgic men [59].

Not only the amount and types of fibers but also the areas of the fibers is of interest. An increased muscle fiber area is a response to increased muscle activity. Larsson et al. [80] observed an increased fiber area in the type 2
fibers of patients experiencing myalgia. Other studies have shown increased fiber area of type 1 fibers [60; 4]. Increased fiber area of both type 1 and 2 fibers has been shown in work exposed groups [59] and groups with myalgia [60]. Larsson et al [78], did not find a significant differences in myosin heavy chain content when comparing healthy and myalgic muscle. Results regarding fiber type composition/myosin heavy chain composition in the myalgic muscle are relatively few and inconclusive.

Morphological irregularities

Alterations in the metabolic mitochondrial apparatus of the muscle have been investigated using immunohistochemistry on biopsy samples. Moth eaten fibers are muscle fibers lacking nicotinamide adenine dinucleotidenicotine hydrogen-tetrazolium reductase (NADH-TR), or have irregularities in its expression [48]. NADH₂ is a metabolite produced primarily in the citric acid cycle and used in the respiratory chain. Both processes take place inside the mitochondrion. A number of studies [80; 81; 59; 77] show the presence of moth eaten fibers in both myalgic and healthy muscle [59; 77], although, moth eaten fibers have a tendency to be more prevalent in myalgic muscle [77]. In a study by Kadi et al 1998 [59] on male forest machine workers, the magnitude of the moth eaten fibers correlated to work exposure. Ragged red (RR) fibers are muscle fibers with aggregations of mitochondria and broken cell membranes, seen using Gomori trichrome staining [145]. An excessive occurrence of RR fibers in myalgic muscle has been reported [80; 81]. Later experiments have not been able to verify these findings regarding abundance of RR fibers and myalgic muscle, instead studies have shown a correlation between work exposure and the occurrence of RR fibers [77]. A relation between RR fibers and insufficient blood supply has been shown in animal experiments by Heffner and Barron 1978 [48]. The presence of moth-eaten and RR fibres indicates uneven distribution and high proliferation of mitochondria. Mitochondrial proliferation may be a compensatory phenomenon in disorders or pathophysiological states, such as local muscle hypoxia, affecting oxidative metabolism.

Immunohistochemical findings

Cytochrome c oxidase (COX)-negative muscle fibers, i.e. muscle fibers lacking complex IV in the mitochondrial respiratory chain are found in muscle biopsies from women suffering from severe trapezius myalgia [60]. Since COX is active in oxidative metabolism, these results suggest altered metabolic activity in the myalgic muscle. In a study on male forest machine operators, these metabolic alterations are concluded to be related to work exposure, since muscle fibers lacking COX prevail in both machine operators
experiencing pain and those who do not experience pain, in comparison to the control group \[59\]. Contradictory results are shown \[77\], where no relationship between COX and low load work exposure was found. There is a need for more comprehensive studies where all subunits of the different complexes in the respiratory electron transport chain are considered, before making a definitive conclusion regarding the oxidative metabolism in myalgic muscle.

**Biochemical findings**

Other metabolic alterations studied in patients suffering from trapezius myalgia have concerned metabolites active prior to the respiratory chain (figure 3). Lactate, pyruvate and glucose have been studied in the muscle interstitium using microdialysis technique \[134\]. The results from these experiments show that patients suffering from trapezius myalgia have increased concentrations of pyruvate and lactate both at rest and after exercise \[111; 120\]. Also, sustained increased levels of lactate are seen in the 20 minute recovery period after low load work \[111\]. From these results an increased anaerobic metabolism in the myalgic muscle is suggested \[111; 33; 120\]. When there are insufficient amounts of oxygen lactate is used as a cellular energy source; otherwise, pyruvate enters the mitochondria for oxidative metabolism following the citric acid cycle. Pyruvate is produced in the glycolysis as glucose is metabolized in different enzymatic steps in the glycolysis (figure 3). These previous studies regarding the metabolism of the myalgic muscle, show no evidence of an overall increase in anaerobic metabolism, since no protein related to oxidative metabolism or fatty acid metabolism are investigated.
Figure 3. Schematic figure of metabolic processes: glycogenolysis, glycolysis, fatty acid beta-oxidation, TCA cycle and respiratory chain. Pyruvate produced in the glycolysis is either enzymatically transformed into lactate via lactate dehydrogenase; or consumed in the TCA cycle in the same manner as the fatty acids from the beta-oxidation. Produced FADH$_2$ and NADH are consumed in the different complexes of the electron transport chain, creating an intermembrane proton gradient which with the help of ATP synthase produces ATP.
**Muscle damage**

Different substances have been found to leak out of the sarcoplasmatic reticulum, as a direct effect of muscle damage. Systemically increased levels of lactate dehydrogenase (LDH) and creatine kinase are clinically used as markers of muscle injury, reflecting membrane damage and leakage [44]. Although lactate levels are elevated in myalgic muscle, a statistically significant difference in LDH levels in human myalgic muscle compared to healthy has not been found [112]. This may indicate that there is no muscle damage in chronic myalgia. On the contrary, animal studies have shown both an increase in LDH and Ca$^{2+}$ leaking during low frequency electrical stimulation [45]. The electrical stimulation resembles the nerve activation during repetitive work tasks. Studies exposing rats to repetitive voluntary reach and grasps movements show indications of inflammation and muscle damage [8; 9]. As these animals are in the early phase of developing myalgia, this might be applicable to humans in the early onset phase of trapezius myalgia. However, these results need to be verified in humans since animal physiology and anatomy differ to human. Inflammatory substances have been argued to play a role in the genesis and maintenance of myalgia [146]. The main focus has been on cytokines, bradykinin, interleukins (IL), IL-6, IL-8 and IL-1β and tumor necrosis factor α (TNFα), and also C-reactive protein (CRP). Cytokines are active in many different processes in the body, systemic as well as local. In a study by Carp et al. [21], possible biomarkers were investigated using blood serum from patients suffering from different kinds of over-use related musculoskeletal disorders. Results show a significant association with pain reported inconvenience and increased levels of CRP and TNFα. Another study investigated the cytokine expression of females suffering from trapezius myalgia [39]; levels of IL-1β bradykinin and IL-8 did not differ significantly between the healthy and myalgic group. Neither interstitial IL-6 levels differ when comparing healthy and myalgic muscle [112]. Since studies concerning cytokines are few, no conclusion regarding the relevance of cytokines can be made.

**Protein and metabolite analyses**

“Omics” is the common suffix for a number of technically advanced methods that provides vast amounts of data [127]. The range of proteins (in proteomics), metabolites (in metabolomics) and genes (in genomics) that can be detected depend on the choice of protocol and statistical method. The most common application of these techniques has been to screen for differences in profiles between groups, as large numbers of genes, proteins and metabolites are analyzed simultaneously. These methods provide fast and efficient analysis in the search for new hypothesis, biomarkers or
biological fingerprints. A biomarker is a protein or metabolite that is considered to be of importance in a pathophysiological condition. The term biological fingerprint is used when several genes, proteins or metabolites as a group determine a pathophysiological condition. The combination of proteomics on muscle biopsies and metabolomics on microdialysate will provide a comprehensive analysis of the trapezius muscle. Biopsies provide information about the ongoing intracellular and extracellular processes in the muscle. Microdialysate obtained from the muscle interstitium provides information about the extracellular processes of the muscle. Proteomics determines the protein content within a wide size range whereas metabolomics reveals small size (> 1.5 kDa) metabolites. As the first steps to better understand possible muscle related underlying mechanisms behind trapezius myalgia the proteomics method of choice was evaluated for reliability. A validation of the method is required to confirm that proteins of interest are included in the analysis, taking in to consideration the choice of buffer solutions and gel compositions [2; 16]. Protein analysis requires consideration to the specific properties of each protein. Different proteins have different molecular weight, acidity, neutral or alkaline properties depending on charge, and are hydrophilic or hydrophobic. Metabolomic and proteomic methods also require consideration of the rapid reaction times of metabolites and enzymes, as well as intrinsic properties of each molecule. The analytical method is hence adjusted to all the different traits of the protein to enable its detection. Combination with other techniques is necessary to verify this type of screening results.

**Metabolomics**

Metabolomics allows a non-targeted survey of the small molecules in biological samples. The approach is based on the systematic study of the unique small-molecule chemical fingerprints that specific cellular processes leave behind [31]. The metabolites are the end products of the cellular processes. With metabolomics, a snap-shot of the physiology of the cell is provided. This technology, ideal for identifying diagnostic biomarkers, consists of two sequential steps: An experimental technique, based on mass spectrometry or nuclear magnetic resonance (NMR) spectroscopy, designed to profile low molecular weight compounds - and a multivariate data analysis. Metabolomic analysis of biofluids or tissues has been successfully used in the fields of physiology, diagnostics, functional genomics, pharmacology, toxicology and nutrition [31]. Gas chromatography-mass spectrometry (GC-MS) is the most comprehensive technical approach, although it is not currently possible to analyze the entire range of metabolites with one single analytical method. Using microdialysate obtained from healthy and myalgic donors further scrutinizes the
possibilities of metabolic differences in the extracellular processes of the myalgic muscle.

**Two-dimensional differential gel electrophoresis (2D-DIGE)**

Two-dimensional difference gel electrophoresis (2D-DIGE) [135] is a quantitative proteomic method that allows simultaneous screening for differences in substantially large numbers of proteins of molecular weight 150 kDa to 10 kDa. With this method two distinct sample groups are compared. One sample from each group is separated on one gel together with an internal standard. The three samples are labeled using one fluorescent dye for each group (Cy2, Cy3 and Cy5). In 2D-DIGE, proteins are after fluorescent labeling separated in two different dimensions. In the first dimension proteins are separated according to isoelectric charge (pI) on a gel strip, as an electric current is added. After separation in the first dimension the gel-strip is attached to a larger gel where the proteins enter, and are separated after molecular weight in the second dimension. A gel with a spotted pattern is hereby obtained, and spots are visible after staining with dye or laser scanning. Proteins deriving from each group are detected using a Typhoon laser scanner at three different wavelengths corresponding to each dye present in a spot. A software program coupled to scanner data enables visualization of spots and intensity parameters, subsequent statistical analysis. The separate spots of interest are excised and digested into small peptides which are identified using mass spectrometry. Using the 2D-DIGE approach a comprehensive picture of the intracellular processes in the muscle of healthy and myalgic donors is obtained. In 2003 a 2-D map of the human vastus lateralis muscle with 107 identified proteins was available [37], hence the present studies were initiated. Thereafter, a number of proteomic studies on human skeletal muscle have been published, showing differences in protein profile in the human vastus lateralis muscle with ageing [38], and exposure to high altitude [144], as well as differences between the human deltoideus and vastus lateralis muscles [20]. Protein changes as an effect of bed rest [94; 14] and interval training [52] have also been studied using different proteomic approaches. Metabolic, structural and regulatory proteins have been picked up using these analyses. These types of studies are expected to contribute significantly to fill the knowledge gap regarding the pathophysiology of trapezius myalgia.
Aims of the thesis

The aims of the thesis were to elucidate the usability and scope of a proteomic method in human skeletal muscle research and together with a baseline study and a complementary metabolomics analysis, provide new clues and perspectives regarding the biochemical state of the myalgic trapezius muscle.

The specific aims of the studies were:

**Paper I**

To develop a sample preparation protocol for human skeletal muscle and investigate the repeatability of the Ettan DIGE system.

**Paper II**

To screen for differences in protein profile between the trapezius muscle and the vastus lateralis muscle by using 2D-DIGE.

**Paper III**

To explore the proteomic differences between healthy and myalgic trapezius muscle using 2D-DIGE and human biopsies.

**Paper IV**

To investigate the extracellular metabolite content in the interstitium of myalgic muscle using microdialysis before and after a work and stress test.
Materials and methods

Subjects

In paper I, one healthy male volunteer participated. The subject did not regularly take any kind of medication.

In paper II, five male volunteers in the age 25-28 participated. Subjects were healthy and did not regularly take any kind of medication.

In paper III, cleaners with myalgia (n=12) and those pain free (n=12) with manual floor cleaning as their main duty participated. Cleaners with chronic trapezius myalgia were in the age range 34-50 years (mean 42 years), and healthy cleaners were in the range 33-49 years (mean 41 years). Cleaning staircases, mainly using a floor mop is monotonous and high work demand with sustained static or highly repetitive contractions of the trapezius muscle [133]. The myalgia was considered work-related when it was reported to have started in connection to the cleaning work, and worsened during the work day. Initially the myalgic group was free of pain during leisure time, but gradually most of the subjects felt pain at all times. If leisure activities were a possible reason for pain, the subjects were excluded from the study. Myalgia was considered chronic if it had lasted longer than one year. Pain duration for the cleaners was on average 8 years (±5 years). The participants in the pain group were not pain free during the sampling. Cleaners not experiencing any pain were pain free at the time of the experiment and only reported a maximum of 2-3 days of pain during the latest 12 months. None of the cleaners took any kind of steroids or anti-inflammatory drugs, and they were not diagnosed with diabetes, articular diseases, fibromyalgia, systemic inflammatory disease or neuromuscular disorders. Also, subjects who had suffered trauma to the neck region were excluded from the study. Previously published papers originate from the same group of subjects [79; 76-78].

In paper IV, eleven women with a mean age of 42 years reporting pain in the descending portion of the trapezius muscle during the last seven days, and reported neck and shoulder pain more than 90 days the last 12 months, were included. Pain duration was on average 7 years. The same exclusion criteria as in paper III were used. For a more detailed description of the recruitment of myalgic patients see Sjörs et al 2009 [122]. The control groups consisted of healthy women with a mean age of 40 years, reporting no neck-shoulder pain. Exclusion criteria in papers III and IV, for the control group, in
addition to above mentioned, was presence of pain in the neck-shoulder region for more than 2-3 days the last 12 months.

**Clinical assessment**

In both papers III and IV the subjects underwent a standardized clinical examination [97] to register inclusion criteria. In addition, using digital palpation with an approximate force of 40 Newton, a tender point was sought in the trapezius two centimeters lateral from the midpoint between the seventh cervical spine and the lateral part of the acromnuim. The patient was instructed to indicate pain at palpation. Biopsies in paper III and microdialysate in paper IV were collected from these sites.

**Biopsies**

In papers I and II, muscle biopsies were obtained surgically from the midpart of the vastus lateralis muscle and in paper II and III biopsies were taken from the descending portion of the trapezius muscle (table 1). Procedures were performed under local anesthesia and with ethical approval. Muscle samples were rapidly mounted in Tissue-Tek medium (Miles laboratories, Ind, USA), and immediately frozen in isopropanol chilled in liquid nitrogen, and stored at −80°C until use. For biochemistry (2D-DIGE and Western Blot), Tissue-Tek was thoroughly removed from the part of the sample to be homogenized.

**Microdialysate**

Microdialysis is an in vivo sampling technique which allows collection of fluid from the muscle interstitium over time [134]. In paper IV, microdialysates were used for the analysis. Microdialysis is performed by insertion of a small tubular semi permeable membrane just beneath the muscle fascia. Inside the test tube a physiological liquid is flushed. Depending on the permeability of the membrane (the pore size) molecules will diffuse into the collective tube and as large molecules are prevented from entering the probe and chemical analysis will be simplified. In paper IV, dialysate from eleven healthy and ten myalgic women were collected from the trapezius muscle. To reach a baseline level after insertion of catheter, subjects rested for 120 minutes before sampling started. After the initial period of rest, a baseline sample was obtained. The subjects performed a standardized repetitive work and stress test intending to mimic a work day. Microdialysis was ended with a 80 minutes recovery at rest. Sampling was performed at every 20 minutes. In paper IV, the baseline and recovery
samples were analyzed using GC-MS. A more detailed description is available in paper IV.

**Work and stress test**

In paper IV, patients and healthy controls performed a work and stress test to determine the effect on the extracellular metabolite content. Initially, a baseline sample was obtained, as microdialysate sampling reached steady state, 120 minutes past probe insertion. The standardized repetitive work was divided into three sessions using two standardized Valpar Component Work Stations (VCWS08 and VCWS204, Valpar Tucson, USA) and one peg-board exercise, previously described [121; 122; 40]. The stations were selected to simulate sedentary or assembly work that requires unilateral or bilateral use of upper extremities. The purpose was to exacerbate pain in the trapezius myalgia group by performing repetitive exercises predominantly with their most painful side. During the work period, the exercises were performed at a standardized work pace and alternated in 20-minute

<table>
<thead>
<tr>
<th>Paper</th>
<th>Subject/group</th>
<th>Mean age</th>
<th>Muscle</th>
<th>Protocol</th>
<th>Sample</th>
<th>Experimental setup</th>
</tr>
</thead>
<tbody>
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<td>I</td>
<td>1</td>
<td>-</td>
<td>Vastus lateralis</td>
<td>2D-DIGE MALDI-TOF Western blot</td>
<td>Biopsy</td>
<td>-</td>
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<tr>
<td>II</td>
<td>5+5</td>
<td>26</td>
<td>Trapezius +Vastus lateralis</td>
<td>2D-DIGE MALDI-TOF Western blot</td>
<td>Biopsy</td>
<td>-</td>
</tr>
<tr>
<td>III</td>
<td>12+12</td>
<td>41+42</td>
<td>Trapezius</td>
<td>2D-DIGE LC-MS/MS</td>
<td>Biopsy</td>
<td>-</td>
</tr>
<tr>
<td>IV</td>
<td>11+10</td>
<td>42+40</td>
<td>Trapezius</td>
<td>GC-MS</td>
<td>Microdialysate</td>
<td>Work and stress test</td>
</tr>
</tbody>
</table>
intervals in the sequence; VCWS08 – peg-board - VCWS204 – peg-board - VCWS204 – peg-board. The repetitive work test was immediately followed by the Trier social stress test (TSST) a standardized and well validated psychosocial stressor [69]. The TSST-test protocol consists of a 10-minute preparatory and information period followed by a five minute speech and five minute verbal arithmetric task [122]. For all subjects, the TSST took place between 1.30 PM and 3.30 PM to minimize confounders from diurnal variation in hormone levels. The experimental sessions were scheduled on day 1-10 in the menstrual cycle (i.e. the follicular phase).

**2D-DIGE**

Human skeletal muscle proteomics were conducted on human biopsies. Biopsies were obtained from vastus lateralis (papers I and II) and the descending part of trapezius (papers II and III) (figure 2). Unless otherwise stated, all chemicals used for 2D-DIGE were from GE healthcare, (Uppsala, Sweden) and of proteomic grade quality. Frozen muscle samples were suspended in lysis buffer (9,5 M Urea, 4% (v/w) CHAPS and 30 mM Tris Base). The protein content of the homogenized samples was quantified using a 2D-Quant Kit. Homogenated sample aliquots from each group were separately labelled with fluorescent dyes: Cy2, Cy3 or Cy5. In papers II and III, the internal standard method was used [3], incorporating a pooled internal standard dyed with Cy2. Details about the gel running conditions are provided in papers I, II and III. Gels were scanned in a Typhoon™ scanner where laser detects the emission from each fluorescent dye separately. Images were processed using ImageQuant™. Protein abundance was determined and statistical evaluation was made using DeCyder™ V6.5.

**MALDI-TOF**

Protein spots were manually excised from gels stained with coomassie brilliant blue in papers I and II, and protein spots in gels stained with Cy2 were excised using Ettan™ Spot Handling Workstation in papers II and III. Excised spots were destained and digested by trypsin [117] before analysis (papers II and III). The tryptic peptides were mixed with an alpha-cyano-4-hydroxycinnamic acid and 2.5-dihydroxybenzoic acid matrix, applied to the sample plate using droplet preparations [74] and analyzed using MALDI-TOF Voyager STR-DE, Applied Biosystems, Stockholm, Sweden. Post source decay (PSD) was used to determine the amino acid sequences of the peptides. In the desorption step, Voyager emits samples by a UV-laser, making the matrix solution together with the analyte to ablate from the dried droplet spot. Ionisation takes place in the hot plume emitted by the laser from the sample plate. The analytes are charged by proton transfer from the
matrix solution. The observed analyte then consists of a neutral molecule, with one or more protons added or removed. MALDI TOF instruments are equipped with reflection mirrors which reflect ions using an electric field, thereby doubling the ion flight path and increasing resolution. Sample mass is detected when the sample hits the detector. The sizes of the fragments were determined by their time of flight which correlates to sample mass. Since each protein was digested into small peptide fragments, many small fragments were analysed creating a mass-spectra. The mass of each fragment, the peptide mass fingerprint generated a peak list from the mass spectrometry and the post-source decay MS/MS which was entered into a database. The databases searches using IPI-human and Swiss-Prot were conducted using Mascot database; search parameters are described in paper II. The peptide mass fingerprint was assigned a specific protein. A score number was obtained, with a value that depended on the number of proteins that matched the suggested protein.

**LC-MS-MS**

Protein spots were destained and digested with trypsin (for a more detailed description see paper III) before analysis. Peptides were analysed using an on-line nano-flow HPLC system (EASY-nLC; Proxeon, Bruker Daltonics) in conjugation with the mass spectrometer HCTultra PTM Discovery System (Bruker Daltonics). This type of mass spectrometer creates ionized peptides in the gas phase from a fine liquid spray. Using liquid chromatography (LC) mass spectrometry peptides will be separated by hydrophobicity before ionization and analysis by mass spectrometer. Here peptides collide with an inert gas and dissociate into fragments (collision induced fragmentation; CID) producing the resulting MS/MS spectrum from which the amino acid sequence of the peptide can be determined. Dissociation of peptide ions was performed using automated online tandem MS analysis. The mass spectra obtained with the analysis were processed by Bruker Daltonics DataAnalysis 3.4 (Bruker Daltonics, Bremen, Germany) and resulting MS/MS data were searched in NCBInr and Swiss-Prot database on MASCOT server.

**GC-MS**

In paper IV, gas-chromatography mass spectrometry (GC-MS) was used to analyze metabolite content in microdialysate from myalgic and healthy subjects. GC-MS offers very high chromatographic resolution, but requires chemical derivatization for many biomolecules. Samples were injected into the GC-MS and transported by the flow of an inert gas (e.g. helium) through a silica capillary chromatographic column. A temperature program ranging from 70°C -320 °C separates the different compounds at 30°C/min (where it
was kept at 2 min.) according to their retention time in the capillary column. The retention time is determined by the physico-chemical properties of each compound. As the compounds are separated in the column, they subsequently get transferred to the mass spectrometer (MS). In the MS, compounds are fragmentized and ionized by electron impact of 70 eV at a current of 2.0 mA. Ions with mass-to-charge ratios \((m/z)\) 50 to 800 depending on the MS settings, pass to the detector were the signals are recorded. The retention indexes are calculated from the retention times, from the injection of a homologous series of n-alkanes \((\text{C}_{12}-\text{C}_{32})\) for each batch. All samples were run in randomized order. The GC-MS analysis generates a vast amount of data, as 30 spectras are recorded every second. Hierarchical multivariate curve resolution is a mathematical technique used to distinguish between signals when two or more metabolites elute simultaneously; provided that they have different mass spectra and concentration profiles [27]. Hierarchical multivariate curve resolution is implemented together with baseline correction and alignment. The identities of the metabolites were obtained based on their retention indices using National Institute of Standards and Technology (NIST) mass spectral search program. The obtained mass spectras were compared to those of known compounds stored in commercial and in house spectral libraries [124].

**Western blot**

In papers I and II, Western blots were used to verify the results from the 2D-DIGE analysis. Protein homogenates of human muscle were separated on 11 cm 12% SDS-PAGE gels (Readygels; Biorad laboratories AB, Sweden) and transferred to a PVDF membrane. Non-specific binding of PVDF membranes was blocked [96]. Membranes were incubated with antibodies against creatine kinase, \(\alpha\)-crystalline \(\beta\)-chain, carbonic anhydrase 3, beta enolase, phosphoglycerate mutase and HSP 27, (for further description see paper I and II). After washing, a secondary antibody was applied, also described in paper I, and incubated 1 hour. Following washing (4x15 minutes), membranes were incubated at room temperature for 5 minutes with chemiluminescence reaction detection reagents (ECL, Amsterdam, Netherlands). Membranes were exposed to autoradiography film (Hyperfilms ECL, Amsterdam). The relative protein content was calculated based on the detection of pixel volumes of the photographed membranes. Background pixel volume was subtracted from each protein band. Relative intensities of bands were quantified with densiometric analysis (Quality One, Bio Rad) in paper I and ChemiDoc XRS with Quantity One software version 4.6.6 in paper II.
**Immunohistochemistry**

In paper II, together with Western blot, immunohistochemistry (IHC) was used as a verification of the results from the 2D-DIGE analysis. Serial cryostat sections of human muscle, 8µm thick, were air-dried and then rehydrated in 0.01 M PBS, immersed in 5% non-immune serum and incubated with primary antibodies for 60 min at 37°C or overnight at 4°C. Antibodies against myotilin [93], alpha-crystalline beta [82] and NADH ubiquinone oxireductase [19], Slow myosin heavy chain [53] and fast myosin heavy chain [53] were used. Visualization of bound antibodies was performed with indirect fluorescence, as described in paper II. The sections were studied under a Nikon eclipse E 800 microscope (Nikon Inc., Melville, NY, USA) equipped with a SPOT RT Color camera (Diagnostic Instruments Inc., Sterling Heights, MI, USA). Digital images were processed using the Adobe Photoshop software (Adobe Systems Inc., Mountain View, CA, USA).

**Statistics**

**Univariate analysis**

DeCyder™ V6.5 software detects spots using data imported from ImageQuant™ with the automated spot detection algorithm in the DIA (differential in-gel analysis) module. Filter settings were adjusted to reduce background noise and maximum peak and minimum area were adjusted to avoid dust particles to be included in the analysis (for more details see papers II and III). The BVA (biological variation analysis) module matches the different gels and calculates the proteins ratios by dividing the spot volumes of the fluorescence signals from Cy3 and Cy5 with the signal from Cy2. In Paper I additional analysis with data directly imported from DIA was done using JMP 5.0.1 (SAS Institute Inc., SAS Campus Drive, Cary, N. C., USA). In paper II all statistical calculations were done in the BVA module using FDR correlated one-way ANOVA [11] on spots present in 5 of 5 gels.

**Multivariate analysis**

Multivariate analysis using SIMCA™ version 11.0 (paper I) and 12.0 (paper II, III, IV) were performed. In paper I, a principal component analysis (PCA) [147] was performed to visualize variation in the data provided with the 2D-DIGE method. In papers II and III, partial least square discriminant analysis (PLS-DA) [148], and in paper IV orthogonal partial least-squares discriminant analysis (OPLS-DA) [131] were performed. In the models, protein spots (papers II and III) and metabolites (paper IV) considered of interest, had a variable of interest (VIP) value over 1.0. In paper IV the
loadings value ($w^*$) of the predictive component was also taken into consideration.

**Results**

**Paper I**

Aliquots from the same muscle extract homogenate were labelled with fluorescent dyes (Cy2, Cy3 and Cy5) and separated on the same gel. Three different gels were analyzed simultaneously and validated. Most important of all, to exclude false positives, was the strict inclusion of spots that were present in 9 out of 9 spot maps. Manual editing of spots, to guarantee that the spots analyzed were not autofluorescence or contaminants, was also of importance. For further details see paper I.

**Figure 4.** Difference in signal between Cy3 (left box) and Cy5 (right box) volumes compared to Cy2 volume. Mean and range, n=3/spot.
The difference in signal between different dyes was described in figure 4, where the variances in signal intensity of Cy3 and Cy5 from identified proteins (paper I), were presented. Also a PCA model explaining the variance in the data (figure 5) reveals that the major variation was between gels. Additional confirmation of the 2D-DIGE results was done using Western blot analysis on four proteins (paper I).

![PCA model](image)

**Figure 5.** PCA model of the three samples from the one single muscle biopsy separated on three different gels. The model shows that most of the variance comes from gel to gel differences.

**Paper II**

Tissue homogenates from trapezius and vastus lateralis were analyzed using 2D-DIGE. Protein spots were detected with different types of set-up. Protein spots present in every gel were analyzed using the DeCyder biological variation analysis (BVA) one-way ANOVA. Initially, 104 protein spots (Fig 6A) differed significantly (p<0.05), but when applying the recommended FDR correlation only 26 spots differed significantly (figure 6B). When filtering the data in the differential in gel analysis (DIA), performing FDR correlated one-way ANOVA, several spots were excluded from the analysis. Interestingly, spots not being significant in the primary one-way ANOVA analysis were considered significant (arrow in figure 6D), when the filter
settings were changed and less background noise were included. The protein phosphoglycerate mutase 2 was analyzed using Western blot to verify the data. The Western blot showed a significant difference between healthy and myalgic muscle (figure 7). When applying a second, multivariate approach, PLS-DA, 104 protein spots were considered of interest having VIP value over 1.0 and a regression coefficient with a jack-knifed 95% confidence interval not including 0 (figure 6C).

Figure 6. Appearance of protein spots of interest from the 2D-DIGE analysis depending on statistical method: A, one-way ANOVA; B, one-way ANOVA with FDR-correlation. C, Applying a multivariate approach, PLS-DA with VIP over1, jack-knifed 95% confidence interval not including 0; D, filtered data with cut-off of 200,000 au using one-way ANOVA and FDR correlation. Arrows in D the protein spots of phosphoglycerate mutase 2.
Muscle homogenates from 12 healthy and 12 myalgic female cleaners were analyzed using 2D-DIGE and LC-MS. Totally 1330 protein spots were detected. Protein spots present in at least 50% of the gels (N=847) with ratio of between subject variance vs. within subject variance falling within the 15th percentile of an F-distribution (equal to using a cut-off at p-value = 0.3 in a one-way ANOVA) were included in the PLS-DA analysis (figure 8). Protein spots were considered of interest when having variable of interest (VIP) value over 1.0 and with a jack-knifed 95% confidence interval.

**Figure 7.** Western blot analysis of phosphoglycerate mutase 2. Pixel volume shows a significant increase of the protein in trapezius muscle.

**Paper III**
Metabolic proteins separating healthy trapezius from myalgic trapezius (table 2) were: glycogen phosphorylase (muscle form), pyruvate kinase isoenzymes M1/M2, beta enolase, fructose bisphosphate adolase A&C, aconitate hydratase (mitochondrial), 2-oxoglutarate dehydrogenase (mitochondrial), creatine kinase M-type, ATP synthase subunit alpha, and glyceraldehyde-3-phosphate dehydrogenase. Contractile proteins found in the analysis were actin, cytoplasmic 1, actin aortic smooth muscle, myosin binding protein C slow type, myosin-7 slow, myosin regulatory light chain 2 fast skeletal muscle isoform, myosin-6 fast, myosin-2, and myosin-7 slow. The identified acute response proteins were heat shock 70 kDa protein, carbonic anhydrase 3 and alpha-1-antitrypsin. Structural proteins separating the groups were keratin type II cytoskeletal 1, LIM domain-binding protein 3, tubulin beta chain, adenylyl cyclase associated protein 2 and desmin.

Figure 8. PLS-DA loadings plot of healthy and myalgic muscle: Analyzed spots (♦) with VIP over 1.
Table 2: Identified proteins separating myalgic and healthy muscle in the recovery phase. Protein spots with Variable of importance (VIP) value > 1.0 in the PLS-DA multivariate model.

<table>
<thead>
<tr>
<th>ID (spotnr)</th>
<th>Protein ID (Swiss Prot)</th>
<th>Protein</th>
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<th>VIP PLS-DA</th>
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<tr>
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<td>462</td>
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<td>Keratin, type II cytoskeletal 1</td>
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<td>1.7</td>
</tr>
<tr>
<td>259</td>
<td>O75112</td>
<td>LIM domain-binding protein 3</td>
<td>-1.40</td>
<td>1.2</td>
</tr>
<tr>
<td>525</td>
<td>P07437</td>
<td>Tubulin beta chain</td>
<td>-1.17</td>
<td>1.2</td>
</tr>
<tr>
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<td>P40123</td>
<td>Adenylyl cyclase associated protein 2</td>
<td>1.20</td>
<td>1.1</td>
</tr>
<tr>
<td>490</td>
<td>Q8TD99</td>
<td>Desmin</td>
<td>1.28</td>
<td>1.1</td>
</tr>
<tr>
<td>179</td>
<td>P02768</td>
<td>Serum albumin</td>
<td>-1.13</td>
<td>1.4</td>
</tr>
<tr>
<td>807</td>
<td>P82650</td>
<td>28S ribosomal protein S22, mitochondrial</td>
<td>-1.27</td>
<td>1.3</td>
</tr>
</tbody>
</table>
Microdialysates from healthy and myalgic trapezius muscle were collected before, during and after a work and stress test session [121; 40]. Microdialysate from “baseline”, before the test, and from the “recovery”, 60 and 80 minutes after the test were analyzed using metabolomics (GC-MS). The identified metabolites were then analyzed using multivariate and univariate analysis: orthogonal partial least square analysis (OPLS) and Mann Whitney U tests. At baseline two metabolites differed significantly in abundance ($p>0.05$): pyroglutamic acid and l-leucine. Both metabolites were more abundant in the interstitium of myalgic trapezius. At recovery (table 3), myristic acid and putrescine were significantly more abundant in the interstitium of myalgic muscle and beta-D-glucopyranose in the interstitium of healthy muscle. The OPLS analysis failed to reveal any discrimination between the healthy and myalgic groups at baseline. A model was presented based on the OPLS analysis performed on the samples obtained from the recovery phase (figure 9). Metabolites of interest are presented in table 3 with VIP values and loadings value ($w^*$).

Figure 9. OPLS-DA loadings plot of the analyzed metabolites: fatty acids, sugars and protein metabolites were of interest according to the model ($w^*$±0.1). Red dots represent protein metabolites, yellow squares fatty acids and green squares sugar metabolites: Metabolites more abundant ($w^*$>0.1) in myalgic muscle: 23) glycerol; 59) L-glutamine; 75) alpha-ketoglutarate; 97) arabitol; 98) Putrescine; 109) myristic acid; 112) Glucose; 137) Stearic acid. Metabolites more abundant ($w^*<$-0.1) in healthy muscle: 125) beta-D-glucopyranose. Round circles represent unidentified metabolites.
Table 3. Identified metabolites of interest in the OPLS-model Significant according to Mann Whitney U test bolded and marked with *.

<table>
<thead>
<tr>
<th>ID</th>
<th>Metabolite</th>
<th>( w^* ) (MYA/CON)</th>
<th>VIP</th>
</tr>
</thead>
<tbody>
<tr>
<td>66</td>
<td>Salicylic acid</td>
<td>0.195</td>
<td>2.57</td>
</tr>
<tr>
<td>98</td>
<td>Putrescine *</td>
<td>0.177</td>
<td>2.33</td>
</tr>
<tr>
<td>109</td>
<td>Myristic acid *</td>
<td>0.163</td>
<td>2.14</td>
</tr>
<tr>
<td>112</td>
<td>Glucose</td>
<td>0.140</td>
<td>1.84</td>
</tr>
<tr>
<td>75</td>
<td>Alpha-Ketoglutarate</td>
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<td>1.67</td>
</tr>
<tr>
<td>23</td>
<td>Glycerol</td>
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</tr>
<tr>
<td>59</td>
<td>L-Glutamine</td>
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<td>137</td>
<td>Stearic acid methyl ester</td>
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<tr>
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<td>Arabitol</td>
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<td>1.33</td>
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<tr>
<td>110</td>
<td>Myristic acid</td>
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</tr>
<tr>
<td>114</td>
<td>Glucose</td>
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</tr>
<tr>
<td>27</td>
<td>L-Proline</td>
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<tr>
<td>68</td>
<td>L-Glutamic acid</td>
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<td>130</td>
<td>Palmitic acid</td>
<td>0.079</td>
<td>1.04</td>
</tr>
<tr>
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<td>Glycerol</td>
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</tr>
<tr>
<td>21</td>
<td>Urea</td>
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</tr>
<tr>
<td>120</td>
<td>Myo-Inositol</td>
<td>-0.087</td>
<td>1.14</td>
</tr>
<tr>
<td>107</td>
<td>Glucose</td>
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</tr>
<tr>
<td>124</td>
<td>D-Galactono-1,4-lactone</td>
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<tr>
<td>125</td>
<td>beta-D-Glucopyranose *</td>
<td>-0.164</td>
<td>2.16</td>
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</table>
Discussion

This exploratory screening of muscle biopsy and microdialysate has provided a comprehensive analysis of the intra- and extracellular processes of chronic myalgic muscle. The major finding in this thesis was that the proteomic method (2D-DIGE) was valid and applicable on muscle biopsy samples (paper I). The method was further applied comparing biopsies from human trapezius and human vastus lateralis muscles (paper II), where a baseline was created for a further analysis of differences between healthy and myalgic trapezius (paper III). Also, differences in extracellular metabolite content between healthy and myalgic muscle were apparent when applying a metabolomics method (paper IV). The obtained results from these screening methods provide new clues regarding the pathophysiology of trapezius myalgia.

The validated 2D-DIGE method (paper I) was used in the baseline study (paper II) where 14 identified proteins differed in abundance between trapezius and vastus lateralis, and were statistically significant according to the FDR correlated one-way ANOVA. Metabolic regulatory and contractile proteins were detected in the analysis. Two different statistical approaches, FDR correlated one-way ANOVA and PLS-DA, were tested for their applicability on 2D-DIGE data. In the analysis, filters were used to reduce background noise and eliminate dust particles from the analyses. When the filter settings were altered, one protein that was not of interest in the initial analysis, appeared as statistically significant. The protein (phosphoglyceratemutase 2) was verified in a Western blot analysis and detected as significantly altered. This indicates that there is a risk of losing important data when applying stringent parametric tests on large biological data sets. This strategy and statistical approach was further adopted in paper III, where PLS-DA was used in the comparison of healthy and myalgic muscle. Here, 28 different identified proteins were of interest in the model, having a VIP value above 1. The proteins were related to muscle contraction, structure, metabolism and acute stress response. In paper IV, the extracellular processes of the muscle were examined. Interstitial fluid obtained from healthy and myalgic muscle was studied using metabolomics. Microdialysate samples were obtained at baseline, prior to a work and stress test, and during recovery, after the work and stress test. The identification of the extracellular metabolites provided information about the metabolic processes of the muscle, which was put in relation with the abundance of proteins detected in the proteomic study (paper III).
Reliability of the 2D-DIGE method

2D-DIGE provides a broad analysis of proteins with different isoelectric charge and molecular weight. In paper I, the method was validated for its suitability to analyze human skeletal muscle biopsies, and in paper II different types of analytical approaches and statistical methods were used. The results from the reliability study of our protocol showed that in order to decrease the risk of detecting false positive changes when using univariate statistics, only spots present in all gels which had been manually edited should be included. In other words, provided that these criteria were met, our protocol was reliable for small samples sizes of human muscle. The main variations in spot intensities between gels (figure 5) are due to experimental factors that arise after the loading of samples on gel strips. Fluorescent labeling of samples occur prior to electrophoresis as the samples are mixed before isoelectric focusing and thereafter are resolved in the same gel [135]. Hence, protein loss during sample entry into the gel strip will be the same for each sample within a gel. Conclusively, the main difference between samples of the same homogenate are gel to gel differences, as also shown by Karp et al [64] in bacteria. This can be compensated for using a pooled internal standard in all gels [3]. A difference in the signal obtained from the different CyDyes (figure 4) was detected, and therefore to discard the analytical errors that may rise from these differences in signal, muscle sample aliquots should not be labeled with the same dye in all gels. In other words, the same aliquot needs to be run labeled with Cy3 in one gel and with Cy5 in another, and vice versa, for the paired aliquots to be compared [128]. Analyzing human skeletal muscle biopsies using 2D-DIGE was considered a good semiquantitative screening method. As technical variation are related to specific laboratory settings and equipment, these type of analyses are important quality controls [16]. The main methodological issues that lead to variations in the present 2D-DIGE data were gel to gel differences and differences in signal from the fluorescent CyDyes.

Statistical relevance or biological relevance

Most often proteomic and metabolomic methods are used to screen for proteins of interest to a certain pathological condition. Depending on the aim of these screenings, the choice of statistical method is of importance. Using univariate analysis a statistically significant difference in abundance is required in order to present the protein of interest. In a biological system a statistically significant difference in abundance of one protein might not be relevant, as the functions of the protein might not be related to the pathophysiological condition. Instead, as systematic changes in proteins appear clues can be given about the conditions and biomarkers are to be
verified in further analyses. If the proteins are relevant for the pathological condition they may be called a biomarker. Previously published data concerning human skeletal muscle 2D-DIGE proteomics have mainly focused on stringent univariate analysis [37; 20; 38; 144; 94; 22], which has also been the type of analysis built into the DeCyder software. Hence, the main focus in paper II was on results deriving from the univariate one-way ANOVA analysis correlated for FDR. In paper II a comparison between the outcomes of FDR correlated one-way ANOVA analysis and PLS-DA was made. Univariate analysis will provide protein spots of interest with a high abundance and a high difference in expression. When a multivariate analysis was added and filter settings in the DIA module were adjusted, proteins lost in the initial stringent univariate analysis appeared as significant. The significance of this protein, phosphoglyceratemutase 2, was further verified in an additional Western blot analysis (figure 7). Therefore, when performing proteomics and metabolomics, one must relate to the question of statistical significance and biological significance and how it is applied to the analytical method. A stringent univariate approach is not the preferable best way to tackle these types of analysis, when dealing with heterogeneous biological material. Being stringent when performing these types of overview analysis may even risk losing important information [17]. A protein or a specific protein pattern, not showing a typical statistical significance could be the true biological marker of the pathological condition. Combinations of univariate and multivariate analysis are preferred to provide an extensive analysis of the data. Multivariate analysis is preferably used as overview analysis presenting clues on proteins of importance, to be verified using further analysis different methodology. With this in mind, in paper III a less stringent multivariate approach was taken when analyzing the 2D-DIGE data. In paper III, protein spots that were present in 50% of the gels or more were included in the multivariate analysis as new clues were provided for further verification.

Proteins related to fiber type

In our proteomic baseline study (paper II) there was a relatively small number of proteins that differed between trapezius and vastus lateralis muscles as only proteins significant according to the FDR correlated one-way ANOVA were presented. The total number of protein spots detected was in parity with other human studies [37; 38]. Individual variation was disregarded since both the trapezius and vastus lateralis muscles were from the same person. Slow myosin light chain isoforms were more abundant in the trapezius muscle; a finding that correlated well with the fiber type composition of the two muscles since the trapezius has a higher occurrence of type 1 fibers compared to vastus lateralis [58; 84-86]. Other contractile
proteins that were also correlated to fiber type composition and differed significantly were actin and myotilin. Differences in content of these proteins were in agreement with earlier studies [100; 23].

As previous results have presented an increased abundance in type 1 fibers or hypertrophy of type 1 fibers [60; 4], expectations when comparing healthy and myalgic muscle (paper III) were that fiber type specific proteins would correspond with slow contracting fibers. In the baseline study (paper II), the proportions of myosin light chain (MyLC) fast and slow corresponded to muscle fiber type; also shown in previous results [126; 101; 20]. On the contrary, no significant difference in MyHC content was shown when biopsies from healthy and myalgic muscle were compared [78]. Also, other studies on fiber type composition of myalgic muscle in comparison to healthy report divergent results [59; 60]. Previous results would be confirmed in protein analysis if proteins related to type 1 fibers would occur in a higher extent in myalgic muscle, in the same manner as in paper II, however, this was not the case. Instead the present 2D-DIGE analysis showed a higher occurrence of a regulatory fast MyLC 2 protein in the myalgic muscle. Together the MyHCs and the MyLCs dictate the contractile function of the myosin molecule. So-called hybrid fibers are fibers that express one type of MyHC or MyLC (i.e. slow) and another type of MyHC or MyLC (i.e. fast) [126]. Here we can assume that there might be an increased occurrence of so-called hybrid fibers in the myalgic trapezius. Laboratory experiments on rat conclude that hybrid fibers appear in muscle transition [106] when a muscle remodel from slow to fast.

A hypothesis presented by Gissel et al 2005 [46] suggests an altered Ca\textsuperscript{2+} sensitivity in myalgic muscle. The Ca\textsuperscript{2+}-accumulation theory [45] is an explanatory model suggesting increased Ca\textsuperscript{2+} concentrations in the muscle due to constant muscle contraction. As Ca\textsuperscript{2+} activates the muscle, signaling the ATPase on the myosin head to initiate muscle contraction, there is an increased need for cellular energy (ATP). This starts the glycolysis producing pyruvate. Pyruvate enters the mitochondria and is used as energy in the tricarboxylic acid cycle (TCA cycle). An investigation made by Kallenberg and Hermens 2006 [62] showed a higher contractile contribution of type 2 fibers during low-intensity work in chronic pain patients, which coheres with our results of an increased abundance of Ca\textsuperscript{2+} sensitive fast regulatory MLCs. Another protein that has been connected to fiber type is creatine kinase M-type. According to the proteomic analysis (paper III) there was a lower abundance of creatine kinase M in myalgic muscle. Since creatine kinase M-type is known to occur with a two to three times higher abundance of in type 2 fibers compared to type 1 fibers [149], the result would indicate a higher presence of type 1 fibers in the myalgic muscle. In the baseline study (paper
II), creatine kinase M-type was more abundant in the vastus lateralis which is accordance with the fiber type distribution of the muscles. Creatine kinase M-type is involved in one of the major mechanisms coupled to the general ATP content of the muscle where phosphocreatine is produced from creatine and creatine kinase [73].

**Inflammation and muscle degradation**

One previously proposed mechanism behind trapezius myalgia is the occurrence of an ongoing inflammation. Inflammation occurs as cells are exposed to prolonged stress due to changed conditions in the biochemical milieu. Infiltration of macrophages [8; 9], enhanced levels of immunological substances such as cytokines [9] and muscle degradation [1] have been reported in previous animal studies on rats performing repetitive tasks. In human studies, lactate dehydrogenase (LDH) has been studied as well as IL-6 and IL-1 beta using microdialysate from muscle interstitium. IL-6 has amongst other functions a metabolic function, signaling the liver to release glycogen [66]. No cytokines were detected in the present omics analyses. However, proteins related to acute stress responses, heat shock protein (HSP) 70, carbonic anhydrase 3 and anti-alpha trypsin were detected in paper III. HSP 70 stabilizes proteins against aggregation, prevents mislocation and facilitates protein folding [52; 95]. These processes are induced by hyperthermia [123], energy depletion [115], hypoxia [55] and reactive oxygen species [137]. HSP 70 was detected with a higher abundance in myalgic muscle (paper III). Saturated fatty acids (stearic acid and palmitric acid) detected in the metabolomics analysis after the work and stress test (paper IV). Palmitric acid is substrate for synthesis of N-palmitoylethanolamide (PEA), an anti-inflammatory and anti-nociceptive agent that is accumulated during cellular stress and inflammation [18]. PEA has been found to be significantly elevated in microdialysate from myalgic patients compared to healthy controls [41].

Muscle stiffness has been connected to the abundance of desmin in human muscle. Desmin is a structural protein that builds up the intermediate filaments. Muscles of knock-out mice lacking desmin become progressively stiffer and accumulate collagen in a degenerating process [91]. Desmin was more abundant in the biopsies from myalgic patients, a finding that requires further investigation with morphological methods in order to evaluate whether there is ongoing remodeling of the cytoskeleton [150]. The difference in desmin may also reflect sample bias related to blood-vessels, as these contain desmin. Polyamines are markers for muscle turnover and were more abundant in microdialysate from myalgic patients obtained during the recovery period (paper IV), and more specifically, putrescine showed a
statistically significant higher abundance in the interstitium of myalgic trapezius compared to the healthy. Increased levels of polyamines have been found in patients suffering from Duchenne muscle dystrophy and limb girdle muscle dystrophy [63], two conditions with high muscle turnover [99]. Carbonic anhydrase 3 is also suggested to be a potential marker of muscle turnover in response to prolonged exercise [13]. There was a decreased level of carbonic anhydrase 3 in myalgic muscle biopsies (paper III) where in that study, the muscle is not provoked by an intervention. Also, carbonic anhydrase 3 is a metabolic protein as it is suggested to be related to the obtaining the redox potential of the muscle [152]. In paper II, comparing healthy trapezius to vastus lateralis muscle, there was an increased abundance of carbonic anhydrase 3 in the trapezius muscle. The possible explanation can be that there is an increased oxidative metabolism in trapezius, as also one protein of the respiratory chain was more abundant in trapezius compared to vastus lateralis.

**Metabolic proteins**

An altered metabolism has been proposed to be one of the underlying mechanisms causing trapezius myalgia. The proteins that related to metabolism and appeared as of interest in the proteomic study reflect the intra and extracellular processes. The metabolomics preformed on interstitial fluids obtained by microdialysis reflect the extracellular metabolic processes of the muscle. In paper II, proteins involved in oxidative metabolism (NADH ubiquinone oxidoreductase 30kDa subunit, carbonic anhydrase 3), glycolysis (beta enolase, phosphoglycerate mutase) and anaerobic metabolism (creatine kinase M-type) differed significantly in abundance. Although there were only a few metabolic proteins identified as significant according to the ANOVA and the FDR correlated analysis, results indicate that there was a higher oxidative metabolism in the trapezius. This is most probably due to its higher content of mitochondria, which in turn correlates to the predominance of a higher abundance of type 1 muscle fibers in the trapezius compared to vastus lateralis. The male vastus lateralis muscle has approximately 50% type 1 fibers [58; 84], whereas the descending part of the trapezius muscle has approximately 65% type 1 fibers [85]. In paper III a multivariate approach was taken when analyzing the proteomic data set, which enhanced the number of proteins detected. In total 10 different metabolic proteins were detected: glycogen phosphorylase muscle form, pyruvate kinase isoenzymes M1/M2, beta enolase, fructose bisphosphate adolase A&C, aconitate hydratase mitochondrial, 2-oxoglutarate dehydrogenase mitochondrial, creatine kinase M-type, ATP synthase subunit alpha, glyceraldehyde-3-phosphate dehydrogenase and phosphoglucomutase-1. Proteins active in the oxidative metabolism (the
respiratory chain) were not considered of interest in the proteomic analysis using a multivariate approach (paper III). The earlier findings where COX activity was considered related to work exposure were in agreement with these results [77]. In both studies, papers III and IV, myalgic muscle was compared to the healthy muscle without any type of provocation. In paper III work exposure was identical for both groups and in paper IV groups are primarily separated according to pain perception. In paper IV additional samples obtained after a work and stress test was analyzed and systematic changes in metabolites were observed.

**Glycogen utilization**

In the proteomic investigation (paper III) an increased abundance of glycogen phosphorylase and phosphoglucomutase-1 (figure 3) was shown in the myalgic muscle. This indicates that myalgic muscle is utilizing muscle glycogen storages to a higher extent than healthy muscle. Glycogen is synthesized from glucose and stored in muscle and liver cells. Glycogen stores are secondary to adipose tissue; fat is the primary source of stored energy. Muscle fiber glycogen is an immediate energy reserve for the muscle, as muscle cells lack the enzyme glucose-6-phosphatase, which is required to pass glucose into the blood [43]. This is in contrast to liver cells, which break down its glycogen and send it out in the blood stream [139]. Excess glycolytic energy (pyruvate), may be transformed into lactate [140]. Lactate buffers for acidification as it is transported out of the cell [109] serving as fuel elsewhere or being stored as liver glycogen.

The activity of glycogen phosphorylase has been suggested to be regulated by availability of inorganic phosphate, insulin and adrenalin (reviewed by Jensen & Richter [56]). The regulation of glycogen breakdown due to adrenergic stimulation is not conclusive. Some studies present evidence of glycogen phosphorylase activation during exercise after adrenalin infusions; while others do not [70; 141]. This might in part be related to the intensity of the exercise, as the availability of inorganic phosphate is a controlling factor [141]. The mechanism is also argumented to be due to substrate availability, as high initial concentrations of muscle glycogen stimulates glycogen breakdown [51]. The availability of glycogen increases during stress as cortisol increases insulin secretion. Insulin stimulates glucose uptake from the blood incorporating it into the muscle as glycogen [28; 118].

A shift from fatty acid metabolism towards glycogen utilization is known to occur during high intensity exercise [50; 110]. An early depletion of glycogen storages causes muscle fatigue; for example, an exercising athlete will stop the race or continue at much lower pace. When there is a cellular adaptation
from carbohydrate to fat utilization this conserves the glycogen stores in the body. Hence, there is an increase in the ability to perform at high work intensities for a longer period of time [67]. The utilization of glycogen storages is also dependant on the type of muscle. The abundance of glycogen phosphorylase differed between muscles but not between the specific muscle fiber types within the muscles. This is shown when performing single fiber extractions of different muscle groups [26].

The synthesis of glycogen is also connected to the presence of certain amino acids. Leucine is an amino acid that stimulates glycogen synthesis through activation of glycogen synthase kinase-3 [102]. Leucine was significantly more abundant (Mann Whitney U test p<0.05) in the interstitium of myalgic muscle compared to healthy muscle at baseline (paper IV). Excessive glutamine intake [87] or glutamate [151] have shown to increase skeletal muscle glycogen storages. In subjects with McArdles disease, unable to break down glycogen to produce pyruvate, muscle glutamate concentrations fall to lower levels than in healthy subjects during exercise [113]. A possible explanation is that glutamate is being used as fuel as it can be converted into alpha ketoglutarate, a key intermediate in the TCA cycle [113]. Alpha-ketoglutarate is an anaplerotic protein and functions the metabolic chains (i.e. TCA cycle) as well as in other biosynthetic reactions. Synthesisation of α-ketoglutarate occurs by transamination of glutamate or through the action of glutamate dehydrogenase on glutamate, replenishing TCA cycle intermediates that have been extracted for biosynthesis. In paper III, α-ketoglutarate dehydrogenase (2-oxo glutarate dehydrogenase) was more abundant in myalgic muscle compared to healthy, and in paper IV, α-ketoglutarate was more abundant in myalgic muscle.

Pyroglutamic acid, a ring-like structure of glutamate, was found in a significantly higher concentration in the interstitium of the myalgic muscle samples compared to healthy subjects (paper IV). Phosphorylated or activated glutamate is highly unstable and if an acceptor molecule is not present, spontaneous cyclization into pyroglutamic acid occurs [65]. Pyroglutamic acid has been considered a metabolic intermediate acting as a reservoir for glutamate. In addition to the metabolic actions, glutamate is also a long-term potentiation neurotransmitter released into the synaptic cleft (reviewed by Miller et al 2011 [92]). To yield glutamate, 5-oxoprolinase is the only enzyme known to act on pyroglutamic acid [138]. Studies regarding the actions of pyroglutamic acid are sparse (reviewed by Kumar & Bachhawat 2012 [72]) but an association of accumulations of pyroglutamic acid and a decrease in the utilization of glutamate has been shown.
**Fatty acid metabolism**

In the metabolomic study (paper IV) fatty acids and glycerol were generally more abundant in the interstitium of myalgic muscle at the recovery phase 60-80 minutes past the work and stress test. Hence, upon systemic provocation, as the work and stress test can be attributed, metabolic differences became apparent between the healthy and myalgic patients. This systemic difference is not apparent between groups at baseline, i.e. samples taken prior to the stress and work tests. One of the many factors involved in the genesis of chronic myalgia is stress [88; 119]. During acute physiological stress metabolism is altered, as an increase in the breakdown of fat (the lipolysis) due to adrenergic mechanisms has been described [47]. An increased drive of the sympathetic nervous system (SNS) facilitates lipid mobilization [24; 12]. However, *in vitro* studies have shown that sustained increased SNS activity (or plasma catecholamine levels) as in chronic stress, are associated with desensitization in the target cells [75; 130]. In paper IV, the excess amount of fatty acids in the muscle interstitium was in agreement with the explanation that there is a desentization of the target cells (the muscle cells). During recovery (paper IV) a large amount of free fatty acids and glycerol are present in the extracellular space of the myalgic muscle. Although, the systemic increased amount of fatty acids are only visualized as the muscle was provoked, i.e. after the work and stress test.

**Metabolic inhibition**

In paper III, identified enzymatic proteins below glucose 6-phosphate (figure 3) were less abundant in myalgic muscle. The results from paper III could be explained by an inhibitory model called “The Randle cycle” (figure 10) [107]. This explanatory model explains how enhanced fatty acid metabolism inhibits the anaerobic and aerobic utilization of glucose. The Randle feedback cycle explains how enhanced acetyl-CoA and/or reduced free CoA levels in the muscle cell inhibits pyruvate dehydrogenase by phosphorylation of the enzyme pyruvate dehydrogenase kinase. This slows down the conversion of glycolytically derived pyruvate to acetyl CoA [107; 36; 108]. A further accumulation of citrate, consequent to fatty acid oxidation, hampers the activity of phosphofructokinase which results in elevated cytoplasmic glucose 6-phosphate levels inhibiting hexokinase (figure 3 and 10). The overall effect of the Randle cycle is a reduced utilization of glucose as oxidable substrate. Unfortunately, not all proteins regulating the different steps in the Randle cycle were identified, leaving room for future research regarding the metabolism of the myalgic muscle.
Figure 10. The inhibitory steps of the Randle cycle (glucose-fatty acid cycle).

**Oxidative metabolism**

In paper II one protein, NADH oxyreductase complex 1 (30 kDa subunit) was statistically significant in the FDR correlated one-way ANOVA. This single protein subunit may indicate a higher oxidative metabolism in the trapezius muscle. Oxygenation is required in order to produce ATP in the mitochondrial respiratory chain. The results from paper III indicate that there was no difference in the oxygen supply of the muscle cells of patients suffering from trapezius myalgia, as no protein from the complexes of the respiratory chain emerged as being of interest in the PLS-DA model. Research and interpretation regarding the function and regulation of single protein subunits, protein complexes and the interactions of the different complexes in order to produce ATP are in progress and reviewed by Lenaz & Genova [83] and Ghezzi & Zevani [42]. For example: NADH ubiquinone oxyreductase (complex 1) contain approximately 45 different subunits [25]. Previous immunohistochemical data (i.e. COX negative fibers) are dependent on epitope specific antibodies. The binding of these antibodies in relation to the protein subunits involved in the different respiratory
complexes and the specific functions of the subunits are an issue of consideration. As the results from paper III indicates, there may not be any difference in the activity of the respiratory chain in myalgic compared to healthy subjects since no protein differed in abundance. This would coincide with the findings that there are no increased oxygenation of the trapzius muscle in myalgia; although, no conclusion regarding the actions of the respiratory chain can be made from this type of analysis. Another substance that has been considered as energy substrate corresponding to anaerobic metabolism is lactate. Lactate is either produced intracellular from pyruvate by lactate dehydrogenase, or transported from other tissues systemically to the cell [103]. Earlier studies have shown an enhanced occurrence of extracellular pyruvate and lactate in the interstitium of the myalgic muscle as the muscle were activated to a certain level. Whether the lactate was coming from the muscle cells or has a systemic origin is not known. In a study by Flodgren et al [34], systemic lactate decreases as local lactate in the muscle interstitium increases; this might be an indication of the redistribution of energy substrates in the body [103]. If lactate levels are not increased in muscle due to energy redistribution, another possible explanation could be the intracellular inhibitory effect of NADH$_2$ (figure 10). NADH$_2$ inhibits pyruvate dehydrogenase, leaving excess pyruvate which is transformed to stored lactate (figure 3). NADH$_2$ is produced in the fatty acid metabolism but also in the TCA cycle. Metabolic steps beyond the formation of Acetyl CoA (TCA cycle and respiratory chain) are similar in the carbohydrate and fatty acid oxidation.

Conclusions regarding metabolism in myalgic muscle

The present studies (papers III and IV) may be regarded as exploratory screenings of muscle biopsies and microdialysates proving comprehensive analyses of the intra and extracellular processes of chronic myalgic muscle. Myalgic muscle exhibits an altered muscle metabolism, suggesting an enhanced metabolism. A previously described feedback mechanism (The Randle cycle) explains the differences in enzymes connected to glycolysis, inhibited by fatty acid metabolism (figure 10). A probable explanation to this altered metabolism might be increased neuronal signaling [44] with increased levels of Ca$^{2+}$ influx to the interstitium thereby altering regulatory myosin light chains (paper III) as a result of chronic low level signaling [62]. A probable increased neuronal signaling (Ca$^{2+}$ influx) or adrenergic stimulation might be an explanation of why the glycogen metabolism was evoked. Stress increases glyconeogenesis, via increased glucose uptake due to increased levels of insulin. Excess levels of glutamate, also acting as a neurotransmitter, also enhance glycogen storage. The increased production of glycogen might then be resulting in an increased utilization. However,
glycogen and its relation to myalgic muscle need to be further elucidated. Also, the significantly increased abundance of pyroglutamic acid in the muscle interstitium at baseline could be a potential marker of increased nerve signaling. The increased levels of extracellular fatty acids after the exercise and stress tests (paper IV) might be an explanation of long term physiological stress. No differences between healthy and myalgic muscle in abundance of proteins related to the mitochondrial respiratory chain was obtained in this exploratory investigation. There are many protein complexes of the respiratory chain and their function are complex. Though, as none of the subunits are considered as of interest in the proteomic study (paper III), a possible conclusion can be that when comparing myalgic and healthy muscle; no specific adaptation to a decreased supply of oxygen in the myalgic muscle is obtained. Results obtained from these screening analyses, together with undetected metabolic proteins related to discussed mechanisms, requires further analysis.

Differences in metabolites between healthy controls and myalgic patients were detected upon provocation. There was a prolonged recovery period after physical activity in myalgic patients \[111; 120\]. The same type of prolonged recovery has been shown in subjects due to physical inactivity; whereas for well trained subjects have a shorter recovery period. Systematic changes in metabolic patterns were noticeable after a work test together with a stress test (paper IV). These changes could be explained by either the stress or the pain sensations or both. Conclusively, there was an altered metabolism in the muscle which was apparent upon provocation.

The functions of the different metabolic proteins (paper III) are diverse, and pattern of proteins related to the metabolism are regulated by different feedback mechanisms where a high amount of substrate (i.e. ATP, NADH2) or enzymes repress the activity of other metabolic proteins or enzymes. These different feedback loops render the interpretation of comprehensive metabolic data.

**Method improvements**

The 2D-DIGE analysis is a very sensitive method enabling detection of small size proteins. Although, higher amounts of proteins detected would be desirable, though the protein yield is dependent on the consideration taken to each proteins specific properties, as different proteins have different molecular weight, acidity, neural or alkaline properties depending on charge, and are hydrophilic or hydrophobic. Also, not all proteins that were visualized in the gel after labeling were able to be analyzed with MALDI-TOF mass spectrometry (paper II). LC-MS (paper IV) is a method that is more
sensitive and was used on protein spots in paper III. Using LC-MS it is revealed that one protein spot in the two dimensional gel can contain more than one protein. To obtain better resolution of protein spots a narrower pI interval in the first dimension could be the solution. Also, a combination with other techniques is necessary to verify these screening results.

Biopsies consist of muscle proteins as well as blood vessels and connective tissue which are included in the analysis. Immunohistochemistry can be used as a complement enabling a visualization of the location of the protein. Also, to obtain a more refined measure only including muscle tissue, single fiber extractions can be made [27]. Mitochondria enriched fractions can be obtained from muscle biopsies or single fiber extractions [129] in order to determine the metabolic activity of muscle tissue only. However, one important aspect to consider is, as large proteins are being filtered away small molecules adhered to them also disappear. In microdialysis this type of filtering appears already at sampling as one of the major substance transporters: albumin, is not included. In paper IV a microdialysis probe with 3000 kDa cut off were used, which provides a broader set of metabolites which fits the metabolomics approach. The OPLS model in the metabolomics study has a low level of explained variance. This could be due to biological variation. To obtain less variance, same subject studies, as in paper II would be preferable. Metabolomic processes are also rapid, with a reaction time of less than one second. A rapid inhibition of these processes is hence needed and is to be accounted for when planning the experiments. Also, dietary intake and physical activity days prior to the experiments needs to be considered.

**Future aspects**

The two explorative studies performed using metabolomics and proteomics provide clues regarding the pathophysiology of chronic trapezius myalgia that need to be further verified. Future analyses regarding energy depletion of the muscle due to insufficient energy supply; altered metabolism, possible muscle acidity, cellular stress responses and altered sarcomeric composition are needed. There is ongoing research regarding the proteins of metabolism as new methods (i.e. omics methods) enable a more thorough analysis. Further studies comparing healthy muscle, myalgic muscle and muscles suffering from other pain conditions such as fibromyalgia are needed; in order to explore whether the differences in protein content are similar in different painful conditions. Results will enable to detect a possible correlation to the perception of muscular pain. Also, more thorough investigations regarding the ATP and phosphor content of the myalgic
trapezius muscle in comparison to healthy are needed in order to make any statement about energy status of the myalgic muscle.

The studies included in this thesis were not able to present any clues about the specific susceptibility of the trapezius muscle to myalgia, thus this is to be further elucidated. As the studies included here are cross-sectional providing a snap-shot of the myalgic muscle, a direct explanation of what causes the myalgic condition could not be obtained. The proteins and metabolites detected to differ between myalgic and healthy muscle are associated with the specific chronic condition. Future hypothesis can be defined from the metabolites and proteins presented here; to study how and if these are involved in the conditions of pain, muscle fatigue and stiffness that is characteristic for myalgic trapezius muscle.

**Conclusions**

A reliable protocol for skeletal muscle sample preparation and analysis using 2D-DIGE was developed and validated, and successfully applied to the comprehensive analysis of normal and pathological muscle. The proteomic approach used allowed the detection of differences in content between the trapezius and vastus lateralis muscles and of normal and myalgic subjects. Further differences between healthy and myalgic subjects were revealed by the use of microdialysis based metabolomics analysis. Both these methods are potent and adequate for a comprehensive survey of myalgic conditions. New clues to enable future hypothesis can be created on the basis of the results from these exploratory studies.
Acknowledgements

Håkan Johansson the driving force of this research and my first supervisor. Fatima Pedrosa-Domellöf, the reason I started in this line of research. Albert Crenshaw for enabling the completion of this thesis.

Fredrik Hellström for all you have done for me that ended with this thesis. Bijar Ghafouri for your excellence in methodology and hospitality. Ulf Lerner for the time I spend in your lab.

Björn Gerdle and Britt Larsson for good discussions, drive and enthusiasm.

Christer Malm, Lars Frängsmyr and Thomas Kieselbach for help with methodology concerning 2D-DIGE and MALDI-TOF.

Henrik Antti and his group for helping with the methodology concerning metabolomics.

Anatomy, all skillful people assisting in the laboratory: Jing-Xia Liu, Margaretha Enerstedt, Anna-Karin Olofsson; Lars-Eric Thornell and Per Stål for good discussions and feedback; and all fellow PhD students.

CBF, thanks to all of you for many good years of laughter and friendship.

Fellow PhD students through the times: Marina Heiden, Eva Json Lönn, Jonas Sandlund, Maria Högvall Nordin, Ulrika Aasa, Martin Björklund, Nebosja Kalezic, Ivana Kalezic, Dmitry Domkin, Gerd Flodgren, Per Liv, Thomas Rudolfsson, Guilheme Elcadi, Fredrik Sjödin, Nazdar Ghafouri, Åsa Svedmark, Jennie Jackson, Camilla Lodin, Mahmoud Rezagholi, Hasse Nordlöf, David Hallman.

To all my dear friends, relatives and in-laws for being here for me.

Min familj: Mamma som jag är så glad är med mig; och min älskade Anders och mina små godisar Irma och Olivia. Ni påminner mig varje dag om vad som är viktigast här i livet.
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