Molecular mechanisms of muscle pain associated with myotonic dystrophy type II

Inaugural-Dissertation

to obtain the academic degree

Doctor rerum naturalium (Dr. rer. nat.)

submitted to

the Department of Biology, Chemistry and Pharmacy

of Freie Universität Berlin

by

VINKO PALADA

from Split, Croatia

November 2016

This work was performed from December 2013 till October 2016 under the supervision of Prof. Simone Spuler (Experimental and Clinical Research Center, Berlin, Germany) and Prof. Gary R. Lewin (Max-Delbrück Center for Molecular Medicine, Berlin, Germany). The experiments were conducted in the laboratory of Prof. Spuler.

1st reviewer: **Prof. Dr. med. Simone Spuler**

Institute for Chemistry and Biochemistry

Department of Biology, Chemistry and Pharmacy

Freie Universität Berlin

and

Department of Muscle Sciences

University Outpatient Clinic for Muscle Disorders

Experimental and Clinical Research Center, Berlin

2nd reviewer: **Prof. Dr. Gary R. Lewin**

Charité University Medical Faculty, Berlin

and

Molecular Physiology of Somatic Sensation Group

Max-Delbrück Center for Molecular Medicine, Berlin

Date of defense: 13.03.2017.

Preface

Recruiting of the patients for the study was performed by Prof. Simone Spuler, dr. Ulrike Grieben and me. I performed quantitative sensory testing (QST) together with Dr. Rabih Moshourab, from the laboratory of Prof. Gary R. Lewin. Muscle biopsy specimens were harvested by dr. Andreas Spuler from the Helios Clinic, Berlin, Germany. I performed RNA extraction from muscle biopsy specimens and preparation of samples for RNA sequencing. RNA sequencing was conducted at dr. Wei Chen´s lab. Validation of RNA sequencing data with quantitative PCR was done by me. Microdialysis was performed in collaboration with dr. Michael Boschmann´s lab. I performed the microdialysis experiments with our patient cohort. Microdialysis vials were tested with MS proteomics in collaboration with Dr. Marieluise Kirchner, from the laboratory of Dr. Gunnar Dittmar. We also analyzed eicosanoid species in DM2 dialysates with LC-MS/MS at Lipidomix Gmbh, Berlin, Germany. I performed the data analysis and interpretation of QST, RNA sequencing, lipidome and proteome data. EBioMedicine manuscript was written by me, Dr. Moshourab, Prof. Lewin and Prof. Spuler.

Acknowledgements

First, I would like to thank my supervisor Prof. Simone Spuler for giving me the opportunity to conduct my PhD thesis in her lab, for always being there for me with scientific advice and encouragement and for helping me to become a better scientist during my PhD time.

I would also like to thank Prof. Gary Lewin for co-supervising my project and for sharing his invaluable expertise in the pain research.

I would like to thank dr. Rabih Moshourab for being an excellent mentor for somatosensory profiling of our patient cohort and for improving my statistical skills.

I want to thank dr. Stefanie Grunwald for the bioinformatic advices and for the vivid discussions of my data.

I would especially like to thank Susanne Wissler for her kind help with administrative and organizational issues.

I would also like to thank dr. Michael Boschmann and dr. Anja Mähler for the help with microdialysis.

I want to thank dr. Dittmar Gunnar and dr. Marieluise Kirchner for our fruitful collaboration and for all the advice regarding proteomic analysis.

I want to thank Kornelia Gräning, Stefanie Belz and Adrienne Rothe for their excellent technical support of my project.

I want to thank all my colleagues from Spuler´s lab for making it such a nice working environment and for making my PhD a fun experience.

I would like to thank all DM2 patients and healthy subjects that volunteered to participate in my project.

I want to thank Myograd for the financial support and for making it possible to attend the international and local scientific events.

Finally, I would like to thank my loving family, my mom Biserka Palada and brother Ivan Palada for their unconditional support to achieve my goals and for always being there.

I would like to dedicate this thesis to my dear cousin Antonio Radacic, I wish you a fast recovery!

Table of contents

Summary

Muscle pain is a frequent clinical symptom that impairs the quality of life but the molecular mechanisms behind the onset of pain remain unknown. Patients with myotonic dystrophy type 2 (DM2) often develop muscle pain of moderate severity which is resistant to conventional analgesics. DM2 is a promising model for studying muscle pain since it is a monogenetic disorder caused by CCTG repeats expansion in intron 1 of transcription factor encoding *CNBP* which alters gene expression and splicing of downstream genes. We assumed that the expansion of repeats in *CNBP* could alter the gene expression and splicing of pain regulating genes in DM2. Additionally, certain myokines secreted from DM2 affected muscles during contraction could contribute to the onset of muscle pain. Considering that hyperlipidemia is frequent in DM2 and lipids are known mediators of peripheral nociception, altered levels of lipids might also modulate the nociception in DM2. In our study, we examined a cohort of 42 DM2 patients and 20 healthy controls. Patients were characterized for somatosensory profiles with a standardized QST protocol. We obtained biopsy specimens from 12 DM2 patients (6 with and 6 without muscle pain) in order to analyze their transcriptome profiles and performed microdialysis with 14 patients (7 with and 7 without muscle pain) for proteomic and lipidomic profiling of muscle pain in DM2. QST revealed significantly decreased pressure pain thresholds over proximal and distal muscles only in DM2 patients with pain indicating that the cause of the muscle pain is within the muscle. DM2 patients had distinct transcriptome profiles based on the presence of muscle pain and RNA sequencing identified 14 differentially expressed genes between patients with and without pain. Shotgun proteomic analysis of DM2 interstitial fluid revealed 22 differentially secreted proteins based on the presence of muscle pain. There was no association between muscle pain in DM2 and the known pro-inflammatory cytokines IL-1, IL-6 and NGF. LC-MS/MS analysis of DM2 lipidome profiles identified the presence of anti-inflammatory eicosanoids 11,12-DHET only in patients without pain. These results are the first evidence that the muscle pain in DM2 is caused by peripheral nociception. Furthermore, this study shows

that by using OMICS techniques in homogenous groups of patients with chronic pain, it is possible to identify novel genes and molecules that might contribute to the onset of pain. Our study might also explain the pathogenic mechanisms of more common disorders with chronic muscle pain such as fibromyalgia.

Zusammenfassung

Muskelschmerzen sind ein häufiges klinisches Symptom, das die Lebensqualität beeinträchtigt. Trotzdem bleiben die Molekularmechanismen, die dahinterstehen, unbekannt. Bei den Patienten mit der Myotonen Dystrophie Typ 2 (DM2) treten oft Muskelschmerzen mittleren Schweregrades auf, die gegen konventionelles Analgetikum resistent sind. DM2 ist ein viel versprechendes Modell für die Studie über Muskelschmerzen, weil es eine monogenetische Krankheit ist, die durch die Zunahme der CCTG Wiederholungen, die im Intron 1 von CNBP, das die Genexpression und das Spleißen der Downstream-Gene ändert, stattfindet. Wir haben angenommen, dass die Zunahme der Wiederholungen in CNBP bei DM2 die Genexpression sowie das Spleißen der Gene, die die Schmerzen regulieren, ändern könnte. Darüber hinaus könnten gewisse Myokine, die aus den von DM2 betroffenen Muskeln während Kontraktion abgesondert wurden, zu dem Auftreten der Schmerzen beitragen. Da bei DM2 die Hyperlipidämie oft vorkommt und die Lipide bekannte Mediatoren der peripheren Nozizeption sind, könnten die geänderten Lipidspiegel auch die Nozizeption bei DM2 regeln. In unserer Studie haben wir eine Kohorte von 42 DM2 Patienten und 20 gesunden Kontrollprobanden untersucht. Die Patienten wurden für somatosensorische Profile mit standarisiertem QST Protokoll charakterisiert. Wir haben von 12 DM2 Patienten (6 mit und 6 ohne Muskelschmerzen) Biopsieproben entnommen, um ihre Transkriptomprofile zu analysieren und haben bei 14 Patienten (7 mit und 7 ohne Muskelschmerzen) die Mikrodialyse für die proteomische und lipämische Profilierung von Muskelschmerzen bei DM2 durchgeführt. QST zeigte deutlich reduzierte Grenzwerte von Druckschmerzen in proximalen und distalen Muskeln nur bei den DM2 Patienten mit Schmerzen, was darauf hinweist, dass die Ursache für die Muskelschmerzen innen im Muskel liegt. Die DM2 Patienten hatten verschiedene Transkriptomprofile, die auf der Präsenz der Muskelschmerzen basierten und die RNA-Sequenzierung identifizierte 14 differentiell bei den Patienten mit und ohne Muskelschmerzen exprimierte Gene. Die proteomische Shotgun-Analyse von der DM2 interstitiellen Flüssigkeit

offenbarte 22 differentiell abgesonderte, auf der Präsenz der Muskelschmerzen basierende Proteine. Es ergab sich keine Verbindung zwischen Muskelschmerzen in DM2 und bekannten pro-inflammatorischen Zytokinen IL-1, IL-6 und NGF. LC-MS-Analyse von DM2 lipämische Profile identifizierten die Präsenz von entzündungshemmenden Eicosanoiden 11,12-DHET nur bei Patienten ohne Muskelschmerzen. Dieses Ergebnis ist der erste Beweis dafür, dass die Muskelschmerzen bei DM2 ein Resultat der peripheren Nozizeption sind. Außerdem zeigt die Studie, dass durch die Nutzung von OMICS-Techniken bei homogenen Gruppen von Patienten mit chronischen Schmerzen es einfacher ist, neuartige Gene und Moleküle zu identifizieren, die zu dem Auftreten von Schmerzen beitragen können. Unsere Studie könnte auch die pathogenen Mechanismen von gewöhnlicheren Krankheiten mit chronischen Muskelschmerzen wie Fibromyalgie erklären.

List of figures

List of tables

List of abbreviations

Acid sensing ion channels (ASICs)

Adenosine triphosphate (ATP)

Brain-derived neurotrophic factor (BDNF)

Calcitonin gene-related peptide (CGRP)

Central nervous system (CNS)

Chloride voltage-gated ion channel 1 (CLCN1)

Cold detection threshold (CDT)

Cyclic adenosine monophosphate (cAMP)

Cyclooxygenase 2 (COX2)

Differentially expressed gene (DEG)

Dorsal root ganglia (DRG)

Electrospray ionization (ESI)

Extracellular signal-regulated kinase (ERK)

False discovery rate (FDR)

Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)

G-protein coupled receptors (GPCRs)

Heat pain threshold (HPT)

High-performance liquid chromatography (HPLC)

Insulin receptor (IR)

Interleukin-1 (IL-1β)

Interleukin-6 (IL-6)

Label-free quantification (LFQ)

Liquid chromatography–mass spectrometry (LC-MS)

Long-term depression (LTD)

Long-term potentiation (LTP)

Magnetic resonance imaging (MRI)

Mass spectrometry (MS)

Mass/charge ratio (m/Z)

Matrix assisted laser desorption/ionization (MALDI)

McGill Pain Questionnaire (MPQ)

Mechanical pain sensitivity (MPS)

Mechanical pain threshold (MPT)

Medical Research Council scale for muscle strength (MRC)

Mitogen-activated protein kinase (MAPK)

Muscleblind-like proteins (MBNL)

Myotonic dystrophy protein kinase (DMPK)

Myotonic dystrophy type 1 (DM1)

Myotonic Dystrophy Type 2 (DM2)

Nerve growth factor (NGF)

Nitric oxide synthase (NOS)

Non-N-methyl-D-aspertate (NMDAR)

Nuclear magnetic resonance (NMR)

Nucleic acid binding protein (CNBP)

Number of words chosen (MPQ-NWC)

Numeric analogue score (NAS)

Pain rating index (MPQ-PRI)

Paradoxical heat detection (PHS)

Phosphatidylinositol 3-kinase(PI3K)

Phospholipase C-beta (PLC-β)

Polymerase chain reaction (PCR)

Positron emission tomography (PET)

Present pain index (MPQ-PPI)

Pressure pain threshold (PPT)

Protein kinase A (PKA)

Protein kinase C (PKC)

Quantitative sensory testing (QST)

Reverse phase liquid chromatography (RP-LC)

Reverse transcription polymerase chain reaction (RT-PCR)

Room temperature (RT)

S-alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPAR)

Selected reaction monitoring (SRM)

Single nucleotide polymorphism (SNP)

Sodium hydroxide (NaOH)

Stable isotope labeling by amino acids in cell culture (SILAC)

Standard deviation (SD)

Substance P (SP)

Tandem mass spectrometry (MS/MS)

Tetrodotoxin (TTX)

Time of flight (TOF)

Transient receptor potential protein (TRP)

Trimmed mean of M-values (TMM)

TRP anykrin 1 (TRPA1)

TRP melastatin 8 (TRPM8)

TRP vanilloid 1 (TRPV1)

Tumor necrosis factor alpha (TNF-α)

Tyrosine kinase receptor (Trk)

Warm detection threshold (WDT)

Wind-up ratio (WUR)

Zinc-finger domain (ZnF)

γ-Aminobutyric acid (GABA)

1. Introduction

Chronic pain is a major health burden that impairs the quality of life in patients (Breivik et al., 2006.). Chronic muscle pain is a frequent clinical symptom present in the conditions such as fibromyalgia syndrome, myofascial pain syndrome, neck and low back pain of muscular origin, inflammatory myopathies and some hereditary myopathies such as myotonic dystrophy type 2 (Mense and Gerwin, 2010). The cause of muscle pain is unknown. Considering that muscle pain is a nonspecific symptom that can arise from different biological, clinical, psychological and demographic risk factors (van Hecke et al. 2013), it is extremely challenging to study the mechanism of muscle pain and to tailor effective therapies for the patients. Current treatments of muscle pain with conventional analgesics are usually ineffective and muscle pain is the main reason for patients missing work because of disability (George et al, 2004). Because of this, I focused in my project on providing mechanistic insight to muscle pain by studying a homogenous group of patients with myotonic dystrophy type 2 which all have the same genetic cause of disease and at least 50% of the patients develop chronic muscle pain.

1.1 Pain

Pain is an evolutionary conserved physiological sensation which is crucial for the survival of organism by indicating the presence of real or potential tissue damage (Gangadharan and Kuner, 2013).

1.1.1 Pain theories

From the early beginnings of scientific thinking there is a long-lasting debate whether pain is one of the senses or related to emotional states and behavior. Specificity theory refers to presence of sensory receptor and associated sensory fiber which respond to the presence of painful stimuli indicating that there are specific neuronal elements transferring the pain information (Moayedi and Davis, 2013). In 1811. Charles Bell in his essay *Idea of a New Anatomy of*

the Brain suggested the existence of sensory neurons that respond to different types of stimuli (Bell and Shaw, 1868). Between 1894 and 1896, Max von Frey provided the first experimental evidence for Specificity theory by describing 4 somatosensory modalities (cold, heat, pain, and touch) after distinguishing the skin spots which responded to innocuous or noxious pressure when force was applied with von Frey hairs (Rey, 1995). At the beginning of 20th century, Charles Scott Sherrington further advanced the Specificity theory by suggesting the existence of nociceptors, sensory receptors which have lower excitability threshold for one type of noxious stimuli and higher for all the other stimuli (Sherrington, 1906). In 1969, Bessou and Perl revolutionized the pain research with discovery of unmyelinated nociceptive fibers in polymodal nociceptors and high-threshold mechanoreceptors (Bessou and Perl, 1969). Intensity theory of pain suggests that pain occurs in any sensory system when the stimulus reached the sufficient intensity (Perl, 2007). While Specificity theory is trying to explain the pain with specific sensory elements, an alternative theory known as the Pattern theory of pain claimed there is no special neuronal network and pain was defined as a result of interactions between patterns of impulses in nonspecific neuronal networks. To support Pattern theory, Lele demonstrated that distortion of cutaneous sensory nerve fiber is causing a discharge in any nerve fiber regardless if the fiber is myelinated or not (Lele et al, 1954).

In 1965, Ronald Melzack and Charles Patrick Wall (Melzack and Wall, 1965) proposed the Gate control theory of pain which was a combination of Specificity and Pattern Theories and provided a model to explain their contradicting findings (Figure 1.1). They proposed a gate in the substantia gelatinosa of the dorsal horn which would modulate the sensory transmission between the site of injury to transmission cells in the spinal cord. Large nonnociceptive fibers close or inhibit the gate while small nociceptive fibers open the gate and facilitate the sensory transmission. When nociceptive stimuli reached an intensity higher than inhibition, the gate opens and pain pathways are activated leading to pain sensation. Since it was introduced, the Gate control theory remains the only theory which accounts for both biological and psychological aspects of the pain (Craig and Rollman, 1999).

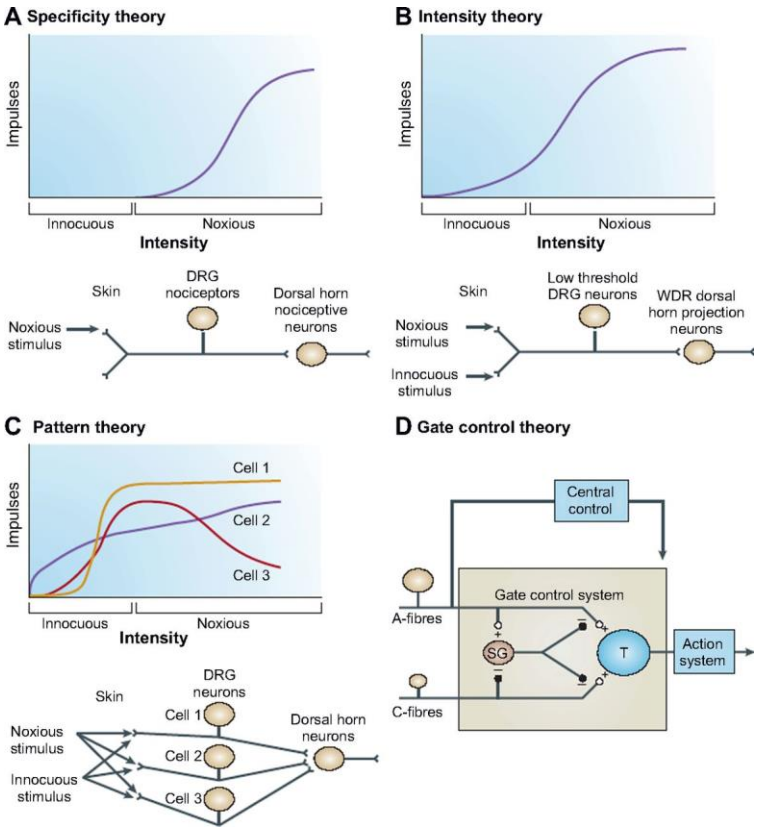


Figure 1.1: Schematic diagrams of pain theories.

A) Specificity Theory of Pain claims that touch and pain are transmitted with separate sensory pathways which project to touch and pain centers in the brain. B) Intensity theory suggests that there are no separate pathways for noxious and innocuous stimuli. The primary afferent neurons form synapses on wide-dynamic range (WDR) neurons in the dorsal horn of the spinal cord. High levels of spinal cord neuron activity lead to noxious sensation while low activity is related to innocuous sensation. C) Pattern Theory of Pain predicts that different somatic organs have different levels of responsivity to noxious stimuli. D) Gate Control Theory of Pain suggests that large (A-fibers) and small (C-fibers) form synapses with neurons in the substantia gelatinosa (SG) and the transmission (T) cells. The inhibitory effect of SG cells on the primary afferent terminals of T cells is increased by activity in A-fibers and decreased by activity in C-fibers. The T cells transduce the action potential.

Adopted and modified from Perl, 2007.

1.1.2 Types of pain

There are different types of pain. Based on duration, pain can be classified as acute and chronic pain. Acute pain is a short-lasting pain which is a result of tissue damage and resolves over time as tissue heals. During pathological conditions, prolonged acute pain can cause the long-lasting changes in brain

which lead to transition from acute to chronic pain (Voscopoulos and Lema, 2010). Pain is classified as chronic pain if it is present for longer than 6 months (Russo and Brose, 1998). Depending on the cause, pain can be defined as nociceptive, inflammatory and neuropathic pain. Nociceptive pain is caused by activation of nociceptors in the peripheral tissues which are sensitized by noxius stimuli, such as mechanical pressure, thermal and chemical stimuli. In patients, nociceptive pain may be present as spontaneous pain (pain without any intentional stimuli) or as allodynia and hyperalgesia. Allodynia is a painful sensation triggered by stimuli which usually does not cause pain (light touch and warmth) while hyperalgesia refers to pain hypersensitivity where noxious stimuli is perceived as even more painful. There are two types of hyperalgesia, primary and secondary hyperalgesia. Primary hyperalgesia is induced at the site of injury and is associated with pain hypersensitivity of peripheral nociceptors. Secondary hyperalgesia occurs in uninjured surrounding tissue at the site of injury and was associated with increased excitability of neurons in CNS (Sandkuhler, 2009). Inflammatory pain is caused by tissue inflammation due to changes in chemical environment of nociceptive neurons and secretion of pro-inflammatory mediators (growth factors, cytokines, prostaglandins, neurotransmitters, peptides) from recruited inflammatory cells (Basbaum et al, 2009). Unlike nociceptive pain which is induced by sensitization of afferents in peripheral tissues, neuropathic pain is caused by neuronal damage in the central or peripheral nervous system (Schaible, 2006). Patients with neuropathic pain can also develop allodynia and hyperalgesia (Petersen et al, 2000).

1.1.3 Nociceptors

Nociceptors are free nerve endings of sensory neurons that are connected with central nervous system (CNS) by nerve fibers. Cell bodies of both nociceptive and proprioceptive sensory neurons are located in the dorsal root ganglia (DRG) from where they have the bidirectional projections to nerve endings in the peripheral tissues and to central nerve endings in the superficial spinal dorsal horn. Peripheral nociceptive endings are present in almost all tissues and

organs except for the brain, parenchyma of the lung, cartilage and liver (Mense, 2009; Gangadharan and Kuner, 2013).

Nociception is a process in which noxious stimuli such as mechanical, thermal and chemical stimuli are detected by peripheral nerve fibers (Basbaum and Jessell, 2000). Detection of the noxious stimuli is mediated by diverse group of ion channels which are expressed at the peripheral nerve endings of nociceptive neurons and mediate the transduction of noxious stimuli to changes in membrane potential (Gold and Gebhart, 2010; Raouf et al, 2010). The influx of cations through ion channels leads to membrane depolarization. Once the depolarization is sufficient to open Na^+ channels, the action potential is triggered. Ca^{2+} and K^+ ion channels modulate the excitability of nociceptive neurons. The gating of ion channels is regulated by inflammatory mediators and the signal transduction is carried via secondary messenger pathways (Schaible et al, 2011).

Most of nociceptors are polymodal which means that they can get activated by more than one type of noxious stimuli (Belmonte and Cervero, 1996). Based on diameter and conduction velocity, peripheral nerve fibers can be classified as C, A-delta and A-beta fibers (Figure 1.2). Medium diameter myelinated A-delta fibers mediate acute or fast pain. Small diameter unmyelinated C fibers are involved in conduction of poorly localized slow pain. Third type are larger diameter myelinated A-beta fibers with fast conduction that respond to nonnociceptive mechanical stimuli such as light touch (Meyer et al, 2008). There are also nociceptors insensitive to mechanical stimuli called silent or mechanically insensitive afferents (MIA) (Meyer et al, 1991). Subpopulations of nociceptors are usually classified based on the peptide content and receptor expression. A peptidergic nociceptors contain one or both neuropeptides substance P (SP) and calcitonin gene-related peptide (CGRP) and express the nerve growth factor (NGF) receptor trkA (McMahon et al. 1995, Ma et al, 2001). A nonpeptidergic nociceptors do not contain neuropeptides and express isolecten B4 (IB4) instead (Molliver et al, 1997).

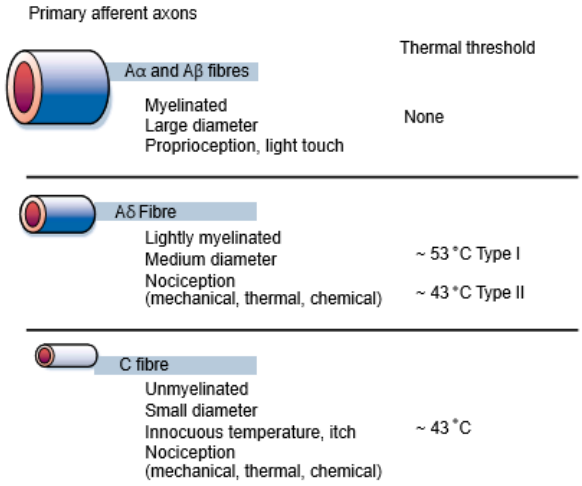


Figure 1.2: Types of nociceptors based on diameter and conduction velocity.
Modified from Julius and Basbaum, 2001.

In the experimental studies of nociceptors, it is necessary to identify the receptive field of the nociceptor which is an area of skin where the afferent is responsive to stimulation. The location of receptive field can be determined by mechanical stimuli (pinching, von Frey filaments). In the case of MIA, the location of mechanically insensitive afferents can be detected by electrostimulation (Amstrong-James, 1975). The most common types of experiments include patch clamp recording performed in cultured DRG neurons, behavioral studies in animal models *in vivo* and invasive *in situ* experiments (Schaible et al, 2011).

1.2 Peripheral and central mechanisms of nociception

Nociception is a bidirectional process since proteins and signal molecules synthesized in DRG nociceptive neurons can be distributed to both peripheral and central terminals (Figure 1.3; Basbaum et al, 2009).

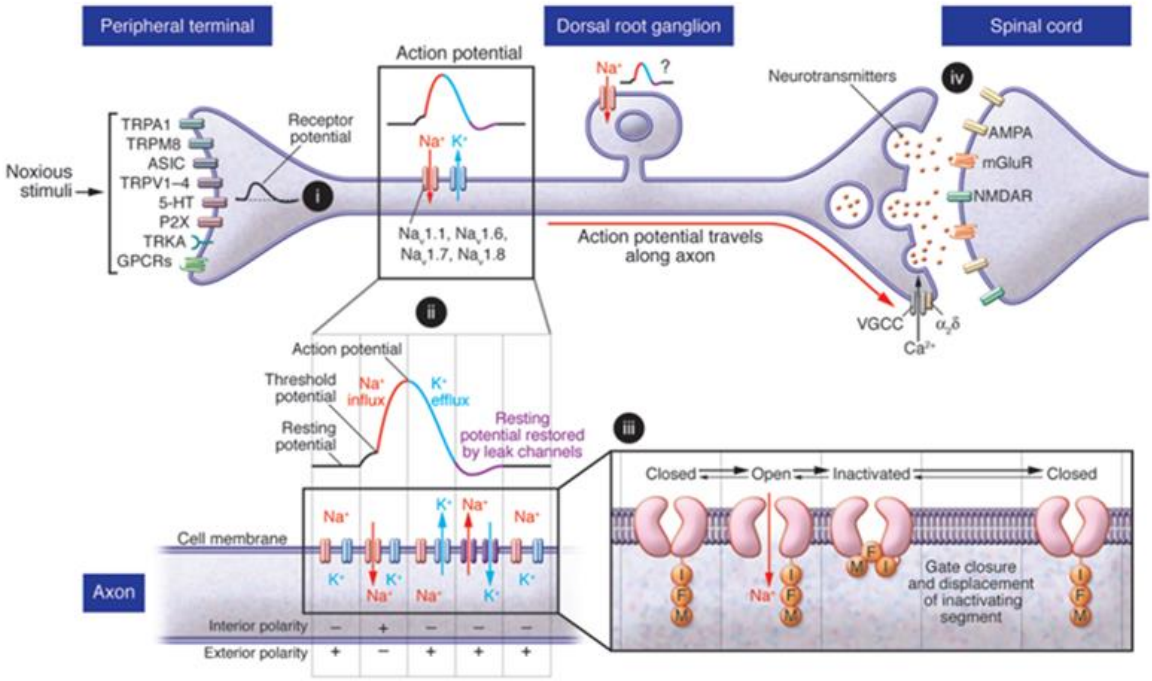


Figure 1.3: Model of nociception.

Cell bodies of nociceptors are located in the dorsal root ganglion with projections to the peripheral tissues (peripheral terminals) and to the spinal cord (central terminals). Ion channels and receptors expressed on the peripheral terminals are activated by noxious stimuli and trigger the membrane depolarization (i). When the threshold potential is reached, the voltage-gated sodium channels are activated which generates the action potential (ii). The action potential is transmitted over the axon to the central terminals in the dorsal horn of spinal cord. The pre-synaptic central terminals form synapses with post-synaptic spinal neurons. Calcium influx through voltage-gated calcium channels (VGCC) facilitates the release of neurotransmitters from presynaptic terminals which activate ionotropic (AMPAR, NMDAR) and metabotropic (mGluR) glutamate receptors on the postsynaptic terminals of spinal neurons that transduce the noxious information to the pain centers in CNS (iii).

Adopted and modified from Raouf et al, 2010.

1.2.1 Peripheral mechanisms of nociception

Peripheral nerve endings of nociceptive sensory neurons have different types of cell membrane receptors that are sensitized in the presence of external noxious stimuli or during tissue damage and inflammation. After tissue damage multiple pro-inflammatory mediators that are released from damaged tissue and immune cells, such as ATP, kinins, purines, protons, cytokines, neuropeptides, neurotrophins and prostaglandins, bind to receptors on nociceptive terminals and activate intracellular pathways that lead to increased sensitization of

peripheral terminals (Hucho and Levine, 2007; Gangadharan and Kuner, 2013). There are 3 classes of peripheral terminal receptors which are involved in pain transduction: ion channels, metabotropic G-protein coupled receptors (GPCRs) and receptors for neurotrophins, neuropeptides and cytokines (Gold and Gebhart, 2010). When noxious stimuli and inflammation are persistent, the expression of the receptors is upregulated which can contribute to the maintenance of pain (Schaible, 2006). Targeting some of these receptors might be an effective way to reduce nociception and provide pain relief.

1.2.1.1 Ion channels

Thermal stimuli are detected by ion channels of the transient receptor potential (TRP) protein family (Papapoutian et al, 2003). TRP proteins contain six transmembrane domains with cytoplasmic N and C-termini which are assembled to homo and heterotetramers to form cation-permeable ion channels (Voets et al, 2005). TRP vanilloid 1 (TRPV1) is a receptor for capsaicin, ingredient of hot chili peppers, and it is a putative transducer of noxious heat (>42°C) (Caterina et al, 2000). TRPV2 receptor is activated by very high threshold of heat temperature (>50°C) (Caterina et al, 1999). Responses to cold stimuli are mediated by menthol receptor TRP melastatin 8 (TRPM8) (McKemy et al, 2002).

Mechanical stimuli are transduced by Ca^{2+} permeable Piezo1 and Piezo2 channels (Coste et al, 2010). The activation of TRPV1 inhibits Piezo channels and controls mechanosensation (Borbiro et al, 2015). The mustard oil receptor TRP anykrin 1 (TRPA1) is also proposed to play a role in transduction of mechanical stimuli and inhibition of TRPA1 with small molecule inhibitor reversed mechanical hyperalgesia induced by inflammation in mice (Petrus et al, 2007).

Acid sensing ion channels (ASICs) are H^+ gated Na^+ channels which are activated by the local decrease in extracellular pH during tissue damage and inflammation (Wemmie et al, 2013). In mammals, four genes (*ACCN 1-4*) encode for six different ASIC subunits (ASIC1a, ASIC1b, ASIC2a, ASIC2b,

ASIC3 and ASIC4) (Baron and Lingueglia, 2015). Most of the subtypes are expressed in DRG but ASIC1a and ASIC3 are expressed only on nociceptive neurons (Olson et al, 1998, Xie et al, 2003). ASIC3 shows higher expression in nociceptive neurons innervating muscle (50%) than skin (10%) which indicates that ASIC3 is involved in detection of muscle acidosis (Molliver et al, 2005). Disruption of ASIC1a and ASIC3 in mice was reported to eliminate mechanical hyperalgesia caused by muscle inflammation (Walder et al, 2010).

Purinergic ion channels P2X2 and P2X3 are ligand-gated Ca^{2+} channels which are sensitized by ATP released from keratinocytes of inflamed tissue. Like ASICs, purinergic channels also modulate development of hyperalgesia during muscle inflammation (Schaible et al, 2011).

Two-pore domain potassium channels (K2P) contribute to resting membrane potential (Bayliss et al, 2008). TREK-1 and TRAAK are K2P channels expressed on nociceptive DRG neurons that are associated with elevated nociception in knockout animal models (Alloui et al, 2006; Linden et al, 2006; Noel et al, 2009).

Voltage gated Na^+ channels (Na_v) are tetrodotoxin (TTX) sensitive ion channels which are activated by depolarization of membrane after noxious stimuli and lead to upstroke of action potential. $Na_v1.7$, $Na_v1.8$ and $Na_v1.9$ are Na_v isoforms expressed in nociceptive neurons (Rush et al, 2007; Fukuoka et al, 2008). A gain of function $Na_v1.7$ mutations cause the inherited painful channelopathies, for example erythromelalgia (Cummins et al, 2004) and paroxysmal extreme pain disorder (Fertleman et al, 2006). Deletion of $Na_v1.7$ in nociceptive neurons protects against hyperalgesia in mice (Nassar et al, 2004) while naked-mole rat specific variant of $Na_v1.7$ contributes to acid pain insensitivity in this species (Smith et al, 2011).

Finally, there is a group of ion channels which contribute to action potential repolarization such as voltage gated K^+ channels, Ca^{2+} modulated K^+ channels (BK), Ca^{2+} dependent Cl^- channels and hyperpolarization-activated cyclic nucleotide–gated (HCN) channels. Distribution of these channels on

nociceptive neurons modulates the duration of action potential upstroke and interspike intervals (Gold and Gebhart, 2010).

1.2.1.2 G-protein coupled receptors

G-protein coupled receptors (GPCRs) have an important role in regulation of nociceptive excitability and peripheral nociception. GPCRs are expressed on nociceptive neurons and activated by peptides, bioactive lipids and metabolites. There are 2 types of GPCRs: excitatory and inhibitory GRCRs. Excitatory receptors are CGRP receptor, B1 and B2 bradykinin receptors, EP1, EP3C and EP4 receptors for prostaglandin while inhibitory receptors are opioid receptors. These receptors are coupled by different G-proteins such as G_q, G_s and G_{11}. G_q/G_{11} signaling pathway leads to activation of phospholipase C-beta (PLC-β), protein kinase C (PKC) and to release of Ca^{2+} from intracellular stores which influences the membrane potential. G_s signaling leads to increase in cellular cAMP levels and activates protein kinase A (PKA) which elevates the excitability of ion channels on nociceptor membrane (Gangadharan and Kuner, 2013; Gold and Gebhart, 2010). It has been shown that antagonists of B1 bradykinin receptor can reduce thermal hyperalgesia while B2 receptors are upregulated during inflammation (Poole et al, 1999; Segond von Banchet et al, 2000).

1.2.1.3 Receptors for neurotrophins, neuropeptides and cytokines

Development and survival of DRG sensory neurons is mediated by neurotrophins such as nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3) which are ligands of tyrosine kinase receptors (trk) expressed on sensory neurons (Fitzgerald, 2005). NGF is a ligand of TrkA receptors which are expressed on nociceptive C fiber and A-delta neurons and it enhances the response of these neurons to noxious stimuli (Ritter et al, 1991, Lewin et al, 1993; Averill et al, 1995; Malik-Hall et al, 2005). BDNF is the ligand of TrkB receptors and plays a role in inflammatory pain hypersensitivity (Groth and Aanonsen, 2002). NT-3 binds to TrkC receptors which are mostly expressed on proprioceptive DRG neurons (Kucera et al, 1998). NGF is an interesting target for pain relief. Targeting of NGF with anti-

NGF antibodies eliminates inflammation-induced hypersensitivity while prolonged treatment with NGF leads to increased expression of TRPV1, Na^+ channels, P2X and bradykinin receptors together with increased synthesis of CGRP and substance P (Stein et al, 2009). Furthermore, it was shown that hypofunctional TrkA contributes to reduced sensitization of TRPV1 channels in naked mole-rats and the absence of NGF induced thermal hyperalgesia (Omerbašić et al, 2016).

Receptors for excitatory neuropeptides (substance P and CGRP) and inhibitory peptides (opioids, somatostatin and neuropeptide Y) are also expressed on peripheral nociceptive terminals. These receptors are autoreceptors since they are expressed on the same DRG neurons that also synthetize their ligands (Bär et al, 2004).

Pro-inflammatory cytokines interleukin-1 (IL-1β), interleukin-6 (IL-6) and tumor necrosis factor alpha (TNF-α) are released from injured tissue and infiltrating immune cells. Cytokines contribute to peripheral nociception by binding to their receptors on peripheral terminals and increasing the excitability of nociceptors (Binshtok et al, 2008; Gudes et al, 2015; Zhou et al, 2016). TNF-α increases the expression of Nav1.8 channels in DRG neurons (Cummins et al, 2000) and boosts the nociceptive excitability by modulating the activity of P38 mitogen-activated protein kinase (p38 MAPK) which leads to hyperpolarization of nociceptive neurons (Gudes et al, 2015). TNF-α stimulates secretion of IL-6 and IL-1 from immune cells while IL-6 increases the production of IL-1. IL-1β modulates cyclooxygenase 2 (COX2) activity which leads to increased production of prostaglandin PGE2 that sensitize nociceptive terminals by binding to E-type prostaglandin receptors (Samad et al, 2001). IL-1β also contributes to peripheral nociception by induction of NGF (Safieh-Garabedian et al, 1995) and by increasing nociceptive excitability in p38 MAPK manner (Binshtok et al, 2008). The administration of IL-6 leads to thermal hyperalgesia and mechanical allodynia while anti-IL-6 antibodies decrease pain in rheumatoid arthritis and juvenile idiopathic arthritis (Choy et al, 2002; Yakota et al, 2008). IL-6 binds to membrane bound IL-6 receptor which activates the intracellular signaling pathways including the Janus-activated kinase/signal

transducer activator of transcription (JAK/STAT), mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) and phosphatidylinositol 3-kinase/protein kinase B (PI3K/AKT) (Zhou et al, 2016).

1.2.2 Central mechanisms of nociception

Central sensitization is an amplification of neural signaling leading to hypersensitivity of central nervous system (CNS) to noxious stimuli during peripheral inflammation and nerve injury (Woolf, 2011). Central terminals of DRG nociceptive neurons form synaptic connections with projection neurons in the dorsal horn of spinal cord that transduce pain signal to spinal cord. Projection neurons transduce pain information along the spinothalamic tract to thalamus or to brainstem over the spinoreticulothalamic tract (Malfait and Schnizer, 2013). Central nociception is a result of plasticity of CNS and is characterized by decrease in threshold of nociceptive spinal cord neurons, expansion of their receptive fields and development of hyperalgesia and allodynia (Schaible et al, 2006). Spinal neurons and glia cells produce prostaglandins, cytokines, neurotrophins and neurotransmitters that enhance synaptic transmission of noxious stimuli from the periphery followed by loss of inhibition mechanism (Marchard et al, 2005). Central sensitization can be monitored with electrostimulation by measuring wind-up (WU), long-term potentiation (LTP) and long-term depression (LTD) of spinal neurons. Wind-up is short-lasting increase in spinal neuron responses when electrostimulation of C fibers is repeated in intervals of 1s (Medell and Wall, 1965). LTP is a long lasting change of responses after stimulation of C fibers with disruption of inhibition while LTD is a long lasting change of synaptic activity after stimulation of A-delta fibers (Rygh et al, 1999). There are 3 types of mechanisms regulating central nociception: glutamate/NMDA receptor-mediated sensitization, loss of GABAergic and glycinergic inhibition and neuro-glia interactions.

1.2.2.1 Glutamate/NMDA receptor-mediated sensitization

Glutamate is a neurotransmitter released from central terminals of DRG nociceptors which has an excitatory effect on dorsal horn neurons. It activates

postsynaptic S-alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPAR) and non-N-methyl-D-aspertate (NMDAR) ionotropic glutamate receptors on spinal cord neurons (Woolf and Salter, 2000). In normal conditions NMDAR are silent but during the tissue injury, increased secretion of glutamate from central nociceptive terminals will depolarize postsynaptic spinal cord neurons and lead to activation of NMDAR, elevated level of cytosolic Ca^{2+} in postsynaptic neurons and central hypersensitivity (Basbaum et al, 2009). Sensitization of spinal cord neurons is additionally achieved by activation of metabotropic glutamate, neurokinin-1 (NK-1) and SP receptors in dorsal horn that also contribute to increased influx of calcium (Dubner, 2005). The increasing level of intracellular calcium activates calcium-dependent protein kinases such as MAPK, PKA, PKC, PI3K, Src and CaMKIIα which contribute to insertion of AMPAR to postsynaptic membrane and modulation of NMDAR activity (Kuner, 2010). MAPKs ERK1 and ERK2 enhance AMPAR and NMDAR activities in spinal dorsal horn neurons (Kohno et al, 2008). Spinal dorsal horn neurons are also sensitized by activity of cyclooxygenase 2 (COX2) and nitric oxide synthases (NOS) which generate prostaglandin E2 and nitric oxide that enhance the release of glutamate from central terminals (Kuner, 2010).

1.2.2.2 Loss of GABAergic and glycinergic inhibition

γ-Aminobutyric acid (GABA)ergic inhibitory neurons are located in the dorsal horn of the spinal cord and can reduce the response to noxious stimuli from the periphery. $GABA_A$ and $GABA_B$ receptors are expressed on both DRG nociceptive neurons and dorsal horn neurons (Persohn et al, 1991; Towers et al, 2000). The function of GABA in spinal cord is to achieve the presynaptic inhibition on central terminals of nociceptors and postsynaptic inhibition of dorsal horn neurons (Basbaum et al, 2009). The inhibitory effect is also achieved by glycine that modulates the activity of glycinergic neurons in dorsal horn. There is a co-localization of GABA and glycine in central terminals and fibers descending from brain stem (Todd, 1996; Kato et al, 2006). During peripheral nerve injury, decrease in GABAergic and glycinergic inhibitory currents from dorsal horn neurons is observed and this contributes to development of central hypersensitivity (Moore et al, 2002). It was shown that

nerve injury induced loss of the postsynaptic potassium chloride exporter KCC2 leads to decrease in Cl^- currents and disinhibition of dorsal horn neurons (Coull et al, 2003). Disinhibition can be also achieved by the action of prostaglandins that selectively block inhibitory neurotransmission in dorsal horn neurons (Ahmadi et al, 2002).

1.2.2.3 Neuro-glia interactions

Interactions between spinal neurons and glial cells can also contribute to central sensitization (Gangadharan and Kuner, 2013). During peripheral nerve injury, microglia infiltrate the dorsal horn of spinal cord in injured peripheral nerve fibers where they release pro-inflammatory cytokines (IL-1, IL-6, TNFα, BDNF and cathepsin S) that lead to the development of central hypersensitivity (DeLeo et al, 2005). Spinal p38 MAPK isoforms are involved in activation of microglia after peripheral nerve injury (Svennson et al, 2004). A member of the MAPK pathway, ERK, is only activated in microglia during first few days of nerve injury (Zhuang et al, 2005). Recruitment and activation of microglia are regulated by purinergic receptors P2X4, P2X7 and P2Y12 that are sensitized by release of ATP at the site of nerve injury (Tsuda et al, 2003; Clark et al, 2010; Haynes et al, 2006). Toll-like receptors (TLRs) can contribute to activation of microglia after nerve injury and it was shown that inhibition of TLRs in mice leads to decrease in microglial activation and central hypersensitivity (Kim et al, 2007). Microglia secrete BDNF which binds to TrkB receptors in spinal cord neurons. This leads to reduced expression of the potassium chloride co-transporter KCC2 in the neurons nearby and makes them more sensitive to the noxious stimuli (Coull et al, 2003). IL-1β released from microglia binds to receptors on central terminals and spinal neurons. This modulates release of neurotransmitters and was associated with enhanced activation of NMDAR and AMPAR (Gao and Ji, 2010). Glial IL-1β can also contribute to loss of inhibition mechanism during onset of neuropathic pain by inhibition of GABAergic and glycinergic spinal currents (Kawasaki et al, 2008).

1.3 Skeletal muscle and associated muscle diseases

Skeletal muscle is a tissue responsible for movement. It plays an important role in basal energy metabolism as a main tissue of oxygen consumption and heat production during physical activity and as the storage place for amino acids and carbochydrates (Wolfe, 2006).

1.3.1 Structure and physiology of skeletal muscle

The structural units of skeletal muscle are elongated multinucleated cells called muscle fibers which are produced by fusion of individual myoblasts during development and regeneration. Groups of muscle fibers are surrounded by a layer of connective tissue called perimysium while the whole muscle is surrounded with additional connective tissue layer known as epimysium. Muscle fibers have different types of organells such as mitochondria for energy production, the sarcoplasmic reticulum for calcium storage and peripherally localized nuclei for gene regulation (Folker and Baylies, 2014). The cell membrane of myofiber is called sarcolemma and consists of structural proteins which are involved in the excitation and muscle contraction which generates muscle force. Cytoplasm of myofibers known as sarcoplasm is organized to functional units of sarcomeres which are parallel to the length of myofibers (McNally and Pyte, 2007). Sarcomers are built by thin actin and thick myosin filaments that form cross-bridges between Z bands containing attachment points for actin filaments on the border of each sarcomere (Figure 1.4; Sherwood et al, 2010; Lieber, 2009). During the contraction, ATP is hydrolyzed by the heavy chain of myosin which provides the energy for contraction and generates the muscle force that is transmitted along the fibers in order to produce movement. There are different types of muscle fibers based on different myosin isoforms which can be distinguished by histological stainings of ATPase reaction: type 1 fibers (slow oxidative fibers), type 2A and 2B fibers (fast oxidative fibers) and type 2X fibers (fast glycolytic fibers; Schiaffino and Reggiani, 2011). The fiber type is determined by motor neuron which together with innervated fibers forms a functional motor unit (Greising et al, 2012). Motor

units can be distinguished based on the mechanical properties of muscle fibers as slow and fast-twitch motor units. Different motor units work together in coordination of muscle contraction by delivering action potention in order to excite innervated muscle fibers (Farina and Negro, 2015).

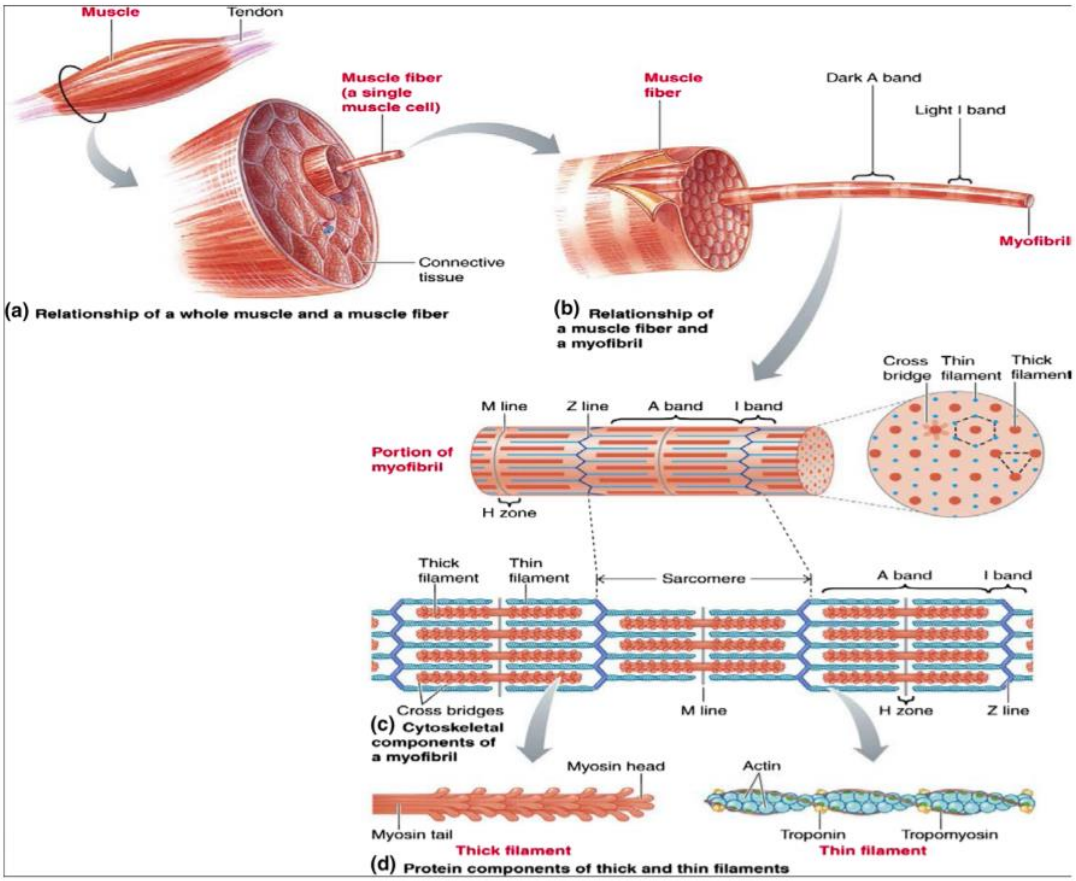


Figure 1.4: Structure of skeletal muscle.

Adopted from Sherwood et al, 2010.

1.3.2 Muscle diseases

Abnormalities in structure and function of skeletal muscle lead to the onset of muscle diseases such as muscular dystrophies, congenital myopathies, myotonic disorders (see details at 1.4), storage diseases, mitochondrial diseases and inflammatory myopathies (McNally and Pyte, 2007).

Muscular dystrophies are a heterogenous group of muscle diseases which are characterized by progressive muscle weakness. Muscle weakness is usually a result of muscle atrophy or muscle hyperthrophy and it is more severe in specific

group of muscles depending of type of dystrophy (Mercuri and Muntoni, 2013). Duchenne muscular dystrophy is X-linked disease caused by mutations in dystrophin gene (DMD) that affects boys in the first years of life. Dystrophin is a structural protein which is a part of the dystrophin-associated glycoprotein complex that plays an important role in stabilization of muscle fibers during muscle contraction by connecting cytoskeleton and extracellular matrix. Mutations in the DMD gene lead to depletion of dystrophin in Duchenne patients while partial depletion of DMD expression leads to milder phenotype in the patients with Becker muscular dystrophy (Bushby et al, 2010). Emery Dreifuss muscular dystrophy is another X-linked muscular dystrophy caused by mutations in Emerin gene (EMD) encoding for nuclear membrane potein which leads to the onset of muscle weakness (Helbling-Leclerc et al, 2002). Limb-girdle muscular dystrophies are caused by mutations in genes encoding sarcoglycans, transmembrane proteins that are part of the dystrophin-associated glycoprotein complex (Wheeler et al, 2002). Dysferlin is another sarcolemmar protein which is often mutated in patients with limb-girdle muscular dystrophy and leads to defective membrane repair (Bansal et al, 2003; Schoewel et al, 2012). Fascioscapulohumeral muscular dystrophy is autosomal dominant muscular dystrophy caused by decreased number of D4Z4 repeats in 4q35 region and toxic gain of function of DUX4 gene which leads to progressive muscle weakness (Lemmers et al, 2010).

Congential myopathies are a group of muscle diseases characterized by hypotonia and muscle weakness with the onset usually from birth. Specific clinical features of congenital myopathies such as the presence of facial weakness, hypotonic posture, weakness of respiratory muscles and normal cognitive functions that make it possible to distinguish congenital myopathies from other similar muscle disorders such as congenital muscular dystrophies and congenital myotonic dystrophies. The early onset of congenital myopathies was associated with mutations in myotubularin (MTM1), dynamin 2 (DNM2), ryanodine receptor 1 (RYR1) and actin (ACTA1) which lead to increased fibrosis and fat infliltration (North et al, 2014).

Inflammatory myopathies are muscle diseases characterized by muscle weakness and inflammation of the muscle. The most common inflammatory myopathies are polymyositis (PM), dermatomyositis (DM), necrotizing autoimmune myositis (NAM) and sporadic inclusion body myositis (sIBM). The pathogenic mechanism of inflammatory myopathies is autoimmune response mediated by T cells in PM and sIBM, complement-mediated microangiopathy in DM and by autoantibodies and macrophages in NAM (Dalakas, 2011).

Muscle diseases and hereditary myopathies are usually pain free but in some disorders, such as polymyositis, myotonic dystrophy type 2 and caveolin 3 (CAV3)-related distal myopathies, patients frequently develop muscle pain (Chawla, 2011; George et al, 2004; Aboumousa et al, 2008).

1.4 Myotonic Dystrophy Type 2 (DM2)

Myotonic dystrophies are autosomal dominant monogenetic diseases and the most prevalent type of muscular dystrophies in adults. Combined prevalence of myotonic dystrophies is estimated at 1 in 8000 (Meola and Cardani, 2015). Two distinct forms of myotonic dystrophy have been identified so far. Myotonic dystrophy type 1 (DM1, Steinert´s Disease) was first described in 1909 by Steinert and colleagues and it is caused by expansion of CTG repeats in the 3´untranslated region of the myotonic dystrophy protein kinase gene (DMPK) (Mahadevan et al, 1992). Myotonic dystrophy type 2 (DM2, proximal myotonic myopathy or PROMM) was described in 1994 and is caused by expansion of CCTG repeats in the intron 1 of the nucleic acid binding protein gene (CNBP, previously known as ZNF9) (Thornton et al, 1994; Liquori et al, 2001). Both mutations induce the aggregation of elongated mutant transcripts and RNA biding proteins inside nuclear foci which leads to missplicing of several genes and multisystemic phenotype (Figure 1.5). Considering that most of DM1 and DM2 clinical symptoms are similar, the biggest difference between two types of myotonic dystrophy is that congenital form is present only in DM1 while muscle pain has an onset in DM2 exclusively (Udd and Krahe, 2012).

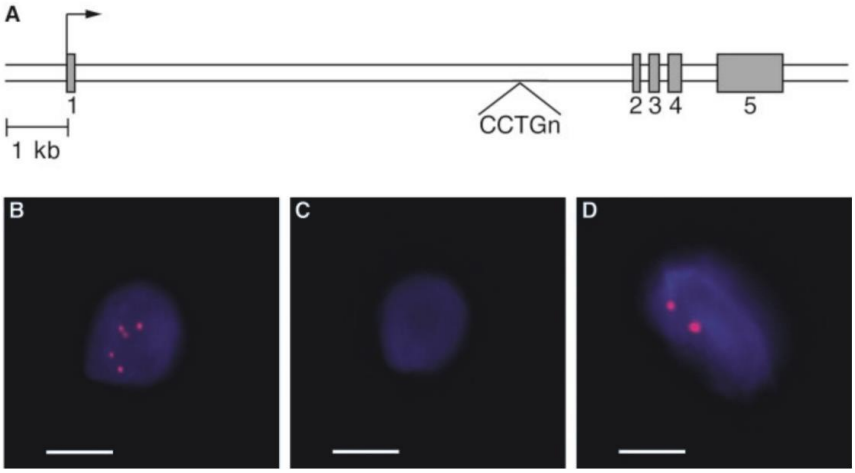


Figure 1.5: RNA in situ hybridization of the repeats expansion in DM2.
A) Structure of CNBP gene with marked position of CCTG repeats expansion in the intron 1 B) In situ hybridization of CAGG probe to DM2 muscle. C) In situ hybridization of CAGG probe to normal muscle. D) In situ hybridization of CAG probe to DM1 muscle. Bar is 5 mM.
Adopted from Liquori et al, 2001.

1.4.1 Clinical aspects of DM2

Myotonic dystrophy type 2 is a multisystemic disease with high variability of clinical phenotypes. The disease onset is between 20 and 70 years of age and usually the first typical symptoms are proximal limb muscle weakness leading to difficulties with climbing stairs or muscle pain (Udd and Krahe, 2012). DM2 patients develop a mild weakness of hip extension, thigh flexion and finger flexion. Myotonia, involuntary muscle contraction and slower relaxation caused by hyperexcitability of muscle (Figure 1.6), is present in almost all DM2 patients but is very mild and hard to detect with electromyography (Young et al, 2010). Cardiac arrhythmias, atrioventricular (AV) conduction defects and cardiomyopathy occur in a small number of patients (Sansone et al, 2013). Some patients develop early-onset, posterior subcapsular cataracts which are surgically removed (Rhodes et al, 2012). CNS abnormalities are present in DM2 patients including white matter lesions observed on structural brain magnetic resonance imaging (MRI), decreased cerebral blood flow in frontal and temporal regions on positron emission tomography (PET) scans and associated behavioral changes with increased number of patients suffering from depression (Meola et al, 1999; Minnerop et al, 2011). Cognitive functions and

social skills are usually unaffected (Weber et al, 2010). More than 50% of DM2 patients develop chronic muscle pain which is resistant to conventional treatments and affects the quality of life and professional performance (George et al, 2004). Sleeping disturbances are also reported including excessive day time sleepiness (EDS), restless leg syndrome (RLS), rapid eye movement (REM) sleep abnormalities and sleep apnea that were associated with the onset of muscle pain (Lam et al, 2013; Romigi et al, 2014). Other less frequent symptoms include hypogonadism, excessive sweating, dysphagia, insulin resistence and hyperlipidemia (Savkur et al, 2001; Day et al, 2003; Heatwole et al, 2011). The increased risk of developing cancer was also reported in DM2 (Win et al, 2012).

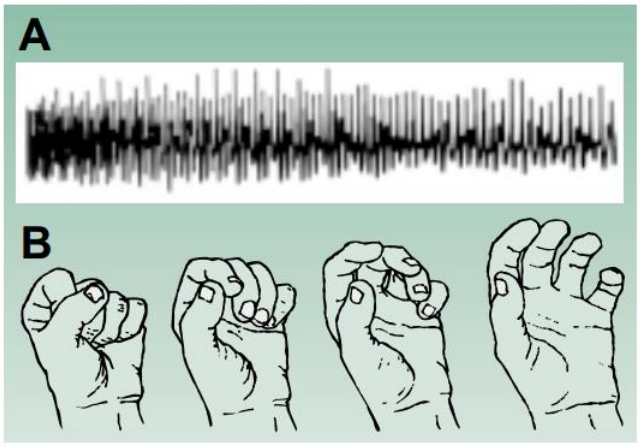


Figure 1.6: Myotonia in DM2.

A) Electromyogram (EMG) showing myotonia, prolonged relaxation of the muscle after contraction; B) Grip myotonia as a difficulty to open the hand after making a fist.

Adopted from O'Sullivan Smith et al, 2000.

Histopathology of DM2 muscle biopsy specimens (Figure 1.7) reveals the atrophy of type 2 muscle fibers and increased number of fibers with centralized nuclei (Pisani et al, 2008). Routine blood tests in DM2 usually reveal elevated serum levels of creatine kinase which is associated with muscle weakness and atrophy, elevated liver enzymes (transaminases, lactate dehydrogenase, γ-glutamyltransferase and aspartate aminotransferase) and increased total cholesterol levels (Heatwole et al, 2011).

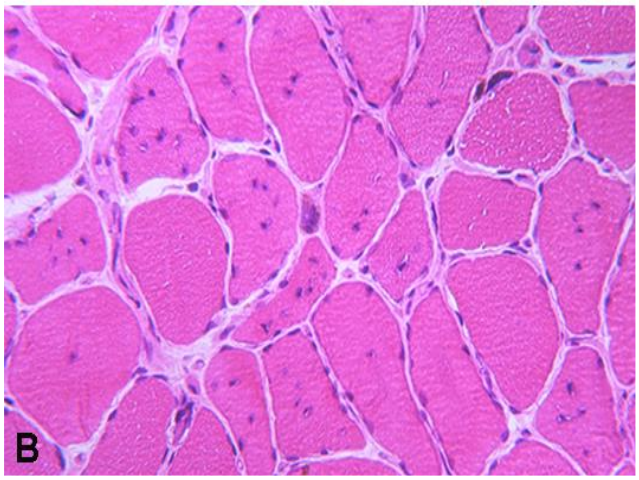


Figure 1.7: Histopathology of DM2 muscle section.
Typical increased number of central nuclei and atrophy of type 2 muscle fibers can be observed on Hematoxylin and Eosin staining.
Staining from AG Spuler.

Genetic diagnosis of DM2 is based on PCR amplification of CCTG repeats in intron 1 of CNBP gene followed by Southern Blot which can distinguish normal from the expanded CCTG repeats (Kamsteeg et al, 2012). Healthy CNBP alleles contain less than 30 copies of the CCTG repeats while mutant alleles contain large expansions between 55 and 11000 repeats (Liquori et al, 2001). While DM1 causing mutation is increasing in size over generations, CCTG expansion in DM2 can lead both to expansion and contraction of repeats size in the successive generations and this lack of progression can explain the absence of congenital form and generally milder phenotype in DM2 compared to DM1 (Table 1.1; Udd and Krahe, 2012). The exact prevalence of DM2 is not yet estimated but it is considered to be similar to DM1. DM2 mutations are predominantly identified in the northern European populations with the prevalence of 1:8000 in Finland (Suominen et al, 2011). Due to mild symptoms, late onset DM2 remains underdiagnosed which makes it harder to estimate the exact prevalence of the disease (Meola and Cardani, 2015).

	Adult-onset myotonic dystrophy type 1	Myotonic dystrophy type 2
Genetics		
Inheritance	Autosomal dominant	Autosomal dominant
Anticipation	Pronounced	Exceptionally rare
Congenital form	Yes	No
Chromosome	19q13.3	3q21.3
Locus	*DMPK*	*CNBP*
Expansion mutation	$(CTG)_n$	$(CCTG)_n$
Location of the expansion	3′ untranslated region	Intron 1
Core features		
Clinical myotonia	Typical in adult onset	Present in less than 50%
Myotonia on electromyography	Generally present	Absent and variable in many patients; needs detailed investigation
Muscle weakness	Disability often by age 30–50 years	Disability at age 60–85 years
Cataracts	Generally present	Present in a few patients at diagnosis
Localisation of muscle weakness		
Face or jaw	Generally present	Usually absent
Ptosis	Often present	Rare, mild, or moderate
Bulbar (dysphagia)	Generally present later in life	Not present
Respiratory muscles	Generally present later in life	Exceptionally rare cases
Distal limb muscle	Generally prominent	Flexor digitorum profundus in some patients
Proximal limb muscle	Can be absent for many years	Main disability in most patients, late onset
Sternocleidomastoid muscle	Generally prominent	Prominent in few patients
Muscular symptoms		
Myalgic pain	Absent or moderate	Most disabling symptom in many patients
Muscle strength variations	Occasional	Can be considerable
Visible muscle atrophy	Face, temporal, distal hands, and legs	Usually absent
Calf hypertrophy	Absent	Present in at least 50%
Laboratory findings		
Concentration of creatine kinase in serum	Normal-to-moderate increase	Normal-to-moderate increase
Muscle biopsy findings		
Fibre atrophy	Smallness of type 1 fibres	Highly atrophic type 2 fibres
Nuclear clump fibres	In late stage only	Scattered early before weakness
Sarcoplasmic masses	Very frequent in distal muscles	Very rare
Ring fibres	Frequent	May occur
Internal nuclei	Massive in distal muscle	Variable and mainly in type 2 fibres
Cardiac symptoms		
Conduction defects	Common	Highly variable, absent to severe
Other neurological symptoms		
Tremors	Absent	Prominent in many patients
Behavioural changes	Common	Not apparent
Hypersomnia	Prominent	Infrequent
Other features		
Manifest diabetes	Occasional	Infrequent
Frontal balding in men	Generally present	Exceptional
Incapacity (work and activities of daily living)	Typically after age 30–35 years	Rarely younger than 60 years, unless severe pain
Life expectancy	Reduced	Normal

Table 1.1: Comparison of clinical symptoms in myotonic dystrophies.
Adopted from Udd and Krahe, 2012.

1.4.2 Molecular pathogenesis of DM2

Molecular pathogenesis in DM2 is a combination of many pathogenic mechanisms (Figure 1.8). The main pathogenic feature of DM2 is a toxic RNA gain of function mechanism in which expanded CCUG *CNBP* transcripts accumulate inside of ribonuclear inclusions known as nuclear foci. Mutant RNAs sequester RNA binding proteins such as muscleblind-like proteins (MBNLs) which are involved in the regulation of alternative splicing. Sequestration of RNA

binding proteins inside of nuclear foci leads to missplicing of several downstream genes (Ferdaei et al, 2002; Meola and Cardani, 2015). MBNLs are a protein family of tissue-specific RNA metabolism regulators with three distinct isoforms MBNL1, MBNL2 and MBNL3. All three isoforms contain four zinc-finger (ZnF) domains that recognize a consensus sequence in pre-mRNA and mRNA targets. MBNL1 and MBNL2 show ubiquitous expression with MBNL2 predominantly expressed in brain while MBNL3 expression is restricted to skeletal muscle (Konieczny et al, 2014). MBNL proteins are involved in the regulation of alternative pre-mRNA splicing, alternative polyadenylation, circular RNA biogenesis, pre-microRNA processing and RNA localization (Ho et al, 2004; Rau et al, 2011; Batra et al, 2014; Ashwal-Fluss et al, 2014). In DM2 brains there is a direct binding of brain dominant isoform MBNL2 to CCUG expanded transcripts exactly in intron 1 of *CNBP* containing expanded repeats which leads to depletion of MBNL2 from normal targets, RNA splicing defects and change from adult to embryonic pattern of splicing (Goodwin et al, 2015). MBNL loss of function is further supported by animal models since *MBNL1* and *MBNL2* knockout mice show characteristic DM2 phenotypes. *MBNL1* knockout mice develop cataracts, muscle weakness, myotonia and splicing abnormalities while loss of *MBNL2* function leads to splicing abnormalities in brain with normal splicing pattern in the skeletal muscle (Kanadia et al, 2003; Suenaga et al, 2012). Deletion of *MBNL3* exon 2 in mice leads to onset of insulin resistance, cardiac defects, cataracts and retention of embryonic splicing forms (Choi et al, 2016).

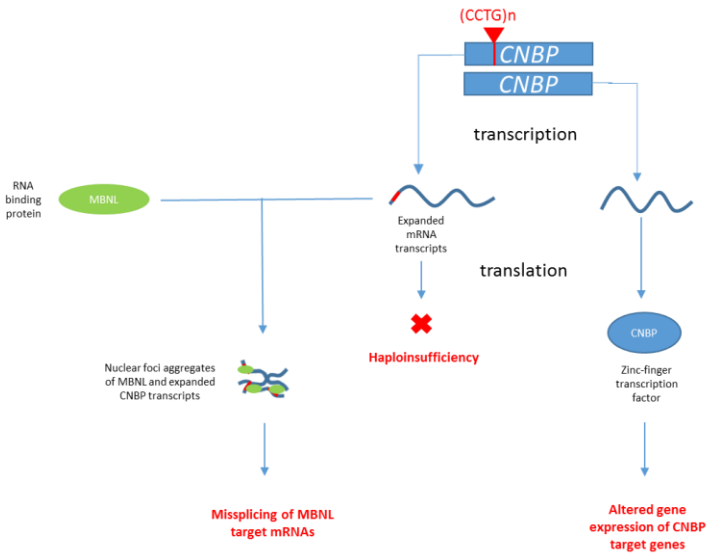


Figure 1.8: Model of DM2 pathogenic mechanisms.

So far, splicing abnormalities have been identified in 218 genes in DM2 (Perfetti et al, 2014). Missplicing of the chloride voltage-gated ion channel 1 (CLCN1) is associated with the onset of myotonia while splicing abnormalities in the amphiphysin (BIN1) are associated to changes in T tubule networks regulating muscle contractions (Wheeler et al, 2007; Fugier et al, 2011). CaV1.1 calcium channel (CACNA1S) which is involved in Ca^{2+} signaling, neurotransmitter synthesis and release is also misspliced in DM2 which is associated with the onset of muscle weakness (Tang et al, 2012). Splicing abnormalities are present in DM2 sarcolemmal proteins such as PDZ domain and a LIM domain 3 (PDLIM3) (Figure 1.9; Perfetti et al, 2014). Typical DM2 symptoms of insulin resistance and cardiac defects could be due to missplicing of the insulin receptor (IR) and the cardiac troponin T (TNN3), respectively (Savkur, 2001; Philips et al, 1998). Regarding pain regulation, splicing abnormalities were detected in neural precursor cell expressed developmentally down-regulated protein 4 (NEDD4) which is an E3 ubiquitin ligase associated with simvastatin-induced muscle pain (Screen et al, 2014).

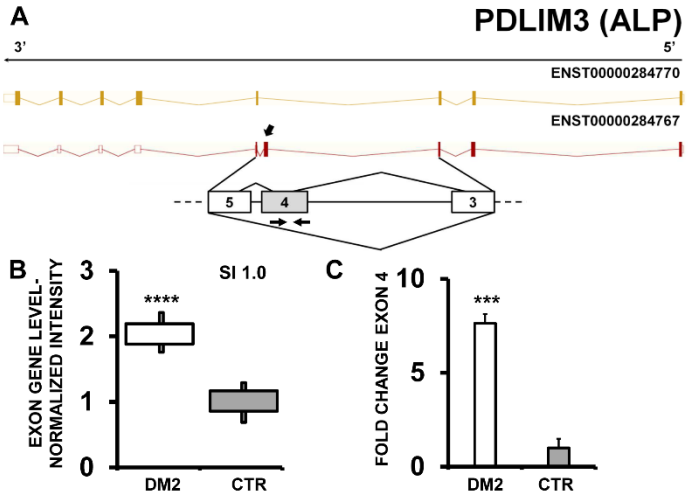


Figure 1.9: Splicing abnormalities in DM2.

Increased inclusion of PDLIM3 exon 4 in DM2 patients.

A) Increased inclusion of exon 4 (marked in gray) of PDLIM3 mRNA transcript in DM2 patients. B) The box plot shows the increased expression of Affymetrix probe recognizing exon 4 in DM2 patients compared to controls (CTR, n=10, **** p<0.0001). Values are normalized for the levels of the whole transcript. The splice index (SI) is indicated. C) Validation of Affymetrix data with qPCR assays (DM2=19, CTR=15; *** p<0.001).

Adopted and modified from Perfetti et al, 2014.

CNBP encodes for CCHC-type zinc finger transcription factor involved in the regulation of proliferation and cell death (Calcaterra et al, 2010). Considering that DM2 patients carry only one wild type allele of *CNBP*, haploinsufficiency could be another pathogenic mechanism in DM2. Indeed, *CBNP*$^{+/-}$ mice lacking one *CNBP* allele develop typical DM2 phenotype with the onset of muscle wasting, cardiac defects and cataracts (Chen et al, 2007). *CNBP* levels are reduced in cytoplasm from muscle satellite cells and also in biopsy specimens obtained from DM2 patients which is associated with reduced level of ribosomal proteins such as poly(A)-binding protein 1 (PABP1), eukaryotic translation elongation factor 1 alpha 1 (eEF1A) and eukaryotic translation elongation factor 2 (eEF2) (Pelletier et al, 2009; Huichalaf et al, 2009). This global reduction in protein synthesis may contribute to the onset of DM2 (Huichalaf et al, 2009). The transcriptional regulation of other genes through *CNBP* regulatory action and cis-alteration of *CNBP* function by neighboring genes could play a role in DM2 pathogenesis (Mateos-Aierdi et al, 2015).

Some muscle specific microRNAs are dysregulated in DM2 contributing to DM2 pathogenesis. Downregulation of MiR-1 in cardiac DM2 muscle is associated with cardiac defects due to dysregulation of gap junction protein alpha 1 (GJA1) and calcium channel protein CACNA1C (Rau et al, 2011). miRNAs were dysregulated in the peripheral blood plasma from DM1 patients where the level of miR-133a showed the correlation with muscle strength measurements (Perfetti et al, 2014). Abnormal protein degradation by ubiquitin proteasome system (UPS) is also part of DM2 pathogenesis. Protein degradation is altered in DM2 myotubes with the reduced levels of ubiquitinated proteins (Rusconi et al., 2010). Dysregulation of ubiquitin ligase NEDD4 is a further evidence of UPS involvement in DM2 (Screen et al, 2014). Finally, mitochondrial dysfunction could lead to DM2 onset since the elongation factors Tu and Ts that participate in the mitochondrial translational machinery are reduced in DM2 myotubes and reduced levels of these proteins were previously associated with muscle hypotonia and motoric abnormalities (Valente et al, 2007; Rusconi et al, 2010)

1.5 Muscle pain in DM2

Muscle pain (or myalgia) is a common clinical symptom and leading cause of working disability; however, the mechanism of pain is unknown (Weigl et al, 2007). In comparison to cutaneous pain, muscle pain is poorly localized which might be due to the lower innervation of the muscle tissue. Muscles are innervated both by nociceptive and proprioceptive neurons. Muscle nociceptors are mostly group III and group IV afferents corresponding to A-delta and C fibers and contain neuropeptides SP and CGRP (Figure 1.10; Mense and Gerwin, 2010). Typical muscle proprioceptors are muscle spindle and tendon which sense the changes in muscle length and tension, respectively (Graven-Nielsen and Mense, 2001). Nociceptive neurons that innervate muscle respond to mechanical and chemical noxious stimuli. The mechanical forces during exercise can contribute to sensitization of muscle nociceptors (Adreani et al, 1997; Hoheisel et al, 2004). Chemical noxious stimuli are produced by protons, lactate and ATP released during muscle contraction which are detected by ASIC, P2X, and TRPV1 receptors on muscle nociceptors (Light et al, 2008). Upregulation of inflammatory cytokines is proposed to contribute to muscle pain in the injured muscle (Warren et al, 2002). After muscle injury in rats, there is an increase of inflammatory cytokines (NGF, TNF-α, IL-6) and upregulation of neuropeptide CGRP in DRG neurons innervating the injured muscle which is persistent up to 2 weeks and might contribute to the onset of chronic muscle pain (Sakuma et al, 2016).

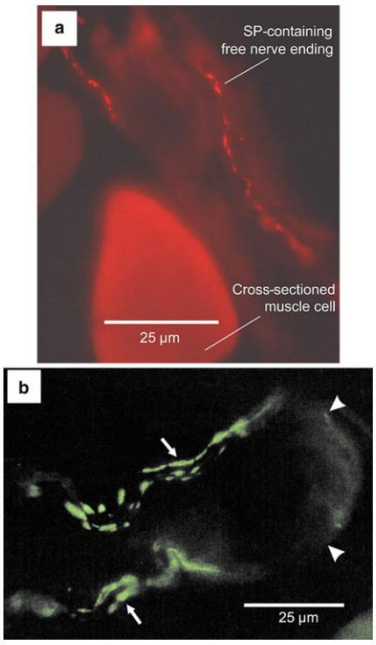


Figure 1.10: Histology of free nerve endings in the muscle.
Histological sections of the rat gastrocnemius muscle show free nerve endings that contain: A) the neuropeptide substance P (SP) and B) calcitonin gene-related peptide (CGRP). The fibers were visualized with antibodies for SP and CGRP coupled to a fluorescent marker.
Adopted and modified from Mense and Gerwin, 2010.

DM2 patients frequently develop widespread chronic muscle pain which differs in duration, location and intensity, impairs the quality of life and is usually resistant to conventional analgesics (George et al, 2004). The prevalence of muscle pain in DM2 is estimated at 76% in Finland (Suokas et al, 2012). Many DM2 patients with muscle pain are misdiagnosed as patients with fibromyalgia (Auvinen et al, 2008). Similar to fibromyalgia, muscle pain in DM2 is widespread and increased by heavy exercise (George et al, 2004; Suokas et al, 2012).

1.6 Systems biology approaches to study chronic pain

In the recent years, development of high-throughput Omics techniques such as transcriptomics, proteomics and lipidomics helped to make the major breakthroughs in systems biology which is a field of biology trying to explain complex biological systems by integration of experimental and computational biology (Kitano, 2002; Tyers and Mann, 2003). Omics technologies are now being used to identify pain causing molecules (RNA, proteins and metabolites)

in the cells and tissues derived from the patients with chronic pain and from pain animal models (Antunes-Martins et al, 2013).

1.6.1 Transcriptomics

Transcriptome is a complete set of RNA transcripts including mRNAs, non-coding RNAs and small RNAs that are produced by genome. There are two main groups of methods to study the transcriptome: hybridization based methods such as microarrays and sequence-based methods such as RNA sequencing (Wang et al, 2009).

Microarrays analyze the expression profile of RNA templates isolated from the tissues or cells where RNA is reverse-transcribed to cDNA followed by fluorescent labelling and hybridization of labeled samples with complementary oligonucleotide probes spanning the target sequences. A laser excites the labeled fluorescent dyes and the hybridization intensities provide the estimate of transcript abundance (Trevino et al, 2007). In RNA sequencing, a high throughput next generation sequencing technology is used to analyze expression profiles of RNA templates that are converted to library of cDNA fragments with adapters attached to one or both ends of each fragment (Figure 1.11). Each fragment is sequenced to obtain short sequences called reads (30-400bp) from one end (single-end sequencing) or both ends (pair-end sequencing) followed by the alignment of reads to the reference genome to measure the expression level of each transcript and gene (Wang et al, 2009).

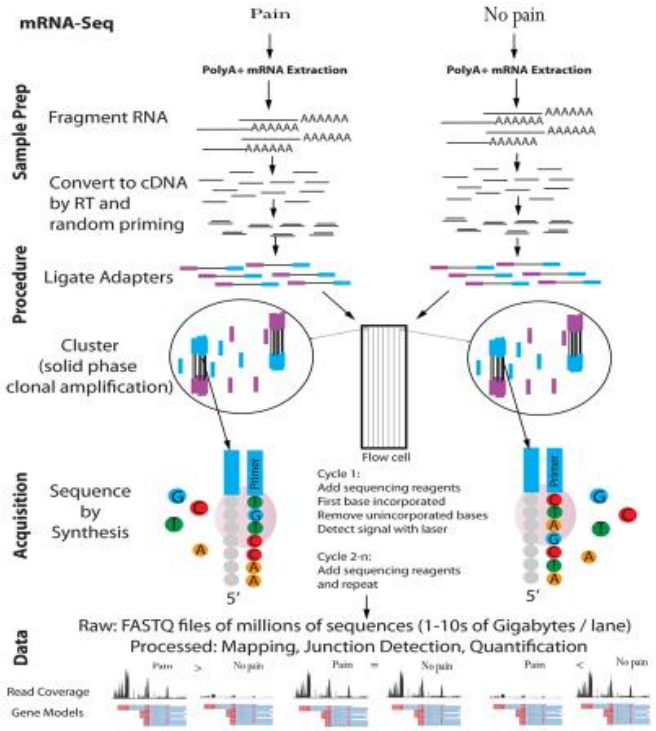


Figure 1.11: Workflow for RNA sequencing.

Adopted and modified from Malone and Oliver, 2011.

RNA sequencing has many advantages over microarrays which has a problem of cross-hybridization decreasing the accuracy of expression estimate, different hybridization properties between the probes and being limited to only studying the genes for which the probes were designed. Unlike microarrays, RNA sequencing has a direct access to the sequence, does not depend on the prior knowledge of gene structure to design the probes, avoids biases from the probe hybridization, it has higher sensitivity and detects more differentially expressed transcripts so it is a preferred method for detection of novel transcripts, SNP discovery and analysis of splicing alterations (Wang et al, 2009; Malone and Oliver, 2011; Zhao et al, 2014). Comparative study of RNA sequencing and microarrays performed in rat model of neuropathic pain concluded that RNA sequencing identified larger number of pain candidate genes and detected a wider range of expression values compared to microarrays (Perkins et al, 2014).

So far, microarrays were used to analyze the gene expression profiles of DRG and spinal cord in rat models of neuropathic pain (Xiao et al, 2002; Lacroix-Fralish et al, 2006), to identify regulated genes in rat DRG and spinal cord after peripheral nerve injury (Costigan et al, 2002; Yang et al, 2004), for studying the

contribution of inflammatory regulators such as complements and cytokines to chronic pain (Griffin et al, 2007; Strong et al, 2012) and to identify the changes of microRNA in mouse prefrontal cortex after inflammatory pain (Poh et al, 2011). In addition, RNA sequencing was performed to analyse the persistent transcriptional alternations in rat model of chronic neuropathic pain after spinal nerve ligation (Hammer et al, 2010), to detect differences in gene expression between DRG and trigeminal sensory ganglia of adult mice (Manteniotis et al, 2013), to analyze the transcriptome of mouse TRPV1-lineage neurons and to compare it with non-TRPV1 neurons (Goswami et al, 2014), for detecting cell specific transcriptional alternations in subtypes of DRG neurons after peripheral nerve injury (Hu et al, 2016) and to analyze differences in gene expression between patients with and without muscle pain (Moshourab, Palada et al, 2016). Typical samples used for transcriptional profiling of pain include the tissue from the animal models obtained from peripheral organs, peripheral nerves, DRG neurons, spinal cord and CNS and the tissue from patients with chronic pain which is usually limited to biopsy specimens from the peripheral organs and blood samples (Antunes-Martins et al, 2013).

1.6.2 Proteomics

Proteome is a complete set of proteins that are synthetized in the organism. There are two main groups of proteomic techniques developed to analyze the proteomes and to provide the insight to gene expression on the protein level: classical gel based methods such as two-dimensional (2D) electrophoresis and novel gel-free methods also known as shotgun proteomics (Altelaar et al, 2013). 2D electrophoresis separates proteins based on the molecular mass and charge which generates protein spots that can be detected as intact proteins or peptides with mass spectrometry (MS). Since 2D electrophoresis is a time-consuming method with poor detection of low abundant proteins, hydrophobic proteins and proteins that are bigger or lower than the gel pore size or pH range, shotgun proteomics was developed to overcome these limitations of gel based approach (Chevalier, 2010). In shotgun proteomics, proteins are isolated from the sample, digested to peptides which get separated by reversed phase high-performance liquid chromatography (HPLC) and analyzed with tandem mass

spectrometry (MS/MS). Tandem MS/MS starts with ionization of peptides by electrospray ionization (ESI) or matrix assisted laser desorption/ionization (MALDI). Ionization is followed by separation of precursor ions in the vacuum of the mass spectrometer which are guided to the mass analyzer to determine mass/charge (m/Z) ratio. The most abundant precursor ions are isolated, fragmentized to daughter ions by collision with the inert gas molecules and recorded as MS/MS spectra (Zhang et al, 2013). This makes it possible to generate the information about amino acid sequence of peptides by assigning the sequence to MS spectrum and correlating them to the spectra libraries. False discovery rate (FDR) can help to distinguish between true and false positive matches that can happen in the case of incorrect assignment of MS spectrum to peptide sequence (Elias and Gygi, 2007). Since it is necessary to compare the abundance of proteins between different samples, two different quantification approaches were developed: label-based and label-free approaches (Bantscheff et al, 2007). Label-based quantification is achieved by labelling peptides from different samples with stable isotopes which causes the mass shift to distinguish the peptides. Labeling can be achieved by metabolic tags such as Stable isotope labeling by amino acids in cell culture (SILAC) where heavy and light isotope amino acids are incorporated to peptides or by chemical tags such as isotope-coded affinity tags (ICAT) and tandem mass tags (iTRAQ or TMT) where tags are covalently bound to peptides (Schwanhäusser et al, 2009; Gygi et al, 1999; Thompson et al, 2003). Another approach is label-free quantification (LFQ) based on the direct measurement of the area under the curve from chromatographic mass peaks which linearly correlates with the protein abundance (Figure 1.12; Megger et al, 2013). Since in LFQ approach the samples are run separately, differences in preparation of the samples between the MS runs can infuence the results. On the other hand, label-based quantification is more expensive, can introduce artefacts and has lower coverage and dynamic range than LFQ (Li et al, 2012). Selected reaction monitoring (SRM) enables detection of low abundance proteins that are not detectable with classic shotgun approaches. SRM starts with spiking of isotopically labelled internal standards to the samples followed by triple quadrupole (QQQ) MS with two levels of mass selection. The intensities of the analyte from sample are compared to the intensities of the internal standard

which allows the measurement of protein abundance with increased sensitivity and higher dynamic range (Lange et al, 2008).

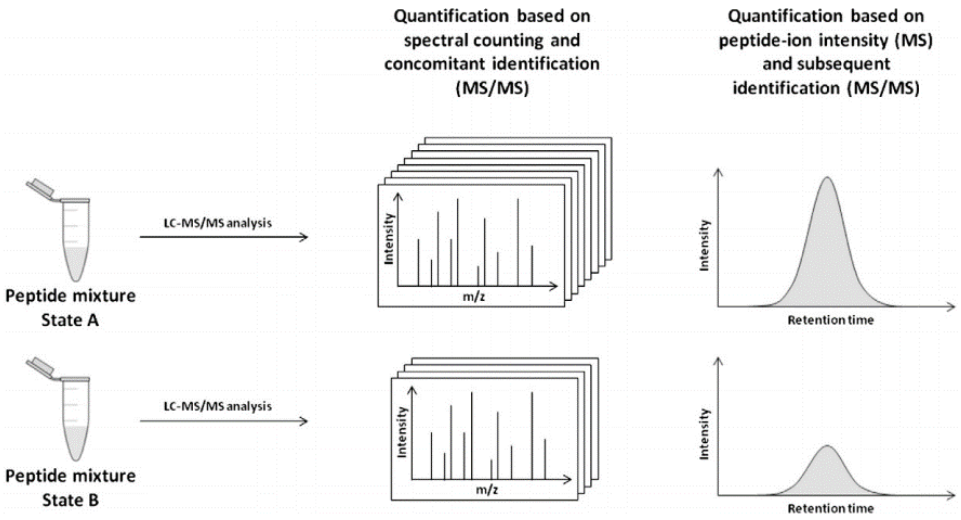


Figure 1.12: Label-free quantification of MS/MS proteome data.
Adopted from Megger et al, 2013.

One of the limitations in applying proteomic techniques to pain research is the low abundance and difficult detection of transmembrane proteins such as ion channels on nociceptive terminals while the advantage is a possibility to study posttranslational modifications which are crucial in regulating the signaling pathways associated with nociception (Antunes-Martins et al, 2013). So far, shotgun proteomics was used to analyze the regulated proteins in rat models of neuropathic pain after peripheral nerve injury (Komori et al, 2007; Melemedjian et al, 2013), for urine screen to detect the regulated proteins in female patients with chronic pain (Goo et al, 2010) and to screen for proteome changes in plasma of patients with chronic lower back pain (Ghafouri et al, 2016). Furthermore, SRM was performed in patients with chronic abdominal pain to detect differences in urinary proteome (Goo et al, 2012).

1.6.3 Lipidomics

Lipidomic techniques combine lipid extraction from the biological sample followed by separation at liquid chromatography (LC) with mass spectrometry (LC-MS) or nuclear magnetic resonance spectroscopy (NMR) to compare the properties of lipids in the sample to internal standards (Li et al, 2014). The

advantage of lipidomic methods is that they require small amounts (1-100 mg of tissue or 10-100 µL of plasma/serum per analysis) of the sample. The most commonly used lipid extraction methods are addition of chloroform or dichloromethane mixture with methanol, addition of methanol and methyl tert-butyl ether to the sample for phase separation (Cajka and Fiehn, 2014). Reverse phase liquid chromatography (RP-LC) is a method of choice for separation of lipids which is based on the hydrophobic interactions between the alkyl chains located on the stationary phase and lipid fatty acids to separate different lipids of the same class or subclass (Han and Gross, 2005). Separated lipids are ionized by ESI which results in positively charged ions or with Atmospheric-pressure chemical ionization (APCI) used for non-polar lipids (e.g. triacylglycerols). LC/MS analysis of ionized lipid species is usually performed on time of flight (TOF) based mass spectrometers and provides the information about retention time, m/z value, and signal intensity of lipid of interest which is compared to intensities of internal standards to estimate the lipid concentrations in the sample (Figure 1.13; Cajka and Fiehn, 2014).

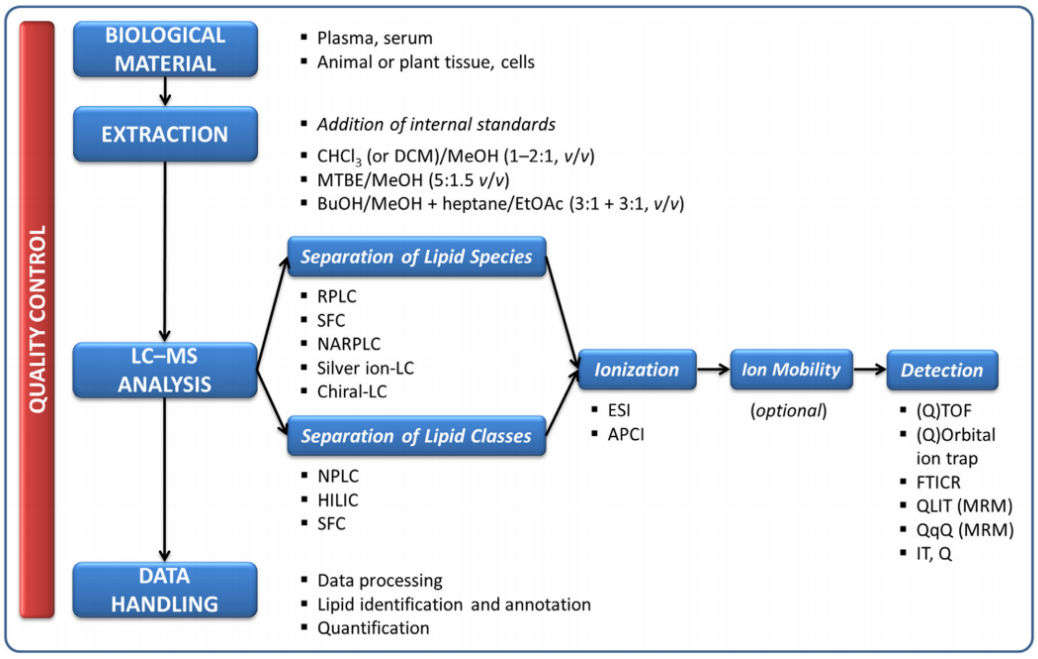


Figure 1.13: Workflow of LC-MS based lipidomics.

Adopted from Cajka and Fiehn, 2014.

Application of LC-MS based lipidomics in pain research has a big potential since lipids are known mediators of peripheral nociception and contribute to the onset

of inflammatory pain (Piomelli and Sasso, 2014). Endogenous lipids 9- and 13-hydroxyoctadecadienoic acid (HODE) are ligands of peripheral TRPV1 receptors and lead to increased activation of TRPV1 which contributes to inflammatory pain (Alsalem et al, 2013). Resolvins D and E show anti-inflammatory effect and contribute to resolution of inflammation and reduced nociception (Ji et al, 2011). Prostaglandins E2 and I2 lead to increased peripheral nociception and hypersensitivity by enhancing the secretion of SP and CGRP at the site of inflammation or tissue injury (Park and Vasko, 2005). Interestingly, analysis of lipidome is easier to perform in patients with chronic pain compared to proteomic and transcriptomic profiling since lipids are commonly present in the circulation which makes it possible to analyze lipid profiles by screening blood, plasma and cerebrospinal fluid samples (Antunes-Martins et al, 2013). To date, LC-MS lipidomics was performed in the dorsal horn of spinal cord, DRG and plasma of rat model for neuropathic pain after peripheral nerve injury which revealed increased sphingolipid levels in the dorsal horn (Patti et al, 2012).

2. Aims of doctoral project

We decided to study the mechanisms of muscle pain in a well-defined cohort of DM2 patients since DM2 is a monogenetic disease and at least 50% of the patients develop the pain. In the first part of the project, we aimed to compare the somatosensory profiles between DM2 with and without pain in order to screen for changes in the nociception and to reveal the pathophysiological mechanisms behind the muscle pain in DM2. In the second part of the project, we wanted to compare DM2 patients with and without pain on the transcriptomic, proteomic and lipidomic level to identify novel genes and myokines that contribute to the onset of muscle pain.

3. Materials and methods

(3.2.-3.5. adopted and modified from Moshourab*, Palada* et al, 2016)

3.1 Materials

3.1.1 List of chemicals and consumables

Name	Company
Ascorbate	Rotex Medica, Germany
Dithiothreitol	Sigma-Aldrich, Germany
Ethanol	Carl Roth, Germany
Iodacetamide	Sigma-Aldrich, Germany
Kapa Sybr Fast qPCR Master Mix	Kapa Bioystems, USA
Lipid Maps MS Standard	Cayman Chemical, USA
Microdialysis probe (100 kDa cutoff)	M Dialysis, Sweden
QuantiTect reverse transcription kit	Qiagen, Germany
Ringer solution	Serumwerk Bernburg AG, Germany
RNeasy Mini Kit	Qiagen, Germany
SpikeTides	JPT Peptide Technologies GmbH, Germany
Trifluoroacetic acid	Merck, Germany
TruSeq Stranded mRNA library preparation kit	Illumina, USA
Trypsin	Promega, USA

3.1.2 List of instruments

Instrument	Company
Agilent 2100 bioanalyzer	Agilent Technologies, USA
CMA/102 microdialysis pump	CMA, Sweden
Eksigent HPLC system	Eksigent, USA
FPIX algometer	Wagner Instruments, USA
Fusion mass spectrometer	Thermo Scientific, USA
HiSeq 2000 sequencing system	Illumina, USA
NanoDrop ND-1000 spectrophotometer	Thermo Scientific, USA
Pinprick stimulators	MRC Systems GmbH, Germany
Quantiva mass spectrometer	Thermo Scientific, USA
Stratagene Mx3000P qPCR cycler	Agilent Technologies, USA
TSA-2001-II NeuroSensory Analyzer	Medoc, Israel
VF3 Optihair set of von frey filaments	Marstock, Germany

3.2 Study design and participants

In our cross-sectional cohort study, we studied a cohort of 42 DM2 patients and 20 age and gender-matched healthy controls. All DM2 patients were recruited from Muscle Disorders Outpatient Clinic at Charité Campus Buch, Berlin, Germany. The local ethical committee (EA1-127-14) approved the study. All patients and healthy subjects signed the written informed consent forms. We also collected signed consent forms from 12 DM2 patients to analyze their stored muscle biopsies specimens with RNA sequencing and from 14 DM2 patients that underwent the microdialysis. Inclusion criteria were age >18 years and molecularly confirmed diagnosis of DM2. Exclusion criteria were additional neurological disorders that could affect sensory function (e.g. stroke) or treatment with opioid analgesics. Healthy volunteers were excluded if they had diabetes, hypertension, neurological disorders affecting sensory function, took analgesics or had muscle pain in the last 3 months. Biopsies were taken between 2003 and 2015 while microdialysis was performed from 2014 till 2016.

3.3 Clinical assessment of DM2 patients

Patients were asked whether: (1) they experience unusual, unpleasant or painful sensations in their muscles; (2) have muscle weakness; and (3) suffer from myotonia. Patients then rated the subjective impairment on daily life activities for each symptom on a visual analogue scale (0-10). Each patient who reported muscle pain gave a clinical rating of the present pain at the beginning of the experiment according to the numeric analogue score (NAS). Patients indicated on a drawing where their pain was localized. Information on the temporal aspects of pain, namely frequency, duration, and modulating factors such as temperature and movement were also collected. Patients were asked to complete the German version of the McGill Pain Questionnaire (MPQ) at the end of sensory testing. Pain rating index (MPQ-PRI); number of words chosen (MPQ-NWC); and present pain index (MPQ-PPI) were calculated. Patients were asked about past medical history: presence of comorbidities such as diabetes, hypertension, arrhythmias, cataracts, allergies, recent laboratory values (CK), current pain medication (statins and analgesics), smoking status, BM, education

and work status. Genetic testing and confirmation of expansion repeat mutation in *CNBP* was performed in the Institute for Medical Genetics, University of Würzburg. Myotonia and muscle weakness (MRC grade, Medical Research council scale) were confirmed in medical charts.

Patients with DM2 were allocated to either the muscle pain or no pain group with the following criteria:

A) Quantitative sensory testing (QST): based on positive history of muscle pain and pain rating (>0) on the testing day
B) Transcriptomic analysis of DM2 muscle biopsy specimens: based on positive history of muscle pain (documented in the medical records)
C) Proteomic and lipidomic analysis of DM2 microdialysis samples: based on Borg scale pain ratings during microdialysis protocol

3.4 Sensory testing protocol

We used the comprehensive, multimodal, quantitative sensory testing (QST) protocol to generate somatosensory profiles for each patient and healthy subjects. We assessed pain thresholds for skin and muscle tissue. Skin thermal and mechanical testing was performed in a unilateral fashion (dominant hand side, two patients were left handed) over the hand dorsum, shoulder and thigh. We excluded vibration detection threshold over these sites. Thresholds for pressure pain were obtained over 8 muscles on the left and right side of the body: extensor digitorum communis, deltoid, quadriceps, and anterior tibialis. The repertoire of pain tests included pressure pain threshold (PPT), mechanical pain threshold (MPT), mechanical pain sensitivity (MPS), dynamic mechanical allodynia and wind-up ratio (WUR). Thermal detection tasks included warm detection threshold (WDT), cold detection threshold (CDT), heat pain threshold (HPT) and the paradoxical heat detection (PHS). Pain testing procedures, instruments and methods were used strictly according to those prescribed by the German research network on neuropathic pain (Rolke et al., 2006).

3.4.1 Pressure pain threshold

The pressure pain threshold (PPT) was measured using a calibrated digital algometer (FPIX, Wagner instruments, USA) that can exert a pressure of up to 2000 kPa. The flat circular rubber tip of the algometer had an area of 1 cm^2. For each tested site 3 stimulations were applied with a slowly increasing ramp of 50kPa/s. Pressure application was stopped when the patient or subject reported pressure pain and the stimulus intensity was noted. The measurements from the left and right side of each muscle group were averaged.

3.4.2 Thermal detection and pain thresholds

A thermode with a contact surface of 9 cm^2 (3x3 cm) of the TSA-2001-II device (Medoc Inc., Israel) was used to deliver heat stimuli. The baseline temperature was 32 °C (0 °C and 50 °C cut-offs) and temperature ramps were delivered with a rate of 1 °C/s. Cold detection threshold (CDT) and warm detection threshold (WDT), were measured first, followed by cold pain threshold (CPT) and heat pain threshold (HPT). The mean of 3 consecutive measurements was calculated as threshold. The thermal sensory limen test – alternating warming and cooling ramps - was utilized to detect paradoxical heat sensation (PHS).

3.4.3 Mechanical detection threshold

Mechanical detection threshold (MDT) was assessed using a set of 23 standardized von frey filaments with rounded tips of 0.5 mm diameter (VF3 Optihair Set, Marstock, Germany) that exerted forces between 0.25 and 512 mN. The one up - one down rule adaptive rule was used which converges to a threshold with a 50% probability of correct detection. The initial stimulus had an intensity of 8 mN. The test was terminated after completion of 10 runs (10 reversals). The final threshold was the geometric mean of stimulus intensities at which a reversal in step direction took place.

3.4.4 Mechanical pain threshold

Pinprick stimulators (MRC systems Gmbh, Germany) with tip diameter 0.25 mm and stimulus intensities (8, 16, 32, 64, 128, 256, and 512) were used to determine the mechanical pain threshold (MPT). The stimulators were applied perpendicularly on the skin for 1 second in an ascending manner and the patient was asked to judge whether the stimulus was perceived as blunt or sharp. The same adaptive method was utilized as for MDT. The mechanical pain threshold for pinprick was the geometric mean of stimulus intensities at which a reversal in step direction took place.

3.4.5 Stimulus/Response function: mechanical pain sensitivity and dynamic mechanical allodynia

Using the same pinprick stimulators, mechanical pain sensitivity (MPS) was assessed by generating a stimulus-response function. Patients were asked to give a pain rating for each pinprick stimulus on a “0-100” numerical rating scale (‘0’ indicating ‘‘no pain’’, and ‘100’ indicating ‘‘most intense pain imaginable’’). Each stimulator was applied a total of 5 times.
Dynamic mechanical allodynia (DMA) was evaluated using a set of light tactile stimulators (cotton wisp ~ 3 mN, cotton wool tip 100 mN, and brush 200-400 mN). The patients rated the painfulness of these stimulations on a “0-100” numerical rating scale.

3.4.6 Wind-up ratio

The wind-up ratio (WUR) test assesses temporal summation. It is the ratio of the pain rating of a series of 10 repeated pinprick stimuli to the single initial pinprick stimulation exerting the same force (256 mN). The interstimulus interval in the series was 1 second and the stimuli were applied to a 1 cm2 skin area.

3.5 Transcriptomic analysis of muscle tissue

Human muscle biopsy specimens were obtained from M. quadriceps femoris in sterile conditions and frozen in liquid nitrogen. Total RNA from 12 DM2 biopsy specimens (6 patients with and 6 patients without muscle pain) was isolated with RNeasy Mini Kit (Qiagen, Germany) according to manufacturer instructions. RNA quantity and purity were determined using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, USA). RNA quality was analyzed using Agilent 2100 bioanalyzer (Agilent Technologies, USA). cDNA library was prepared using TruSeq Stranded mRNA library preparation kit (Illumina, USA). RNA paired-end sequencing was performed at Illumina HiSeq 2000 platform (Illumina, USA).

3.6 Quantitative RT-PCR

Differentially expressed genes were validated by qPCR using Sybr green assay at Stratagene Mx3000P cycler (Agilent Technologies, USA) according to manufacturer instructions. cDNA was produced by QuantiTect reverse transcription kit (Qiagen, Germany). qPCR reactions contained 10ng of cDNA template, Kapa Sybr Fast qPCR Master Mix (Kapa Biosystems, USA), specific primers and water in a total volume of 20μL. PCR cycle conditions were 3 mins at 95°C followed by 40 cycles at 95°C for 3sec and 60°C for 30sec. The melting curve acquisition was performed at 95°C for 1min followed by 50°C for 30sec and temperature increase to 95°C. Each sample was measured in triplicate and GAPDH and cyclophilin A were used as the internal controls to normalize the differences in sample concentration and loading. Negative controls were included for every run. PCR efficiency test was performed for each primer pair. Sequences of the oligonucleotides are provided in Table 3.1.

Gene symbol	Forward primer	Reverse primer	Product size (bp)
GAPDH	GAAGGTGAAGGTCGGAGTC	GAAGATGGTGATGGGATTTC	226
PPIA	CGCCGAGGAAAACCGTGTACTATT	GACCTTGTCTGCAAACAGCTCAAAG	115
MAOA	CAGCCAAAGCATGGAGAATCAA	TGGCAGCAGATAGTCCTGAAA	98
NR4A3	CGGGATTTACTGATCTCCCCAAAG	GTTTGACCTGATGGAAAGTCTGAGG	95
FRMPD1	AACCCCGACAGAAGCAACTT	GGCTCCAACTAGAAGGGCAA	152
SLC16A12	CTGGGAGTTCTTACAGGTCTTGG	GGCTTTCCGTCTGCTGAAGT	87

Table 3.1 Oligonucleotide sequences used in qPCR validation.

3.7 Microdialysis

Microdialysis was performed in 14 DM2 patients (7 with and 7 without muscle pain, age and gender matched) in M. quadriceps femoris (vastus lateralis) during 4h to collect the interstitial fluid. The microdialysis catheter was inserted at the standardized location for all patients. The skin and the subcutaneous tissues at the entrance site of catheter were locally anaesthetized with an injection of Xylocaine (0,5 ml, 20 mg/ml). We used the microdialysis membrane (M Dialysis AB, Sweden) that was permeable for molecules up to 100kDa. The microdialysis catheters were perfused with a CMA/102 microdialysis pump (CMA, Sweden) which delivered flow at the rate of 2 µL/min during first hour and 1 µL/min during the rest of procedure. The perfusate contained Ringer solution (Serumwerk Bernburg AG, Germany) with addition of 50 mM ethanol (Carl Roth, Germany) and 1 µM ascorbate (Rotex Medica, Germany). Diluates were collected in vials during 4x60min protocol (Table 3.2). After catheter insertion, the patients rested for 60 mins to reach steady state conditions and to allow tissue to recover from any possible damage induced by catheter insertion (F1, F2 and F3 phase). Second hour of protocol was performed at the room temperature (F4 phase) followed by 30 mins of local cooling of the quadriceps by applying cold bandage during third hour of protocol (F5 phase) and recovery at room temperature during forth hour of microdialysis (F6 phase). Temperature of cold bandage (+4°) was measured with a thermometer before the start of dialysis and treated area of the quadriceps was kept uncovered during the procedure. The collected vials with dialysate were kept on the dry ice during the microdialysis and stored at -80ºC. Vials F4-F6 were used for further proteomic and lipidomic analysis while vials from F1-F3 were used for optimization of omics detection. Patients were allocated to groups with and without muscle pain based on Borg ratings of perceived exertion scale from 1 to 10 during microdialysis where 0 indicated no pain, 5 correlated to the moderate pain and 10 was maximal imaginable pain (Borg et al, 1981).

Vial	Interval	Intervention	Flow rate (µL/min)
1	30´	Tissue recovery (F1)	2
2	15´	Baseline calibration (F2)	2
3	15´	Baseline calibration (F3)	2
4	60´	Room temperature (F4)	1
5	60´	30´ cooling (+4°) followed by 30´ tissue recovery (F5)	1
6	60´	Room temperature (F6)	1

Table 3.2: Microdialysis protocol for DM2 patients.
The microdialysis vials containing dialysate are sorted in the chronological order with intervention and flow rate noted.

3.8 Preparation of microdialysis samples for lipidome analysis

Microdialysis samples (30µL of dialysate) were mixed with 0.5 ml of distilled water, 0.5 ml of methanol, and 0.1 ml of internal standard containing 10 ng of each 20-HETE-d6, 14,15-EET-d8, 14,15-DHET-d11, PGE2-d4, LTB4-d5 and 15-HETE-d8 (Cayman Chemical, USA) for the quantification of groups of similar metabolites. Alkaline hydrolyses of the samples was performed with addition of 300 µl of 10M sodium hydroxide for 20 min at 60 °C. 300 µl of 60% acetic acid and 2 ml of 1M sodium acetate buffer (pH 6.0) were added to neutralize the solution. The pH was measured and adjusted to pH 6.0 with 0.1M NaOH or 10% acetic acid. The hydrolyzed samples were centrifuged and the metabolites were extracted from the supernatant. Subsequent solid-phase extraction was performed using Varian Bond Elut Certify II columns. The eluate was dried on a heating block at 40 °C under a stream of nitrogen. Residues were then dissolved in 50 µl of acetonitrile and applied to LC-MS/MS.

3.9 LC-MS/MS analysis of eicosanoid species (lipidomics)

LC-MS/MS analysis of the extracted eicosanoids was performed at Agilent 6490 Triplequad mass spectrometers (Agilent Technolgies, USA) coupled with Agilent 1200 HPLC (degasser, binary pump, well-plate sampler, thermostated column compartment). The HPLC system contained Phenomenex Kinetex Column (150 mm x 2.1 mm, 2.6 µm, Phenomenex, Germany). Chromatography

was performed under gradient conditions with acetonitrile/0.1% formic acid in water as mobile phase. Initial gradient was at 5% acetonitrile, increased to 55% after 0.5 min, to 69% after 14.5 min, and to 95% after 14.6 min. The flow rate was 0.3 mL/min during 20 min of total run time. The injection volume was 7.5 µL. Drying gas was adjusted at 250°C/10L/min, sheath gas at 380°C/12L/min. Capillary and nozzle voltage were optimized at -4500V and -1500V.

3.10 Preparation of microdialysis samples for proteome analysis

Microdialysis samples (2µg of proteins/ injection) were treated with tryptic digestion for 30min at room temperature (RT) in 10 mM dithiothreitol solution (Sigma-Aldrich, Germany), followed by alkylation by 55 mM iodacetamide (Sigma-Aldrich, Germany) for 20min in the dark at RT. The endoproteinase LysC (Wako, Japan) was added following a protein/enzyme ratio of 50:1 and incubated for 4h at RT. After dilution of the sample with 4x digestion buffer (50 mM ammonium bi-carbonate in water (pH 8.0), sequence grade modified trypsin (Promega, USA) was added (same protein/enzyme ratio as for LysC) and sample was digested overnight. Finally, trypsin and Lys-C activity were quenched by acidification of the reaction mixtures with trifluoroacetic acid (Merck, Germany) to pH ~2. Afterwards, peptides were extracted and desalted using Stage Tips (Thermo Scientific, USA).

3.11 Selected reaction monitoring (targeted proteomics)

We performed selected reaction monitoring (SRM) at Quantiva mass spectrometer (Thermo Scientific, USA) using 37,5 fmol of each heavy standard peptide (IL-1, IL-6, TNFα and NGF; SpikeTides, JPT Peptide Technologies GmbH, Germany) spiked into 2µg of peptide sample (Table 3.3). Gradient separation was performed during 60min (8-30% Acetonitril, 0.5% formic acid) at reversed phase chromatography using the Eksigent HPLC (Eksigent, USA) on in-house manufactured 20 cm fritless silica microcolumns with an inner diameter of 75µm. Columns were packed with ReproSil-Pur C18-AQ 3µm resin, at a nanoflow rate of 250nl/min. Eluted peptides were directly ionized by electrospray ionization and transferred into Quantiva mass spectrometer

(Thermo Scientific, USA). Resolution was 0.7 for Q1 and Q3. Duration of cycle was 3 seconds with dwell time of 50ms for transitions of heavy standard precursor and 200ms for transition of light (sample) precursor.

Target protein	SpikeTides TQL - peptides
IL-1β	DDKPTLQLESVDP-K*-
IL-6	EALAENNLNLP-K*-
NGF	SSSHPIFH-R*-

Table 3.3: SpikeTide heavy standard peptides for SRM.
R* = Arg U-13C6; U-15N4; K*= Lys U-13C6; U-15N2. All peptides were isotopically labeled at incorporated C-terminal Arg or Lys.

3.12 Label free quantification (shotgun proteomics)

Label free quantification (LFQ) was performed at Fusion mass spectrometer (Thermo Scientific, USA) using 2 µg of peptide sample. Gradient separation was performed during 155min (8-30% Acetonitril, 0.5% formic acid) at reversed phase chromatography using the Eksigent HPLC on in-house manufactured 20 cm fritless silica microcolumns with an inner diameter of 75 µm. Columns were packed with ReproSil-Pur C18-AQ 3 µm resin at a nanoflow rate of 250 nl/min. Eluted peptides were directly ionized by electrospray ionization and transferred into Fusion mass spectrometer (Thermo Scientific). Mass spectrometry was performed in the data-dependent positive mode. One MS1 full scan (range 400-1500 m/z, resolving power 120k, AGC target 4E5, and maximum injection time of 50ms) was followed by 3s Top Speed precursor precursors selection. Only precursors with an assigned monoisotopic m/z and a charge state of 2-6 were selected. Dynamic exclusion was set to 60s and intensity threshold of 5000 was used. For MS2 analyses the following parameters were used: rapid scan rate, CID NCE 30%, 1.6 m/z isolation window, AGC target of 1E2 and maximum injection time of 250ms.

3.13 Statistical analysis

All statistical calculations were performed using R software. The QST parameters - CDT, WDT, PPT, WUR, MPS, MDT, and MPT - are usually normally distributed in log-space and thus were log-transformed. The QST-profiles of DM2 patients were compared to controls using repeated measure two factorial (for group and tested site) ANOVA. All QST measures from each patient were then standardized by z-transformation with respect to the age- and sex-matched healthy subject group.

$$\text{Z-score} = (\text{Mean}_{\text{DM2}} - \text{Mean}_{\text{healthy subjects}})/\text{SD}_{\text{healthy subjects}}$$

Where Mean_{DM2} is the value of the QST parameter in a DM2 patient, and $\text{Mean}_{\text{healthy subjects}}$ and $\text{SD}_{\text{healthy subjects}}$ are mean and standard deviation of the corresponding QST parameter in the healthy control group. Z-scores signs were adjusted so that a z-score > 0 indicated means a gain of sensory function (lower threshold), and the z-score < 0 means loss of function (increase in threshold). The advantages of graphical representation of QST profiles as z-transformed data was to directly compare between sensory modalities of different units and ranges between groups and tested sites.

Gene expression data was analyzed with CLC Genomic Workbench v7.0 (Qiagen, Germany) and Qlucore Omics Explorer v3.1 (Qlucore, Denmark). Samples obtained from patients without muscle pain were considered as a reference group. Preprocessed raw sequences were imported and trimmed in CLC Genomics Workbench and all trimmed reads were aligned to the human reference genome (GRCh37) and mapped back to the human transcriptome (v.19). Mapped read counts were normalized using Trimmed mean of M-values (TMM) method implemented in Edge-R package. Normalized read counts were used for analysis of differential gene expression at Qlucore Omics Explorer. P values were calculated by two group comparison T-test. Genes with P-value < 0,05 and fold change >±1,8 were considered to be differentially expressed and were presented as a heatmap with hierarchical clustering of the samples. Differentially expressed genes from RNAseq data were additionally confirmed with qPCR using $\Delta\Delta$Ct method with the average of Ct values for GAPDH and

cyclophilin A used as a reference. Ct values were calculated by MxPro qPCR software v4.1 (Agilent Technologies, USA). Gene functions were analysed using Quick GO gene ontology database (http://www.ebi.ac.uk/QuickGO/).

Lipidome data was analyzed from the calibration curves which were based on changes in the relative peak area in response to different target lipid/internal standard concentration ratios. Student T-test (p-value < 0.05) was used for comparison of lipid levels between pain and no pain group and data was visualized with Prism 5.0 software (Graphpad Software Inc, USA).

SRM proteome data was analyzed with Skyline (MacCoss Lab Software, USA) and the average ratio values of light (sample)/heavy (standard) peptide were calculated for each peptide (IL-1, IL-6 and NGF) using “area under the curve” values of 3 transitions. Student T-test (p-value < 0.05) was used for comparison of target protein levels between pain and no pain group and data was visualized with Prism 5.0 software (Graphpad Software Inc, USA).

Shotgun MS/MS data were analyzed by MaxQuant (version 1.5.2.8). The internal Andromeda search engine was used to search MS/MS spectra against a decoy human UniProt database (HUMAN.2014-10) containing forward and reverse sequences. The search included variable modifications of methionine oxidation and N-terminal acetylation and fixed modification of carbamidomethyl cysteine. Minimal peptide length was set to six amino acids and a maximum of two missed cleavages was allowed. The FDR was set to 0.01 for peptide and protein identifications. LFQ analysis was performed in MaxQuant. Unique and razor peptides were considered for quantification with a minimum ratio count of 1. Retention times were recalibrated based on the built-in nonlinear time-rescaling algorithm. MS/MS identifications were transferred between LC-MS/MS runs with the “Match between runs” option in which the maximal retention time window was set to 2 min. LFQ intensity values were logarithmized and missing values were imputed with random numbers from a normal distribution whose mean and standard deviation were chosen to best simulate low abundance values below the noise level (width = 0.3; shift = 1.8). Patient

samples were grouped to muscle pain and no pain group according to Borg scale ratings during microdialysis and significantly enriched proteins for each group were determined by a volcano plot-based strategy, combining standard two-sample t-test p-values with ratio information. A significance cut-off was set to p-value < 0.05 and t-test difference of LFQ intensity mean (no pain group) – LFQ intensity mean (pain group) which was higher than log2 of ±1.75. We used Perseus (version 1.5.5.3) for functional enrichment analysis of Gene Ontology, Pfam and KEGG terms to identify changes in the protein level in patients with muscle pain. Enrichment or depletion of annotation terms was based on p values with significance cut-off < 0.01. The p-values were +/-log10 transformed and visualized in Excel.

4. Results

(4.1-4.3 adopted and modified from Moshourab*, Palada* et al, 2016)

4.1 Patient characteristics

All 42 DM2 patients were ambulatory and attended the clinical and QST assessment session (Table 4.1). Data from 7 patients were excluded from the analysis: six patients took opioid analgesics at the testing day and one patient had sensory deficits due to stroke. Twenty healthy subjects (11 females and 9 males) were recruited with a mean ± SD age of 55 ± 8.3 years (P>0.05 t-test, compared to DM2). The proportion of males and females between DM2 (54% females) and healthy controls (55% females) was comparable. 23 patients reported muscle pain (DM2-Mya group) and 12 patients were without pain (DM2-No Mya group). On a scale of 0-10, muscle pain was subjectively rated as most burdening in everyday activity (5.7 ± 2.3) compared to muscle weakness (5.1 ± 2.8) and myotonia (3.5 ± 2.8). Muscle pain was typically localized symmetrically with the common onset in proximal muscles but the affected body region differed between the patients. Seventeen of 23 DM2 patients reporting muscle pain had experienced pain for more than 5 years. The character of pain (assessed by the MPQ) was: tugging (14/23), cramping (13/23), tiring (12/23), punishing (10/23), annoying (9/23), radiating (8/23), burning (8/23) and dull (6/23). Pain rating index (PRI) and present pain intensity (PPI) indicated that DM2 patients develop muscle pain of moderate severity. Muscle pain was reported as being worse during cold weather in 10 patients. Patients also reported that the muscle pain was increased during the movement and intensive exercise. Of the 10 diabetic patients, one had a diagnosis of diabetic polyneuropathy and trigeminal neuralgia but did not complain of pain. Nine (39%) DM2 patients with muscle pain had other types of concurrent pain (low back pain, arthralgia, and headaches). A higher proportion of patients with muscle pain (15/23, 68%) had clinical evidence of muscle weakness compared to DM2 patients without pain (5/12, 42%) (P>0.05, χ2-test). Other common clinical symptoms were hyperlipidemia, cataracts and hypertension present in

46% of our DM2 cohort while smaller number of patients had cardiac conduction defects (23%) and insulin resistance (14%).

	Muscle Pain	No Pain	Total
N	23	12	35
Age, y	57.3 ± 10.3	48·6 ± 15.2	54.3 ± 12.7
Male/female	9m/14f	7m/5f	16m/19f
BMI, Kg/m2	26.2 ± 4.5	24.4 ± 4.8	25·6 ± 4.6
Muscle features			
Muscle weakness	15 (68%)	5 (42%)	20 (57%)
Myotonia	19 (85%)	7 (58%)	26 (74%)
Systemic features			
Insulin resistance	4 (17%)	1 (8%)	5 (14%)
Hyperlipidemia	11 (48%)	5 (42%)	16 (46%)
Cataract	8 (35%)	3 (25%)	11 (46%)
Hypertension	11 (48%)	5 (42%)	16 (46%)
Cardiac conduction abnormalities	7 (30%)	1 (6%)	8 (23%)
CK	286 ± 196	506 ± 414	
Pain features			
Pain Intensity, NAS	4.2 ± 2.2	NA	
Pain Duration, y	7.4 ± 3.3	NA	
Pain Location			
Thigh	16		
Shoulder	14		
Back	8		
Lower arm	9		
Lower leg	8		
Muscle Pain related to movement	10 (43%)	NA	
Muscle Pain related to cold	14 (61%)	NA	
MPQ			
PRI	8.3	NA	
MWC	16.1	NA	
PPI	3.3	NA	

Table 4.1: Characteristics of patients with Myotonic Dystrophy type 2.

Mean ± SD is shown. MPQ: McGill pain Questionnaire; PRI: Pain rating index; MWC: number of words chosen; PPI, present pain intensity. BMI is body mass index.

Medical research council (MRC) grades of muscle weakness were collected for all DM2 patients who participated in the study both for proximal and distal

muscles (Table 4.2). Based on lower MRC scores, muscle weakness was more pronounced in proximal muscles compared to distal muscles. The myotonia was generally fluctuating, mild (induced by percussion in 22), and severe in 2 patients (difficulty in standing/tumbling). Nine DM2 patients showed myotonia during EMG recording. None of the DM2 patients with myotonia required mexiletine.

ID	Muscle pain	Myotonia	EMG	Muscle weakness	MRC proximal worst value	MRC distal worst value
PROMM002	muscle pain	myotonia	myotonic	no weakness	5	5
PROMM003	no pain	myotonia	-	weakness	5	4
PROMM004	muscle pain	no myotonia	-	weakness	4	5
PROMM005	muscle pain	no myotonia	myotonic	weakness	1	5
PROMM006	no pain	myotonia	-	no weakness	5	5
PROMM008	muscle pain	myotonia	-	weakness	4	3
PROMM009	muscle pain	myotonia	myotonic	weakness	5	4
PROMM010	muscle pain	myotonia	myotonic	weakness	4	5
PROMM011	muscle pain	myotonia	-	no weakness	5	5
PROMM013	no pain	no myotonia	-	weakness	4	5
PROMM014	muscle pain	myotonia	-	weakness	4	5
PROMM015	no pain	myotonia	-	no weakness	5	5
PROMM016	muscle pain	myotonia	-	weakness	4-	4-
PROMM017	no pain	no myotonia	myotonic	weakness	4-	5
PROMM018	no pain	no myotonia	-	no weakness	5	5
PROMM019	no pain	no myotonia	-	no weakness	5	5
PROMM020	muscle pain	myotonia	myotonic	weakness	4+	5
PROMM021	muscle pain	myotonia	-	no weakness	5	5
PROMM023	no pain	myotonia	myotonic	weakness	4+	4+
PROMM024	muscle pain	myotonia	-	weakness	5	4
PROMM025	muscle pain	myotonia	-	no weakness	5	5
PROMM026	muscle pain	myotonia	-	weakness	4	4
PROMM027	muscle pain	myotonia	-	weakness	3	4
PROMM028	muscle pain	myotonia	-	no weakness	5	5
PROMM029	muscle pain	myotonia	myotonic	weakness	3	4
PROMM031	no pain	no myotonia	-	no weakness	5	5
PROMM033	muscle pain	myotonia	-	no weakness	5	5
PROMM034	no pain	myotonia	-	no weakness	5	5
PROMM035	muscle pain	myotonia	myopathic	weakness	4	5
PROMM036	muscle pain	no myotonia	-	weakness	4	4
PROMM037	no pain	myotonia	-	weakness	5	4+
PROMM038	muscle pain	no myotonia	-	weakness	5	4+
PROMM039	muscle pain	myotonia	-	no weakness	5	5
PROMM040	muscle pain	myotonia	-	no weakness	5	5
PROMM041	no pain	myotonia	-	no weakness	5	5

Table 4.2: Characteristics of muscle weakness and myotonia in DM2 patients.

MRC is Medical Research Council scale for muscle strength. Grades 4 -, 4 and 4+ are used to indicate movement against slight, moderate and strong resistance respectively.

Muscle biopsy specimens were collected for RNA sequencing from 6 patients with and 6 DM2 without muscle pain (Table 4.3). Biopsies from female DM2 patients were obtained only from patients with muscle pain.

ID RNA seq	Sex	Age	Muscle pain	Biopsy
1	F	45	Yes	01/2009
2	F	51	Yes	10/2005
3	F	57	Yes	11/2010
4	M	65	Yes	06/2007
5	M	56	Yes	10/2008
6	M	68	Yes	10/2008
7	M	43	No	07/2014
8	M	31	No	04/2005
9	M	67	No	03/2009
10	M	53	No	11/2009
11	M	55	No	04/2004
12	M	44	No	11/2005

Table 4.3: Characteristics of DM2 patients with muscle biopsy specimens.

Histopathological findings from DM2 were similar between patients with and without pain (Table 4.4). All DM2 patients had an atrophy of type 2 fibers. Internal centralized nuclei were present in 10 muscle biopsies while nuclear clumps were detected in 7 biopsy specimens. Atrophy of type 2 fibers was followed by increased fibrosis in 7 biopsies and only 1 biopsy contained infiltrates of immune cells. Necrotic, regenerative and ragged red fibers were mostly absent.

Patient	Fiber diameter (µm)	Type 2 fiber atrophy	Nuclear clumps	Internal nuclei	Inflammatory cells	Increase in connective tissue	Other findings
1	4-100	Yes	Yes	++	No	No	HC
2	6-80	Yes	Yes	No	No	+	No
3	7-150	Yes	No	++	No	+	No
4	4-120	Yes	Yes	No	No	+	Rare NF, RF
5	4-120	Yes	Yes	+++	+	No	No
6	6-120	Yes	Yes	+	No	+	No
7	8-145	Yes	No	++	No	+	No
8	6-100	Yes	Yes	+++	No	+	Rare NF, RF
9	4-100	Yes	Yes	+	No	No	RRF
10	15-130	Yes	No	++	No	++	No
11	10-150	Yes	No	+++	No	No	No
12	18-105	Yes	No	+	No	No	No

Table 4.4: Histopathological findings in DM2 patients with muscle biopsy specimens.

Patients 1-6 had muscle pain, patients 7-12 had no pain. HC, hypercontracted fibers; NF, necrotic fibers; RF, regenerating fibers; RRF, ragged red fiber; + present in < 20% of fibers or mild pathology; ++ present in 20-60% of fibers or moderate severe pathology; +++ present in 60-100% of fibers or severe pathology.

Medication was similar between DM2 patients with and without pain at the time of biopsy (Table 4.5).

DM2 patients with muscle biopsy specimens				
Patient	**Gender**	**Age**	**Muscle Pain**	**Medication**
1	F	45	Yes	None
2	F	51	Yes	None
3	F	57	Yes	Olmesartan, amlodipine, hydrochlorothiazide, lercanidipine, metamizol, colesevelam
4	M	65	Yes	Metaprolole, ramiprile, diltiazeme, ezetimib, pantoprazole, repaglinide
5	M	56	Yes	Metaprolole, perindoprile, indapamide, colesevelam, omega-3 fatty acids
6	M	68	Yes	Valsartane, hydrochlorothiazide, pantoprazole
7	M	43	No	Metohexal, eplerenon, ramiprile, atorvastatin, omega-3 fatty acids, colesevelam
8	M	31	No	Prednisolone
9	M	67	No	N/A
10	M	53	No	Thorasemide
11	M	55	No	Candesartan, metamizol, ibuprofen
12	M	44	No	None

Table 4.5: Medication of DM2 patients at the time of biopsy.

Microdialysis samples were collected from 14 DM2 patients (Table 4.6). Based on Borg scale pain ratings on the testing day, there were 7 patients with muscle pain and 7 patients without pain. Both groups were age and gender matched. Average Borg scale pain ratings in DM2 with pain were 3.75 in F1-4, 5.41 in F5 phase and 3.75 in F6 which indicates the presence of muscle pain with moderate severity. Interestingly, there was a transient increase in Borg scale pain ratings during cooling (F5) phase in 3 patients (PROMM B, E and L). Samples from all 14 patients (PROMM A-N) were analyzed with SRM and LFQ proteomics while samples from 12 DM2 patients (PROMM A-L) were analyzed with LC-MS lipidomics.

No.	Code	Gender	Age	Borg Scale (F1)	Borg Scale (F2)	Borg Scale (F3)	Borg Scale (F4)	Borg Scale (F5)	Borg Scale (F6)	Muscle Pain	Microdialysis
1	PROMM A	f	73	0	0	0	0	0	0	No	07/2014
2	PROMM B	m	61	0	0	0	0	4	0	Yes	07/2014
3	PROMM C	m	55	5	5	5	5	5	5	Yes	10/2014
4	PROMM D	m	54	0	0	0	0	0	0	No	01/2015
5	PROMM E	f	57	5	5	5	5	7	5	Yes	03/2015
6	PROMM F	m	31	0	0	0	0	0	0	No	09/2015
7	PROMM G	m	63	0	0	0	0	0	0	No	10/2015
8	PROMM H	m	56	3.5	3.5	3.5	3.5	3.5	3.5	Yes	10/2015
9	PROMM I	f	55	2	2	2	2	2	2	Yes	10/2015
10	PROMM J	f	46	0	0	0	0	0	0	No	12/2015
11	PROMM K	f	60	0	0	0	0	0	0	No	01/2016
12	PROMM L	f	55	4	4	4	4	8	4	Yes	01/2016
13	PROMM M	f	69	3	3	3	3	3	3	Yes	02/2016
14	PROMM N	m	54	0	0	0	0	0	0	No	03/2016

Table 4.6: Characteristics of DM2 patients that underwent microdialysis.

4.2 Sensory changes in patients with DM2

Among all the QST measures the most prominent abnormality was seen in pressure pain threshold (PPT). Repeated-measures ANOVA indicated statistically significant decrease in pressure pain threshold in the DM2-Mya

group compared with the control groups (Figure 4.1A; Table 4.7). There is a general and strong shift for mean pressure pain thresholds towards hyperalgesia in patients with DM2, with mean z-score values for DM2 patients with myalgia lying beyond the healthy subject range of 2 z-score units. Between-group comparisons (Fig. 4.1) reveal significant differences, in the gain-of-function direction, between the healthy subject groups and the DM2–No Mya and DM2-Mya groups for all muscle groups (P<0.0001, ANOVA). The number (percentage) of DM2-Mya patients with z-scores for PPT lying beyond +1.96 (170KPa for EDC, 200KPa for deltoid, 265KPa for anterior tibialis, and 265KPa for the quadriceps) are 9(39%), 15(65%), 13(57%), 16(70%) for EDC, deltoid, anterior tibialis, and quadriceps muscles respectively. This contrasts with 1(8%), 1(8%), 2(16%), 2(16%) for EDC, deltoid, anterior tibialis, and quadriceps muscles respectively in DM2-No Mya patients.

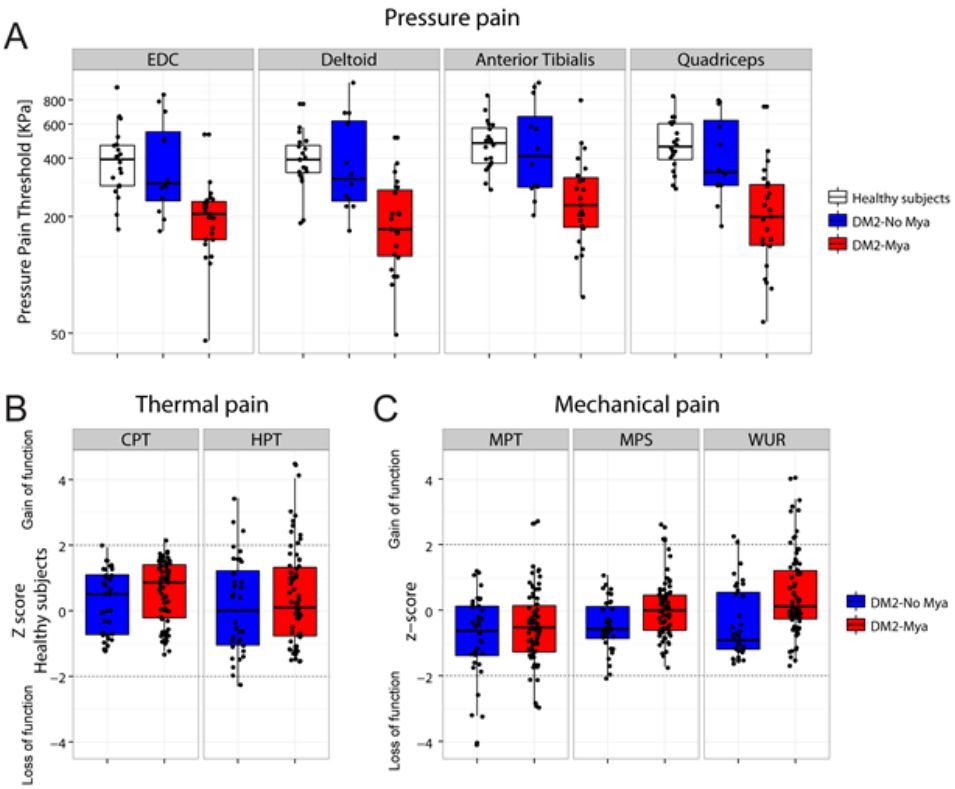


Figure 4.1: Plots of QST parameters for pressure, thermal and mechanical pain.

(A) Scatter and box and whisker plots of pressure pain threshold across the 4 muscle groups in healthy subjects, DM2-Mya and DM2-No Mya groups. (B) Thermal and (C) mechanical pain QST parameters represented as scatter and box and whisker plots of z-scores over the 3 tested areas (Hand, Shoulder, and Thigh). Mean of the healthy subject z-core is represented by “0” and dotted lines designating ± 1.96 SD.

The median z-scores for all thermal and mechanical pain parameters in the DM2 group generally fall within the healthy subject ranges except for a few individuals (Figure 4.1B & C). Within mechanical pain parameters, all DM2 participants showed a slight loss of MPT function, i.e., increased pain threshold to pinprick stimulation, compared to healthy subjects (P=0.03, ANOVA). When comparing DM2 patients, we found no significant differences in MPT, however, we observed enhanced subjective rating to pinprick stimuli (MPS) and increased windup ratio in DM2-Mya compared to DM2-No Mya group (Figure 4.2; Table 4.7). Thus DM2 patients with myalgia exhibited increased MPS and windup ratios (positive z-score, gain of function) typical for patients with generalized central sensitization (P=0.03, ANOVA). Allodynia occurred in one patient with myalgia in the shoulder region.

			DM2 n=35									
	Main effects			**Hand**			**Shoulder**			**Thigh**		
	F-value	*P*		mean	SD		mean	SD		mean	SD	
CDT	0.98	ns		-0.32	1.21		-0.44	0.88		0.01	1.36	
WDT	4.0	0.049		-0.66	1.37		-0.24	1.13		-0.76	1.55	
CPT	3.98	0.051		0.43	1.05		0.38	0.92		0.58	0.96	
HPT	0.9	ns		0.57	1.68		0.33	1.25		0.06	1.19	
MPT	4.8	0.03		-0.49	0.89		1.02	1.37		-0.2	1.1	
MPS	1.9	ns		-0.23	0.79		-0.06	0.91		0.06	0.79	
WUR	0.4	ns		0.24	1.31		0.00	1.06		0.36	1.44	
MDT	10.5	0.002		-1.28	1.2		-1.05	1.52		-0.6	1.17	
				EDC			Deltoid			Quadriceps			Ant. Tibialis	
PPT	13.8	<0.0001		1.18	1.43		1.57	1.92		2.13	2.24		1.62	2.10

Table 4.7: ANOVA comparison of sensory profiles between DM2 patients and healthy subjects on hand, shoulder and thigh.

Mean and SD values of QST parameters after z-score transformation for the 3 tested sites· ns= not significant (p>0·05). ANOVA done on log-transformed data (CDT, WDT, PPT, MPT, MDT, SR, WUR). CDT indicates cold detection threshold; WDT, warmth detection threshold; CPT, cold pain threshold; HPT, heat pain threshold; PPT, pressure pain threshold; MPT, mechanical pain threshold; MPS, mechanical pain sensitivity; WUR, wind-up ratio; MDT, mechanical detection threshold.

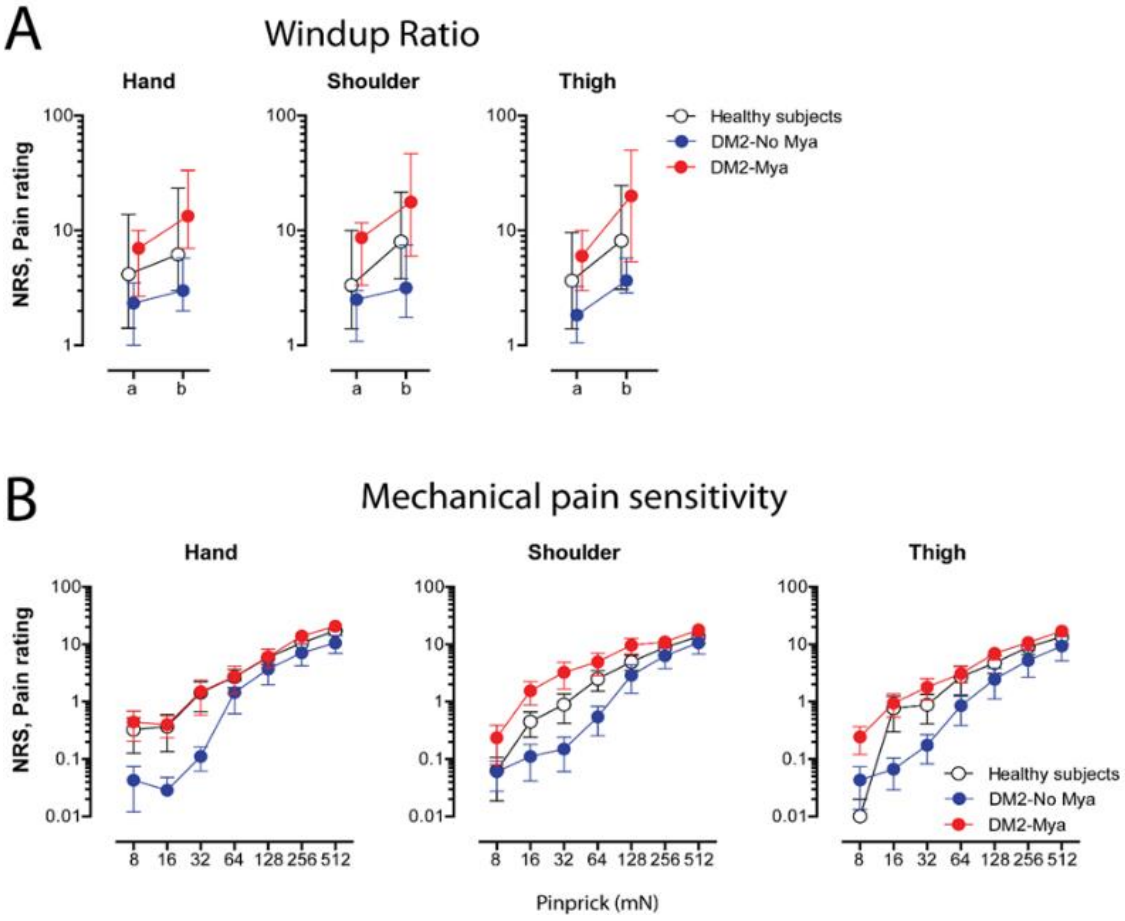


Figure 4.2: Pain intensity response for Windup ratio and stimulus response tests using pinprick stimulators in healthy subjects and DM2 myalgia and no myalgia patients.

(A) Windup ratio comparison of pain rating of a single (a) pinprick with a series (b) of 10 repetitive stimuli. (B) Pain evoked by pinprick stimulators was rated more intensely in DM2 patients with myalgia. NRS; numerical rating scale (0-100). Data are presented as mean and SD.

Interestingly, mechanical detection threshold was significantly increased in DM2 patients indicating that touch sensation was impaired in all DM2 patients [median/inter quartile range in mN in healthy subject 1.1/0.7 (hand), 2.0/2.0 (Shoulder), and 4.5/4.1 (thigh); in DM2 patients 3.2/3.1 (hand), 4.0/8.9 (shoulder), and 8.0/7.8 (thigh)]. The mean z-score values for MDT reveal a loss of function in the DM2 group compared to healthy subjects (Table 4.8). This effect held true after excluding the 10 diabetic patients (data not shown). We observed loss of thermal warming discrimination and therefore a loss of sensory function in 4 DM2 patients (2 with and 2 with no myalgia). Paradoxical heat

sensation was observed in 2 DM2-Mya patients, 1 DM2-No Mya patient and 1 healthy control subject.

			Myalgia n = 23						No Myalgia n = 12					
	main effects		Hand		Shoulder		Thigh		Hand		Shoulder		Thigh	
	F	*P*	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
CDT	0.2	ns	-0.29	1.16	-0.55	0.91	-0.06	1.48	-0.38	1.33	-0.24	0.84	0.14	1.17
WDT	0·4	ns	-0.79	1.20	-0.15	0.99	-0.98	1.51	-0.41	1.67	-0.42	1.39	-0.36	1.61
CPT	1·3	ns	0.73	0.91	0.43	0.97	0.59	0.99	-0.14	1.08	0.28	0.84	0.58	0.93
HPT	0·4	ns	0.57	1.7	0.54	1.26	0.15	1.31	0.57	1.71	-0.06	1.18	-0.1	1.42
MPT	0·7	ns	-0.29	0.95	-0.85	1.3	-0.26	1.08	-0.87	0.64	-1.32	1.5	-0.07	1.17
MPS	4·8	0·03	-0.16	0.73	0.14	0.88	0.26	0.82	-0.62	0.76	-0.43	0.89	-0.31	0.62
WUR	5·4	0·03	0.51	1.36	0.36	1.02	0.72	1.38	-0.26	1.09	-0.65	0.8	-0.3	1.37
MDT	0·9	ns	-1.35	1.89	-1.19	1.49	-0.81	1.0	-1.15	1.26	-0.80	1.64	-0.21	1.39

			EDC		Deltoid		Quadriceps		Ant Tibialis		EDL		Deltoid		Quadriceps		Ant Tibialis	
			Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
PPT	14.2	<0.0001	1.73	1.13	2.31	1.62	2.95	2.05	2.35	1.82	0.12	1.4	0.16	1.67	0.55	1.74	0.22	1.93

Table 4.8: ANOVA comparison of z-score profiles between DM2 patients with and without myalgia.

ns= not significant (p>0.05). ANOVA done on log-transformed data (CDT, WDT, PPT, MPT, MDT, SR, WUR). CDT indicates cold detection threshold; WDT, warmth detection threshold; CPT, cold pain threshold; HPT, heat pain threshold; PPT, pressure pain threshold; MPT, mechanical pain threshold; MPS, mechanical pain sensitivity; WUR, wind-up ratio; MDT, mechanical detection threshold. EDC (extensor digitorum communis); Quad (Quadriceps); TA (tibialis anterior).

4.3 Myalgic and non-myalgic DM2 muscle has distinct transcriptome profiles

We analysed transcriptome profiles of muscle biopsies obtained from myalgic and non-myalgic, age-matched DM2 patients (n=6 for each group, Table 4.3). Bioinformatic analysis of muscle RNAseq data revealed 14, muscle-specific, differentially expressed (DE) genes based on presence or absence of myalgia

(Table 4.9 and Figure 4.3). Due to limited muscle tissue material, only five of these genes were confirmed by qPCR; however, all five confirmed the RNASeq analysis (Table 4.9).

Gene Symbol	Gene Name	Average Total Gene Reads (myalgic)	Average Total Gene Reads (non-myalgic)	P-value	RNAseq Fold change	qPCR Fold change
MAOA	Monoamine oxidase A	446.67	2441.67	0	-5.47	-2.33
ARC	Activity-regulated cytoskeleton-associated protein	14	34.83	2.83*E(-5)	-2.49	
CYB5D1	Cytochrome B5 domain 1	50.33	28	0.01	1.8	
GSTCD	Glutathione S-Transferase, C-Terminal Domain Containing	58.17	32	0.01	1.82	
GRB14	Growth factor receptor-bound protein 14	763	408	0.01	1.87	5.67
PANK1	Pantothenate kinase 1	924.83	453.67	2.1*E(-3)	2.04	
ZNF711	Zinc finger protein 711	155.5	75.5	9.02*E(-4)	2.06	
FAM26E	Family with sequence similarity 26, member E	187.33	90.67	9.41*E(-4)	2.07	
PFKFB2	Fructose-2,6-biphosphatase 2	3894.67	1820	7.21*E(-4)	2.14	
ZNF841	Zinc finger protein 841	25.83	11.67	2.05*E(-3)	2.21	
HECW2	HECT, C2 and WW domain containing E3 ubiquitin protein ligase 2	325.67	140.17	1.33*E(-4)	2.32	
SLC16A12	Solute carrier family 16, member 12	150.67	60	1.96*E(-5)	2.51	3.95
FRMPD1	FERM and PDZ domain 1	63.33	17.5	4.21*E(-8)	3.62	4.17
NR4A3	Neuron-derived orphan receptor 1	1115.17	271	6.30*E(-12)	4.11	5.64

Table 4.9: List of differentially expressed genes based on the presence of myalgia (myalgic vs non-myalgic).

Normalized reads per gene for the differentially expressed genes (DEGs).

The highest differential expression found was a decrease in the levels of monoamine oxidase A (MAOA) in myalgic DM2 patients. MAOA encodes an enzyme that degrades amine neurotransmitters, such as dopamine, norepinephrine, and serotonin. Another gene with decreased expression in myalgic DM2 was activity-regulated cytoskeleton-associated protein (ARC).

Significantly increased expression in myalgic DM2 muscle were found for CYB5D1, GSTCD, GRB14, PANK1, ZNF711, FAM26E, PFKFB2, ZNF841, HECW2, SLC16A12, FRMPD1, NR4A3 and SLC16A12. Analysis of gene functions showed that these genes are involved in transcriptional regulation (NR4A3, ZNF711 and ZNF841), metabolic regulation (PANK1, PFKFB2), ubiquitin ligation (HECW2), signal transduction (GRB14), iron binding (CYB5D1), creatine transmembrane transport (SLC16A12) or predicted cation channel activity (FAM26E).

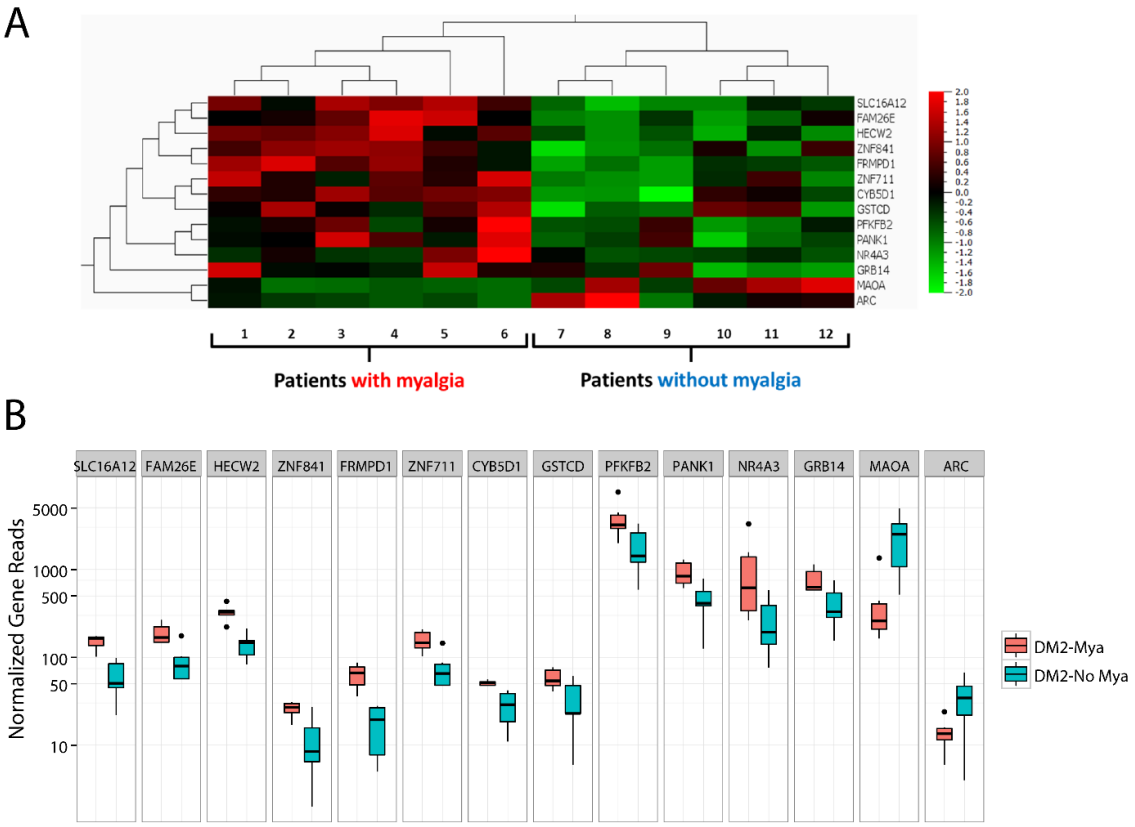


Figure 4.3: Transcriptome profile differences between muscle tissues derived from DM2 patients with myalgia and without myalgia.

(A) Heat map showing expression patterns of differentially expressed genes (DEG) based on presence or absence of myalgias. Each row in the heat map indicates gene expression values for one of 14 differentially expressed genes while every column shows gene expression profile for one of 12 tested DM2 patient. Areas in green correlate with low and areas in red correlate with high gene expression (see scale). (B) Normalized reads per gene for the DEGs.

4.4 DM2 patients with and without muscle pain have different muscle secretome profiles

In total, LFQ analysis identified 909 proteins in DM2 interstitial fluid. There were 85 differentially regulated proteins (Table 4.10) between DM2 with and without pain (P-value < 0.05; T-test Difference >log2 of ±1.75). Thirty-two proteins were differentially regulated in the F4 phase (room temperature), 36 proteins in the F5 phase (cooling) and 17 proteins in the F6 phase (recovery, room temperature). In the F4 phase, the biggest increase in protein level was observed for CYB5R3 in patients with muscle pain and ABHD14B in patients without pain. In the F5 phase, the highest upregulation was detected for CYB5R3 in DM2 with muscle pain and HCLS1 in DM2 without pain. In the F6 phase, the highest increase in level was observed for BLVRB in DM2 patients with muscle pain and APOD in DM2 without pain.

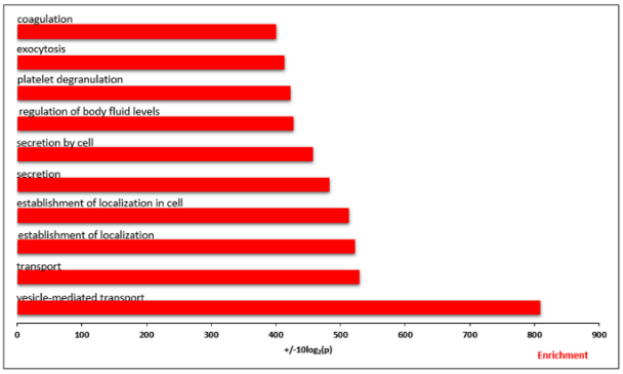
Dialysis phase	Gene names	Protein names	T-test difference nopain vs pain (F4)	P-value nopain vs pain (F4)
F4	A1BG	Alpha-1B-glycoprotein	-2.50207	0.007816948
	ABHD14B	Alpha/beta hydrolase domain-containing protein 14B	2.957785714	0.036909961
	ACTB	Actin, cytoplasmic	2.425018571	0.014621678
	APOC2	Apolipoprotein C-II	-2.686761429	0.001465719
	ARF1	ADP-ribosylation factor	2.007665714	0.043979163
	ATP2A1	Sarcoplasmic/endoplasmic reticulum calcium ATPase 1	1.843357143	0.024759014
	BPGM	Bisphosphoglycerate mutase	-2.334497143	0.035789545
	C8G	Complement component C8 gamma chain	-2.379005714	0.000141866
	CAT	Catalase	-2.110668571	0.011075826
	CFB	Complement factor B	2.152025714	0.025768096
	CFHR3	Complement factor H-related protein 3	-3.246462857	0.038879118
	CKM	Creatine kinase M-type	-2.267378571	0.043688598
	CYB5R3	NADH-cytochrome b5 reductase	-3.603641429	0.008674411
	DBNL	Drebrin-like protein	-3.100672857	0.024772444
	DCD	Dermcidin	-2.676808571	0.037582706
	DDAH2	N(G),N(G)-dimethylarginine dimethylaminohydrolase 2	-2.137685714	0.015859201
	DEFA1	Neutrophil defensin 1	-3.162278571	0.029373488
	DMD	Dystrophin	-3.445531429	0.023267062
	DNAJB4	DnaJ homolog subfamily B member 4	-2.414624286	0.043460886
	DSC3	Desmocollin-3	-3.000097143	0.037339919
	DUPD1	Dual specificity phosphatase DUPD1	-2.818994286	0.038492866
	EEF1A2	Elongation factor 1-alpha 2	-2.527071429	0.012455364
	EFEMP1	EGF-containing fibulin-like extracellular matrix protein 1	-2.960865714	0.042033593
	EPB41L2	Band 4.1-like protein 2	-1.843035714	0.040753975
	FKBP3	Peptidyl-prolyl cis-trans isomerase FKBP3	-2.47182	0.000689465
	FN3K	Fructosamine-3-kinase	-2.62725	0.006391578
	GLOD4	Glyoxalase domain-containing protein 4	-2.316147143	0.017005587
	GOT1	Aspartate aminotransferase	2.223967143	0.032483284
	H2AFY	Histone H2A	-1.91288	0.049775117
	HIST1H2AJ	Histone H2A type 1-J	1.823688571	0.003251067
	HSPA8	Heat shock cognate 71 kDa protein	2.249907143	0.032291968
	HSPB1	Heat shock protein beta-1	2.16664	0.039705067

Dialysis phase	Gene names	Protein names	T-test difference nopain vs pain (F5)	P-value nopain vs pain (F5)
F5	ABLIM1	Actin-binding LIM protein 1	1.882195714	0.008435931
	ACTC1	Actin	3.539304286	0.020868884
	ANG	Angiogenin	1.771021429	0.003781146
	BIN2	Bridging integrator 2	2.928488571	0.003787542
	C1QB	Complement C1q subcomponent subunit B	2.233118571	0.021805036
	C9	Complement component C9	2.294882857	0.021857954
	CFH	Complement factor H	2.11878	0.033979
	CFHR1	Complement factor H-related protein 1	1.955738571	0.000520154
	CKB	Creatine kinase B-type	2.834154286	0.020389692
	CKM	Creatine kinase M-type	3.46966	0.040799786
	CORO1A	Coronin-1A	2.445735714	0.042871644
	CTSD	Cathepsin D	2.707754286	0.036123752
	CYB5R3	NADH-cytochrome b5 reductase 3	-2.840304286	0.043521844
	DPT	Dermatopontin	-2.112432857	0.027652695
	DSC1	Desmocollin-1	-2.793068571	0.046729698
	EEF1A2	Elongation factor 1-alpha 2	-2.21576	0.009502375
	EIF5B	Eukaryotic translation initiation factor 5B	1.803652857	0.023985223
	EPB41	Protein 4.1	2.273281429	0.025207004
	F9	Coagulation factor 9	2.592181429	0.005298553
	FAM184B	Protein FAM184B	1.773267143	0.016273934
	FN1	Fibronectin	2.181228571	0.016747661
	GNAI2	Guanine nucleotide-binding protein G(i) subunit alpha-2	2.989058571	0.040197308
	GOT1	Aspartate aminotransferase	2.31887	0.00919705
	GSTP1	Glutathione S-transferase P	2.268845714	0.028173307
	H2AFV	Histone H2A	3.110904286	0.015196115
	HBA2	Hemoglobin	3.343605714	0.018044716
	HCLS1	Hematopoietic lineage cell-specific protein	4.274798571	0.006206001
	HEBP2	Heme-binding protein 2	2.131244286	0.013390785
	HMGA1	High mobility group protein HMG-I/HMG-Y	1.779872857	0.002622851
	HMGB1	High mobility group protein B1	2.403875714	0.005154777
	HMGN1	Non-histone chromosomal protein HMG-14	2.081658571	0.017993903
	HMGN2	Non-histone chromosomal protein HMG-17	1.812832857	0.022303186
	HNRNPH1	Heterogeneous nuclear ribonucleoprotein H	2.49678	0.00147058
	HSPA2	Heat shock-related 70 kDa protein 2	2.756625714	0.023615085
	RPS8	40S ribosomal protein S8	1.88109	0.005903603
	SKAP2	Src kinase-associated phosphoprotein 2	1.874631429	0.047822149
Dialysis phase	**Gene names**	**Protein names**	**T-test difference nopain vs pain (F6)**	**P-value nopain vs pain (F6)**
F6	APOD	Apolipoprotein D	2.315968333	0.029159354
	BLVRB	Flavin reductase (NADPH)	-4.788356667	0.002297079
	CLC	Eosinophil lysophospholipase	-2.885677619	0.028866396
	CPN2	Carboxypeptidase N subunit 2	-3.05715	0.00792184
	CPPED1	Calcineurin-like phosphoesterase domain-containing protein 1	-2.595659762	0.018179933
	CRABP2	Cellular retinoic acid-binding protein 2	-2.295518571	0.00459767
	CRIP2	Cysteine-rich protein 2	-2.118150476	0.031525604
	CRKL	Crk-like protein	-1.923835476	0.015344244
	CTSG	Cathepsin G	2.128955238	0.046156683
	CTTN	Src substrate cortactin	-3.222860476	0.0086431
	DAG1	Dystroglycan	-2.40334381	0.030255388
	DCUN1D1	DCN1-like protein 1	-2.111585238	0.041457568
	DDAH1	N(G),N(G)-dimethylarginine dimethylaminohydrolase 1	-2.079326429	0.031988537
	DDT	D-dopachrome decarboxylase	-2.043715238	0.040720681
	DDX17	Probable ATP-dependent RNA helicase DDX17	-2.034209762	0.017894039
	DDX39B	Spliceosome RNA helicase DDX39B	-2.027887143	0.0265841
	DEK	Protein DEK	-2.016922381	0.027119451

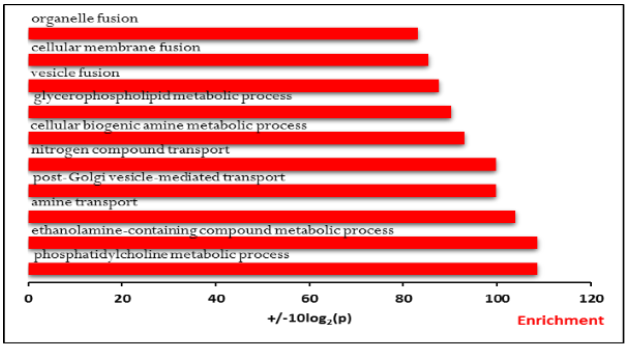
Table 4.10: Differentially regulated proteins in DM2.

In the patients with muscle pain, proteins with the highest turnover rate during F4 phase at RT were proteins involved in SNAP receptor and chloride channel regulator activities while proteins with the lowest turnover rates were involved in protein self-association and cytoskeleton binding (Figure 4.4). In F5 cooling phase, patients with pain had a reduced level of proteins with arylesterase activity whereas in F6 recovery phase there was increased level of proteins involved in polyubiquitin binding.

Enrichment of GOBP terms in muscle pain (F4 phase)



Enrichment of GOBP terms in muscle pain (F5 phase)



Enrichment of GOBP terms in muscle pain (F6 phase)

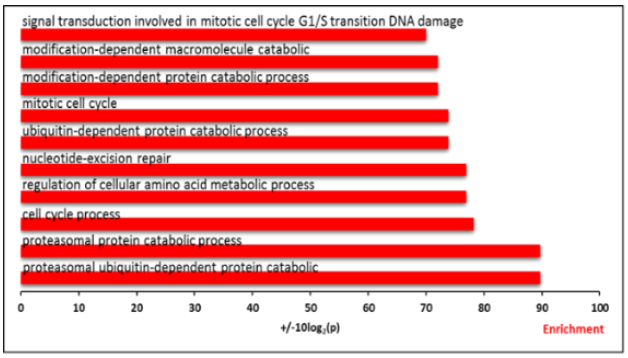


Figure 4.4: Functional enrichment analysis of proteome in DM2 patients with muscle pain.

Enrichment in GOBP terms for F4 (room temperature), F5 (cooling) and F6 (recovery) phase are listed for pathways with $p<0.01$.

Interestingly, we observed strong enrichment of proteins involved in ketone body metabolism in DM2 patients without pain compared to patients with pain (Figure 4.5). The enrichment of ketone body metabolism in DM2 without pain was significant during F5 cooling phase (*P*=0.0062462; Enrichment factor = 4.848).

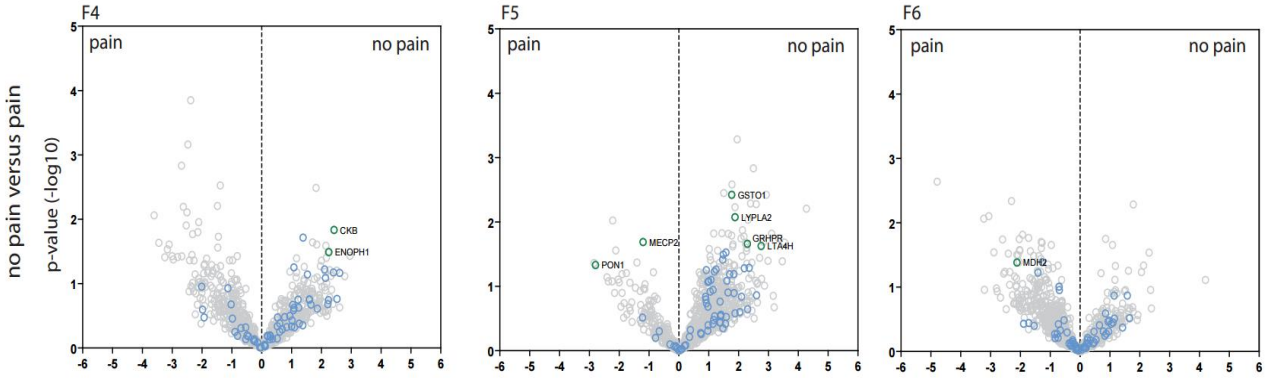


Figure 4.5. Vulcano plot for enrichment of ketone body metabolism in DM2 without muscle pain.

Blue dots in volcano plot are proteins with GOBP annotation for ketone body metabolism. Green dots in volcano plots are proteins with GOBP annotation for ketone body metabolism which were significantly upregulated in pain or no pain group (p value < 0.05; T-test difference >log2 of ± 1.75). Proteins in pain group are shown on left side while proteins in no pain group are shown on the right side of volcano plot. P values (-log10) are plotted on Y-axis and t-test difference values are plotted on X-axis.

Out of 909 proteins, 22 secreted proteins A1BG, ANG, APOC2, APOD, ARF1, C1QB, C8G, CTSD, CTSG, C9, CFB, CFH, CFHR1, CFHR3, CPN2, DAG1, DCD, DEFA1, DPT, EFEMP1, F9 and FN1 had significantly different level between DM2 patients with and without pain (Table 4.11). Among 22 differentially secreted proteins, 9 were regulated at room temperature (F4 phase), 9 proteins during cooling (F5 phase) and 4 proteins at room temperature after cooling (F6 phase). Proteins with highest upregulation in DM2 muscle pain group were CFHR3 (F4 phase), DPT (F5 phase) and CPN2 (F6 phase) while downregulation of proteins in patients with pain was observed for CFB (F4 phase), CTSD (F5 phase) and APOD (F6 phase).

Gene name	Protein name	T-test difference nopain vs pain (F4)	P-value nopain vs pain (F4)	T-test difference nopain vs pain (F5)	P-value nopain vs Pain (F5)	T-test difference nopain vs pain (F6)	P-value nopain vs pain (F6)
A1BG	**Alpha-1B-glycoprotein**	**-2.50207**	**0.007816948**	0.652277143	0.300522866	-0.388760476	0.712547493
ANG	**Angiogenin**	0.940485714	0.317343213	**1.771021429**	**0.003781146**	-0.082136905	0.915393002
APOC2	**Apolipoprotein C-2**	**-2.686761429**	**0.001465719**	-0.248415714	0.69982463	-0.578453095	0.409361592
APOD	**Apolipoprotein D**	1.763454286	0.084481154	0.587377143	0.210872367	**2.315968333**	**0.029159354**
ARF1	**ADP-ribosylation factor 1**	**2.007665714**	**0.043979163**	0.030685714	0.979184197	-1.03655619	0.423500457
C1QB	**Complement C1q subcomponent subunit B**	-1.016667143	0.141390964	**2.233118571**	**0.021805036**	-0.933032619	0.406394595
C8G	**Complement component C8 gamma chain**	**-2.37901**	**0.000142**	0.96006	0.10257	0.00828	0.982979
CTSD	**Cathepsin D**	0.093981429	0.953950397	**2.707754286**	**0.036123752**	1.936937381	0.271930317
CTSG	**Cathepsin G**	-2.0634	0.165364177	0.63702	0.589303183	**2.128955238**	**0.046156683**
C9	**Complement component C9**	-0.134018571	0.829924181	**2.294882857**	**0.021857954**	0.113914524	0.872719156
CFB	**Complement factor B**	**2.152025714**	**0.025768096**	-0.182191429	0.835339632	0.315234286	0.797380731
CFH	**Complement factor H**	-1.34065	0.157388865	**2.11878**	**0.033979**	-0.772508095	0.481267998
CFHR1	**Complement factor H-related protein 1**	-0.817338571	0.216744626	**1.955738571**	**0.000520154**	-0.539685714	0.509239077
CFHR3	**Complement factor H-related protein 3**	**-3.246462857**	**0.038879118**	-1.259547143	0.377300995	-2.043612143	0.203652754
CPN2	**Carboxypeptidase N subunit 2**	-0.294822857	0.825046828	0.037532857	0.974866185	**-3.05715**	**0.00792184**
DAG1	**Dystroglycan**	-0.471042857	0.636769578	-0.766991429	0.434091288	**-2.40334381**	**0.030255388**
DCD	**Dermcidin**	**-2.676808571**	**0.037582706**	0.88187	0.126623648	-2.194144286	0.068545804
DEFA1	**Neutrophil defensin 1**	**-3.162278571**	**0.029373488**	-1.252545714	0.244382062	-2.017752857	0.155909478
DPT	**Dermatopontin**	-2.009255714	0.100971248	**-2.112432857**	**0.027652695**	-1.80891881	0.132788279
EFEMP1	**EGF-containing fibulin-like extracellular matrix protein 1**	**-2.960865714**	**0.042033593**	-0.772942857	0.493806164	-1.252595238	0.356897719
F9	**Coagulation factor 9**	-0.497191429	0.637477266	**2.592181429**	**0.005298553**	-0.49719881	0.655813236
FN1	**Fibronectin**	-0.100618571	0.919066417	**2.181228571**	**0.016747661**	-0.26929381	0.825631995

Table 4.11: List of differentially secreted proteins based on presence of muscle pain.
No pain vs pain. P value < 0,05; T-test difference >log2 of ± 1.75. Positive t-test difference indicates increase while negative t-test difference indicates decrease in the protein level. F4 phase (room temperature); F5 phase (cooling); F6 phase (recovery, room temperature).

Functional enrichment analysis of Gene Ontology (GO) was performed to check which biological processes (GOBP), molecular functions (GOMF) and cellular localization (GOCC) characterize differentially secreted proteins based on the presence of muscle pain (Table 4.12). From 22 differentially secreted proteins, 12 proteins are involved in the regulation of immune system (CFB, CFHR1, C8G, CTSG, FN1, ANG, C1QB, C9, ARF1, CFH, DEFA1 and CTSD). Six of

them are known to play a role in the complement activation (CFB, CFHR1, C8G, C1QB, C9 and CFH). Five secreted proteins have the serine-type endopeptidase activity (CFB, CTSG, C1QB, F9 and CTSD).

GO biological process complete	#	#	expected	Fold Enrichment	+/-	P value
complement activation, alternative pathway	13	4	.01	> 100	+	1.07E-05
↳innate immune response	622	7	.68	10.26	+	2.70E-02
↳immune response	1130	9	1.24	7.26	+	1.30E-02
↳immune system process	2056	12	2.25	5.32	+	3.13E-03
↳defense response	1260	10	1.38	7.24	+	2.81E-03
↳complement activation	86	6	.09	63.62	+	3.75E-06
↳positive regulation of immune response	595	7	.65	10.73	+	2.02E-02
↳immune effector process	501	8	.55	14.56	+	3.13E-04
↳protein activation cascade	109	7	.12	58.56	+	1.94E-07
↳humoral immune response	187	8	.21	39.01	+	1.44E-07
killing of cells of other organism	26	4	.03	> 100	+	1.70E-04
↳cell killing	56	4	.06	65.13	+	3.59E-03
↳disruption of cells of other organism	26	4	.03	> 100	+	1.70E-04
↳modification of morphology or physiology of other organism	105	5	.12	43.42	+	8.15E-04
regulation of complement activation	33	4	.04	> 100	+	4.40E-04
↳regulation of acute inflammatory response	76	4	.08	47.99	+	1.20E-02
↳regulation of humoral immune response	51	4	.06	71.52	+	2.48E-03
↳regulation of protein activation cascade	35	4	.04	> 100	+	5.56E-04
↳regulation of protein processing	84	4	.09	43.42	+	1.78E-02
↳regulation of protein maturation	85	4	.09	42.91	+	1.86E-02
defense response to fungus	37	4	.04	98.58	+	6.93E-04
↳response to fungus	53	4	.06	68.82	+	2.88E-03

GO molecular function complete	#	#	expected	Fold Enrichment	+/-	P value
serine-type endopeptidase activity	235	5	.26	19.40	+	1.34E-02
↳serine-type peptidase activity	262	5	.29	17.40	+	2.26E-02
↳serine hydrolase activity	265	5	.29	17.20	+	2.39E-02

GO cellular component complete	#	#	expected	Fold Enrichment	+/-	P value
membrane attack complex	7	2	.01	> 100	+	3.62E-02
blood microparticle	139	10	.15	65.60	+	2.24E-13
↳extracellular region part	3825	22	4.19	5.24	+	1.35E-12
↳extracellular region	4539	22	4.98	4.42	+	5.61E-11
↳extracellular space	1424	21	1.56	13.45	+	8.42E-20
basement membrane	99	4	.11	36.84	+	5.29E-03
↳extracellular matrix	522	9	.57	15.72	+	2.82E-06
↳extracellular matrix component	132	4	.14	27.63	+	1.63E-02
extracellular exosome	2735	22	3.00	7.33	+	8.96E-16
↳extracellular vesicle	2749	22	3.01	7.30	+	1.00E-15
↳vesicle	4024	22	4.41	4.99	+	4.09E-12
↳extracellular organelle	2750	22	3.02	7.29	+	1.01E-15

Table 4.12: GO annotations of differentially secreted proteins between DM2 with and without muscle pain.

We also analyzed the changes in secreted protein levels during F4-F5 (room temperature-cooling) and F5-F6 (cooling-room temperature) phase transitions (Table 4.13). During F4-F5 transition, there was a significant change in level of A1BG, APOC2, APOD, C1QB in DM2 with muscle pain and CFB, CFH, CFHR1, CFHR3, CFL1, CKB, DCD in patients without pain. For F5-F6 transition, we observed a significant change in level of APOD and HMGB1 in patients with muscle pain and CFH, CFHR1, CLC, CLEC3B, CPB2, DCD in DM2 without pain.

Transition F4-F5							
Muscle pain				**No pain**			
Gene name	**Protein name**	**T-test difference F4 vs F5**	**P-value F4 vs F5**	**Gene name**	**Protein name**	**T-test difference F4 vs F5**	**P-value F4 vs F5**
A1BG	Alpha-1B-glycoprotein	2.7130484	0.0028012	CFB	Complement factor B	2.3087398	0.0131479
APOC2	Apolipoprotein C-II	1.7705748	0.0240346	CFH	Complement factor H	-2.406864	0.016743
APOD	Apolipoprotein D	-1.975851	0.0118859	CFHR1	Complement factor H-related protein 1	-2.11548	0.0022552
C1QB	Complement C1q subcomponent subunit B	2.5341361	0.0177825	CFHR3	Complement factor H-related protein 3	-2.070097	0.0230128
				CFL1	Cofilin-1	-2.758995	0.0054389
				CKB	Creatine kinase B-type	-2.345042	0.0309408
				DCD	Dermcidin	-1.801797	0.0357017
Transition F5-F6							
Muscle pain				**No pain**			
Gene name	**Protein name**	**T-test difference F5 vs F6**	**P-value F5 vs F6**	**Gene name**	**Protein name**	**T-test difference F5 vs F6**	**P-value F5 vs F6**
APOD	Apolipoprotein D	2.4512245	0.0206226	CFH	Complement factor H	2.7029411	0.006646
HMGB1	High mobility group protein B1	-1.797024	0.0482424	CFHR1	Complement factor H-related protein 1	2.5019727	0.0009737
				CLC	Eosinophil lysophospholipase	2.5448489	0.0479037
				CLEC3B	Tetranectin	2.7706612	0.0366963
				CPB2	Carboxypeptidase B2	-5.255737	0.0319357
				DCD	Dermcidin	1.9768184	0.0399381

Table 4.13: Differentially secreted proteins during phase transitions (F4-F5 and F5-F6).
P value < 0,05; T-test difference >log2 of ± 1.75. Positive t-test difference indicates increase while negative t-test difference indicates decrease in the protein level. F4 phase (room temperature); F5 phase (cooling); F6 phase (recovery, room temperature).

Finally, we determined the average of LFQ intensities for all 3 phases of microdialysis (F4, F5 and F6) and 2 secreted proteins DEFA1 and DPT had siginificantly increased average level in DM2 patients with pain compared to DM2 without pain (Table 4.14).

Gene name	Protein name	Mean LFQ Nopain F4	Mean LFQ Pain F4	Mean LFQ Nopain F5	Mean LFQ Pain F5	Mean LFQ Nopain F6	Mean LFQ Pain F6	Mean LFQ no pain (F4-F6)	Mean LFQ pain (F4-F6)	Mean T-test difference Pain vs nopain (F4-F6)	P-value
A1BG	Alpha-1B-glycoprotein	18.412	20.914	18.854	18.201	18.540	18.928	18.602	19.348	0.746	ns
ANG	Angiogenin	22.089	21.148	22.639	20.868	21.250	21.333	21.993	21.116	-0.876	ns
APOC2	Apolipoprotein C-2	17.538	20.224	18.205	18.454	17.871	18.450	17.872	19.043	1.171	ns
APOD	Apolipoprotein D	21.103	19.339	21.902	21.315	21.180	18.864	21.395	19.839	-1.556	ns
ARF1	ADP-ribosylation factor 1	21.626	19.618	20.269	20.238	18.502	19.539	20.132	19.799	-0.334	ns
C1QB	Complement C1q subcomponent subunit B	21.271	22.288	21.987	19.754	20.718	21.651	21.325	21.231	-0.094	ns
CTSD	Cathepsin D	20.141	20.047	20.769	18.061	20.519	18.582	20.476	18.897	-1.580	ns
CTSG	Cathepsin G	19.825	21.888	21.649	21.012	22.008	19.879	21.161	20.926	-0.234	ns
C9	Complement component C9	19.064	19.198	19.743	17.448	18.358	18.244	19.055	18.297	-0.758	ns
CFB	Complement factor B	21.340	19.188	19.031	19.214	20.363	20.048	20.245	19.483	-0.762	ns
CFH	Complement factor H	19.268	20.608	21.675	19.556	18.972	19.744	19.971	19.969	-0.002	ns
CFHR1	Complement factor H-related protein 1	18.461	19.278	20.576	18.620	18.074	18.614	19.037	18.837	-0.200	ns
CFHR3	Complement factor H-related protein 3	19.053	22.299	21.123	22.382	21.979	24.022	20.718	22.901	2.183	ns
CPN2	Carboxypeptidase N subunit 2	19.793	20.088	19.175	19.137	17.788	20.845	18.919	20.024	1.105	ns
DAG1	Dystroglycan	21.060	21.531	20.738	21.505	19.641	22.045	20.480	21.694	1.214	ns
DCD	Dermcidin	19.947	22.624	21.749	20.867	19.772	21.966	20.489	21.819	1.330	ns
DEFA1	Neutrophil defensin 1	**19.344**	**22.506**	**21.398**	**22.651**	**20.917**	**22.934**	**20.553**	**22.697**	**2.144**	**0.0278**
DPT	Dermatopontin	**19.968**	**21.978**	**20.105**	**22.218**	**19.511**	**21.320**	**19.862**	**21.838**	**1.977**	**0.0036**
EFEMP1	EGF-containing fibulin-like extracellular matrix protein 1	19.797	22.758	21.184	21.957	21.749	23.002	20.910	22.572	1.662	ns
F9	Coagulation factor 9	19.212	19.709	20.974	18.382	19.554	20.051	19.913	19.381	-0.533	ns
FN1	Fibronectin	19.933	20.034	20.583	18.402	19.780	20.050	20.099	19.495	-0.604	ns

Table 4.14: Average LFQ intensities for differentially secreted proteins in DM2 with and without pain.

P value < 0,05; T-test difference >log2 of ± 1.75. Positive t-test difference indicates increase while negative t-test difference indicates decrease in the protein level. F4 phase (room temperature); F5 phase (cooling); F6 phase (recovery, room temperature); ns=not significant.

4.5 No association of inflammatory cytokines IL-1, IL-6 and NGF with muscle pain in DM2 patients

The measured concentrations of known inflammatory cytokines IL-1, IL-6 and NGF with SRM were below 2 fmol/µL in most of the samples (Figure 4.6). Outliers were present mostly in DM2 patients with muscle pain. SRM

measurement of all 3 cytokines did not reveal significant differences in the concentration and ratio of light and heavy standard peptides between DM2 patients with and without muscle pain (P-value > 0.05).

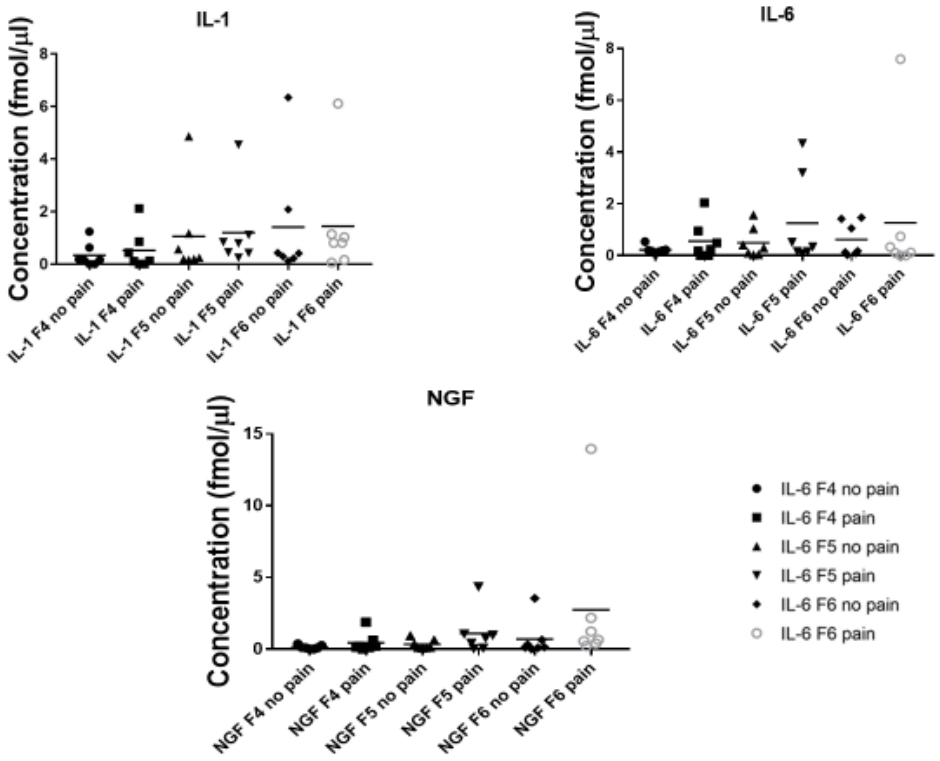


Figure 4.6: SRM concentrations of known inflammatory cytokines in DM2.
Interleukin-1 (IL-1); Interleukin-6 (IL-6); Nerve growth factor (NGF); F4 phase (room temperature); F5 phase (cooling); F6 phase (recovery, room temperature).

4.6 Anti-inflammatory eicosanoid 11,12-DHET present only in DM2 without pain

LC-MS/MS analysis of DM2 interstitial fluid from 12 DM2 patients (6 with and 6 without muscle pain) identified 58 eicosanoids out of 80 eicosanoids measured (Table 4.15). We identified 21 derivates of arachidonic acid, 21 derivates of eicosapentaenoic acid, 12 derivates of docosahexaenoic acid and 5 derivates of linoleic acid. There was no difference (P-value > 0.05) between DM2 with and without pain for any of measured eicosanoids, both for inflammatory and anti-inflammatory eicosanoids (Figure 4.7). Interstingly, we detected anti-inflammatory eicosanoid 11,12-DHET only in 3 DM2 patients without pain (2 females and 1 male) while it was not present in any DM2 with pain (Table 4.16).

	Arachidonic acid	***Eicosapentaenoic acid***	***Docosahexaenoic acid***	***Linoleic acid***
LOX	5-HETE 12-HETE 15-HETE LTB_4 LXA_4 (n.d)	5-HEPE 12-HEPE LTB_5 Resolvin E1 15-HEPE 18-HEPE	4-HDHA 7-HDHA 16-HDHA 10-HDHA 13-HDHA 14-HDHA 17-HDHA (n.d) 20-HDHA 10,17-DiHDHA (n.d) Resolvin D1 (n.d)	13-HODE
CYP	5,6-EET 8,9-EET 11,12-EET 14,15-EET 5,6-DHET 8,9-DHET 11,12-DHET 14,15-DHET 19-HETE 20-HETE	5,6-DiHETE 8,9- DiHETE 11,12- DiHETE 14,15- DiHETE 17,18-DiHETE 8,9-EEQ 11,12-EEQ 14,15-EEQ 17,18-EEQ 19-HEPE 20-HEPE	7,8-EDP 10,11-EDP 13,14-EDP 16,17-EDP 19,20-EDP 7,8-DiHDPA (n.d) 10,11-DiHDPA (n.d) 13,14-DiHDPA (n.d) 16,17-DiHDPA (n.d) 19,20-DiHDPA (n.d) 21-HDHA (n.d) 22-HDHA (n.d)	9,10-EpOME 12,13-EpOME 9,10-DiHOME 12,13-DiHOME
COX	PGH_2 (n.d) PGD_2 PGE_2 PGE_1(n.d) 15-keto-PGE_2 (n.d) $PGF_2\alpha$ (n.d) d17-6-keto- $PGF_1\alpha$ (n.d) 2,3-dinor-6-keto-$PGF_1\alpha$ TXB_2 (n.d) 11-dehydro-TXB_2 (n.d) PGJ_2 (n.d) 15-deoxy-delta-12,14-PGJ_2 (n.d)	PGE_3 (n.d) d17-6-keto-$PGF_1\alpha$ TXB_3 (n.d) 11-dehydro-TXB_3		
autoxidative metabolites	8-HETE 9-HETE 11-HETE	8-HEPE 9-HEPE		

Table 4.15: Profile of measured eicosanoids in DM2 intersticial fluid.

n.d.=not detectable

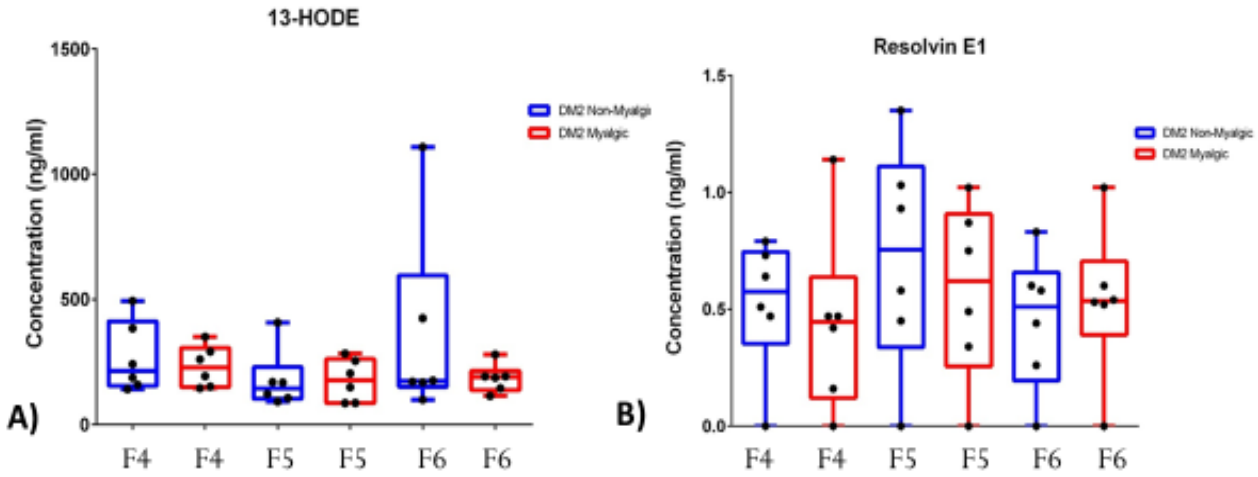


Figure 4.7: Eicosanoid profiles in DM2 based on onset of myalgia.

A) Inflammatory eicosanoid 13-HODE; B) Anti-inflammatory eicosanoid Resolvin E1.

			11,12-DHET (ng/ml)					
Patient	**Gender**	**Muscle pain**	**F4**	**F5**	**F6**	**P-value (F4)**	**P-value (F5)**	**P-value (F6)**
PROMM A	F	No	0.05	0.06	0.15	0.0780	0.1871	0.1918
PROMM J	F	No	0.00	0.00	0.00			
PROMM K	F	No	0.03	0.02	0.06			
PROMM D	M	No	0.08	0.22	0.00			
PROMM F	M	No	0.00	0.00	0.00	**Mean F4 No pain**	**Mean F5 No pain**	**Mean F6 No pain**
PROMM G	M	No	0.00	0.00	0.00	0.03	0.05	0.03
PROMM E	F	Yes	0.00	0.00	0.00			
PROMM I	F	Yes	0.00	0.00	0.00			
PROMM L	F	Yes	0.00	0.00	0.00			
PROMM B	M	Yes	0.00	0.00	0.00			
PROMM C	M	Yes	0.00	0.00	0.00	**Mean F4 Pain**	**Mean F5 pain**	**Mean F6 Pain**
PROMM H	M	Yes	0.00	0.00	0.00	0.00	0.00	0.00

Table 4.16: Concentration of 11,12-DHET in DM2 with and without pain.

F4 phase (room temperature); F5 phase (cooling); F6 phase (recovery, room temperature).

4.7 Correlation between RNA sequencing and proteome data

There was no correlation between RNA sequencing and LFQ proteome data. 14 differentially expressed genes based on onset of muscle pain from RNA sequencing of muscle biopsy specimens were not identified with sufficient intensity in LFQ proteome analysis of DM2 interstital fluid. None of 22 differentially secreted proteins were differentially expressed in RNA sequencing DM2 cohort with and without muscle pain (Table 4.17). Fifteen differentially secreted proteins were expressed in DM2 muscle (total gene reads >10).

Gene Name	P-value (No-Myalgia vs Myalgia)	Fold change (No-Myalgia vs Myalgia)	FDR P-value correction	No Myalgia Total Gene Reads (mean)	Myalgia Total Gene Reads (mean)
APOD	0.2	-1.283	1	14503	12061.83
ARF1	0.84	-1.04	1	8451.5	8693.5
CTSD	0.941	1.014	1	8422.167	9208.167
FN1	0.837	-1.041	1	7716.167	7867.5
DAG1	0.419	1.17	1	7448.667	9306.667
DPT	0.192	-1.289	1	3152	2645.167
CFH	0.367	-1.192	1	2814	2515.333
EFEMP1	0.221	-1.269	1	2005	1667.167
C1QB	0.811	-1.048	1	791.5	806.6667
CTSG	0.1809	-2.137	0.04	199	98
C8G	0.878	1.032	1	134.5	151.1667
C9	0.855	-1.039	1	83.83333	87.33333
CFB	0.014	1.669	0.782	51.33333	95.66667
ANG	0.132	-1.385	1	45.83333	34.83333
A1BG	0.819	1.063	1	24.16667	28.33333
CFHR1	0.403	-1.324	1	6.666667	5.333333
DEFA1	0.33	-1.45	1	5.166667	3.833333
APOC2	0.396	1.476	1	2.333333	3.833333
CPN2	0.23	-2	1	2.333333	1.166667
CFHR3	0.047	4.732	1	0.166667	1.333333
DCD	0.998	11.382	0.637	0	1.333333
F9	1	1	1	0	0

Table 4.17: Gene expression profile of differentially secreted proteins in DM2.

5. Discussion

Studying of chronic pain mechanisms and development of effective pain medication is hindered by complex etiology of chronic pain. In addition to genetic risk factors and heritability, there are different demografic and environmental factors associated with chronic pain such as lower pain thresholds in females, increased onset of chronic pain in older age, higher incidence of chronic pain in people with history of abuse and violence, greater pain sensitivity in patients with lower economic income and increased onset of pain due to lack of sunshine and lower temperatures (van Hecke et al, 2013). Stratification of patients with chronic pain for clinical trials is further complicated by differences on the molecular, cellular and neuronal level between the patients which lead to different pain sensitivity, high rate of placebo responses and the absence of clinically validated biomarkers for pain (Dib-Hijj and Waxmann, 2014). In recent years, rare monogenetic pain disorders are recognized as a model to identify pain pathogenic mechanisms due to a strong genotype-phenotype correlation especially among the families with both symptomatic and asymptomatic family members (Bennet and Woods, 2014). Emerging high-throughput Omics technologies made it possible to screen for novel pain targets and to tailor new and more effective therapies for patients with chronic pain (Antunes-Martins et al, 2013; Dib-Hajj and Waxmann, 2014). Based on this, we decided to study the mechanism of chronic muscle pain by performing Omics screen in patients with myotonic dystrophy type 2 (DM2). DM2 is a promising model for investigating muscle pain since it is a monogenetic disorder, patients frequently develop widespread chronic muscle pain and they have similar socio-demografic and environmental backgrounds (George et al, 2004; Suokas et al, 2012). We hypothesized that since DM2 is a spliceopathy caused by the expansion of CCTG repeats in *CNBP* encoding for a transcription factor (Liquori et al, 2001; Perfetti et al, 2014), this could lead to missregulation of gene expression and splicing in pain regulating genes. Peripheral nociception is mediated by different types of ion channels expressed at peripheral terminals of nociceptors and some types of ion channels were found to be misspliced in myotonic dystrophies (Gangadharan and Kuner, 2013;

Charlet-B et al., 2002; Tang et al., 2012). Certain myokines secreted from DM2 affected muscles could also contribute to the onset of muscle pain. Additionally, hyperlipidemia and insulin resistence are common in DM2 patients suggesting that elevated endogenous lipid levels could contribute to muscle pain in DM2 (Heatwole et al, 2011, Piomelli and Sasso, 2014).

5.1 Somatosensory profiling of muscle pain in DM2

Somatosensory profiling can be used in pain clinical trials to reduce the heterogeneity and to group the patients in homogenous pain subgroups which makes it easier to identify the underlying pathophysiological mechanisms and to tailor the effective pain therapies (Baron et al, 2012; von Hehn et al, 2012). Quantitative sensory testing (QST) is a standardized method for somatosensory profiling in the patients with chronic pain which makes it possible to distinguish between localized and generalized pain and peripheral and central nociceptive mechanisms (Rolke et al, 2006). QST profiles were previously obtained for the patients with muscle disorders that have an onset of chronic muscle pain such as fibromyalgia and chronic lower back pain which revealed shared and distinct pathophysiological mechanisms (Pfau et al., 2009). Thus, we performed QST profiling of muscle pain in our DM2 cohort in order to examine wheather muscle pain in DM2 is caused by peripheral or central mechanisms. QST data found decreased pressure pain thresholds (PPTs) in DM2 patients with muscle pain indicating that the pain might be triggered by the peripheral mehanisms within the muscle (Graven-Nielsen and Arendt-Nielsen, 2010). Decreased PPTs were previously reported in DM2 patients compared to patients with chronic noninflammatory muscle disorders (George et al., 2004). We also observed a slight increase in wind-up ratio (WUR) in DM2 patients with muscle pain compared to DM2 without pain. Since WUR is a QST measurement of temporal summation, these results show that some DM2 patients with pain develop central sensitization which might be induced by prolonged peripheral nociception (Graven-Nielsen and Mense, 2001; Lewin et al, 2014). Interstingly, we also observed allodynia in one patient with muscle pain and significantly decreased mechanical detection thresholds in all DM2 patients. Complete QST profiling of muscle pain in DM2 might help to distinguish muscle pain in DM2

from fibromyalagia which is important considering that DM2 patients are frequently misdiagnosed as fibromyalgic patients (Auvinen et al., 2008). We did not observe increased mechanical and thermal hypersensitivity in DM2 patients which is characteristic for fibromyalgia while elevated WURs were detected both in DM2 and fibromyalgia (Staud et al, 2001; Blumenstiel et al., 2011). It should be further examined whether observed differencies in somatosensory profiles are the result of different pain processing mechansims or they might be related to disease progression.

5.2 Distinct DM2 muscle transcriptome profiles based on onset of muscle pain

Transcriptomic profiling of DM2 muscle biopsy specimens identified 14 differentially expressed genes in the skeletal muscle based on the presence of muscle pain. Some of them were previously associated with pain regulation. Monoamine oxidase gene (MAOA) was investigated in fibromyalgic clinical trial were specific MAOA inhibitor moclobemide was administered to female patients with fibromyalgia (Hannonen et al., 1998). Moclobemide significantly reduced muscle pain in fibromyalgic patients at the end of a 12 weeks intervention compared to placebo group. Polymorphisms in MAOA were associated with increased cortical pain processing and with post-surgical pain (Di Lorenzo et al., 2014; Kim et al., 2006). Increased gene expression of MAOA in human muscle biopsies was previously associated with insulin resistence (Mason et al., 2011; Elgzyri et al., 2012). Regarding insulin resistence which is frequently observed in DM2, there was upregulation of growth factor receptor-bound protein 14 (GRB14) in DM2 patients with muscle pain which is a known selective inhibitor of insulin receptor (Depetris et al., 2005). DM2 with muscle pain had increased expression of creatine transporter SLC16A12 which might be associated with muscle wasting and inflammatory pain (Abplanalp et al., 2013). Small molecule inhibitor is already identified for 6-phosphofructo-2-kinase (PFKFB2) enzyme which should facilitate the functional examination of this differentially expressed gene in the animal models (Telang et al., 2012). DM2 patients that were recruited for transcriptomic profiling did not show any difference in age, medication at the time of biopsy and histopathological findings regardless of the onset of muscle pain. Thus, we do not have any evidence that

the differences in the transcriptome of DM2 patients with and without muscle pain are based on anything else besides the presence of muscle pain. Identified differentially expressed genes based on the onset of muscle pain in DM2 require further functional characterization to validate if they could be used as effective biomarkers for chronic muscle pain and to investigate their involvement in pathogenesis of other common conditions with muscle pain. The future studies should consider recruiting the bigger cohort of the patients which might increase the sensitivity of transcriptomic profiling and allow detection of higher number of pain candidate genes which were slightly below the significance threshold in the current study.

5.3 Differences in DM2 muscle secretome associated with muscle pain

Shotgun LFQ proteomic profiling of DM2 interstitial fluid detected 85 differentially regulated proteins based on the presence of muscle pain. A large fraction of differentially regulated proteins were intercellular proteins which indicates the possible leakage of intracellular content due to damaged muscle fibers during insertion of microdialyis catheter to the muscle. Out of 85 proteins, 22 proteins were differentially secreted proteins in DM2 patients with and without muscle pain. Some of the secreted proteins were previously associated with pain regulation. Alpha-1B-glycoprotein (A1BG) was elevated in urine LC-MS proteome analysis of 10 female patients with chronic pain (Goo et al., 2010). There was overexpression of apolipoprotein D (APOD) in rat DRG neurons following peripheral nerve injury (Kim et al., 2001). Complement C1q (C1QB) was upregulated on mRNA level in the cortex of a mouse model for chronic inflammatory pain (Poh et al., 2012). Rats with persistent hyperalgesia had increased level of cathepsin G (CTSG) in spinal cord neurons (Liu et al., 2015). Patients with chronic low back pain had elevated plasma levels of complement factor B (CFB) in LC-MS proteome analysis (Ghafouri et al., 2016). Complement factor H (CFH) was upregulated in serum LC-MS proteome analysis of patients with Legg-Calve-Perthes disease which is an osteonecrosis of the developing femoral head complicated by pain (Liu et al., 2015). Neutrophil defensin 1 (DEFA1) was increased on mRNA level in patients with abdominal pain compared to patients with appendicitis (Chawla et al., 2016). Finally, it was

reported that fibronectin (FN1) inhibits the development of chronic pain in rats after spinal cord injury (Lin et al., 2012). Remarkably, six differentially secreted proteins (CFB, CFHR1, C8G, C1QB, C9 and CFH) were part of the complement system which facilitates the migration of neutrophils and monocytes to the inflammed tissue and might contribute to inflammatory pain (Frank and Fries, 1991). Complement C5a is known to stimulate the release of histamine and TNF-α and it contributes to post-surgical pain in mice (Jang et al, 2011).

Furthermore, we observed a big change in the protein level after cooling (F5) microdialysis phase for most of the regulated proteins. The recovery of proteins after cooling and extracellular concentrations might be influenced by different factors such as flow rate, uptake of solutes to the cells, rapid fluctuations in blood temperature and perfusion (Mendelowitsch et al, 1998). The ideal protein candidate for the functional analysis of pain biomarkers should have the constant level based on the onset of muscle pain with minimal changes in concentration during microdialysis protocol as it was observed for dermatopontin (DPT), DEFA1, complement factor H-related protein 3 (CFHR3) and EGF-containing fibulin-like extracellular matrix protein 1 (EFEMP1). We also detected some additional regulated secreted proteins after analysing the changes in protein level during F4-F5 (room temperature-cooling) and F5-F6 (cooling-room temperature) phase transitions which might modulate the onset of muscle pain in DM2. The enrichment analysis of DM2 proteome data revealed the enrichment of proteins involved in ketone body metabolism in DM2 patients. Considering that ketogenic diet reduces inflammatory pain in rats (Ruskin et al, 2009), this finding indicates that ketogenic diet might have a potential to decrease the muscle pain levels in DM2.

Since known mediators of inflammatory pain IL-1, IL-6 and NGF have low abundance in the physiological conditions, we decided to use serial reaction monitoring (SRM) to measure their association with muscle pain in DM2 (Samad et al, 2001; Stein et al, 2009; Sakuma et al, 2016). Suprisingly, we did not find the significant difference in the level of IL-1, IL-6 and NGF based on the onset of muscle pain in DM2. Considering that SRM has greater sensitivity and reproducibility for detection of low abundant proteins compared to high-

throughput immunoassays such as the enzyme-linked immunosorbent assay (Shi et al, 2012), this finding provides a strong evidence that known chronic pain regulators IL-1, IL-6 and NGF do not contribute to the muscle pain in DM2 patients.

5.4 The role of lipids in the onset of muscle pain in DM2

Elevated levels of lipids and insulin resistence are common features of DM2 pathophyisiology (Heatwole et al, 2011). Insulin resistence might be a result of missplicing of insulin receptor described in myotonic dystrophies (Savkur et al, 2001). Considering that endogenous lipids are known mediators of peripheral nociception and regulators of inflammatory pain (Alsalem et al, 2013), we performed extensive lipidomic LC-MS/MS profiling of DM2 interstitial fluid. We did not find any significant difference in the level of 58 eicosanoid derivates from arachidonic, eicosapentaenoic, docosahexaenoic and linoleic acid based on the presence of muscle pain in DM2. However, we detected 11,12-dihydroxy-5Z,8Z,14Z-eicosatrienoic acid (11,12-DHET) only in 3 DM2 patients without muscle pain. 11,12-DHET is a cytochrome P450 (CYP) derivate of arachidonic acid which was previously shown to induce anti-inflammatory effects by inhibition of TNF-α signalling that might protect DM2 patients from developing muscle pain (Node et al, 1999). In general, we detected high patient-to-patient variation in the level of lipids for both groups of DM2 patients. Thus, for the future lipidomic studies of chronic pain, it would be required to take in account the dietary habits of the patients in order to group patients in more homogenous subgroups based on the metabolism of lipids. Lipidomic studies should ideally be performed with the patients of approximately same age since it is known that basal metabolic rate declines with aging at the rate of 1-2% per decade which also might have an effect on the lipid metabolism (Roberts and Rosenberg, 2006). Similar to ketogenic diet intervention, nuticionistic addition of 11,12-DHET to diet of DM2 patients should be considered for a clinical trial in future in order to decrease muscle pain in DM2.

5.5 Correlation between DM2 transcriptome and proteome profiling of muscle pain

Correlation between the transcriptome and proteome data depends on different biological and technical factors and recent studies concluded that the correlation is usually poor (de Sousa et al, 2009; Maier et al, 2009). Global quantification of mammalian gene expression control revealed that the protein abundance is predominantly regulated by translational control and genes with similar combinations of mRNA and protein half-life share similar functional properties (Schwanhäusser et al, 2011). Translational efficiency depends on mRNA structure, regulatory proteins, ribosomal density, ribosomal occupancy and protein half-life and it can influence mRNA-protein correlation (Marier et al, 2009). We did not observe any correlation between DM2 transcriptome and proteome data sets. Genes encoding for differentially secreted proteins in DM2 based on the onset of muscle pain were not among differentially expressed genes in DM2 transcriptome. Similarly, differentially expressed genes based on the onset of muscle pain were not detected with sufficient intensity in shotgun MS proteomics. This lack of correlation between DM2 transcriptome and proteome data is not surprising considering that the biological samples used in the experiments were different (muscle biopsy specimens in RNA sequencing and muscle interstitial fluid in MS proteomic analysis), the patient cohorts were not completely identical and experimental conditions are hard to compare.

5.6 Relevance of the study

This study provides the first mechanistic insight to chronic muscle pain, the frequent clinical symptom with unknown cause. Decreased pressure pain thresholds in DM2 patients with muscle pain indicate that the cause of muscle pain is within the muscle. This finding is further supported by distinct muscle transcriptome and proteome profiles between DM2 patients with and without muscle pain. Identified differentially expressed genes and differentially secreted proteins based on the onset of muscle pain in DM2 are potential biomarkers for chronic muscle pain and they present novel pain targets which should be further functionally examined in order to develop more effective and patient tailored

pain therapeutics. In addition, we observed the increased level of anti-inflammatory lipids and proteins involved in ketogenic signaling in DM2 patients without pain which suggests that diatery interventions might lead to pain relief in DM2.

Our project had some limitations that should be avoided in the next clinical trials of chronic pain. Sensory and molecular phenotyping of muscle pain were not performed at the same time. The future studies should consider performing sensory and molecular profiling of muscle pain on the same day in order to make a direct correlation between the changes of sensory phenotypes and molecular changes in patients with muscle pain. Sample size used for Omics experiments was small since DM2 is a rare disorder. Increasing of the sample size should significantly improve the sensitivity of molecular pain profiling.

Overall, this study shows that the analysis of somatosensory and molecular pain profiles in homogenous groups of DM2 patients based on the onset of muscle pain can identify novel targets for pain therapies and might help to better understand the mechanisms of more common conditions with muscle pain such as fibromyalgia.

6. Future perspectives

In this study we identified 14 differentially expressed genes and 22 differentially secreted proteins in DM2 muscles based on the onset of muscle pain. Some of them were previously associated with the pain regulation in other disorders with chronic pain. The next step would be to perform the functional validation of identified pain targets in the animal models. Suitable animal models could be *CNBP*$^{-/-}$ or heterozygous *CNBP*$^{+/-}$ knockout mice since haploisufficieny is enough for the onset of DM2 multisystemic phenotype (Chen et al, 2007). In addition, functional validation of targets could be performed in knockout mice models of MBNL1-3 which are reported to develop typical clinical features of myotonic dystrophies (Kanadia et al, 2003; Suenaga et al, 2012, Choi et al, 2016). The involvement of regulated genes and proteins in the onset of muscle pain could be validated in animal models by measuring the changes in pain sensitivity with beahvioural pain tests and by electrophysiological recordings of primary afferents innervating muscles.

RNA binding proteins from MBNL protein family were recently recognized as mediators of circular RNA (circRNA) splicing and biogenesis (Ashwal-Fluss et al, 2014). Considering that mislocalization of MBNL plays a central role in DM2 pathogenesis and leads to missplicing of several genes (Goodwin et al, 2015), it would be interesting to screen for changes in splicing of circRNA in DM2 and whether they contribute to the onset of muscle pain in the patients.

Epigenetic mechanisms such as changes in DNA methylation, histone acetylation and miRNA expression are known to contribute to the onset of chronic pain by modulating gene expression in spinal nociceptive neurons and pain centers in CNS. Targeting of epigenetic mechanisms shows potential for development of more effective analgesics that do not cause the onset of tolerance (Descalzi et al, 2015). Considering that pain therapeutics are ineffective in reducing pain in DM2, it would be desirable to study epigenetic mechanisms of muscle pain in DM2 in order to tailor more effective pain medication for the patients.

7. Publications

Moshourab, R.*, **Palada, V.***, Grunwald, S., Grieben, U., Lewin, G.R.*, Spuler S.* (2016). A Molecular Signature of Myalgia in Myotonic Dystrophy 2. *EBioMedicine*, 7: 205-211. doi: 10.1016/j.ebiom.2016.03.017. (* contributed equally).

8. Bibliography

Aboumousa, A., Hoogendijk, J., Charlton, R., Barresi, R., Herrmann, R., Voit, T., Hudson, J., Roberts, M., Hilton-Jones, D., Eagle, M., Bushby, K., Straub, V. (2008). Caveolinopathy--new mutations and additional symptoms. *Neuromuscul Disord*, 18(7): 572-578. doi: 10.1016/j.nmd.2008.05.003.

Adreani, C.M., Hill, J.M., Kaufman, M.P. (1997) Responses of group III and IV muscle afferents to dynamic exercise. *J Appl Physiol*, *82*: 1811-1817.

Ahmadi, S., Lippross, S., Neuhuber, W.L., Zeilhofer, H.U., (2002). PGE2 selectively blocks inhibitory glycinergic neurotransmission onto rat superficial dorsal horn neurons. *Nat Neurosci*, *5*: 34-40.

Alloui, A., Zimmermann, K., Mamet, J., Duprat, F., Noel, J., Chemin, J., Guy, N., Blondeau, N., Voilley, N., Rubat-Coudert, C., Borsotto, M., Romey, G., Heurteaux, C., Reeh, P., Eschalier, A., Lazdunski, M. (2006). TREK-1, a K+ channel involved in polymodal pain perception. *EMBO J*, *25*: 2368-2376.

Alsalem, M., Wong, A., Millns, P., Arya, P.H., Chan, M.S., Bennett, A., Barrett, D.A., Chapman, V., Kendall, D.A. (2013). The contribution of the endogenous TRPV1 ligands 9-HODE and 13-HODE to nociceptive processing and their role in peripheral inflammatory pain mechanisms. *Br J Pharmacol*, *168*(8): 1961-1974. doi: 10.1111/bph.12092.

Altelaar, A.F., Munoz, J., Heck, A.J. (2013). Next-generation proteomics: towards an integrative view of proteome dynamics. *Nat Rev Genet*,*14*(1): 35-48. doi: 10.1038/ nrg3356.

Abplanalp, J., Laczko, E., Philp, N.J., Neidhardt, J., Zuercher, J., Braun, P., Schorderet, D.F., Munier, F.L., Verrey, F., Berger, W., Camargo, S.M.R., Kloeckener-Gruissem, B. (2013). The cataract and glucosuria associated

monocarboxylate transporter MCT12 is a new creatine transporter. *Hum Mol Genet*, *22*, 3218-3226. doi:10.1093/hmg/ddt175.

Armstrong-James, M. (1975). The functional status and columnar organization of single cells responding to cutaneous stimulation in neonatal rat somatosensory cortex S1. *J Physiol, 246*: 501-538.

Antunes-Martins, A., Perkins, J.R., Lees, J., Hildebrandt, T., Orengo, C., Bennett, D.L. (2013). Systems biology approaches to finding novel pain mediators. *Wiley Interdiscip Rev Syst Biol Med*, *5*(1): 11-35. doi: 10.1002/ wsbm.1192.

Ashwal-Fluss, R., Meyer, M., Pamudurti, N.R., Ivanov, A., Bartok, O., Hanan, M., Evantal, N., Memczak, S., Rajewsky, N., Kadener, S. (2014). circRNA biogenesis competes with pre-mRNA splicing. *Mol Cell*, *56*(1): 55-66. doi: 10.1016 /j.molcel.2014.08.019.

Auvinen, S., Suominen, T., Hannonen, P., Bachinski, L.L., Krahe, R., Udd, B. (2008). Myotonic dystrophy type 2 found in two of sixty-three persons diagnosed as having fibromyalgia. *Arthritis Rheum*, *58*(11): 3627-3631. doi: 10.1002/art.24037.

Averill, S. McMahon, S.B., Clary, D.O., Reichardt, L.F., Priestley, J.V. (1995). immunocytochemical localization of trkA receptors in chemically identified subgroups of adult rat sensory neurons. *Eur J Neurosci*, 7: 1484-1494.

Banik R.K., Kozaki, Y., Sato, J., Gera, L., Mizumura, K. (2001). B2 receptor-mediated enchanced bradykinin sensitivity of rat cutaneous C-fiber nociceptors during persistent inflammation. *J Neurophysiol*, *86*(6), 2727-2735.

Bansal, D., Miyake, K., Vogel, S.S., Groh, S., Chen, C.C., Williamson, R., McNeil, P.L., Campbell, K.P. (2003). Defective membrane repair in dysferlin-deficient muscular dystrophy. *Nature*, *423*: 168-172.

Bantscheff, M., Schirle, M., Sweetman, G., Rick, J., Kuster, B. (2007). Quantitative mass spectrometry in proteomics: a critical review. *Anal Bioanal Chem*, *389*(4): 1017-1031.

Bär, K.J., Schurigt, U., Scholze, A., segond von Banchet, G., Stopfel, N., Bräuer, R., Halbhuber, K.J., Schaible H.G. (2004). The expression and localization of somatostatin receptors in dorsal root ganglion neurons of normal and monoarthritic rats. *Neuroscience*, *127*: 197-206.

Baron, A., Lingueglia, E. (2015). Pharmacology of acid-sensing ion channels - Physiological and therapeutical prospectives. *Neuropharm, 94*: 19-35.

Baron, R. (2006). Mechanisms of Disease: neuropathic pain—a clinical perspective. *Nat Clin Pract Neurol*, *2*(2): 95-106.

Baron, R., Förster, M., Binder, A. (2012). Subgrouping of patients with neuropathic pain according to pain-related sensory abnormalities: a first step to a stratified treatment approach. *Lancet Neurol*, *11*(11): 999-1005. doi: 10.1016/S1474-4422(12)70189-8. Review.

Basbaum, A.I., Bautista, D.M., Scherrer, G., Julius, D. (2009). Cellular and Molecular Mechanisms of Pain. *Cell, 139*: 267-284. doi: 10.1016/j.cell. 2009.09.028.

Basbaum, A.I., Jessell, T. (2000). The perception of pain. In Principles of Neuroscience, *Appleton and Lange*, 472-491.

Batra, R., Charizanis, K., Manchanda, M., Mohan, A., Li, M., Finn, D.J., Goodwin, M., Zhang, C., Sobczak, K., Thornton, C.A., Swanson, M.S. (2014). Loss of MBNL leads to disruption of developmentally regulated alternative polyadenylation in RNA-mediated disease. *Mol Cell*, *56*: 311-322.

Bayliss, D.A., Barrett, P.Q. (2008). Emerging roles for two-pore-domain potassium channels and their potential therapeutic impact. *Trends in Pharma Sci*, *29*(11): 566-575. doi: 10.1016/j.tips.2008.07.013.

Bell, C., Shaw, A. (1868). Reprint of the “Idea of a New Anatomy of the Brain,” with Letters, &c. *J Anat Physiol, 3*: 147-182.

Belmonte, C., Cervero, F. (1996). Neurobiology of nociceptors. *Oxford University Press*

Bennett, D.L., Woods, C.G. (2014). Painful and painless channelopathies. *Lancet Neurol*, *13*(6): 587-599. doi: 10.1016/S1474-4422(14)70024-9.

Bessou, P., Perl, E.R. (1969). Response of cutaneous sensory units with unmyelinated fibers to noxious stimuli. *J Neurophysiol*, *32*: 1025-1043.

Binshtok, A.M., wang, H., Zimmermann, K., Amaya, F., Vardeh, D., Shi, L., Brenner, G.J., Ji, R., Bean, B.P., Woolf, C.J., Samad, T.A. (2008). Nocieptors Are Interleukin-1β Sensors. *J Neurosci, 28*(52): 14062-14073.

Blumenstiel, K., Gerhardt, A., Rolke, R., Bieber, C., Tesarz, J., Friederich, H.C., Eich, W., Treede, R.D. (2011). Quantitative sensory testing profiles in chronic back pain are distinct from those in fibromyalgia. *Clin J Pain*, 27(8): 682-690. doi: 10.1097/AJP.0b013e3182177654.

Borbiro, I., Badheka, D., Rohacs, T. (2015) Activation of TRPV1 channels inhibits mechanosensitive Piezo channel activity by depleting membrane phosphoinositides. *Sci Signal, 8*(363): ra15. doi: 10.1126/scisignal.2005667.

Borg, G., Holmgren, A., Lindblad, I. Quantitative evaluation of chest pain. (1981). *Acta Med Scand Suppl*, *644*:43-45.

Breivik, H., Collett, B., Ventafridda, V., Cohen, R., Gallacher, D. (2006). Survey of chronic pain in Europe: prevalence, impact on daily life and treatment. *Eur J Pain*, *10,* 287-333. doi: 16/j.ejpain.2005.06.009.

Bushby, K., Finkel, R., Birnkrant, D.J., Case, L.E., Clemens, P.R., Cripe, L., Kaul, A., Kinnett, K., McDonald, C., Pandya, S., Poysky, J., Shapiro, F., Tomezsko, J., Constantin, C.; DMD Care Considerations Working Group. (2010). Diagnosis and management of Duchenne muscular dystrophy, part 2: implementation of multidisciplinary care. *Lancet Neurol*, *9*(2):177-189. doi: 10.1016/S1474-4422(09)70272-8.

Calcaterra, N.B., Armas, P., Weiner, A.M., Borgognone, M. (2010). CNBP: a multifunctional nucleic acid chaperone involved in cell death and proliferation control. *IUBMB Life*, *62*(10): 707-714. doi: 10.1002/iub.379.

Caterina, M.J., Leffler, A., Malmberg, A.B., Martin, W.J., Trafton, J., Petersen-Zeitz, K.R., Koltzenburg, M., Basbaum, A.I., Julius, D. (2000). Impaired nociception and pain sensation in mice lacking the capsaicin receptor. *Science*, *288*: 306-313.

Caterina, M.J., Rosen, T.A., Tominaga, M., Brake, A.J., Julius, D. (1999). A capsaicin-receptor homologue with a high threshold for noxious heat. *Nature*, *398*: 436-441.

Charlet-B, N., Savkur, R.S., Singh, G., Philips, A. V., Grice, E.A., Cooper, T.A., Charlet-B., N., Savkur, R.S., Singh, G., Philips, A. V., Grice, E.A., Cooper, T.A. (2002). Loss of the Muscle-Specific Chloride Channel in Type 1 Myotonic Dystrophy Due to Misregulated Alternative Splicing. *Mol Cell*, *10*: 45-53. doi:10.1016/S1097-2765(02) 00572-5.

Chawla, L.S., Toma, I., Davison, D., Vaziri, K., Lee, J., Lucas, R., Seneff, M.G., Nyhan, A., McCaffrey, T.A. (2016). Acute appendicitis: transcript profiling of

blood identifies promising biomarkers and potential underlying processes. *BMC Med Genomics*, *9*(1): 40. doi: 10.1186/s12920-016-0200-y.

Chen, W., Wang, Y., Abe, Y., Cheney, L., Udd, B., and Li, Y. P. (2007). Haploinsuffciency for Znf9 in Znf9+/− mice is associated with multiorgan abnormalities resembling myotonic dystrophy. *J Mol Biol*, *368*: 8-17. doi:10. 1016/j.jmb.2007.01.088.

Choi, J., Dixon, D.M., Dansithong, W., Abdallah, W.F., Roos, K.P., Jordan, M.C., Trac, B., Lee, H.S., Comai, L., Reddy, S. (2016). Muscleblind-like 3 deficit results in a spectrum of age-associated pathologies observed in myotonic dystrophy. *Sci Rep*, *6*: 30999. doi: 10.1038/srep30999.

Chevalier, F. (2010). Highlights on the capacities of “Gel-based” proteomics. *Proteome Sci*, *8*: 23. doi: 10.1186/1477-5956-8-23.

Choy, E.H., Isenberg, D.A., Garrood, T., Farrow, S., Ioannou, Y., Bird, H., Cheung, N., Williams, B., Hazleman, B., Price, R., Yoshizaki, K., Nishimoto, N., Kishimoto, T., Panayi, G.S. (2002) Therapeutic benefit of clocking interleukin-6 activity with an anti-interleukin-6 receptor monoclonal antibody in rheumatoid arthritis: a randomized, double-blinded, placebo-controlled, dose-escalation trial. *Arthritis Rheum, 46*: 3143-3150.

Clark, A.K., Staniland, A.A., Marchand, F., Kaan, T.K., McMahon, S.B., Malcangio, M. (2010). P2X7-dependent release of interleukin-1beta and nociception in the spinal cord following lipopolysaccharide. *J Neurosci*, *30*(2): 573-582. doi: 10.1523/ JNEUROSCI. 3295-09.2010.

Coste, B., Mathur, J., Schmidt, M., Earley, T.J., Ranade, S., Petrus, M.J., Dubin, A.E., Patapiutian, A. (2010). Piezo1 and Piezo2 are essential components of distinct mechanically activated cation channels. *Science 330*: 55-60.

Costigan, M., Befort, K., Karchewski, L., Griffin, R.S., D'Urso, D., Allchorne, A., Sitarski, J., Mannion, J.W., Pratt, R.E., Woolf, C.J. (2002). Replicate high-

density rat genome oligonucleotide microarrays reveal hundreds of regulated genes in the dorsal root ganglion after peripheral nerve injury. *BMC Neurosci*, 3:16.

Coull, J.A., Boudreau, D., Bachand, K., Prescott, S.A., Nault, F., Sik, A, De Koninck, P., De Koninck, Y. (2003). Trans-synaptic shift in anion gradient in spinal lamina I neurons as a mechanism of neuropathic pain. *Nature, 424*: 938-942.

Coull, J.A., Boudreau, D., Bachand, K., Prescott, S.A., Nault, F., Sík, A., De Koninck, P., De Koninck, Y. (2003). Trans-synaptic shift in anion gradient in spinal lamina I neurons as a mechanism of neuropathic pain. *Nature*, *424*(6951): 938-942.

Craig, J.C., Rollman, G.B. (1999). SOMESTHESIS. *Annu Rev Psychol*, *50* (1): 305-331. doi: 10.1146/annurev.psych.50.1.305.

Cummins, T.R., Black, J.A., Dib-Hajj, S.D., Waxman, S.G. (2000). Glial-derived neurotrophic factor upregulates expression of functional SNS and NaN sodium channels and their currents in axotomized dorsal root ganglion neurons. *J Neurosci*, *20*: 8754-8761.

Cummins, T.R., Dib-Hajj, S.D., Waxman, S.G. (2004). Electrophysiological properties of mutant Nav1.7 sodium channels in a painful inherited neuropathy. *J Neurosci*, *24*(38):8232-8236.

Dalakas, M.C. (2011). An update on inflammatory and autoimmune myopathies. *Neuropathol Appl Neurobiol*, *37*(3): 226-242. doi: 10.1111/j.1365-2990.2010. 01153.x. Review.

Day, J.W., Ricker, K., Jacobsen, J.F., Rasmussen, L.J., Dick, K.A., Kress, W., Schneider, C., Koch, M.C., Beilman, G.J., Harrison, A.R., Dalton, J.C., Ranum, L.P. (2003). Myotonic dystrophy type 2: molecular, diagnostic and clinical spectrum. *Neurology*, 60(4): 657-664.

de Sousa Abreu, R., Penalva, L.O., Marcotte, E.M., Vogel, C. (2009). Global signatures of protein and mRNA expression levels. *Mol Biosyst*, *5*: 1512-1526.

DeLeo, J.A., Sorkin, L.S., Watkins, L.R. (2007). Immune and Glial Regulation of Pain. Seattle: IASP.

Depetris, R.S., Hu, J., Gimpelevich, I., Holt, L.J., Daly, R.J., Hubbard, S.R. (2005). Structural basis for inhibition of the insulin receptor by the adaptor protein Grb14. *Mol Cell*, *20*: 325-333. doi: 10.1016/j.molcel.2005.09.001.

Descalzi, G., Ikegami, D., Ushijima, T., Nestler, E.J., Zachariou, V., Narita, M. (2015). Epigenetic mechanisms of chronic pain. *Trends Neurosci*, *38*(4): 237-246. doi: 10.1016/j.tins.2015.02.001. Review.

Di Lorenzo, C., Daverio, A., Pasqualetti, P., Coppola, G., Giannoudas, I., Barone, Y., Grieco, G.S., Niolu, C., Pascale, E., Santorelli, F.M., Nicoletti, F., Pierelli, F., Siracusano, A., Seri, S., Di Lorenzo, G. (2014). The upstream Variable Number Tandem Repeat polymorphism of the monoamine oxidase type A gene influences trigeminal pain-related evoked responses. *Eur J Neurosci*, *39*: 501-507. doi: 10.1111/ejn.12458.

Dib-Hajj, S.D., Waxman, S.G. (2014). Translational pain research: Lessons from genetics and genomics. *Sci Transl Med*, *6*: 249sr4. doi:10.1126/scitranslmed.3007017.

Dubner, R. (2005). Plasticity in central nociceptive pathways. In: Merskey, H., Loeser, J.D.; Dubner, R. (eds) The paths of pain 1975-2005. *IASP Press*, 101-115.

Elgzyri, T., Parikh, H., Zhou, Y., Dekker Nitert, M., Rönn, T., Segerström, Å.B., Ling, C., Franks, P.W., Wollmer, P., Eriksson, K.F., Groop, L., Hansson, O. (2012). First-Degree Relatives of Type 2 Diabetic Patients Have Reduced

Expression of Genes Involved in Fatty Acid Metabolism in Skeletal Muscle. *J Clin Endocrinol Metab*, jc.2011-3037. doi:10.1210/jc.2011-3037.

Elias, J.E., Gygi, S.P. (2007). Target-decoy search strategy for increased confidence in largescale protein identifications by mass spectrometry. *Nat Methods*, *4*(3): 207-214.

Fardaei, M., Rogers, M.T., Thorpe, H.M., Larkin, K., Hamshere, M.G., Harper, P.S., Brook, J.D. (2002). Three proteins, MBNL, MBLL and MBXL, colocalize in vivo with nuclear foci of expanded-repeat transcripts in DM1 and DM2 cells. *Hum Mol Genet*, *11*: 805-814.

Farina, D, Negro, F. (2015). Common synaptic input to motor neurons, motor unit synchronization, and force control. *Exerc Sport Sci Rev*, *43*(1): 23-33. doi: 10.1249/JES.0000000000000032. Review.

Fitzgerald, M. (2005). The development of nociceptive circuits. *Nat Rev Neurosci, 6*: 507-520. doi: 10.1038/nrn1701.

Folker, E.S., Baylies, M.K. (2013). Nuclear positioning in muscle development and disease. *Front Physiol*, *4*: 363. doi: 10.3389/fphys.2013.00363. Review.

Frank, M.M., Fries, L.F. (1991). The role of complement in inflammation and phagocytosis. *Immunol Today*, *12*: 322-326. doi: 10.1016/0167-5699(91)90009-I.

Fugier, C., Klein, A.F., Hammer, C., Vassilopoulos, S., Ivarsson, Y., Toussaint, A., Tosch, V., Vignaud, A., Ferry, A., Messaddeq, N., Kokunai, Y., Tsuburaya, R., de la Grange, P., Dembele, D., Francois, V., Precigout, G., Boulade-Ladame, C., Hummel, M.C., Lopez de Munain, A., Sergeant, N., Laquerrière, A., Thibault, C., Deryckere, F., Auboeuf, D., Garcia, L., Zimmermann, P., Udd, B., Schoser, B., Takahashi, M.P., Nishino, I., Bassez, G., Laporte, J., Furling, D., Charlet-Berguerand, N. (2011). Misregulated alternative splicing of BIN1 is

associated with T tubule alterations and muscle weakness in myotonic dystrophy. *Nat Med*, *17*(6): 720-725. doi: 10.1038/nm.2374

Fukuoka, T., Kobayashi, K., Yamanaka, H., Obata, K., Dai, Y., Noguchi, K. (2008). Comparative study of the distribution of the alpha-subunits of voltage-gated sodium channels in normal and axotomized rat dorsal root ganglion neurons. *J Comp Neurol*, *510*(2):188-206. doi: 10.1002/cne.21786.

Gangadharan, V., Kuner, R. (2013). Pain hypersensitivity mechanisms at a glance. *Dis Model Mech*, *6(4)*: 889-895. doi: 10.1242/dmm.011502.

Gao, Y.J., Ji, R.R. (2010). Targeting astrocyte signaling for chronic pain. *Neurotherapeutics*, 7(4): 482-493. doi: 10.1016/j.nurt.2010.05.016.

George, A., Schneider-Gold, C., Zier, S., Reiners, K., Sommer, C. (2004) Musculoskeletal pain in patients with myotonic dystrophy type 2. *Arch Neurol*, *61*(12): 1938-1942.

Ghafouri, B., Carlsson, A., Holmberg, S., Thelin, A., Tagesson, C. (2016). Biomarkers of systemic inflammation in farmers with musculoskeletal disorders; a plasma proteomic study. *BMC Musculoskelet Disord*, *17*: 206. doi: 10.1186/s12891-016-1059-y.

Gold, S.M., Gebhart, G.F. (2010) Nociceptor sensitization in pain pathogenesis. *Nat Med, 16*(11), 1248-1257. doi: 10.1038/nm.2235

Goodwin, M., Mohan, A., Batra, R., Lee, K.Y., Charizanis, K., Fernández Gómez, F.J., Eddarkaoui, S., Sergeant, N., Buée, L., Kimura, T., Clark, H.B., Dalton, J., Takamura, K., Weyn-Vanhentenryck, S.M., Zhang, C., Reid, T., Ranum, L.P., Day, J.W., Swanson, M.S. (2015). MBNL Sequestration by Toxic RNAs and RNA Misprocessing in the Myotonic Dystrophy Brain. *Cell Rep*, *12*(7): 1159-1168. doi: 10.1016 /j.celrep.2015.07.029.

Goo, Y.A., Tsai, Y.S., Liu, A.Y., Goodlett, D.R., Yang, C.C. (2010). Urinary proteomics evaluation in interstitial cystitis/painful bladder syndrome: a pilot study. *Int Braz J Urol*, 36(4): 464-478.

Goo, Y.A., Cain, K., Jarrett, M., Smith, L., Voss, J., Tolentino, E., Tsuji, J., Tsai, Y.S., Panchaud, A., Goodlett, D.R., Shulman, R.J., Heitkemper, M. (2012). Urinary proteome analysis of irritable bowel syndrome (IBS) symptom subgroups. *J Proteome Res*, *11*(12): 5650-5662. doi: 10.1021/pr3004437.

Goswami, S.C., Mishra, S.K., Maric, D., Kaszas, K., Gonnella, G.L., Clokie, S.J., Kominsky, H.D., Gross, J.R., Keller, J.M., Mannes, A.J., Hoon, M.A., Iadarola, M.J. (2014). Molecular signatures of mouse TRPV1-lineage neurons revealed by RNA-Seq transcriptome analysis. *J Pain*, *15*(12): 1338-1359. doi: 10.1016/j.jpain.2014.09.010.

Graven-Nielsen, T., Mense, S. (2001) The peripheral apparatus of muscle pain: evidence from animal and human studies. *Clin J Pain*, *17*: 2-10,

Graven-Nielsen, T., Arendt-Nielsen, L., (2010). Assessment of mechanisms in localized and widespread musculoskeletal pain. *Nat Rev Rheumatol*, *6*: 599-606. doi:10.1038/nrrheum.2010.107.

Greising, S.M., Gransee, H.M., Mantilla, C.B., Sieck, G.C. (2012). Systems biology of skeletal muscle: fiber type as an organizing principle. *Wiley Interdiscip Rev Syst Biol Med*, *4*(5): 457-473. doi: 10.1002/wsbm.1184.

Griffin, R.S., Costigan, M., Brenner, G.J., Ma, C.H., Scholz, J., Moss, A., Allchorne, A.J., Stahl, G.L., Woolf, C.J. (2007). Complement induction in spinal cord microglia results in anaphylatoxin C5a-mediated pain hypersensitivity. *J Neurosci*, 27(32): 8699-8708.

Groth, R., Aanonsen, L. (2002). Spinal brain-derived neurotrophic factor (BDNF) producs hyperalgesia in normal mice while antisense directed against

either BDNF or trkB, prevent inflammation-induced hyperalgesia. *Pain, 100*: 171-181.

Gudes, S., Barkai, O., Caspi, Y., Katz, B., Lev, S., Binshtok, A.M. (2015). The role of slow and persistent TTX-resistant sodium currents in acute tumor necrosis factor-α-mediated increase in nociceptors excitability. *J Neurophysiol, 113*: 601-619. Doi: 19.1152/jn.00652.2014.

Gygi, S.P., Rist, B., Gerber, S.A., Turecek, F., Gelb, M.H., Aebersold, R. (1999). Quantitative analysis of complex protein mixtures using isotope-coded affinity tags. *Nat Biotechnol*, 17: 994-999.

Hammer, P., Banck, M.S., Amberg, R., Wang, C., Petznick, G., Luo, S., Khrebtukova, I., Schroth, G.P., Beyerlein, P., Beutler, A.S. (2010). mRNA-seq with agnostic splice site discovery for nervous system transcriptomics tested in chronic pain. *Genome Res*, *20*(6): 847-860. doi: 10.1101/gr.101204.109.

Han, X., Gross, R.W. (2005). Shotgun lipidomics: electrospray ionization mass spectrometric analysis and quantitation of cellular lipidomes directly from crude extracts of biological samples. *Mass Spectrom Rev*, *24*(3): 367-412. doi: 10.1002/ mas.20023.

Hannonen, P., Malminiemi, K., Yli-Kerttula, U., Isomeri, R., Roponen, P. (1998). A randomized, double-blind, placebo-controlled study of moclobemide and amitriptyline in the treatment of fibromyalgia in females without psychiatric disorder. *Br J Rheumatol*, *37*: 1279-1286.

Haynes, S.E., Hollopeter, G., Yang, G., Kurpius, D., Dailey, M.E., Gan, W.B., Julius, D. (2006). The P2Y12 receptor regulates microglial activation by extracellular nucleotides. *Nat Neurosci*, *9*(12):1512-1519.

Heatwole, C., Johnson, N., Goldberg, B., Martens, W., Moxley, R. (2011) Laboratory abnormalities in patients with myotonic dystrophy type 2. *Arch Neurol*, *68*(9):1180-1184. doi: 10.1001/archneurol.2011.191.

Helbling-Leclerc, A., Bonne, G., Schwartz, K. (2002). Emery-Dreifuss muscular dystrophy. *Eur J Hum Genet*, *10*:1 57-161.

Ho, T.H., Charlet-B, N., Poulos, M.G., Singh, G., Swanson, M.S., Cooper, T.A. (2004). Muscleblind proteins regulate alternative splicing. *EMBO J*, *23*: 3103-3112.

Hoheisel, U., Reinohl, J., Unger, T., Mense, S. (2004). Acidic pH and capsaicin activate mechanosensitive group IV muscle receptors in the rat. *Pain*, *110*: 149-157.

Hucho, T., Levine, J.D. (2007). Signaling pathways in sensitization: toward a nociceptor cell biology. *Neuron*, *55*: 365-376.

Huichalaf, C., Schoser, B., Schneider-Gold, C., Jin, B., Sarkar, P., Timchenko, L. (2009). Reduction of the rate of protein translation in patients with myotonic dystrophy 2. *J Neurosci*, *29*, 9042-9049.

Jang, J.H., Liang, D., Kido, K., Sun, Y., Clark, D.J., Brennan, T.J. (2011). Increased local concentration of complement C5a contributes to incisional pain in mice. *J Neuroinflammation*, *8*: 80. doi: 10.1186/1742-2094-8-80.

Ji, R.R., Xu, Z.Z., Strichartz, G., Serhan, C.N. (2011). Emerging roles of resolvins in the resolution of inflammation and pain. *Trends Neurosci*, 34(11): 599-609. doi: 10.1016/j.tins.2011.08.005. Review.

Kamsteeg, E.J., Kress, W., Catalli, C., Hertz, J.M., Witsch-Baumgartner, M., Buckley, M.F., van Engelen, B.G., Schwartz, M., Scheffer H. (2012). Best practice guidelines and recommendations on the molecular diagnosis of myotonic dystrophy types 1 and 2. *Eur J Hum Genet*, *20*(12): 1203-1208. doi: 10.1038/ejhg.2012.108.

Kanadia, R.N., Johnstone, K.A., Mankodi, A., Lungu, C., Thornton, C.A., Esson, D., Timmers, A.M., Hauswirth, W.W., Swanson, M.S. (2003). A muscleblind knockout model for myotonic dystrophy. *Science*, *302*(5652):1978-1980.

Kato, G., Yasaka, T., Katafuchi, T., Furue, H., Mizuno, M., Iwamoto, Y., Yoshimura, M. (2006). Direct GABAergic and glycinergic inhibition of the substantia gelatinosa from the rostral ventromedial medulla revealed by in vivo patchclamp analysis in rats. *J Neurosci*, *26*: 1787-1794.

Kawasaki, Y., Zhang, L., Cheng, J.K., Ji, R.R. (2008). Cytokine mechanisms of central sensitization: distinct and overlapping role of interleukin-1beta, interleukin-6, and tumor necrosis factor-alpha in regulating synaptic and neuronal activity in the superficial spinal cord. *J Neurosci*, *28*: 5189-5194.

Kitano, H. (2002). Computational systems biology. *Nature*, 420 (6912): 206-210.

Kim, D., Kim, M.A., Cho, I.H., Kim, M.S., Lee, S., Jo, E.K., Choi, S.Y., Park, K., Kim, J.S., Akira, S., Na, H.S., Oh, S.B., Lee, S.J. (2007) A critical role of toll-like receptor 2 in nerve injury-induced spinal cord glial cell activation and pain hypersensitivity. *J Biol Chem*, *282*(20): 14975-14983.

Kim, D.S, Lee, S.J., Park, S.Y., Yoo, H.J., Kim, S.H., Kim, K.J., Cho, H.J. (2001). Differentially expressed genes in rat dorsal root ganglia following peripheral nerve injury. *Neuroreport*, *12*(15): 3401-3405.

Kim, H., Lee, H., Rowan, J., Brahim, J., Dionne, R.A. (2006). Genetic polymorphisms in monoamine neurotransmitter systems show only weak association with acute post-surgical pain in humans. *Mol Pain*, *2*: 24. doi:10.1186/1744-8069-2-24.

Kohno, T., Wang, H., Amaya, F., Brenner, G.J., Cheng, J.K., Ji, R.R., Woolf, C.J. (2008) Bradykinin enhances AMPA and NMDA receptor activity in spinal

cord dorsal horn neurons by activating multiple kinases to produce pain hypersensitivity. *J Neurosci*, *28*: 4533-4540.

Komori, N., Takemori, N., Kim, H.K., Singh, A., Hwang, S.H., Foreman, R.D., Chung, K., Chung, J.M., Matsumoto, H. (2007). Proteomics study of neuropathic and nonneuropathic dorsal root ganglia: altered protein regulation following segmental spinal nerve ligation injury. *Physiol Genom*, *29*: 215-230.

Konieczny, P., Stepniak-Konieczna, E., Sobczak, K. (2014). MBNL proteins and their target RNAs, interaction and splicing regulation. *Nucleic Acids Res*, *42*(17): 10873-10887. doi: 10.1093/nar/gku767.

Kucera, J., Fan, G., Walro, J., Copray, S.; Tessarollo, L., Jaenisch, R. (1998). Neurotrophin-3 and trkC in muscle non-essential for the development of mouse muscle spindles. *9*(5): 905-909.

Kuner, R. (2010). Central mechanisms of pathological pain. *Nat Med*, *16*: 1258-1266.

Lacroix-Fralish, M.L., Tawfik, V.L., Tanga, F.Y., Spratt, K.F., DeLeo, J.A. (2006). Differential spinal cord gene expression in rodent models of radicular and neuropathic pain. *Anesthesiology*, *104*(6): 1283-1292.

Lam, E.M., Shepard, P.W., St Louis, E.K., Dueffert, L.G., Slocumb, N., McCarter, S.J., Silber, M.H., Boeve, B.F., Olson, E.J., Somers, V.K., Milone, M. (2013). Restless legs syndrome and daytime sleepiness are prominent in myotonic dystrophy type 2. *Neurology*, *81*(2): 157-164. doi: 10.1212/WNL.0b013e31829a340f.

Lange, V., Picotti, P., Domon, B., Aebersold, R. (2008). Selected reaction monitoring for quantitative proteomics: a tutorial. *Mol Syst Biol*, *4*: 222. doi: 10.1038/msb.2008.61.

Lele, P.P., Sinclair, D.C., Weddell, G. (1954) The reaction time to touch. *J Physiol, 123*: 187–203.

Lemmers, R.J., van der Vliet, P.J., Klooster, R., Sacconi, S., Camaño, P., Dauwerse, J.G., Snider, L., Straasheijm, K.R., van Ommen, G.J., Padberg, G.W., Miller, D.G., Tapscott, S.J., Tawil, R., Frants, R.R., van der Maarel, S.M. (2010). A Unifying Genetic Model for Facioscapulohumeral Muscular Dystrophy, *Science*, *329*(5999): 1650-1653. doi:10.1126/science.1189044.

Lewin, G.R., Ritter, A.M., Mendell, L.M. (1993). Nerve growth factor-induced hyperalgesia in the neonatal and adult rat. *J Neurosci*, *13*(5): 2136-2148.

Lewin, G.R., Lechner, S.G., Smith, E.S.J. (2014). Nerve growth factor and nociception: from experimental embryology to new analgesic therapy. *Handb Exp Pharmacol*, *220*: 251-282. doi:10.1007/978-3-642-45106-5_10.

Li, L., Han, J., Wang, Z., Liu, J., Wie, J., Xiong, S., Zhao, Z. (2014). Mass spectrometry methodology in lipid analysis. *Int J Mol Sci*, *15*(6): 10492-10507. doi: 10.3390/ ijms150610492.

Li, Z., Adams, R.M., Chourey K., Hurst, G.B., Hettich, R.L., Pan, C. (2012). Systematic comparison of label-free, metabolic labeling, and isobaric chemical labeling for quantitative proteomics on LTQ Orbitrap Velos. *J Proteome Res*, *11*: 1582-1590.

Lieber, R.L. (2009). Skeletal Muscle Structure, Function, and Plasticity. *LWW*.

Lin, C.Y., Lee, Y.S., Lin, V.W, Silver, J. (2012). Fibronectin Inhibits Chronic Pain Development after Spinal Cord Injury. *J Neurotrauma*, *29*(3): 589-599. doi: 10.1089/neu.2011.2059.

Linden, A.M., Aller, M.I., Leppa, E., Vekovischeva, O., Aitta-Aho, T., Veale, E.L., Mathie, A., Rosenberg, P., Wisden, W., Korpi, E.R. (2006). The in vivo contributions of TASK-1-containing channels to the actions of inhalation

anesthetics, the alpha (2) adrenergic sedative dexmedetomidine, and cannabinoid agonists. *J Pharmacol Exp Ther*, *317*: 615–626. doi: 10.1124/jpet.105.098525

Liu, R., Fan, L., Yin, L., Wang, K., Miao, W., Song, Q., Dang, X., Gao, H., Bai, C. (2015). Comparative study of serum proteomes in Legg-Calve-Perthes disease. *BMC Musculoskelet Disord*, *16*: 281. doi: 10.1186/s12891-015-0730-z.

Liu, X., Tian, Y., Meng, Z., Chen, Y., Ho, I.H., Choy, K.W., Lichtner, P., Wong, S.H., Yu, J., Gin, T., Wu, W.K., Cheng, C.H., Chan, M.T. (2015). Up-regulation of Cathepsin G in the Development of Chronic Postsurgical Pain: An Experimental and Clinical Genetic Study. *Anesthesiology*, *123*(4): 838-850. doi: 10.1097/ALN. 0000000000000828.

Liquori, C.L., Ricker, K., Moseley, M.L., Jacobsen, J.F., Kress, W., Naylor, S.L., Day, J.W., Ranum, L.P. (2001) Myotonic dystrophy type 2 caused by a CCTG expansion in intron 1 of ZNF9. *Science*, *293*(5531): 864-867.

Ma, Q.P., Hill, R., Sirinathsinghji D. (2001) Colocalization of CGRP with 5-HT1B/1D receptors and substance P in trigeminal ganglion neurons in rats. *Eur J Neurosci, 13*(11): 2099-2104.

Mahadevan, M., Tsilfidis, C., Sabourin, L., Shutler, G., Amemiya, C., Jansen, G., Neville, C., Narang, M., Barceló, J., O'Hoy, K., et al. (1992). Myotonic dystrophy mutation: an unstable CTG repeat in the 3' untranslated region of the gene. *Science*, *255*(5049): 1253-1255. doi: http://dx.doi.org/10.1126/science.1546325.

Maier, T., Guell, M., Serrano, L. (2009). Correlation of mRNA and protein in complex biological samples. *FEBS Lett*, *583*: 3966-3973.

Malfait, A.M., Schnitzer, T.J. (2013). Towards a mechanism-based approach to pain management in osteoarthritis. *Nat Rev Rheumatol*, *9*: 654-664. Doi: 10.1038/nrrheum. 2013.138.

Malik-Hall, M., Dina, O.A., Levine, J.D. (2005) Primary afferent nociceptor mechanisms mediating NGF-induced mechanical hyperalgesia. *Eur J Neurosci, 21*: 3387-3394.

Malone, J.H., Oliver B. (2011). Microarrays, deep sequencing and the true measure of the transcriptome. *BMC Biol*, *9*: 34. doi: 10.1186/1741-7007-9-34. Review.

Manteniotis, S., Lehmann, R., Flegel, C., Vogel, F., Hofreuter, A., Schreiner, B.S., Altmüller, J., Becker, C., Schöbel, N., Hatt, H., Gisselmann, G. (2013). Comprehensive RNA-Seq expression analysis of sensory ganglia with a focus on ion channels and GPCRs in Trigeminal ganglia. *PLoS One*, *8*(11): e79523. doi: 10.1371/journal.pone. 0079523.

Marchand, F., Perretti, M., McMahon, S.B. (2005) Role of the immune system in chronic pain. *Nat Rev Neurosci*, *6*(7): 521-532.

Mason, C.C., Hanson, R.L., Ossowski, V., Bian, L., Baier, L.J., Krakoff, J., Bogardus, C. (2011). Bimodal distribution of RNA expression levels in human skeletal muscle tissue. *BMC Genomics*, *12*: 98. doi:10.1186/1471-2164-12-98.

Mateos-Aierdi, A.J., Goicoechea, M., Aiastui, A., Fernández-Torrón, R., Garcia-Puga, M., Matheu, A., López de Munain, A. (2015). Muscle wasting in myotonic dystrophies: a model of premature aging. *Front Aging Neurosci*, *7*: 125. doi: 10.3389/fnagi. 2015.00125.

McKemy, D.D., Neuhausser, W.M., Julius, D. (2002). Identification of a cold receptor reveals a general role for TRP channels in thermosensation. *Nature, 416*: 52-58.

McMahon, S.B., Bennett, D.L., Priestley, J.V., Shelton, D.L. (1995). The biological effects of endogenous nerve growth factor on adult sensory neurons revealed by a trkA-IgG fusion molecule. *Nat Med, 1* (8): 774-780.

McNally, E.M., Pytel, P. (2007). Muscle diseases: the muscular dystrophies. *Annu Rev Pathol*, *2*: 87-109. Review.

Megger, D.A., Bracht, T., Meyer, H.E., Sitek, B. (2013). Label-free quantification in clinical proteomics. *Biochim Biophys Acta*, *1834*(8):1581-90. doi: 10.1016/j.bbapap. 2013.04.001.

Mendelowitsch, A., Mergnerb, G.W., Shuaiba, A., Sekhara, L.N. (1998). Cortical brain microdialysis and temperature monitoring during hypothermic circulatory arrest in humans. *J Neurol Neurosurg Psychiatry*, *64*: 611-618. doi: 10.1136/jnnp.64.5.611.

Mense, S. (2009). Anatomy of nociceptors. In Science of pain, *Elsevier*

Mense, S., Gerwin, R.D. (2010). Muscle Pain: Understanding the Mechanisms. *Springer* doi: 10.1007/978-3-540-85021-2_2.

Melemedjian, O.K., Yassine, H.N., Shy, A., Price, T.J. (2013). Proteomic and functional annotation analysis of injured peripheral nerves reveals ApoE as a protein upregulated by injury that is modulated by metformin treatment. *Mol Pain*, *9*: 14. doi: 10.1186/1744-8069-9-14.

Meola, G., Cardani, R. (2015). Myotonic dystrophies: An update on clinical aspects, genetic, pathology, and molecular pathomechanisms. *Biochim Biophys Acta*, *1852*(4): 594-606. doi: 10.1016/j.bbadis.2014.05.019.

Meola, G., Sansone, V., Perani, D., Colleluori, A., Cappa, S., Cotelli, M., Fazio, F., Thornton, C.A, Moxley, R.T. (1999). Reduced cerebral blood flow and impaired visual-spatial function in proximal myotonic myopathy. *Neurology*, *53*: 1042-1050.

Mercuri, E., Muntoni, F. (2013). Muscular dystrophies. *Lancet*, *381*(9869): 845-860. Review.

Meyer, R.A., Davis, K.D., Cohen, R.H., Treede, R.D., Campbell, J.N. (1991). Mechanically insensitive afferents (MIAs) in cutaneous nerves of monkey. *Brain Res*, *561*, 252-161.

Meyer, R.A., Ringkamp, N., Campbell, J.N. and Raja, S.N. (2008). Peripheral mechanisms of cutaneous nociception. In Textbook of Pain. *Elsavier*, 3-34.

Minnerop, M., Weber, B., Schoene-Bake, J.C., Roeske, S. Mirbach, S., Anspach, C., Schneider-Gold, C., Betz, R.C., Helmstaedter, C., Tittgemeyer, M., Klockgether, T., Kornblum, C. (2011). The brain in myotonic dystrophy 1 and 2: evidence for a predominant white matter disease. *Brain*, 134(12): 3527-3543. doi: 10.1093/ brain/awr299.

Moayedi, M., Davis, K.D. (2013). Theories of pain: from specificity to gate control.

Molliver, D.C., Immke, D.C., Fierro, L., Paré, M., Rice, F.L., McCleskey, E.W. (2005). ASIC3, an acid-sensing ion channel, is expressed in metaboreceptive sensory neurons. *Mol Pain*, *1*: 35.

Molliver, D.C., Wright, D.E., Leitner, M.L., Parsadanian, A.S., Doster, K., Wen, D., Yan, Q., Snider, W.D. (1997) IB4-Binding DRG Neurons Switch from NGF to GDNF Dependence in Early Postnatal Life. *Neuron*, *19*(4): 849-861.

Moshourab, R., Palada, V., Grunwald, S., Grieben, U., Lewin, G.R., Spuler, S. (2016). A Molecular Signature of Myalgia in Myotonic Dystrophy 2. *EBioMedicine*, 7: 205-11. doi: 10.1016/j.ebiom.2016.03.017.

Moore, K.A., Kohno, T., Karchewski, L.A., Scholz, J., Baba, H., Woolf, C.J. (2002). Partial peripheral nerve injury promotes a selective loss of GABAergic

inhibition in the superficial dorsal horn of the spinal cord. *J Neurosci*, 22: 6724-6731.

Nassar, M.A., Stirling, L.C., Forlani, G., Baker, M.D., Matthews, E.A., Dickenson, A.H., Wood, J.N. (2004). Nociceptor-specific gene deletion reveals a major role for Nav1.7 (PN1) in acute and inflammatory pain. *Prot Natl Acad Sci USA, 101*: 12706-12711.

Node, K., Huo, Y., Ruan, X., Yang, B., Spiecker, M., Ley, K., Zeldin, D.C., Liao, J.K. (1999). Anti-inflammatory properties of cytochrome P450 epoxygenase-derived eicosanoids. *Science*, *285*(5431): 1276-1279.

Noel, J., Zimmermann, K., Busserolles, J., Deval, E., Alloui, A., Diochot, S., Guy, N., Borsotto, M., Reeh, P., Eschalier, A., Lazdunski, M. (2009). The mechano-activated K+ channels TRAAK and TREK-1 control both warm and cold perception. *EMBO J*, *28*:1308-1318.

O´Sullivan Smith, C., Bennett, R.L., Bird, T.D. (2000). Myotonic Dystrophy: Making an informed choice about genetic testing. *University of Washington Press*

Olson, T.H., Riedl, M.S., Vulchanova, L., Ortiz-Gonzalez, X.R., Elde, R. (1998). An acid sensing ion channel (ASIC) localizes to small primary afferent neurons in rats. *Neuron*, *9*(6):1109–1113.

Omerbašić, D., Smith, E.S., Moroni, M., Homfeld, J., Eigenbrod, O., Bennett, N.C., Reznick, J., Faulkes, C.G., Selbach, M., Lewin, G.R. (2016). Hypofunctional TrkA Accounts for the Absence of Pain Sensitization in the African Naked Mole-Rat. *Cell Rep*, *17*(3): 748-758. doi: 10.1016/j.celrep. 2016.09.035.

Papapoutian, A., Peier, A.M., Story, G.M., Viswanath, V. (2003). ThermoTRP channels and beyond: mechanisms of temperature sensation. *Nat Rev Neurosci, 4*: 529-539.

Park, K.A., Vasko, M.R. (2005). Lipid mediators of sensitivity in sensory neurons. *Trends Pharmacol Sci*, *26*: 571-577.

Patti, G.J., Yanes, O., Shriver, L.P., Courade, J.P., Tautenhahn, R., Manchester, M., Siuzdak, G. (2012) Metabolomics implicates altered sphingolipids in chronic pain of neuropathic origin. *Nature Chem Biol*, *8*: 232-234.

Pelletier, R., Hamel, F., Beaulieu, D., Patry, L., Haineault, C., Tarnopolsky, M., Schoser, B., Puymirat, J. (2009). Absence of a differentiation defect in muscle satellite cells from DM2 patients. *Neurobiol Dis*, *36*: 181-190.

Perfetti, A., Greco, S., Fasanaro, P., Bugiardini, E., Cardani, R., Garcia-Manteiga, J.M., Riba, M., Cittaro, D., Stupka, E., Meola, G., Martelli, F. (2014). Genome wide identification of aberrant alternative splicing events in myotonic dystrophy type 2. *PLoS One*, *9*(4): e93983. doi: 10.1371/journal.pone.0093983.

Perkins, J.R., Antunes-Martins, A., Calvo, M., Grist, J., Rust, W., Schmid, R., Hildebrandt, T., Kohl, M., Orengo, C., McMahon, S.B., Bennett, D.L. (2014). A comparison of RNA-seq and exon arrays for whole genome transcription profiling of the L5 spinal nerve transection model of neuropathic pain in the rat. *Mol Pain*, *10*: 7. doi: 10.1186/1744-8069-10-7.

Persohn, E., Malherbe, P., Richards, J.G. (1991). In situ hybridization histochemistry reveals a diversity of GABAA receptor subunit mRNAs in neurons of the rat spinal cord and dorsal root ganglia. *Neurosci*, *42*: 497-507.

Petrus, M., Peier, A.M., Bandell, M., Hwang, S.W., Huynh, T., Olney, N., Jegla, T., Pataputtian, A. (2007). A role of TRPA1 in mechanical hyperalgesia is revealed by pharmacological inhibition. *Mol Pain, 3*: 40.

Philips, A.V., Timchenko, L.T., Cooper, T.A. (1998). Disruption of splicing regulated by a CUG-binding protein in myotonic dystrophy. *Science*, *280*: 737-741.

Piomelli, D., Sasso, O. (2014). Peripheral gating of pain signals by endogenous lipid mediators. *Nat Neurosci*, *17*(2): 164-174. doi: 10.1038/nn.3612.

Pisani, V., Panico, M.B., Terracciano, C., Bonifazi, E., Meola, G., Novelli, G., Bernardi, G., Angelini, C., Massa, R. (2008). Preferential central nucleation of type 2 myofibers is an invariable feature of myotonic dystrophy type 2. *Muscle Nerve*, *38*(5):1405-1411. doi: 10.1002/mus.21122.

Poh, K.W., Yeo, J.F., Ong, W.Y. (2011). MicroRNA changes in the mouse prefrontal cortex after inflammatory pain. *Eur J Pain*, *15*(8): 801.e1-12. doi: 10.1016/j.ejpain. 2011.02.002.

Poh, K.W., Yeo, J.F., Stohler, C.S., Ong, W.Y. (2012). Comprehensive gene expression profiling in the prefrontal cortex links immune activation and neutrophil infiltration to antinociception. *J Neurosci*, *32*(1): 35-45. doi: 10.1523/ JNEUROSCI. 2389-11.2012.

Poole, S., Lorenzetti, B.B., Cunha, J.M., Cunha, F.Q., Ferreira, S.H. (1999). Bradykinin B1 and B2 receptors, tumour necrosis factor α and inflammatory hyperalgesia. *Br J Pharmacol*, *126*(3): 649-656.

Raouf, R., Quick, K., Wood, J.N. (2010). Pain as a channelopathy. *J Clin Invest*, *120*(11): 3745-3752. doi: 10.1172/JCI43158.

Rau, F., Freyermuth, F., Fugier, C., Villemin, J.P., Fischer, M.C., Jost, B., Dembele, D., Gourdon, G., Nicole, A., Duboc, D., et al. (2011). Misregulation of miR-1 processing is associated with heart defects in myotonic dystrophy. *Nat Struct Mol Biol*, *18*: 840-845.

Rey, R. (1995) The History of Pain. *Harvard University Press*

Ritter, A.M., Lewin, G.R., Kremer, N.E., Mendell, L.M. (1991) Requirement for nerve growth factor in the development of myelinated nociceptors in vivo. *Nature, 350*: 500-502.

Roberts, S.B., Rosenberg, I. (2006). Nutrition and Aging: Changes in the Regulation of Energy Metabolism with Aging. *Physiol Rev*, *86*(2): 651-667. Review

Rolke, R., Baron, R., Maier, C., Tölle, T.R., Treede, R.-D., Beyer, A., Binder, A., Birbaumer, N., Birklein, F., Bötefür, I.C., Braune, S., Flor, H., Huge, V., Klug, R., Landwehrmeyer, G.B., Magerl, W., Maihöfner, C., Rolko, C., Schaub, C., Scherens, A., Sprenger, T., Valet, M., Wasserka, B. (2006). Quantitative sensory testing in the German Research Network on Neuropathic Pain (DFNS): Standardized protocol and reference values. *Pain*, *123*: 231-243. doi: 10.1016/j.pain.2006.01.041.

Romigi, A., Albanese, M., Placidi, F., Izzi, F., Liguori, C., Marciani, M.G., Mercuri, N.B., Terracciano, C., Vitrani, G., Petrucci, A., Di Gioia, B., Massa, R. (2014). Sleep disorders in myotonic dystrophy type 2: a controlled polysomnographic study and self-reported questionnaires. *Eur J Neurol*, *21*(6): 929-934. doi: 10.1111/ene.12226.

Rusconi, F., Mancinelli, E., Colombo, G., Cardani, R., Da Riva, L., Bongarzone, I., Meola, G., Zippel, R. (2010). Proteome profile in Myotonic Dystrophy type 2 myotubes reveals dysfunction in protein processing and mitochondrial pathways. *Neurobiol Dis*, *38*(2): 273-280. doi: 10.1016/j.nbd.2010.01.017.

Rush, A.M., Cummins, T.R., Waxman, S.G. (2007) Multiple sodium channels and their roles in electrogenesis within dorsal root ganglion neurons. *J Physiol*, *579* (Pt 1):1-14.

Ruskin, D.N., Kawamura, M., Masino, S.A. (2009). Reduced pain and inflammation in juvenile and adult rats fed a ketogenic diet. *PLoS One*, *4*(12): e8349. doi: 10.1371/journal.pone.0008349.

Russo, C.M., Brose W.G (1998). Chronic pain. *Annu Rev Med, 49*: 123-133.

Rygh, L.J., Svendson, F., Hole, K., Tjolsen, A. (1999). Natural noxious stimulation can induce long-term increase of spinal nociceptive responses. *Pain, 82*: 305-310.

Safieh-Garabedian, B., Poole, S., Allchorne, A., Winter, J, Woolf, C.J. (1995). Contribution of interleukin-1 beta to the inflammation-induced increase in nerve growth factor levels and inflammatory hyperalgesia. *Br J Pharmacol*, *115*: 1265-1275.

Sakuma, Y., Miyagi, M., Inoue, G., Ishikawa, T., Kamoda, H., Yamauchi, K., Orita, S., Suzuki, M., Oikawa, Y., Inage, K., Kubota, G., Sainoh, T., Sato, J., Takahashi, K., Ohtori, S. (2016). Muscle injury in rats induces upregulation of inflammatory cytokines in injured muscle and calcitonin gene-related peptide in dorsal root ganglia innervating the injured muscle. *Muscle Nerve*, *54*(4): 776-782. doi: 10.1002/mus.25092

Samad, T.A., Moore, K.A., Sapirstein, A., Billet, S., Allchorne, A., Poole, S., Bonventre, J.V., Woolf, C.J. (2001). Interleukin-1beta-mediated induction of Cox-2 in the CNS contributes to inflammatory pain hypersensitivity. *Nature*, *410*(6827): 471-475.

Sandkuhler, J. (2009) Models and mechanisms of hyperalgesia and allodynia. *Physiol Rev, 89*: 707-758.

Sansone, V.A., Brigonzi, E., Schoser, B., Villani, S., Gaeta, M., De Ambroggi, G., Bandera, F., De Ambroggi, L., Meola, G. (2013). The frequency and severity of cardiac involvement in myotonic dystrophy type 2 (DM2): long-term outcomes. *Int J Cardiol*, *168*(2): 1147-1153. doi: 10.1016/j.ijcard.2012.11.076

Savkur, R.S., Philips, A.V., Cooper, T.A. (2001). Aberrant regulation of insulin receptor alternative splicing is associated with insulin resistance in myotonic dystrophy. *Nat Genet*, *29*(1): 40-47.

Schiaffino, S., Reggiani, C. (2011). Fiber types in mammalian skeletal muscles. *Physiol Rev*, *91*:1447-1531.

Schaible, H.G. (2007). Peripheral and central mechanisms of pain generation. *Handb Exp Pharmacol, 177*: 3-28.

Schaible, H.G., Ebersberger, A., Natura, G. (2011). Update on peripheral mechanisms of pain: beyond prostaglandins and cytokines. *Arthritis Research and Therapy*, *13*: 210.

Schoewel, V., Marg, A., Kunz, S., Overkamp, T., Carrazedo, R.S., Zacharias, U., Daniel, P.T., Spuler, S. (2012). Dysferlin-peptides reallocate mutated dysferlin thereby restoring function. *PLoS One*, *7*(11): e49603. doi: 10.1371/journal.pone.0049603.

Schwanhäusser, B., Gossen, M., Dittmar, G., Selbach, M. (2009). Global analysis of cellular protein translation by pulsed SILAC. *Proteomics*, *9*(1): 205-209. doi: 10.1002/pmic.200800275.

Schwanhäusser, B., Busse, D., Li, N., Dittmar, G., Schuchhardt, J., Wolf, J., Chen, W., Selbach, M. (2011). Global quantification of mammalian gene expression control. Nature, 473(7347): 337-342. doi: 10.1038/nature10098.

Screen, M., Jonson, P.H., Raheem, O., Palmio, J., Laaksonen, R., Lehtimäki, T., Sirito, M., Krahe, R., Hackman, P., Udd, B. (2014). Abnormal splicing of NEDD4 in myotonic dystrophy type 2: possible link to statin adverse reactions. *Am J Pathol*, *184*(8): 2322-2332. doi: 10.1016/j.ajpath.2014.04.013

Sherwood, L. (2010) Human physiology. Brooks/Cole-Cengage Learning

Sherrington C.S. (1906) Observations on the scratch-reflex in the spinal dog. *J Physiol, 34*: 1-50.

Shi, T., Su, D., Liu, T., Tang, K., Camp, D.G. 2nd, Qian, W.J., Smith, R.D. (2012). Advancing the sensitivity of selected reaction monitoring-based targeted quantitative proteomics. *Proteomics*, *12*(8): 1074-1092. doi: 10.1002/pmic.201100436.

Smith, E.S., Omerbašić, D., Lechner, S.G., Anirudhan, G., Lapatsina, L., Lewin, G.R. The molecular basis of acid insensitivity in the African naked mole-rat. *Science*, *334*(6062): 1557-1560. doi: 10.1126/science.1213760

Staud, R., Vierck, C.J., Cannon, R.L., Mauderli, A.P., Price, D.D. (2001). Abnormal sensitization and temporal summation of second pain (wind-up) in patients with fibromyalgia syndrome. *Pain*, *91*: 165-175.

Stein, C., Clark, J.D., Oh, U., Vasko, M.R., Wilcox, G.L., Overland, A.C., Vanderah, T.W., Spencer, R.H. (2009). Peripheral mechanisms of pain and analgesia. *Brain Res rev*, *60*: 90-113.

Strong, J.A., Xie, W., Coyle, D.E., Zhang, J.M. (2012). Microarray analysis of rat sensory ganglia after local inflammation implicates novel cytokines in pain. *PLoS One*, *7*(7): e40779. doi: 10.1371/journal.pone.0040779.

Suenaga, K., Lee, K.Y., Nakamori, M., Tatsumi, Y., Takahashi, M.P., Fujimura, H., Jinnai, K., Yoshikawa, H., Du, H., Ares, M. Jr, Swanson, M.S., Kimura, T. (2012). Muscleblind-like 1 knockout mice reveal novel splicing defects in the myotonic dystrophy brain. *PLoS One*, *7*(3): e33218. doi: 10.1371/journal. pone.0033218.

Suokas, K.I., Haanpää, M., Kautiainen, H., Udd, B., Hietaharju, A.J. (2012). Pain in patients with myotonic dystrophy type 2: a postal survey in Finland. *Muscle Nerve*, *45*(1): 70-74. doi: 10.1002/mus.22249.

Suominen, T., Bachinski, L.L., Auvinen, S., Hackman, P., Baggerly, K.A., Angelini, C., Peltonen, L., Krahe, R., Udd, B. (2011). Population frequency of myotonic dystrophy: higher than expected frequency of myotonic dystrophy type 2 (DM2) mutation in Finland. *Eur J Hum Genet*, *19*(7): 776-82. doi: 10.1038/ejhg.2011.23.

Svensson, C. I., Fitzsimmons, B., Azizi, S., Powell, H. C., Hua, X.-Y., Yaksh, T. L. (2004). Spinal p38beta isoform mediates tissue injury-induced hyperalgesia and spinal sensitization. *J Neurochem*, *92*: 1508-1520.

Tang, Z.Z., Yarotskyy, V., Wei, L., Sobczak, K., Nakamori, M., Eichinger, K., Moxley, R.T., Dirksen, R.T., Thornton, C.A. (2012). Muscle weakness in myotonic dystrophy associated with misregulated splicing and altered gating of Ca(V)1.1 calcium channel. *Hum Mol Genet*, *21*(6): 1312-1324. doi: 10.1093/hmg/ddr568.

Telang, S., Clem, B.F., Klarer, A.C., Clem, A.L., Trent, J.O., Bucala, R., Chesney, J. (2012). Small molecule inhibition of 6-phosphofructo-2-kinase suppresses t cell activation. *J Transl Med*. *10*: 95. doi:10.1186/1479-5876-10-95.

Thompson, A., Schafer, J., Kuhn, K., Kienle, S., Schwarz, J., Schmidt, G., Neumann, T., Johnstone, R., Mohammed, A.K., Hamon, C. (2003). Tandem mass tags: a novel quantification strategy for comparative analysis of complex protein mixtures by MS/MS. *Anal Chem*, *75*: 1895-1904.

Thornton, C.A., Griggs, R.C., Moxley, R.T. (1994). Myotonic dystrophy with no trinucleotide repeat expansion. *Ann Neurol*, *35*(3): 269-272.

Todd, A.J. (1996). GABA and glycine in synaptic glomeruli of the rat spinal dorsal horn. *Eur J Neurosci*, *8*: 2492-2498.

Towers, S., Princivalle, A., Billinton, A., Edmunds, M., Bettler, B., Urban, L., Castro-Lopes, J., Bowery, N.G. (2000). GABAB receptor protein and mRNA distribution in rat spinal cord and dorsal root ganglia. *Eur J Neurosci*, *12*, 3201-3210.

Trevino V., Falciani, F., Barrera-Saldaña, H.A. (2007). DNA Microarrays: A Powerful Genomic Tool for Biomedical and Clinical Research. *Mol Med*, *13* (9-10): 527–541. doi: 10.2119/2006-00107.Trevino

Tsuda, M., Shigemoto-Mogami, Y., Koizumi, S., Mizokoshi, A., Kohsaka, S., Salter, M.W., Inoue, K. (2003). P2X4 receptors induced in spinal microglia gate tactical allodynia after nerve injury. *Nature*, *424*: 778-783.

Tyers, M., Mann, M. (2003). From genomics to proteomics. Nature, *422* (6928): 193-197.

Udd, B., Krahe R. (2012). The myotonic dystrophies: molecular, clinical, and therapeutic challenges. *Lancet Neurol*, *11*(10): 891-905. doi: 10.1016/S1474-4422(12)70204-1.

Valente, L., Tiranti, V., Marsano, R.M., Malfatti, E., Fernandez-Vizarra, E., Donnini, C., Mereghetti, P., De Gioia, L., Burlina, A., Castellan, C., Comi, G.P., Savasta, S., Ferrero, I., Zeviani, M. (2007). Infantile encephalopathy and defective mitochondrial DNA translation in patients with mutations of mitochondrial elongation factors EFG1 and EFTu. *Am J Hum Genet*, *80*(1): 44-58.

Voets, T., Talavera, K., Owsianik, G., Nilius, B. (2005). Sensing with TRP channels. *Nat Chem Biol, 1*: 85-92.

von Hehn, C.A., Baron, R., Woolf, C.J. (2012). Deconstructing the neuropathic pain phenotype to reveal neural mechanisms. *Neuron*, *73*(4): 638-652. doi: 10.1016/j.neuron.2012.02.008.

Voscopoulos, C., Lema, M. (2010). When does acute pain become chronic? *Br J Anaesth, 105.* Suppl 1: i69-85. doi: 10.1093/bja/aeq323

Walder, R.Y., Rasmussen, L.A., Rainier, J.D., Light, A.R., Wemmie, J.A., Sluka, K.A. (2010). ASIC1 and ASIC3 play different roles in the development of hyperalgesia after inflammatory muscle injury. J Pain, *11*(3): 210-218. doi: 10.1016/ j.jpain.2009.07.004.

Wang, Z., Gerstein M., Snyder M. (2009). RNA-Seq: a revolutionary tool for transcriptomics. *Nat Rev Genet*, 10(1): 57–63. doi: 10.1038/nrg2484.

Warren, G.L., Hulderman, T., Jensen, N., McKinstry, M., Mishra, M., Luster, M.I., Simeonova, P.P. (2002). Physiological role of tumor necrosis factor alpha in traumatic muscle injury. *FASEB J*, 16(12): 1630-1632.

Weber, Y.G., Roebling, R., Kassubek, J., Hoffmann, S., Rosenbohm, A., Wolf, M., Steinbach, P., Jurkat-Rott, K., Walter, H., Reske, S.N., Lehmann-Horn, F., Mottaghy, F.M., Lerche, H. (2010). Comparative analysis of brain structure, metabolism, and cognition in myotonic dystrophy 1 and 2. *Neurology*, 74(14): 1108-1117. doi: 10.1212 /WNL.0b013e3181d8c35f.

Weigl, M., Cieza, A., Cantista, P., Stucki, G. (2007). Physical disability due to musculoskeletal conditions. *Best Pract Res Clin Rheumatol*, *21*(1):167-190.

Wemmie, J.A., Taugher, R.J., Kreple, C.J. (2013). Acid-sensing ion channels in pain and disease. *Nat Rev Neurosci, 14*(7): 461-471. doi: 10.1038/nrn3529.

Wheeler, T.M., Lueck, J.D., Swanson, M.S., Dirksen, R.T., Thornton, C.A. (2007). Correction of ClC-1 splicing eliminates chloride channelopathy and

myotonia in mouse models of myotonic dystrophy. *J Clin Invest*, 117(12): 3952-3957.

Wheeler, M.T., Zarnegar, S., McNally, E.M. (2002). Zeta-sarcoglycan, a novel component of the sarcoglycan complex, is reduced in muscular dystrophy. *Hum Mol Genet*, *11*: 2147-2154.

Win, A.K., Perattur, P.G., Pulido, J.S., Pulido, C.M., Lindor, N.M. (2012). Increased cancer risks in myotonic dystrophy. *Mayo Clin Proc*, *87*(2): 130-135. doi: 10.1016/j.mayocp.2011.09.005.

Wolfe, R.R. (2006). The underappreciated role of muscle in health and disease. *Am J Clin Nutr*, *84*:475-482.

Woolf, C.J., Salter, M.W. (2000). Neuronal plasticity: increasing the gain in pain. *Science*, *288*: 1765-1768.

Woolf, C.J. (2011). Central sensitization: implications for the diagnosis and treatment of pain. *152*(3 Suppl): S2-15. doi: 10.1016/j.pain.2010.09.030.

Xiao, H.S., Huang, Q.H., Zhang, F.X., Bao, L., Lu, Y.J., Guo, C., Yang, L., Huang, W.J., Fu, G., Xu, S.H., Cheng, X.P., Yan, Q., Zhu, Z.D., Zhang, X., Chen, Z., Han, Z.G., Zhang X. (2002). Identification of gene expression profile of dorsal root ganglion in the rat peripheral axotomy model of neuropathic pain. *Proc Natl Acad Sci U S A*, *99*: 8360-8365.

Xie, J., Prince, M.P., Wemmie, J.A., Askwith, C.C., Welsh, M.J. (2003). ASIC3 and ASIC1 mediate FMRFamide-related peptide enhancement of H+-gated currents in cultured dorsal root ganglion neurons. *J Neurophysiol, 89*(5): 2459-2465.

Yang, L., Zhang, F.X., Huang, F., Lu, Y.J., Li, G.D., Bao, L., Xiao, H.S., Zhang, X. (2004). Peripheral nerve injury induces trans-synaptic modification of

channels, receptors and signal pathways in rat dorsal spinal cord. *Eur J Neurosci*,19: 871–883.

Yokota, S., Imagawa, T., Mori, M., Miyamae, T., Aihara, Y., Takei, S., Iwata, N., Umebayashi, H., Murata, T., Miyoshi, M., Tomiita, M., Nishimoto, N., Kishimoto, T. (2008) Efficacy and safety of tocilizumab in patients with systemic-onset juvenile idiopathic arthritis: a randomised, double-blind, placebo-controlled, withdrawal phase III trial. *Lancet*, *371* (9617): 998-1006. doi: 10.1016/S0140-6736(08)60454-7.

Young, N.P., Daube, J.R., Sorenson, E.J., Milone, M. (2010). Absent, unrecognized, and minimal myotonic discharges in myotonic dystrophy type 2. *Muscle Nerve*, *41*(6): 758-762. doi: 10.1002/mus.21615.

Zhang, Y., Fonslow, B.R., Shan, B., Baek, M.C., Yates, J.R. 3rd (2013). Protein analysis by shotgun/bottom-up proteomics. *Chem Rev*, *113*: 2343-2394. doi: 10.1021/cr3003533.

Zhao, S., Fung-Leung, W.P., Bittner, A., Ngo, K., Liu, X. (2014). Comparison of RNA-Seq and Microarray in Transcriptome Profiling of Activated T Cells. *PLoS ONE*, *9*(1): e78644. doi: 10.1371/journal.pone.0078644.

Zhou, Y., Liu, Z., Liu, Z., Chen, S., Li, M., Shahveranov, A., Ye, D., Tian, Y. (2016). Interleukin-6: an emerging regulator of pathological pain. *J Neuroinflammation*, 13(1): 141. doi: 10.1186/s12974-016-0607-6.

Zhuang, Z.Y., Gerner, P., Woolf, C.J., Ji, R.R. (2005). ERK is sequentially activated in neurons, microglia, and astrocytes by spinal nerve ligation and contributes to mechanical allodynia in this neuropathic pain model. *Pain*, *114*: 149-159.