Role of microRNA-223 in pain modulation

Spinal nociceptive signaling and inflammatory mediators

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Abstract

Aims: Lumbar radicular pain (LRP) is often associated with increased local and systemic synthesis of inflammatory mediators. This process can be modulated by specific microRNAs (miRs). In an animal model, we investigated the effect of miR-223 on the spinal nociceptive signaling and local gene expression. In patients with LRP, we examined the relationship between the expression of miR-223, circulating inflammatory cytokines, the HLA-DQB1 haplotype and pain recovery.

Methods: In anaesthetized Lewis rats, extracellular single unit recordings of spinal nociceptive activity and qPCR were used to explore the effect of miR-223 application onto the dorsal nerve roots (L3-L5). Moreover, in patients with LRP, the relationship between serum miRNAs, serum cytokines and lumbar radicular pain was investigated by qPCR and multiplex proximity extension assay (PEA). Finally, the differences in the expression of miR-223 and cytokines between carriers and non-carriers of HLA-DQB1*03:02 haplotype identified by TaqMan genotyping were studied.

Results: In rats, a significant decrease in the C-fiber response was demonstrated following application of miR-223 onto the dorsal nerve roots. In addition, the gene expression of interleukin-6 (IL-6) was increased in the spinal cord. In patients with acute LRP, a correlation between high expression of miR-223 and better recovery was observed. Patients with high levels of IL-6 reported more pain than patients with low levels of IL-6. In the carriers of HLA-DQB1*03:02 haplotype, higher expression of miR-223 and transforming growth factor-β (TGF-β) was observed.

Conclusions: Our data suggest that miR-223 may reduce nociceptive signaling in the pain pathways, possibly by modulating the expression of inflammatory mediators.

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Abbreviations

5-HT	5-hydroxytryptamine/serotonin
A	Adenosine
AGO	Argonaute protein
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANOVA	Analysis of variance
ATP	Adenosine-tri-phosphate
BDNF	Brain-derived neurotrophic factor
BK	Bradykinin
Bp	Base pair
C	Cytosine
CAMKII	Ca^{2+}-calmodulin dependent kinase II
cAMP	Cyclic adenosine monophosphate
cDNA	Complimentary deoxyribonucleic acid
CNS	Central nervous system
CRE	cAMP response element
CREB	cAMP response element binding protein
C_t-value	Cycle threshold-value
DAG	Dyacylglycerol
DH	Dorsal horn
DNA	Deoxyribonucleic acid
DRG	Dorsal root ganglion
ERK	Extracellular signal related kinase
G	Guanine
GABA	γ-aminobutyric acid

Glu	Glutamate
GluR1	Glutamate receptor 1
gl130	Glycoprotein 130
HLA	Human leukocyte antigen
IASP	International Association for the Study of Pain
IL	Interleukin
IP_3	Inositol-1,4,5-triphosphate
LRP	Lumbar radicular pain
LTP	Long-term potentiation
MAPK	Mitogen-activated kinase
mGlu	Metabotropic glutamate receptor
MHC	Major compatibility complex
miR	Micro RNA
miRISC	miR-induced silencing complex
MMP	Matrix metalloproteinase
MRI	Magnetic resonance imaging
mRNA	Messenger ribonucleic acid
NF-κβ	Nuclear factor kappa-light-chain-enhancer of activated B cells
NGF	Nerve growth factor
NK1R	Neurokinin-1 receptor
NLRP3	Nod-like receptor protein 3
NMDA	N-methyl-D-aspartate
NO	Nitric oxide
NP	Nucleus pulposus
OUH	Oslo University Hospital
PAG	Periaqueductal gray
PCR	Polymerase chain reaction

PEA	Proximity extension assay
PIP_2	Phosphatidylinositol 4,5-biphospate
PKA	Protein kinase A
PKC	Protein kinase C
PLC	Phospholipase C
PG	Prostaglandin
qPCR	Quantitative polymerase chain reaction
RNA	Ribonucleic acid
RNase	Ribonuclease
RVM	Rostroventral medulla
SEM	Standard error of the mean
SLR	Straight Leg Rising test
SNP	Single nucleotide polymorphisms
SP	Substance P
T	Tyrosine
TE-buffer	Tri-ethylenediaminetetraacetic acid-buffer
TGF	Transforming growth factor
TLR	Toll-like receptor
T_m	Melting temperature
TNF	Tumor necrosis factors
TRPV1	Transient receptor potential cation channel subfamily V member 1
UTR	Untranslated region
VAS	Visual analogue scale
VGCC	Voltage-gated calcium channels
WDR	Wide dynamic range

1 Introduction

1.1 Individual and societal burden of chronic pain

Chronic pain is a globally widespread problem both at an individual level and for the society as a whole. It has been estimated that approximately 30% of the adult population in Norway experience chronic pain at some point in their life (Rustoen *et al.*, 2004; Landmark *et al.*, 2013). Musculoskeletal disorders are one of the most prevalent causes leading to chronic pain, where low back pain is one of the most frequent reasons for sick leave (Brage *et al.*, 2010). Treatment of chronic pain leads to considerable costs for society. In Norway, treatment and social help expenses caused by musculoskeletal disorders alone constitute approximately 70 billion NOK yearly (Lærum, 2013). At an individual level, chronic pain is a debilitating health problem that has many repercussions, including reduced life quality, impaired physical function and sick leave. Existing surgical and analgesic treatment methods are not always sufficient or effective for patients with low back pain. Therefore, it is important to investigate mechanisms of chronic pain development in order to evolve new and more specific drug targets. For review see (Breivik *et al.*, 2013).

1.2 Pain versus nociception

One of the most evolutionary important mechanisms of survival is avoidance of dangerous situations, where pain is functioning as an alerting system. Pain is defined as "an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage" by the International Association for the Study of Pain (IASP). According to this definition, pain perception is always subjective due to the complex processing of the given stimuli that is influenced by sensory, cognitive, emotional and contextual factors. Thus, pain is not directly equivalent to initial stimuli magnitude. In contrast, IASP defines nociception as "the neuronal processes of encoding and processing noxious stimuli". Thus, nociception involves activation of specific receptors in response to potentially harmful stimuli and subsequent neuronal signaling. For review see (Loeser and Treede, 2008).

1.2.1 Chronic pain

Chronic pain is defined by IASP as pain that lasts longer than 3 months after the initial tissue damage, i.e., longer than assumed normal duration of tissue healing. Increased pain sensation in response to noxious stimuli is described as hyperalgesia. Allodynia is the phenomenon where experience of pain occurs due to normally innoxious stimuli.

1.3 Nociceptive signaling

Perception of pain begins with activation of nociceptors at the ends of primary afferent nerve fibers. Nociceptors are polymodal high-threshold receptors that are activated by noxious thermal, mechanical or chemical stimuli. These receptors are located on medium diameter Aδ-fibers and small diameter C-fibers. The thinly myelinated Aδ-fibers have a conduction velocity of 5 to 30 m/s, while the unmyelinated C-fibers have a much slower conduction velocity of 0.2 to 2 m/s. Aδ- and C-fibers are responsible for nociceptive signaling, unlike large diameter myelinated Aα- and Aβ-fibers that respond to innoxious stimuli. Sensory information from the primary afferent nociceptive fibers relays to the dorsal horn (DH) of the spinal cord. For review see (Julius and Basbaum, 2001).

Axons of afferent nerve fibers terminate in the different laminae of the DH. Nociceptive Aδ- and C-fibers axons are innervating Rexed's lamina I, the upper part of lamina II and parts of lamina V, whereas most of Aα- and Aβ- (tactile and hair follicle) afferents are ending in lamina III-V (Light and Perl, 1979), but some of Aβ- axons extend to lamina II (Woodbury *et al.*, 2008; Brown *et al.*, 1981). (Laminae were defined by Rexed based on neuron size and density in the different sections of the DH (Rexed, 1952).)

In the DH, the neurotransmitters glutamate (Glu) (Kangrga and Randic, 1991) and substance P (SP) (Kantner *et al.*, 1985) are pre-synaptically released from activated nociceptive afferent fibers. Binding of glutamate and SP to α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor and neurokinin 1 (NK1) receptor, respectively, leads to membrane depolarization of the postsynaptic neurons.

Postsynaptic neurons are divided into 3 classes according to their projection: interneurons, propriospinal neurons and projection neurons. Interneurons are one of the most abundant classes of neurons in the DH and constitute most of the lamina I-III neurons, and can be either excitatory or inhibitory. Inhibitory interneurons use γ-

aminobutyric acid (GABA) and glycin as neurotransmitters to modulate activity of other neurons locally, since interneurons have short and branched axons. Propriospinal neurons have projections that reach to neurons across different spinal levels (Flynn *et al.*, 2011). Projection neurons have extended axons that transfer sensory information from the DH to the brain. For review see (Todd, 2010).

1.3.1 Ascending pathways

Ascending projections from neurons differ between the surface and the deeper regions of the DH. In the surface lamina, projection neurons are nociceptive-specific and respond to noxious input only. These neurons convey nociceptive information first to the lateral parabrachial area and further to the amygdala and the periaqueductal grey (PAG) where emotional and behavioral modulation of pain occurs. They also project to the hypothalamus and the ventrolateral medulla that regulates autonomic homeostasis, and to the cingulate cortex that controls emotional aspects of pain. In the deeper lamina, wide dynamic range (WDR) neurons process both noxious and tactile stimuli. WDR neurons convey information about intensity and location of pain, through the thalamus, to the somatosensory cortex. For review see (Gauriau and Bernard, 2002).

1.3.2 Descending modulation

Serotogenic, noradrenergic and enkephalinergic descending projections modulate the neuronal conveyance in the spinal cord. Activity of these projections is regulated by the PAG, the pons and the rostroventral medulla (RVM). The PAG receives input from the ascending sensory pathway and the prefrontal cortex, the insula, the anterior cingulate cortex, the lateral hypothalamus and the amygdala (Figure 1.1).

ON- and OFF- cells are essential components of the descending modulation in the RVM system, and have pro-nociceptive and anti-nociceptive effects, respectively. Under normal conditions, inhibitory modulation prevails over facilitatory modulation. However, in some neuropathic conditions facilitatory modulation is dominating. For review see (Gjerstad, 2007).

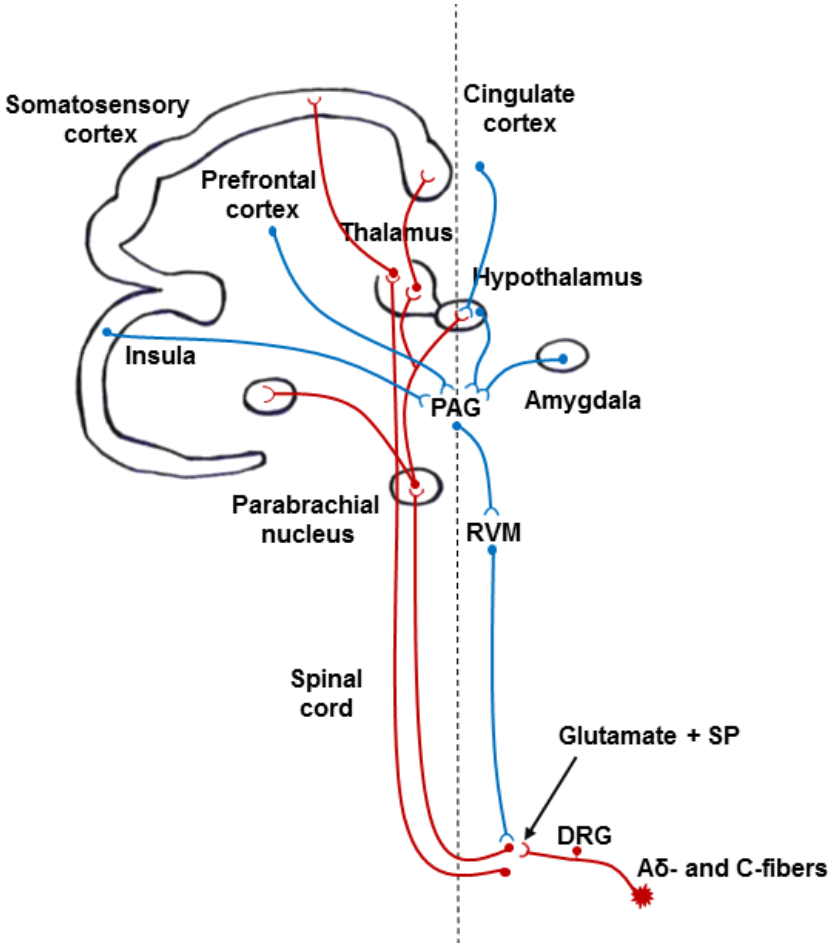


Figure 1.1 A schematic representation of nociceptive signaling and modulatory pathways. Afferent Aδ- and C-fibers and ascending projections are shown in red, while descending projections are shown in blue. Nociceptive information from afferent fibers is conveyed to the dorsal horn (DH) of the spinal cord. From the DH projection neurons transmit the signals to the brainstem and supraspinal centers. The emotional and behavioral modulation involves the lateral parabrachial area, the amygdala, the periaqueductal grey (PAG) and cingulate cortex. Discrimination of pain location and intensity occurs in the somatosensory cortex, where signals are convey through the thalamus. Descending pathways modulate nociceptive activity in the DH and thereafter in the ascending pathways. DRG: dorsal root ganlgion, PAG: peraqueductal grey, RVM: rostral ventromedial medulla, SP: substance P. Adapted from (Gjerstad, 2007)

1.4 Sensitization

Sensitization is defined as an exaggerated nociceptive response to suprathreshold stimuli (hyperalgesia) and/or a lowered threshold for response (allodynia). This may also include spontaneous discharges and an enlarged receptive field. Acute pain normally functions as an alert system for potentially noxious stimuli, whereas chronic pain has no confirmed biological value and leads to unnecessary mental torment. Transition from acute to

chronic pain may occur as a result of neural plasticity. For review see (Loeser and Treede, 2008; Woolf and Salter, 2000)

1.4.1 Peripheral sensitization

Peripheral sensitization leads to local hypersensitivity of the primary afferent nociceptive fibers. This happens due to changes in the chemical environment around the nerve fibers. For instance, a variety of mediators, such as adenosine triphosphate (ATP), neurotrophic factors such as nerve growth factors (NGF) and brain-derived neurotrophic factor (BDNF), prostaglandins (PG), bradykinin (BK), substance P (SP), serotonin (5-HT), cytokines and chemokines are released following tissue damage. This may directly or indirectly affect receptors and ion channels and lead to enhanced responsiveness of the primary afferent neurons. For review see (Julius and Basbaum, 2001).

1.4.2 Central sensitization

Central sensitization is defined as "increased responsiveness of nociceptive neurons in the central nervous system to their normal or subthreshold afferent input" by IASP. Such exaggerated responsiveness to primary afferent input influences lager areas of the body due to the converging nature of nociceptive signaling. Central sensitization includes not only increased responsiveness to afferent signaling, but also reduced action of inhibitory neurons by decreasing synthesis and release of inhibitory transmitters by these cells (Scholz *et al.*, 2005). For review see (Woolf, 2007).

1.4.3 Molecular mechanism of central sensitization

Excitation of afferent nerve fibers by mild noxious stimuli induces presynaptic release of glutamate in the DH, which binds the ionotropic AMPAR in the postsynaptic membrane. This leads to the opening of AMPAR and inflow of Na^+ into the cell, inducing a short-lasting depolarization of the postsynaptic membrane. Glutamate can also bind to metabotropic Ca^{2+} permeable NMDAR. However, NMDAR's ion channel is blocked by a Mg^{2+}-ion at resting membrane potential. Therefore, opening of NMDAR requires both glutamate and removal of Mg^{2+} blockage by membrane depolarization. Opening of voltage-gated calcium channels (VGCCs) also requires membrane depolarization and leads to additional Ca^{2+} inflow.

Excitation of nerve fibers by stronger noxious stimuli includes presynaptic release of SP in addition to glutamate. SP binds to NK1R on the postsynaptic membrane and causes phospholipase C (PLC) activation. PLC cleaves membrane phospholipid phosphatidylinositol 4,5-biphosphate (PIP_2) into dyacylglycerol (DAG) and inositol-3,4,5-triphosphate (IP_3). IP_3 facilitates release of intracellular Ca^{2+} by binding to Ca^{2+} channels on the endoplasmic reticulum (Drdla and Sandkühler, 2008). The following increased cytosolic Ca^{2+} level may lead to long-term potentiation (LTP) through several mechanisms. For review see (Latremoliere and Woolf, 2009).

The increased cytosolic Ca^{2+} level stimulates several Ca^{2+} dependent kinases, including Ca^{2+} calmodulin dependent kinase II (CaMKII) (Pedersen *et al.*, 2005), protein kinase C (PKC) (Lin *et al.*, 1996), protein kinase A (PKA) (Lin *et al.*, 2002) and extracellular signal-regulated kinase (ERK)(Lever *et al.*, 2003). When activated, ERK translocates to the nucleus, where it activates the cyclic adenosine monophosphate (cAMP) response element binding protein (CREB). CREB binds to the regulatory cAMP response element (CRE) and stimulates gene transcription.

1.5 Vertebral column

The human vertebral column consists of 7 cervical, 12 thoracic, 5 lumbar and 5 sacral vertebrae, whereas a rat's spine consists of 7 cervical, 13 thoracic, 6 lumbar, 4 sacral and 27-30 caudal vertebrae. Intervertebral discs are connecting joints between adjacent vertebrae. The main functions of intervertebral discs are to distribute loads arising from body weight and muscle activity across the spine, and to allow movement of vertebrae in relation to each other, facilitating flexibility of the spine. The spinal disc consists of a gel-like substance in the center of the disc termed nucleus pulposus (NP) that is surrounded by a thick layer of annulus fibrosus and outer hyaline cartilage endplate that adjoins to the vertebrae. NP is made of collagen and elastin fibers that are integrated into a highly hydrated aggrecan-containing gel. Elastin fibers are incorporated into lamellae rings that are crossed with collagen fibers inside annulus fibrosus. For review see (Raj, 2008; Urban and Roberts, 2003).

1.5.1 Disc degeneration and herniation

Sciatica and disc herniation or prolapse after disc degeneration is associated with lumbar radicular pain. Disc herniation occurs more frequently in older people due to changes in the morphology of the intervertebral discs with age. Loss of aggrecan leads to lower osmotic pressure and subsequent hydration loss, and makes the discs more rigid and fragile. These alterations may lead to rupture of annulus fibrosus and protrusion of NP from the disc. This may cause mechanical compression of the dorsal nerve roots and may ultimately result in sciatica. For review see (Raj, 2008; Urban and Roberts, 2003).

1.5.2 Nucleus pulposus

Some decades ago, it was suggested that mechanic compression of the nerve roots was the sole cause of the observed sensitization following disc herniation. However, further research has shown that the sensitization of nerve fibers may also arise by local release of pro-inflammatory mediators, i.e. interleukin-6 (IL-6), prostaglandin E2 (PGE2), nitric oxide (NO) and matrix metalloproteinases (MMPs) (Kang *et al.*, 1997), without any mechanical impact (Takebayashi *et al.*, 2001; Anzai *et al.*, 2002). These mediators may be released both by NP and by recruited immune cells. NP is an immunogenic substance, since it is isolated inside the intervertebral disc, and has no contact with the immune system. Following experimental disc herniation, exosomal microRNA content is released from NP (Moen *et al.*, 2017).

1.6 microRNA

MicroRNAs (miRs) are small, up to 25 nucleotides long, functional non-coding RNAs, which mediate post-translational repression by binding to the 3'-untranslated region (UTR) of mRNA targets (Figure 1.2). This binding either blocks the target mRNA from being translated into protein, or targets it for degradation. The sequences of miRs and their target sites in mRNA are highly conserved across species

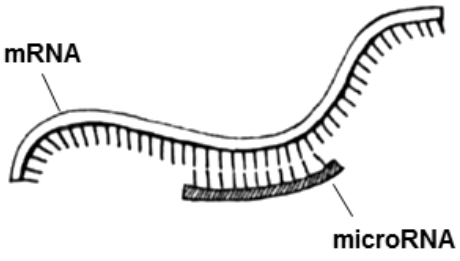


Figure 1.2. A schematic representation of translation repression of complementary mRNA target by microRNA.

(Friedman *et al.*, 2009). For review see (Bartel, 2004).

Argonaute (AGO) proteins assemble with miRs to form miR-induced silencing complexes (miRISCs) in order to perform direct post-translational repression combined with destabilization of fully or partially complementary mRNA targets. Degradation of mRNA targets is achieved in three steps: first a deadenylation step, resulting in shortening of mRNA poly (A) tails; then, the mRNA undergoes decaping by hydrolysation of the mRNA 5'cap; and lastly a 5'-to-3' exonucleolytic decay. For review see (Jonas and Izaurralde, 2015).

In addition, other functional mechanisms of miR action have been discovered: miR-21 and miR-29a have the ability to bind to toll-like receptors (TLRs) and activate immune cells (Fabbri *et al.*, 2012).

Involvement of miRs has been demonstrated in the most of biological processes, including cell proliferation, differentiation and growth, as well as disease initiation and progression(Ameres and Zamore, 2013; Hesse and Arenz, 2014). In humans, more than half of protein-encoding genes can be regulated by miRs (Friedman *et al.*, 2009). Moreover, miRs are found in most of body fluids, e.g. serum and saliva (Gallo *et al.*, 2012), meaning changes in the expression of these regulatory oligonucleotides may have profound systemic effects. Therefore, studying and understanding the regulation of miRs may be important in order to understand the mechanisms behind the development of various diseases.

Circulating miRs are protected from degradation by being packaged into vesicles, by being loaded extracellularly into high-density lipoprotein (HDL) (Vickers *et al.*, 2011), or by binding to AGO2 proteins on the surface of vesicles (Arroyo *et al.*, 2011). The sorting of miRs into vesicles is not a random process (Sato-Kuwabara *et al.*, 2015). In fact, several specific miR sorting mechanisms have been identified; one of them is the miRISC-related pathway (Gibbings *et al.*, 2009).

Alterations of miR expression are reported in a broad range of pain conditions, including inflammation and peripheral nerve and spinal cord injury (Sakai and Suzuki, 2014). For instance, up-regulation of miR-233 may be associated with better recovery in lumbar disc herniation patients (Moen *et al.*, 2017).

1.7 Cytokines

Cytokines are small proteins that are involved in autocrine, paracrine and endocrine cell signaling. A diversity of immune cells produce cytokines in the periphery, whereas in the central nervous system cytokines are secreted from glial cells. Cytokines include several classes: interleukins (ILs), chemokines, tumor necrosis factors (TNFs), interferons (IFNs), colony-stimulation factors (CSFs) and transforming growth factors (TGFs). For review see (Rothwell *et al.*, 1996).

Cytokines are engaged in inflammatory and immune responses, i.e. following tissue damage, and have the ability to regulate (enhance/reduce) their own expression or the expression of other cytokines. They can be divided into pro-inflammatory and anti-inflammatory cytokines depending on their effect on inflammation. The pro-inflammatory cytokine IL-6 and the anti-inflammatory cytokines IL-10 and TGF-β play important roles in the development of inflammatory and neuropathic pain. For review see (Dinarello, 2000).

IL-6 is secreted mainly by macrophages, but also by microglia (Kreutzberg, 1996; Milligan *et al.*, 2003) and is considered to be an important immunological mediator both in the periphery (Murphy *et al.*, 1995) and in the CNS, were it is also engaged in neuroprotective activity, neuronal differentiation, growth and survival (Gruol and Nelson, 1997). Following peripheral nerve damage, the level of IL-6 increases at the dorsal spinal level, particularly in layer I and II (DeLeo *et al.*, 1996), both in microglial cells and in neurons (Yamauchi *et al.*, 2006). Moreover, IL-6 has an important role in inflammatory and neuropathic pain (Kreutzberg, 1996). However, it was also discovered that IL-6 may have a contradictory role. Following peripheral neural damage, exogenous IL-6 showed to have with an anti-nociceptive effect by inhibiting electrically evoked C-fiber responses (Flatters *et al.*, 2003).

IL-10 is considered to be an anti-inflammatory cytokine and may inhibit the release of IL-1β and IL-6 from glial cells, and thus affects development of neuropathic pain (Milligan *et al.*, 2006; Sawada *et al.*, 1999).

Another important anti-inflammatory cytokine may be TGF-β that is produced by astrocytes, leukocytes, dendritic cells and natural killer cells. TGF-β greatly prevents microglial proliferation and activation in vitro (Suzumura *et al.*, 1993) and inhibits the

release of pro-inflammatory cytokines, such as TNF, INF-γ and IL-1β, from astrocytes (Benveniste *et al.*, 1995). Therefore both IL-10 and TGF-β may inhibit inflammatory processes and reduce inflammatory and neuropathic pain.

1.8 Genetic variability

In the human genome, 99.9% of the DNA is identical for all individuals, except monozygotic twins, who share 100% of their DNA. Genetic polymorphisms constitute the remaining 0.1% of the DNA. One type of genetic variability is single-nucleotide polymorphisms (SNPs) that occur when a single base pair is substituted (Kruglyak and Nickerson, 2001). Such substitutions may occur both in the gene coding regions and in the non-coding regions of the DNA. SNPs in the coding sequence may lead to alterations of amino acids, which may cause the synthesis of non-functional protein or protein with changed function. Often two or more SNPs are inherited together from one generation to the next. Such combinations of those SNPs may be defined as haplotypes. This individual gene variability may also lead to increased susceptibility to a variety of diseases, including chronic pain (Klepstad *et al.*, 2004; Solovieva *et al.*, 2004; Zubieta *et al.*, 2003). For review see (Brookes, 1999).

Polymorphisms in genes encoding the major compatibility complex (MHC) may affect neuropathic pain-like behavior in rats after nerve injury (Dominguez *et al.*, 2008). A corresponding correlation has been observed in humans. In humans MHC is termed human leukocyte antigen (HLA). The HLA haplotype DQB1*03:02 may be associated with increased pain in patients with lumbar disc herniation (Dominguez *et al.*, 2013). The HLA-DQ gene region belongs to HLA class II molecules, where the gene is coding for the α- and β-chains of the HLA class II heterodimer. HLA class II molecules are anchored in the membrane of the antigen-presenting cells, such as macrophages.

HLA-DQB1 genes contain polymorphisms establishing the peptide binding specificity of the β-chain of the heterodimer. Alternative splicing leads to further diversity of this heterodimer. HLA-DQB1*03:02 is one of the haplotypes of this gene, and is defined by presence of 3 SNPs: rs9275312, rs2395185, rs39167765 (Dominguez *et al.*, 2013).

2 Aims

The purpose of this master project was to generate new knowledge about the mechanism underlying development of long lasting pain following disc herniation. First, by an animal model mimicking clinical disc herniation, the possible relationships between the local miR-223 and the neuronal activity in the dorsal horn (DH), i.e., in the pain pathways, were examined by extracellular single cell recordings. Next, the effect of miR-223 on the local gene expression in the DH was investigated by qPCR. Moreover, in patients with lumbar radicular pain (LRP), the level of expression of miR-223 and cytokines in the serum was quantified by multiplex proximity extension. The relationship between miR-223, cytokines and pain recovery was studied. Finally, the differences in the expression of the abovementioned between carriers and non-carriers of DQB1*03:02 HLA haplotype were studied. Four sub goals were defined:

In the animal model (rats);

1) Examine the C-fiber response in the spinal dorsal horn after application of miR-223 onto the dorsal nerve roots.

2) Explore the effect of miR-223 on expression of the IL-6, IL-10 and TGF-β in the spinal dorsal horn.

In the patients;

3) Investigate the expression of miR-223, IL-6, IL-10 and TGF-β in serum and recovery of patients with LRP after disc hernation.

4) Examine the expression of miR-223, IL-6, IL-10 and TGF-β in carriers and non-carriers of the DQB1*03:02 HLA haplotype.

3 Materials and Methods

3.1 The animal experiments

The animal experiments were approved by the Norwegian Animal Research Authority and performed in accordance with the laws and regulations controlling experiments and procedures on live animals in Norway. Inbred Lewis female rats (ordered from Janvier Labs, France) weighting 180 to 230g were used.

After arrival, the animals were acclimatized to the animal facility at the Norwegian National Institute of Occupational Health for at least 5 days. The air temperature in the facility was maintained at 20-22°C with relative humidity at 50-55% and an air ventilation rate of 15 x the volume of the room per hour. All experiments were performed during the light phase of an artificial 14 hours light/10 hours dark cycle.

The rats were housed in cages; with maximum four rats per cage. The animals had free access to food and water. The animal facilities were monitored daily by the staff.

3.1.1 Anesthesia

Before surgery, isoflurane gas (Baxter International Inc., USA) was used to sedate the animals in an isolated gas chamber with O_2 supply. After 60-90 seconds, 250mg/ml urethane (Sigma- Aldrich Co., USA) was administrated through intraperitoneal injections to anesthetize the rat. To avoid a lethal overdose, urethane was given in several small doses; the first injection of 0.5 ml was followed by small injections of 0.2-0.3 ml (to a total of approximately 2g urethane/kg bodyweight). Absence of eye reflexes, ear reflexes and paw withdrawal in response to pinch was considered as sufficient anesthesia for surgery. Ointment (Simplex, 80% Vaseline and 20% Paraffin) was used to prevent their eyes from drying during the experiments.

3.1.2 Surgery

Body temperature was maintained at 36-37°C during surgery by a feedback heating pad (homoeothermic blanket control unit, Harvard Apparatus Ltd. Kent, UK). The animals were monitored during the whole experiment. The back and the upper thigh were shaved

before the surgery. The rats were euthanized immediately after the completion of the experiments.

1)Dissecting free the sciatic nerve

An incision was made above the pelvic girdle, and the sciatic nerve was freed and isolated from the adjacent tissue. Retractors were used to hold the incision open, so a bipolar silver hook electrode could be placed in contact with the sciatic nerve for electrical stimulation.

2)Laminectomy procedure

Ear pins and clamps, placed rostral and claudal to the laminectomy site, were used to keep the head and the spinal cord fixed in place.

The laminectomy was performed in the Th13 to L1 region corresponding to the spinal cord segments L3 to S1. The dura mater and arachnoidea were removed with a cannula and two tweezers, under the microscope.

3)Spinal cord tissue harvesting

The animals were euthanized before tissue harvesting. A 10 mm long dorsal lateral quadrant was separated from the rest of the spinal cord and immediately frozen in liquid nitrogen and stored at -80°C. In the miR-223-3p group, the spinal cord tissue was taken out 3 hours after the application of miR-223-3p (solved in Invivofectamine 3.0 reagent), whereas in the control group, the spinal cord tissue was taken out after 3 hours.

3.2 Electrophysiology

A parylene coated tungsten microelectrode with impedance 2-4 MΩ (Frederick Haer & Co., Bowdoinham, USA) was lowered into the left spinal dorsal horn by a micromanipulator (Märzhäuser Wetzlar GmbH & Co., KG, Wetzlar, Germany) and was used to measure the neuronal activity. A reference electrode, placed subcutaneously, served as reference.

The neuronal signals were amplified by an AC preamplifier and further filtered by a band pass filter (Digitimer Ltd, Hertfordshire, UK) with a half-amplitude cut-off of 500-1250

Hz. Digitalization of the signal was performed by a CED 1401µ interface before sampling of data with the software CED Spike 2 (Cambridge Electronic design, Cambridge, UK). The spinal cord segments L3-S1 were identified by tapping on the left paw.

A test stimulus was applied to the sciatic nerve through the silver hook electrode every fourth minute. A pulse buffer was connected to a stimulus isolation unit (NeuroLog System, Digitimer Ltd, UK) to regulate the intensity of the stimulation (Figure 3.1A).

Action potentials with a latency of 0 to 50 ms following each test stimulus were defined as the A-fiber response, whereas action potentials with a latency 50 to 300 ms were defined as the C-fiber response (Figure 3.1B). The C-fiber response threshold was defined as the minimum stimulus intensity required for evoking a single spike at the time interval from 50 to 300ms. The test stimuli intensity was maintained at 1,5x pre-baseline C-fiber threshold throughout the experiment.

The baseline was defined as the mean of 6 stable measurements following test stimuli. Only C-fiber responses with 5-20 spikes and values not diverging more than 20% from the baseline mean were included. Amplitude and shape of the spikes were used to discriminate between signals from different cells to ensure single-cell recording (Figure 3.1C).

Every 30 minutes 0.9% NaCl was applied onto the spinal cord to protect it from drying during the experiment.

miR-223-3p was purchased from Qiagen Sciences Inc. (USA). The 2.4 mg/ml miR-223-3p solution was first mixed 10 µl complexation buffer, and then diluted with 20µl Invivofecatmine 3.0 reagent (Thermo Fisher Scientific Inc., USA) to obtain a concentration of 0.6 mg/ml. The mixture was vortexed and incubated for 30 minutes at 50°C prior to administration onto the dorsal nerve roots. For protocol see appendix 1.

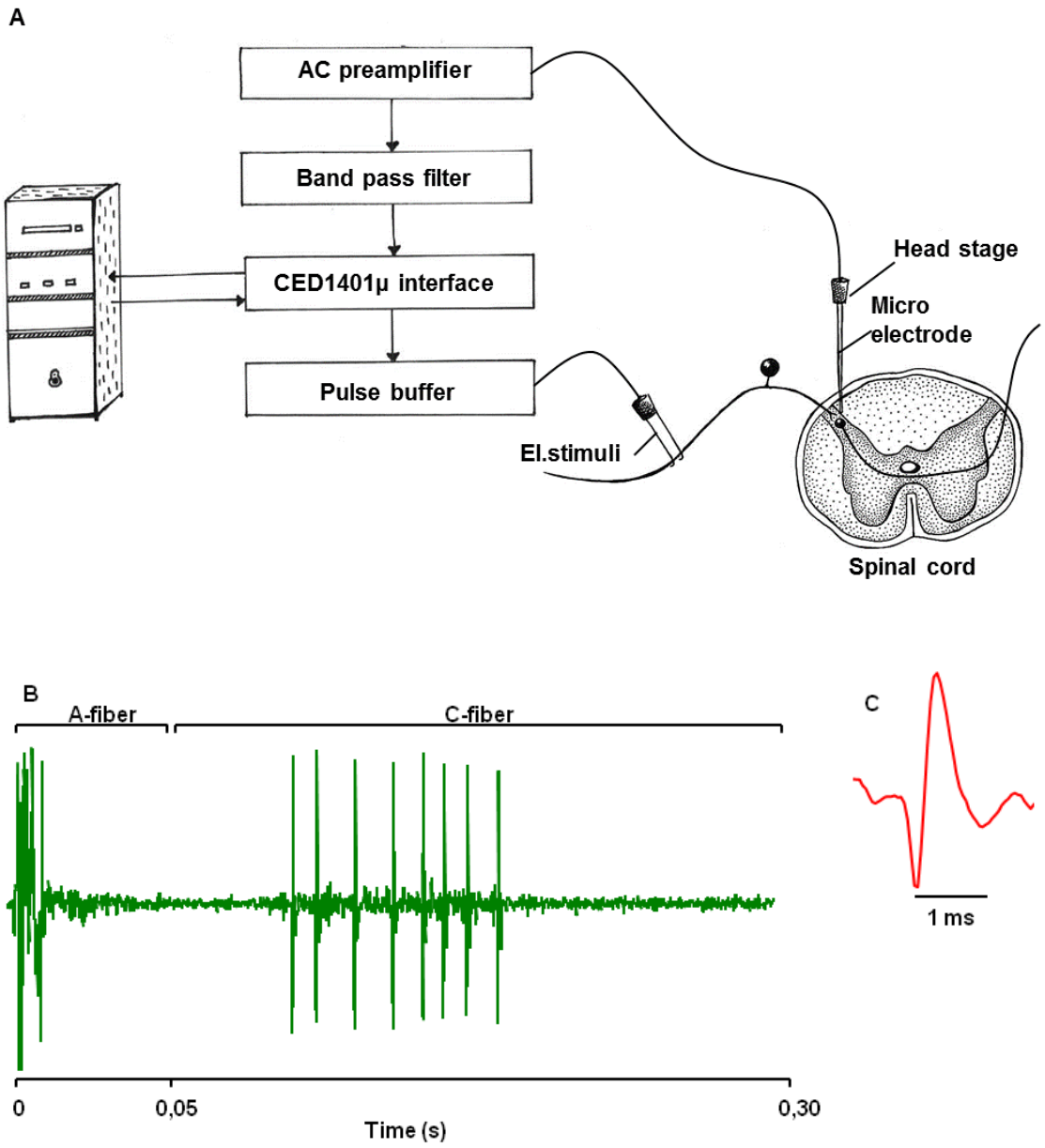


Figure 3.1 A) A representation of the experimental set-up for extracellular single cell recording. The sciatic nerve was electrically stimulated to evoke neuronal activity in the spinal cord, which was recorded by a microelectrode. The neuronal signal was amplified by an AC amplifier and further filtered by a band pass filter before digitalization by a CED1401µ interface. B) An example of an extracellular response after electrical stimulation, showing the temporal difference between A-fiber and C-fiber response. C) Enlarged view of an action potential from B).

3.3 Gene expression

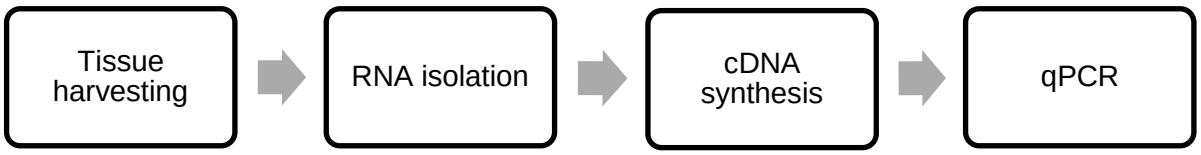


Figure 3.2 Overview of the procedural steps in order to measure gene expression in the dorsal horn in the spinal cord. Spinal cord tissue was harvested after 3 hours of electrophysiological measurements. Ribonucleic acid (RNA) was isolated from the tissue, and used further as a template to synthesize complementary deoxyribonucleic acid (cDNA) by reverse transcriptase. Specific primers were designed to measure expression of the target genes by quantitative polymerase chain reaction (qPCR).

3.3.1 RNA isolation

Isol-RNA Lysis Reagent (5PRIME) was added to each sample of spinal cord tissue. A mixer mill and 3 sterile metal balls (Retsch MM 301, Germany) were used to homogenize the tissue. The samples were then incubated and centrifuged; Cell debris was discarded. Chloroform was added to the remaining supernatant to separate ribonucleic acid (RNA) from deoxyribonucleic acid (DNA), proteins and lipids. The water phase containing RNA was collected after centrifugation, and isopropanol was added to precipitate RNA out of the solution. Following incubation and centrifugation, the supernatant was discarded, and the RNA pellet was washed with 75% ethanol and centrifuged again. The supernatant was removed, and the pellet was dried before it was dissolved in the ribonuclease (RNase) free water and incubated at 65°C. Finally, the samples were frozen at -80°C (Pedersen *et al.*, 2010). For protocol see appendix 2.

The concentrations of the samples were obtained by measuring optical densities with a spectrophotometer (NanoDrop 2000/2000c, version 1.6.198, Thermo Scientific Inc., USA). The samples were diluted to 0.25µg/µl by adding RNase free water.

3.3.2 cDNA synthesis

The isolated mRNA was converted to complementary DNA (cDNA) by using qScript cDNA synthesis Kit (Quanta BioSciences Inc., USA). The mRNA samples and all the components were thawed, mixed thoroughly and kept on ice during the preparation step. A reaction mix was made of optimized buffer (5X concentrated solution), magnesium, random primers, deoxynucleotides (dNTPs) and nuclease free water. This reaction mix

was added to each mRNA sample. Lastly, MMLV reverse transcriptase (50X concentration) and ribonuclease (RNase) inhibitor protein were added to the samples to carry out the convertion of RNA to cDNA. The samples were placed in a thermal Mastercycler nexus v.2.2.0.0 (Eppendorf, USA) with the following program: 22°C for 5 minutes, 42°C for 30 minutes and 85°C for 5 minutes. After the completion of the synthesis, 80µl tris ethylenediaminetetraacetic acid (TE)-buffer was used to dilute the cDNA samples to a concentration of 10ng/µL. The samples were stored at -80°C. For protocol see appendix 3.

3.3.3 qPCR

The expression of the target genes was estimated by quantitative polymerase chain reaction (qPCR). Primers for the target genes were designed by the software Primer Express v.3.0.1 (Applied Biosystems, USA), which helps to eliminate primers with a high number of internal complementation. To ensure specificity for cDNA, primers were designed to yield products that span introns in the genomic DNA. Primer sequences were checked for potential binding to other genes by using a basic local alignment search tool (BLAST). β-actin was used as a reference gene because of its constant high expression, regardless of intervention. Primers were ordered from Sigma-Aldrich (Germany). For primer sequences, see Table 3.1.

Table 3.1 Rat specific primers used for qPCR.

Primer	Sequence 5'→3'	%GC	Bp	Tm (°C)
IL6 Forward	AGG GAG ATC TTG GAA ATG AGA AAA	37.5	27	64.8
IL6 Reverse	TCA TCG CTG TTC ATA CAA TCA GAA	37.5	27	65.8
IL10 Forward	AGA AGG ACC AGC TGG ACA ACA	52.3	21	66.1
IL10 Reverse	CAA GTA ACC CTT AAA GTC CTG CAG TA	42.3	26	64.2
TNFα Forward	GCC ACC ACG CTC TTC TGT CTA	57.1	21	59.1
TNFα Reverse	TGA GAG GGA GCC CAT TTG G	57.9	19	59.6
TGF-β1 Forward	CGT GGA AAT CAA TGG GAT CAG	47.6	21	53.8
TGF-β1 Reverse	TCG GTT CAT GTC ATG GAT GGT	47.6	21	56.4
β-Actin Forward	CTA AGG CCA ACC GTG AAA AGA	47.6	21	58.0
B-Actin Reverse	ACA ACA CAG CCT GGA TGG CAT	52.4	21	59.2

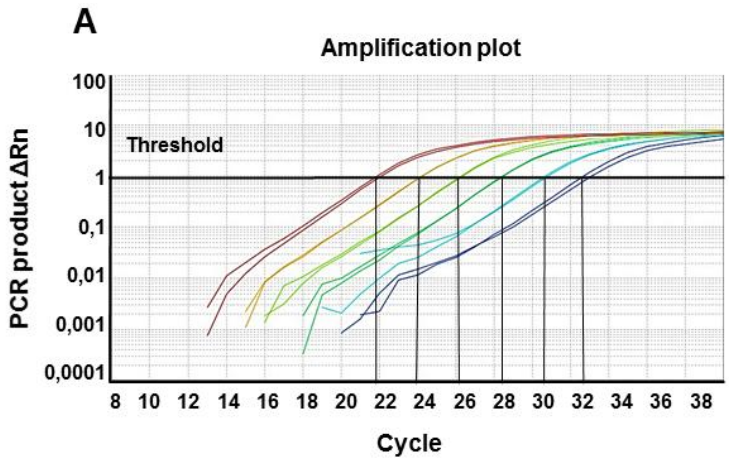
A: Adenosine, C: Cytosine, G: Guanine, T: Thymine, %GC: %Guanine/Cytosine, Bp: base pairs, Tm: melting temperature.

A master mix containing double-distilled water, Perfecta SYBR green dye, ROX dye, dNTPs, primers (forward and reverse) and Taq polymerase was made to perform the

qPCR reaction. SYBR green dye was used to quantify the amount of PCR product at the end of each cycle, because SYBR green dye emits fluorescence at 520nm when it incorporates in double stranded (ds) DNA. ROX dye was used to normalize non-PCR related fluorescence fluctuations of the SYBR green dye, thereby correcting for differences in sample volume in each well. A fourfold dilution series containing cDNA from 3 different samples were used to establish a standard curve for both the target genes and the β-actin. The samples and the dilution series were loaded to the qPCR plate in 2 parallels, sealed with plastic film and spun down (Eriksen *et al.*, 2012).

The qPCR reaction was performed by a StepOnePlus qPCR machine (Applied Biosystems, USA) at the following program: 90°C for 2 minutes followed by 40 cycles of 95°C for 10 seconds and 60°C for 30 seconds. After the completion of the program, a melting curve was established by slowly heating the PCR products up to 95°C while measuring fluorescence. For protocol see appendix 4.

The results of the qPCR reaction were analyzed by the StepOne software v2.3. The standard curve was used to define a threshold value of fluorescence (Figure 3.3B). An amplification plot was created by plotting the normalized fluorescence value from each well against the number of completed cycles. A cycle threshold (Ct) value of each sample was defined by the intercept between the threshold value and the amplification plot of each sample, and was used to quantify the amount of the target gene in each sample (Figure 3.3A). The melting curve was used to ensure that the fluorescence was measured from the quantification of the desired PCR product, rather than by-products (Figure 3.4).



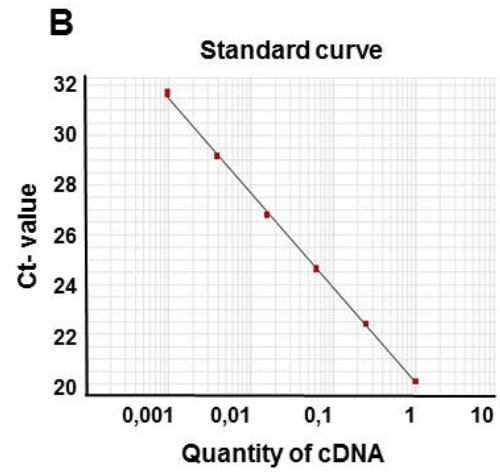


Figure 3.3 A) Example of an amplification plot of the dilution series of the reference gene β-actin. The threshold value of each sample was defined by the StepOne software v2.3. Cycle threshold (Ct) value was determined based on the number of cycles for each sample required to reach the threshold. B) Example of standard curve. The standard curve was used to define a threshold value of each sample.

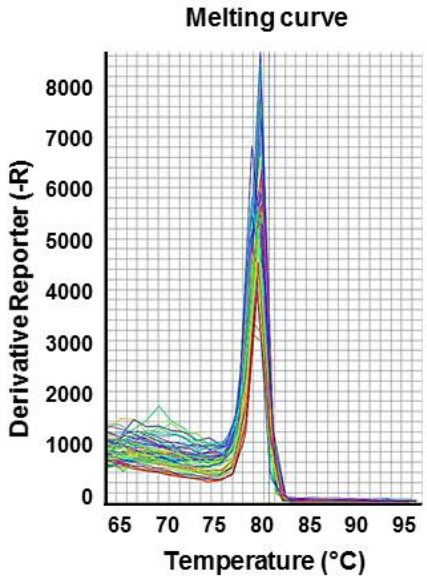


Figure 3.4 Example of a melting curve. The presence of only one peak shows that the fluorescence measured in the samples was obtained from the quantification of the desired product, and not from by-products.

3.4 The clinical study

All participants received written information and signed an informed consent form. The study was approved by the Norwegian Regional Committee for Medical Research ethics and the Norwegian Social Science Data Services.

3.4.1 Patients

Patients with lumbar radicular pain were recruited from Oslo University Hospital (OUH, Ullevål), Norway, during the period of 2007-2009. As described by (Moen *et al.*, 2016) the inclusion criteria for participants were: age between 18 and 60 years, lumbar disc herniation confirmed by magnetic resonance imaging (MRI) with corresponding radicular pain, and positive Straight Leg Raising (SLR) test. The exclusion criteria were: lumbar spinal stenosis, earlier surgery for herniated disc at the same level or fusion at any level in lumbar spine, generalized musculoskeletal pain, inflammatory rheumatic disease, diabetic polyneuropathy, cardiovascular disease (NYHA III and IV), cancer, psychiatric disease, cauda equine syndrome, alcohol or drug abuse, recent surgery (within one month), pregnancy, poor Norwegian language, or non-European-Caucasian ethnicity. In total, 122 patients (82%) of the 148 patients who met the inclusion criteria were included in the intended follow-up assessment.

Surgical treatment was given to patients with persistent radicular pain lasting for more than 8 weeks, neurological deficits (sensory changes, muscle weakness, and depressed or abscent deep tendon reflexes), and corresponding magnetic resonance imaging findings in the anticipated location. Patients who did not clearly fulfill these criteria were managed conservatively by a treatment comprised of a brief cognitive intervention, activity guidance during the acute phase of disc herniation, and, for the majority of patients, physiotherapy.

3.4.2 VAS

Pain perception in patients was determined by Visual Analog Scale (VAS) activity ratings at inclusion and after 12 months. VAS is a 10 cm scale with endpoints “no pain” and “worst possible pain”. Pain recovery rate was defined as delta VAS: the resulting value of the subtraction of the VAS scores at 12 months from the VAS scores at inclusion. The

recovery group was defined by ΔVAS>2, whereas the persistent pain group was defined by ΔVAS<2. The drop-out rate was 8% and conclusively, 112 patients were assessed at 12 months follow-up.

3.4.3 Blood sampling

At 12 months follow-up, venous blood was collected and kept on ice for 45 minutes. The supernatant serum was collected and stored in aliquots at -80°C, after the centrifugation of the blood samples at 2000g for 10 minutes at 4°C. Serum was further used to analyze the biomarkers for the levels of 92 inflammatory proteins (Moen *et al.*, 2016).

3.4.4 Proximity extension assay

As described by (Moen *et al.*, 2016) the multiplex proximity extension assay (PEA) technology was used to analyze a panel of 92 proteins simultaneously. The serum samples were assessed with Proseek Multiplex Inflammation I (Olink Bioscience, Uppsala, Sweden) using the PEA according to the manufacturer's instructions. Briefly, 3µl incubation mix containing 2 PEA probes that is antibodies equipped with single strand DNA oligonucleotide, against each protein was mixed with 1µl serum and the mixture was incubated at 8°C overnight. The mixture was then mixed with 96µl extension mix containing PEA enzyme and PCR reagents and incubated for 5 minutes at room temperature before the plate was transferred to a thermal cycler for 17 cycles of DNA amplification. A 96.96 Dynamic Array IFC (Fluidigm, CA, USA) was prepared and primed according to the manufacturer's instructions. 2,8 µl of sample mixture was mixed with 7,2 µl detection mix in a new 96-well plate and 5µl was loaded into the right side of the primed 96.96 Dynamic Array IFC. The unique primer pairs for each cytokine were loaded into the left side of the 96.96 Dynamic Array IFC, and the protein expression program was run in Fluidigm Biomark reader according to the instructions for Proseek.

3.4.5 TaqMan genotyping

As described by (Dominguez *et al.*, 2013), genomic DNA was extracted from the blood samples the before the genotyping. The TaqMan allelic discrimination method was used to genotype for 3 single-nucleotide polymorphisms (SNPs) (rs927312, rs3916765 and rs2395185). To distinguish between the 2 alleles, the probes were labeled with the FAM

or VIC reporter dye (Applied Biosystems, USA), as previously described by (Olsen *et al.*, 2012). An ABI 7900HT sequence detection system (Applied Biosystems, USA) was used to perform the reactions. Water was used instead of DNA as a negative control in every run. The SDS 2.2 software was used to determine genotypes. Approximately 10% of the samples were re-genotyped, and the concordance rate was 100%.

3.5 Statistics

Statistical analyses were performed in SPSS 22 (IBM SPSS Inc., USA), Stata SE 14 (StataCorp LCC, USA) and Microsoft Office Excel 2007. All graphs were constructed in Sigma plot 12.5 (Systat software Inc., USA). Data are given by examples and by means ± standard error of the mean (SEM). A p-value below 0.05 was considered significant.

3.5.1 Electrophysiology

The recordings of the spinal nociceptive activity were performed for 3 hours after baseline. The baseline was defined as the mean value of 6 stable recordings. The baseline recordings were averaged to 2 values (comprising 3 consecutive recordings each), and the post-baseline recordings were averaged to 5 values (comprising 9 consecutive recordings each).

The effect of the miR-223-3p application onto the spinal cord was analyzed using a repeated measurements analysis of variance (rmANOVA). Since the data violated the sphericity assumption, a Greenhouse-Geisser correction was applied.

3.5.2 Gene expression

The expression of IL-6, IL-10 and TGF-β was normalized to the expression of β-actin and to the mean of the gene expression in the control group. The fold expression of these cytokines in the miR-233 group was compared to the control group by a two-tailed unpaired Student's *t*-test.

The expression level of IL-6 and IL-10 in the control group (0,9% NaCl) was also compared to a invivofectamine control group (invivofectamine solution was applied onto the dorsal nerve roots without miR-223-3p) using a two-tailed unpaired Student's *t*-test (Appendix 5).

3.5.3 The clinical study

The changes in the expression of miR-223-3p, IL-6, IL-10 and TGF-β between inclusion, 6 weeks and 12 months were analyzed using a two-tailed unpaired Student's t-test and a rmANOVA with Greenhouse-Geisser correction.

The differences in the expression of miR-223-3p, IL-6, IL-10 and TGF-β after inclusion between the recovery group and the persistent pain group were analyzed by a mixed effects regression models with a random intercept for subject.

The differences in the expression of miR-223-3p, IL-10, IL-6 and TGF-β at inclusion between non-carriers and carriers of DQB1*03:02 haplotype were analyzed by a two-tailed unpaired Student's *t*-test.

4 Results

4.1 Electrophysiology

Extracellular single cell recordings were performed in the dorsal horn. The C-fiber response was examined in the miR-223-3p group and the control group for 3 hours. A significant difference in the C-fiber response between the miR-223-3p group (n=8) and the control group (n=7) was demonstrated (P=0.037, rmANOVA, Greenhouse-Geisser correction) (Figure 4.1C).

In the miR-223-3p group, the C-fiber response decreased in 5 out of 8 experiments; up to ~20% of baseline in 2 of the cells and ~60%, ~65% and ~80% of baseline in the other 3 cells (Figure 4.1B). However, a minor increase in the C-fiber response was observed in one experiment. An increased variance in the C-fiber response was observed after miR-223-3p application.

In the control group, the C-fiber response in most cells remained stable throughout the whole experiment (Figure 4.1A). In 2 out of 7 cells, however, a minor increase in the C-fiber response was observed.

4.2 Gene expression

To explore how miR-223-3p may influence inflammatory processes in the dorsal horn, the expression of the pro-inflammatory cytokine IL-6 and the anti-inflammatory cytokines IL-10 and TGF-β were studied by qPCR. Fold change expression of the target genes was normalized to the gene expression of β-actin and to the mean of the gene expression in the control group. The expression of IL-6 was significantly increased 3 hours after miR-223-3p application compared to the control group (P<0.001, Student's *t*-test). A minor up-regulation of the expression of IL-10 was observed 3 hours after miR-223-3p application; however the difference between the miR-223-3p group and the control group was not significant. No clear differences in the expression of TGF-β were detected 3 hours after miR-223-3p application. See Figure 4.2

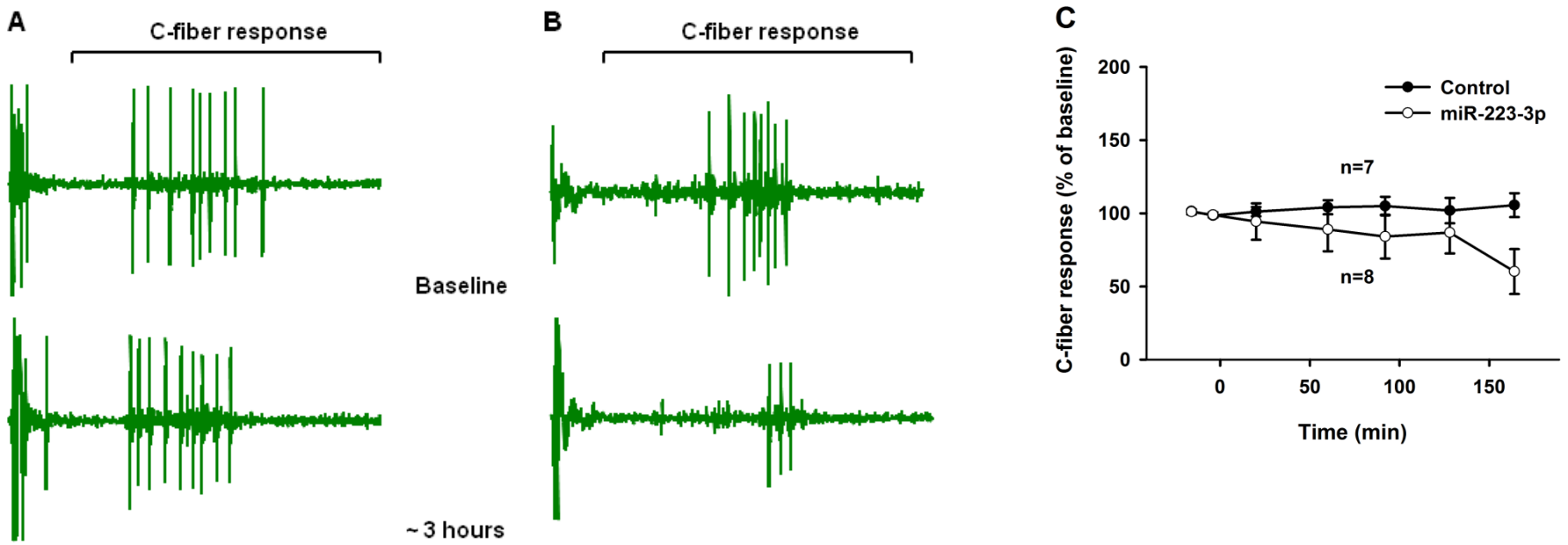


Figure 4.1 A and B) Examples of the electrically evoked C-fiber responses in the dorsal horn neurons at baseline and at the end of the experiment (after 3 hours). The C-fiber response was stable for 3 hours in the control group, but decreased from 8 spikes at baseline to 3 spikes after 3 hours in the miR-223-3p group. C) The C-fiber response in percent of baseline in the miR-223-3p group and the control group; The C-fiber response was significantly decreased after miR-223-3p application (P=0.037, rmANOVA, Greenhouse-Geisser correction). Data are given as means±SEM.

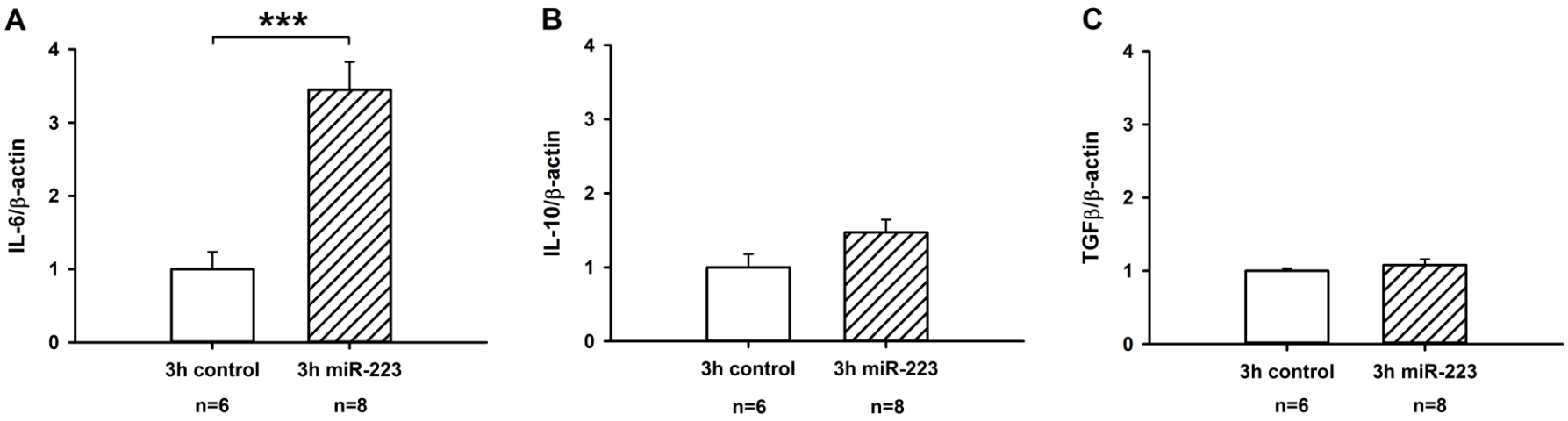


Figure 4.2 Fold expression (normalized to mean of control) of A) IL-6, B) IL-10, D) TGF-β in the dorsal horn in the control group and the miR-223-3p group 3 hours after miR-223-3p application. ***P<0.001, Student's t-test. Data are given as means±SEM.

4.3 Patients

In patients with LRP, the miR-223-3p expression was significantly up-regulated in the serum at inclusion compared to 12 months later (P= 0.004, Student's *t*-test) (Figure 4.3A). The miR-223-3p expression at inclusion in the recovery group (ΔVAS>2) was significantly higher than in the persistent pain group (ΔVAS<2) (Figure 4.3B) (P=0.019, Student's *t*-test).

The expression of IL-6 was significantly reduced following inclusion in the recovery group relative to the persistent pain group (Figure 4.4A) (P=0.011, mixed effects regression models). A minor reduction in the IL-10 expression was also observed in the recovery group (Figure 4.4B) (P=0.12, mixed effects regression models). No differences were observed in the TGF-β expression (Figure 4.4C) (P=0.46, mixed effects regression models).

Data obtained from the serum was analyzed to discover how the expression of IL-6, IL-10 and TGF-β changed. The IL-6 expression was gradually decreased after inclusion (P=0.06, rmANOVA, Greenhouse-Geisser correction) (Figure 4.5A). No clear change in the IL-10 expression was observed (P=0.17, rmANOVA, Greenhouse-Geisser correction) (Figure 4.5B). The TGF-β expression was, however, decreased from inclusion to 12 months (P<0.001, rmANOVA, Greenhouse-Geisser correction) (Figure 4.5C).

No correlation was found between the expression of miR-223-3p at inclusion and the expression of IL-6, IL-10 and TGF-β in the serum of patients with lumbar radicular pain (LRP) (Appendix 6).

Finally, the expression of miR-223-3p, IL-6, IL-10 and TGF-β in the serum at inclusion was examined with regard to non-carriers and carriers of DQB1*03:02 haplotype. The miR-223-3p and TGF-β expression were significantly up-regulated in carriers of the DQB1*03:02 haplotype (P=0.004 and P=0.041, respectively, Student's *t*-test). No significant differences in the IL-6 and IL-10 expression were observed between non-carriers and carriers of DQB1*03:02 haplotype. See Figure 4.6.

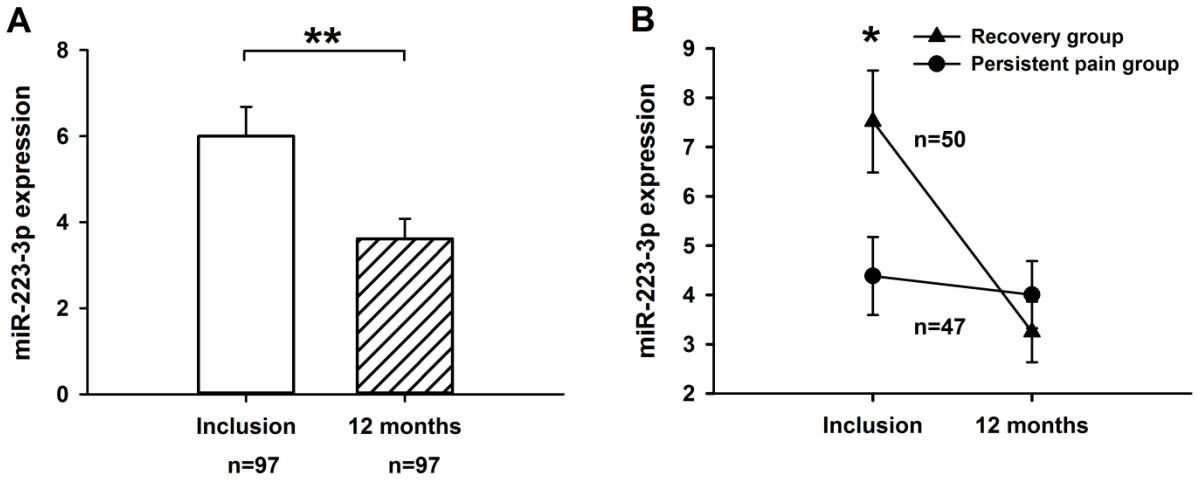


Figure 4.3 Expression of miR-223-3p in the serum of patients with lumbar radicular pain A) at inclusion and after 12 months. B) in the recovery group (ΔVAS>2) and the persistent pain group (ΔVAS<2) at inclusion and after 12 months. *P<0.05, **P<0.01, Student's *t*-test. Data are given as means±SEM.

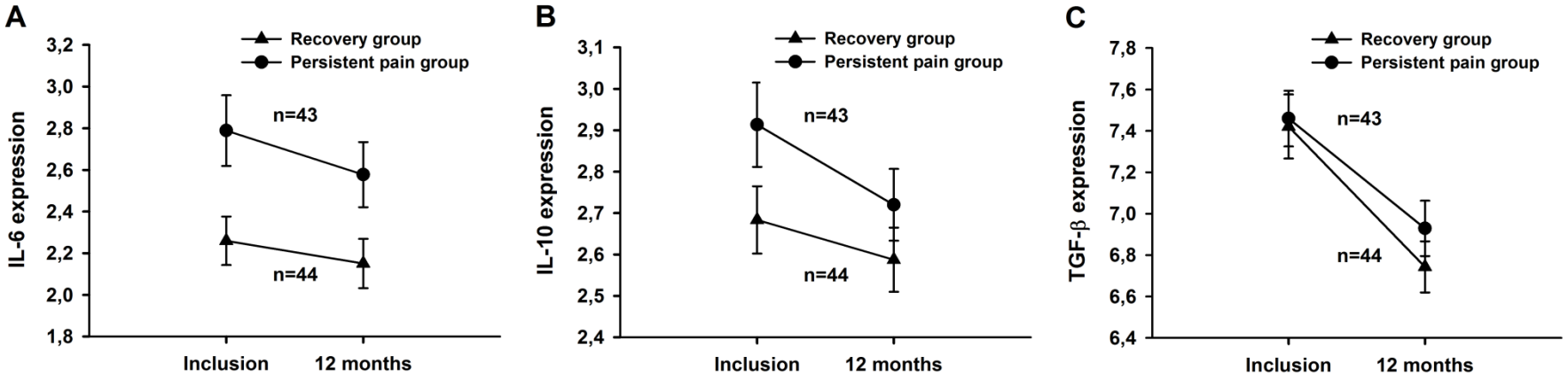


Figure 4.4 A) IL-6 (P=0.01) B) IL-10 (P=0.11) and C) TGF-β, (P=0.46) expression in the serum of patients with lumbar radicular pain at inclusion and 12 months later, divided in the recovery group (ΔVAS>2) and the persistent pain group (ΔVAS<2). Data are given as means±SEM.

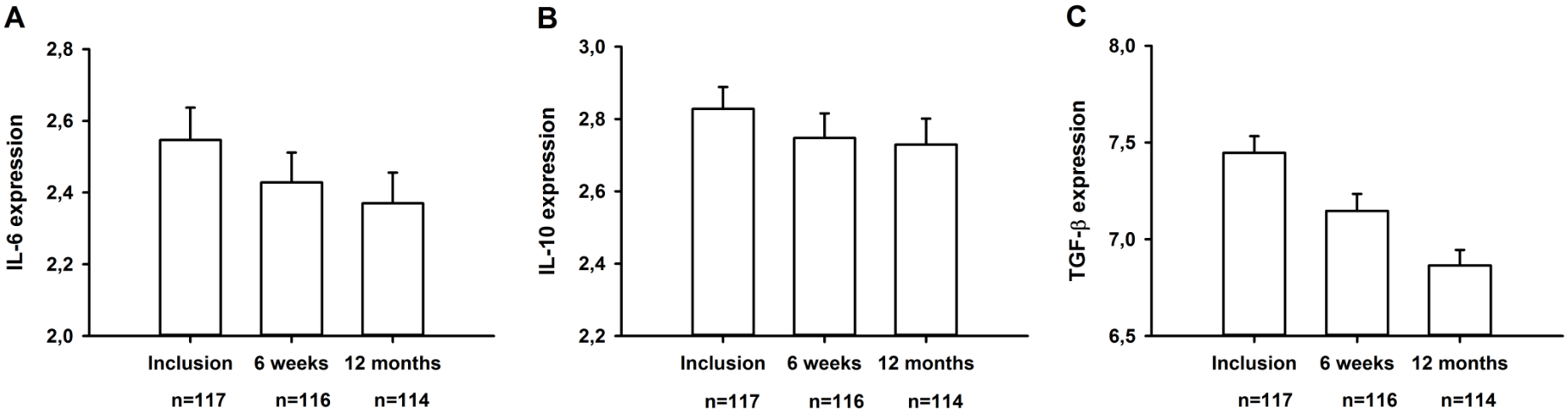


Figure 4.5 A) IL-6 (P=0.06) and B) IL-10 (P=0.18) C) TGF-β (P<0.001) expression in the serum of patients with lumbar radicular pain at inclusion, after 6 weeks and after 12 months. Data are given as means±SEM.

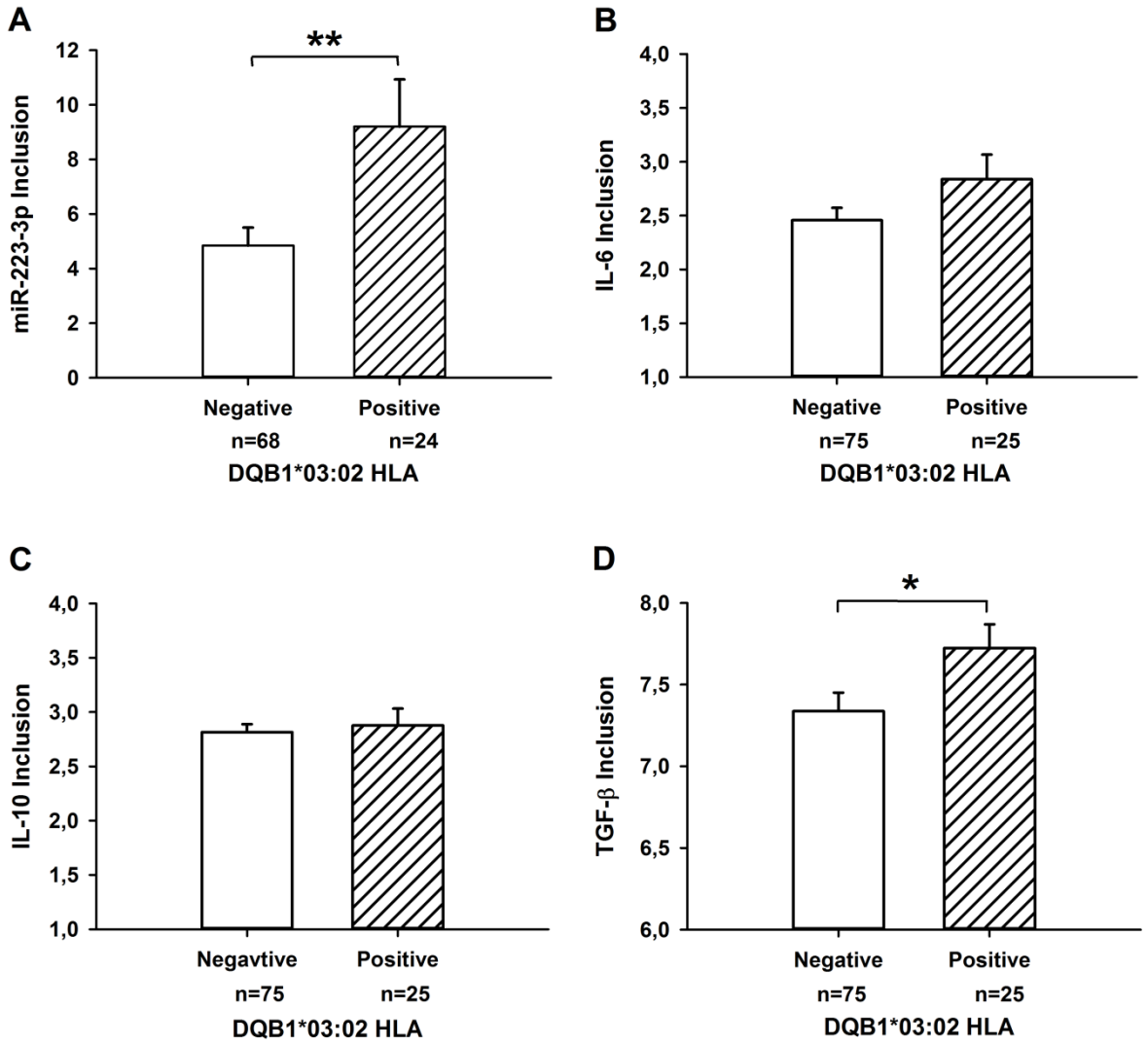


Figure 4.6 Differences in the level of A) miR-223-3p, B) IL-6, C) IL-10 and D) TGF-β in the serum of patients with lumbar radicular pain at inclusion between non-carriers and carriers of the DQB1*03:02 haplotype. *P<0.05, **P<0.01, Student's *t*-test. Data are given as means±SEM.

5 Discussion of methods

5.1 Animals

Inbred female Lewis rats were used in all animal experiments. This strain has been used for research purposes for a long time, and it is a major advantage that all Lewis rats are genetically identical. It is especially common to use this strain to study inflammatory processes. Lewis rats have more pronounced inflammatory responses than other rat strains (Popovich *et al.*, 1997; Perretti *et al.*, 1993) and are therefore suited to study the expression of cytokines in the spinal dorsal horn. Female rats were chosen because of the lower risk of developing laboratory animal allergy than in male rats that have more potent allergens (Renstrom *et al.*, 2001). However, the usage of female rats also has some disadvantage due to the estrous cycle that may affect their pain threshold (Fillingim and Ness, 2000). Therefore the experiments were randomized in time to avoid the influence of the estrous cycle.

5.1.1 Anesthesia

Isoflurane gas was used prior to urethane injections. No limitations in dosage were reported. Urethane (ethyl carbamate $NH_2COOH_2CH_3$) is a common rodent anesthetic, which is best suited for terminal experiments. It has a long-lasting effect, and is suitable as a steady surgical anesthesia. Urethane has minimal impact on the cardio-respiratory functions, which is important when studying neurotransmission (Maggi and Meli, 1986). However, high dosages of urethane may affect multiple neurotransmitter-gated channels (Hara and Harris, 2002). Therefore, the maximum dosage was set to 2g/kg bodyweight, which does not interfere with electrophysiological measurements, as confirmed in earlier experiments (Egeland *et al.*, 2013; Gjerstad *et al.*, 2005; Pedersen *et al.*, 2010). Urethane is considered to be carcinogenic, and requires careful handling with protective gloves and appropriate disposure as a hazardous chemical (Field and Lang, 1988).

5.1.2 Surgery

Laminectomy is an invasive procedure that by itself can lead to inflammation and pain. However, in order to perform electrophysiological recordings and observe the effect of miR-223 the laminectomy was necessary. Since the laminectomy was done both in the miR-223

group and the control group, it was assumed that the procedure had the same effec in both groups.

5.2 Electrophysiology

Several methods, such as patch clamp (Ikeda *et al.*, 2003), extracellular field potential recordings (Eriksen *et al.*, 2012) and extracellular single cell recordings (Pedersen *et al.*, 2010) have earlier been used to measure nociceptive activity in the spinal dorsal horn. All of these methods have their respective advantages and limitations.

Patch clamping is performed *in vitro*, in isolated slices of spinal cord. This method makes it easy to obtain stable long-term measurements and observe immediate effects due to exposure to different chemicals. However, the tissue being examined is isolated from the rest of the body. This implies certain limitations, such as lack of circulating mediators and absence of descending modulation. These essential physiological factors are needed to obtain the complete physiological picture.

Therefore, *in vivo* recordings are required to investigate nociceptive activity under physiological conditions and obtain a more complete picture of physiological changes following chronic pain. Extracellular field potentials are recorded *in vivo*, but it is difficult to identify the source of the recorded potentials because of contribution from several neurons. This makes it difficult to interpret the measurements.

In the extracellular single cell recordings, electrically evoked responses can be easily disturbed by minor vibrations (e.g. from the electromagnetic fields of electrical equipment), producing noise. Hence, a band pass filter was used to reduce the noise by selecting the frequencies corresponding to action potentials frequencies in nerve fibers. To ensure single-cell-recording during the whole experiment, the cells were distinguished by spike amplitude and shape. However, especially in the long-term measurements, it can occur that 2 cells can have quite similar wave shapes (Miki *et al.*, 2002). Therefore, it is difficult to substantiate if the same cell was recorded for the entire duration of the experiment.

C-fiber response was considered as more relevant for the chronic pain conditions than Aδ-fiber response. These two types of fibers were separated by latency following test stimuli.

5.2.1 miR-223 administration

Two parallel groups of experiments were performed in order to investigate the effect of miR-223 application onto the dorsal nerve roots; I) the miR-223 group (miR-233 is dissolved in Invivofectamine 3 Reagent), and II) the control group (0.9%NaCl). Ideally, scrambled miRs dissolved in Invivofectamine 3 Reagent should have been used as an additional control. Due to limitations in time, only the Invivofectamine 3 Reagent control without any miRs and 0.9%NaCl control were performed to confirm the effect of miR-223 application on gene expression.

5.3 Gene expression analysis

It is important to emphasize that changes in the level of mRNA are not directly convertible to changes in the amount of functional protein synthesized. It is only an assumption of the potential changes in protein level.

qPCR is a highly sensitive method, which allows amplification of small amounts of RNA (Bustin, 2000). Other methods, like Northern blotting, RNase protection assay and in situ hybridization, are alternatives for quantifying the amount of mRNA, but these methods are less sensitive than qPCR (Bustin, 2000).

Design of primers with high specificity to the target gene is a critically important step prior to amplification of cDNA by qPCR. Therefore, multiple levels of primer control were applied. To avoid amplification of genomic DNA, primers were designed to span introns. The software Primer Express allowed choosing optimal primers with low self-complementation, e.g. avoiding formation of "hair-pins". Optimal primers were defined by length of 18-30 bp, GC content of 40-60% and Tm at 50-60°C with differences less than 1°C between forward and reverse primers. Supplementary check for identical sequences in other genes was performed by BLAST. In addition, melting curve was used to assure that only desired products were amplified, and no by-products were present.

β-actin was used as a reference gene to normalize expression due to variation in amount of mRNA extracted from tissue, a level of mRNA degradation and cDNA synthesis performance. Earlier studies showed low variation in the β-actin expression among samples, irrespective of experimental conditions in the DH (Pedersen *et al.*, 2010; Egeland *et al.*, 2013).

5.4 The clinical study

Only patients diagnosed with lumbar disc herniation from the OUH were included. As described by (Moen *et al.*, 2016) strict inclusion and exclusion criteria were applied to avoid influence from unrelated disorders, such as cancer and mental diseases. By choosing only individuals with European-Caucasian origin, a less genetically diverse cohort of patients was obtained. In total, 112 patients between the ages of 18 and 60 years with relatively even sex distribution were included.

One limitation of the study was that the drugs used by the patients were not taken into account. The patients received different medicaments that might have affected the intensity of pain and the level of proteins in the blood. Patients with more severe pain received stronger analgesics. In addition, patients with persistent radicular pain lasting for over 8 weeks received surgery. Other factors that are associated with life style, such as smoking and other habits may also influence the pathology of radicular pain.

5.4.1 VAS

VAS scoring has been used in earlier studies, and is considered a reliable method to define pain intensity in patients (Sindhu *et al.*, 2011; Boonstra *et al.*, 2008). In the clinic, clear communication is critical. Hence, only the patients with fluent Norwegian language were included in the present study.

5.4.2 Blood sampling and PEA

Patients may have progressed to different phases of pain development at inclusion, due to the fact that different individuals may have a varying threshold for when to go to hospital. The expression of cytokines and miR-223 were measured at inclusion, at 6 weeks and at 12 months. The measurements at 6 weeks are most likely to be skewed by the influence of medicaments. However, the measurements provide us enough information to discriminate the recovery group from the persistent pain group. The PEA technology was used to study proteins in serum due to its outstanding scalability and high specificity and sensibility related to clinical measurements (Assarsson *et al.*, 2014).

6 Discussion of results

6.1 The animal study

6.1.1 Electrophysiology

Following miR-223 application onto the spinal cord, a significant decrease in the nociceptive signaling, i.e. the electrically evoked C-fiber response, was demonstrated in the spinal DH. In contrast, the C-fiber response remained stable throughout the whole experiment in the control group. These observations suggest that miR-223 may inhibit nociceptive signaling. This may be explained by the miR's ability to mediate post-translation repression or degradation of the target mRNAs by binding to their 3'-UTR.

For instance, miR-223 may provide a neuroprotective effect via targeting glutamate receptor 2 (GluR2) subunit of the AMPAR and N-metyl D-aspartate receptor 2 B (NR2B) subunit of the NMDAR. This may reduce the expression of these receptors. As a result, the neuronal excitation is restricted and post-synaptical influx of Ca^{2+} into neurons is reduced (Harraz *et al.*, 2012). This may explain the observed decrease in C-fiber response due to miR-223 application.

Furthermore, miR-223 may have a role in the modulation of inflammatory processes by post-translational regulation of Nod-like receptor protein 3 (NLRP3), encoding the NLRP3 inflammasome. This inflammasome facilitates the processing of pro-inflammatory IL-1β to its active state by Caspase-1 cleavage, and promotes inflammation. Hence, miR-223 may reduce the inflammatory response after nerve injury (Yang *et al.*, 2015).

Previous data have shown, an up-regulation of miR-223 expression in exosome-like vesicles released from NP following application of NP onto the nerve roots (Moen *et al.*, 2017). These exosomes may deliver their miR content to recipient cells and suppress the expression of target mRNAs (Montecalvo *et al.*, 2012). Therefore, by exosomal transfer to the dorsal nerve roots, miR-223 may affect the gene expression, and the neural activity in the spinal DH.

6.1.2 Gene expression

An increase in the IL-6 expression was observed in the spinal DH tissue 3 hours after miR-223 application. As the IL-6 expression was only measured at 3 hours after miR-223 application, it remains unknown how the IL-6 expression changes over time.

Following spinal nerve ligation in rats, application of IL-6 onto the spinal cord may elicit a dose-related anti-nociceptive effect by inhibiting the initial electrically evoked C-fiber responses, and reduce wind-up (Flatters *et al.*, 2003). The combination of the effect on the initial C-fiber response and the effect on wind-up may suggest that IL-6 act on both pre- and post-synaptic mechanisms. Moreover, application of IL-6 onto rat cortical slices rapidly depresses induced glutamate release and inhibits mitogen-activated kinase (MAPK)/ERK signaling, possibly as a neuroprotective effect to reduce signaling (D'Arcangelo *et al.*, 2000).

To elicit its effect, IL-6 binds to its specific receptor subunit at the cell surface and two signaling glycoprotein 130 (gp130) subunits (Hirano *et al.*, 1994; Paonessa *et al.*, 1995). Normal uninjured neurons show low levels of gp130. However, a persistent up-regulation of IL-6 receptor mRNA and gp130 mRNA at the site of nerve damage (Grothe *et al.*, 2000; Ito *et al.*, 1998) may explain the inhibitory effects of IL-6 in neuropathic rats compared to native rats.

Application of soluble IL-6 receptor (sIL-6R) provides a way for IL-6 to access to neural cells that normally have a low density of membrane-bound IL-6 receptors (mIL-6R) and gp130. In contrast to our findings, previous patch clamp recordings of lamina II neurons show that application of sIL-6R together with IL-6 increases excitatory postsynaptic currents and reduces inhibitory postsynaptic currents. Furthermore, intrathecal administration of IL-6 together with its receptor induces thermal hyperalgesia in rats (Kawasaki *et al.*, 2008).

6.2 The clinical study

In the serum of patients with LRP, high expression of miR-223 was detected in the acute phase. We also observed a correlation between changes in intensity of pain and the level miR-223 expression, where high level of miR-223 at inclusion suggested better recovery of LRP after 12 months.

As previously mentioned, miR-223 may have an anti-inflammatory effect by post-translational repressing NLRP3, encoding the NLRP3 inflammasome and canceling subsequent activation of IL-1β (Yang *et al.*, 2015). Earlier studies have shown that IL-1β may participate in activation of pro-inflammatory immune cells (Verri *et al.*, 2006). Hence, by repressing the NLRP3 inflammasome formation, miR-223 may also down-regulate the inflammation.

A significant reduction of the IL-6 expression over time and a similar tendency to decline in the IL-10 expression was observed in the recovery group. A high expression of IL-6 in the acute phase was associated with a slower recovery in patients. Accordingly, previous studies suggest that inflammation contributes to the development of LRP following disc herniation (Burke *et al.*, 2002; Kang *et al.*, 1996; Kraychete *et al.*, 2010).

IL-6 expression has also been shown to be up-regulated due to disc herniation (Kang *et al.*, 1996; Pedersen *et al.*, 2015). In earlier studies, an association between high baseline levels of IL-6 in the serum of patients and poor recovery of LRP was shown (Schistad *et al.*, 2014). This supports our findings. Moreover, clinical data from serum protein profiling showed that low-grade systemic inflammation may be associated with persistent LRP after disc herniation (Moen *et al.*, 2016).

The expression of IL-6 may be influenced by miR-223, due to its ability to inhibit nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κβ), which is important for production of inflammatory cytokines, including IL-6 (Castro-Villegas *et al.*, 2015), and ultimately activation of macrophages and other immune cells (Wang *et al.*, 2015; Liu *et al.*, 2015). It has also been demonstrated that down-regulation of miR-223 promotes IL-6 and IL-1β release from macrophages (Chen *et al.*, 2012). However, no clear correlation between the IL-6 expression and the miR-223 expression was observed in the present study. The role of IL-6 in the neuropathic pain appears to be contradictory, which may be explained by the different mechanisms of its action in acute versus later phases of LRP.

In our study, a gradual decrease in expression of IL-6, IL-10 and TGF-β was observed after inclusion. This may be related to a falling level of inflammation after the acute phase. No clear correlation was observed between the level of miR-223 and the expression of IL-10 or TGF-β. However, the expression of TGF-β was increased at inclusion. TGF-β has an anti-inflammatory impact and may contribute to the miR-223 action possibly by impeding proliferation and activation of microglia cells (Suzumura *et al.*, 1993) and preventing the release of several pro-inflammatory cytokines from astrocytes (Benveniste *et al.*, 1995).

Higher expression of miR-223 and TGF-β at inclusion was observed in patients carrying the DQB1*03:02 HLA haplotype. Although carriers of this haplotype may have an increased risk of persistent pain one year after disc herniation, these subjects reported a lower intensity of pain at inclusion than non-carriers (Dominguez *et al.*, 2013). Thus higher miR-223 and TGF-β levels at inclusion may support these previous findings.

Accumulating evidence points toward an anti-inflammatory and anti-nociceptive influence of miR-223 and TGF-β. The function of HLA-DQ receptor, which is an αβ-heterodimer located on antigen-presenting cells, however is to present extracellular pathogens to T-cells. Many polymorphisms are observed in the HLA-D region (Trowsdale *et al.*, 1985), and DQB1*03:02 is one of the variants of sequences encoding the β-chain of this receptor. Exactly how this haplotype affects the expression of miR-223 and TFG-β remains to be investigated.

7 Conclusions

In the animal model;

I) Application of miR-233 onto the dorsal nerve roots reduced neuronal activity in the dorsal horn. This indicates that miR-223 may reduce the excitability of the ascending pathways. The effect of miR-223 may be explained by the ability of miR-223 to reduce neuronal transmission possibly by inhibiting post-synaptic Ca^{2+} influx by repressing the expression of receptor subunits of NMDAR and AMPAR.

II) The expression of IL-6 was significantly increased in the spinal dorsal horn 3 hours after miR-223 application. Thus IL-6 may be associated with an early anti-nociceptive influence on the neuronal activity in the dorsal horn by inhibiting electrically evoked C-fiber responses. We did not observe any changes in the expression of IL-10 or TGF-β.

In the patients;

III) In the patients with acute lumbar radicular pain, a correlation between expression of miR-223 at inclusion and better recovery of LRP was observed. This suggests that miR-223 may have an anti-inflammatory effect by post-translational repression of proteins that are involved in the promotion of inflammation. A lower level of IL-6 expression was observed in the recovery group than in the persistent pain group. A similar tendency was also observed in the expression of IL-10. However, the change in the IL-10 expression was not significant. This may indicate a less pronounced inflammation in the recovery group. The expression of TGF-β was significantly decreased 12 months after inclusion. This may potentially be associated with a falling level of inflammation after the acute phase.

IV) In the carriers of HLA-DQB1*03:02 haplotype, a higher expression of miR-223 and TGF-β was demonstrated. It remains to be investigated how this haplotype affects the expression of these genes. No differences were observed in the expression of IL-10 and IL-6 in the carriers of this haplotype.

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Appendices

Appendix 1 Invivofectamine 3.0 reagent complexation protocol (Thermo Fisher Scientific Inc., USA)

1. 20 µl of a 2.4 mg/ml miR solution was prepared by mixing the following components in a 1:1 ratio:

Component	Volume
miR-223-3p solution	10µl
Complexation buffer	10µl

2. Invivofectamine 3.0 Reagent was brought to room temperature and 20 µl added to a 1,5 ml tube.
3. Diluted miR solution was added immediately to Invivofectamine 3.0 Reagent in tube. Remaining reagent was returned to -20°C.
4. The tube was vortexed immediately to ensure Invivofectamine 3.0-miR-223 complexation.
5. The Invivofectamine 3.0-miR-223 mixture was incubated for 30 minutes at 50°C.
6. The tube was centrifuged briefly to collect the sample.

The Invivofectamine 3.0-miR mixture was applied onto the spinal cord

Appendix 2 Procedure for RNA isolation

1. The spinal cord tissue sample was transferred to a pre-cooled 2.0 ml PCR clean eppendorf tube and 8.0 ml Isol-RNA Lysis Reagent (5PRIME) was added.

2. Three sterile metal balls were added to each sample, and the tissue was homogenized by aid of a mixer mill (frequency: 30, 4x30 seconds).

3. The sample was incubated for 5 min at room temperature.

4. The sample was centrifuged at 12 000 g for 5 min at 4 °C. The supernatant was transferred to a new eppendorf tube.

5. 0.2 ml chloroform was added. The sample was shaken vigorously by hand for 15 sec and incubated for 3 min at room temperature.

6. The sample was centrifuged at 12 000 g for 15 min at 4 °C.

7. The water phase was transferred to a new Eppendorf tube. 0.5 ml isopropanol was added. The content was mixed well and incubated for 10 min at room temperature.

8. The sample was centrifuged at 12 000 g for 15 min at 4 °C.

9. The supernatant was removed and the RNA pellet was washed with 1 ml 75 % EtOH, mixed and vortexed.

10. The sample was centrifuged at 12 000 g for 5 min at 4 °C.

11. The supernatant was removed. The pellet was dried for 15-30 min at room temperature, dissolved in 10 µl RNase free water and kept on ice.

12. The sample was incubated for 10 min at 65 °C, placed on ice, spun, placed back on ice and mixed by a pipette.

13. The sample was then frozen and stored at -80 °C.

14. The concentration of the samples was determined by measuring of optical densities with a Nanodrop 2000 spectrophotometer.

15. The sample was diluted to 0.25 µg/µl by adding ((10 µl x concentration µg/µl) / 0.25 µg/µl)) – 10 µl = x.x µl RNase free water.

16. The sample was stored at -80 °C.

Appendix 3 Procedure for cDNA synthesis using "qScript cDNA synthesis kit" (Quanta Biosciences Inc., USA)

All reagents were thawed and kept on ice.

1. The RNA samples were diluted to get 250 ng/µl RNA

2. 4µL qScript reaction mix (5X) and 1µL qScript reverse transcriptase was added to each sample.

Reagent	volume/sample
RNA (1µg)	5.0µl
Nuclease-free water	10.0µl
qScript reaction mix	4.0 µl
qScript reverse transcriptase	1.0 µl
Total	20.0 µl

3. The samples were vortexed and spun down for 10s.

4. The reverse transcription reaction was run on the PCR machine at the following program: 22 °C for 5min, 42 °C for 30 min and 85 °C for 5 min and held at 4^{o}C after the program was finished.

5. Each sample was added 80µl of TE-buffer, mixed and spun down.

6. The samples were stored at – 80 °C.

Appendix 4 Procedure for qPCR

All reagents and samples were kept on ice unless specified otherwise.

1. A master mix was prepared:

Reagent	volume/sample
ddH_2O	5.58 µl
Perfecta SYBR Green FastMix	10.0 µl
Primer forward (25 pmol/ µl)	0.21 µl
Primer reverse (25 pmol/ µl)	0.21 µl
Total	16.00 µl

2. The cDNA samples used for β-actin analysis were diluted: 1 µl cDNA (10 ng/µl) + 9 µl RNase free water.
3. 8 µl from three different cDNA samples (10 ng/µl) were mixed to give a stock cDNA solution. A dilution series used to generate a standard curve for each gene was prepared.
4.

Dilution series nr	cDNA	RNase free water	
1	4.35 µl	undiluted	40ng
2	6 µl	+ 18µl	10ng
3	6 µl from nr 2	+ 18 µl	2.5ng
4	6 µl from nr 3	+ 18 µl	0.63ng
5	6 µl from nr 4	+ 18 µl	0.16ng
6	6 µl from nr 5	+ 18 µl	0.04ng

5. 16.00 µl master mix was loaded to each well on a 96 well plate.
6. 4.00 µl ddH_2O were added to the non-template control (NTC) wells.
7. 4.00 µl sample cDNA or pre-diluted samples for β-actin analysis or dilution series samples were transferred to the PCR-plate in two parallels and mixed well.
8. The PCR plate was sealed with a plastic film and spun down at 2500 rpm. A rubber mat was placed on top of the PCR plate.
9. The qPCR reaction was run at the following schedule: 90 °C for 2 min followed by 40 cycles of 95 °C for 10 sec and finally 60 °C for 30 sec.

Appendix 5 NaCl control

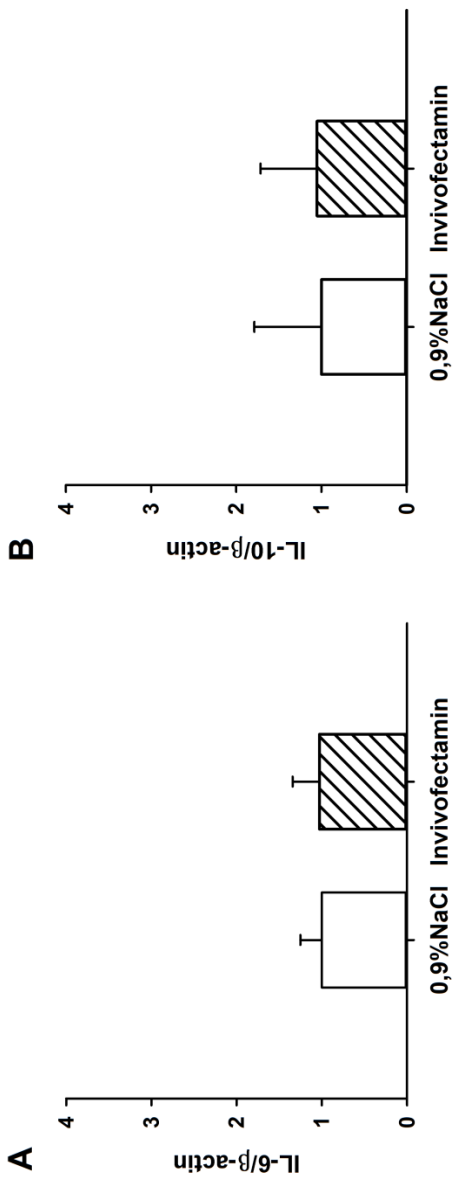

Figure 5.1 Fold expression (normalized to mean of 0,9% NaCl control) of A) IL-6, B) IL-10 in the two groups: 0,9% NaCl control group (n=3) and Invivfectamin group (n=3) 3 hours after Invivofectamin application.

Appendix 6 Expression of miR-223 at inclusion in serum versus cytokine expression in serum at different time points

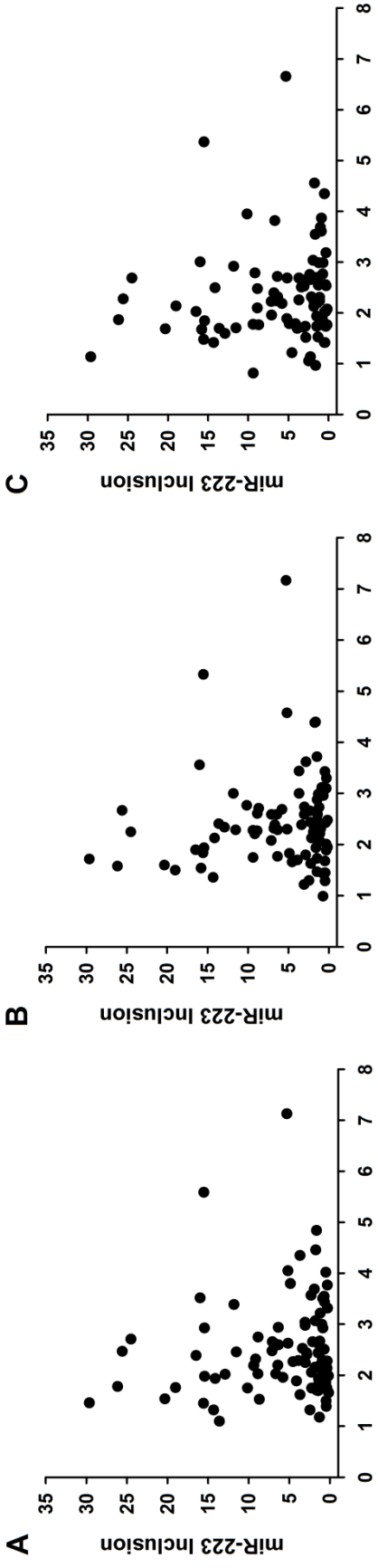

Figure 6.1 Expression of miR-223 at inclusion versus IL-6 expression in the serum from patients with lumbar radicular pain at different time points: A) at inclusion B) after 6 weeks C) after 12 months.

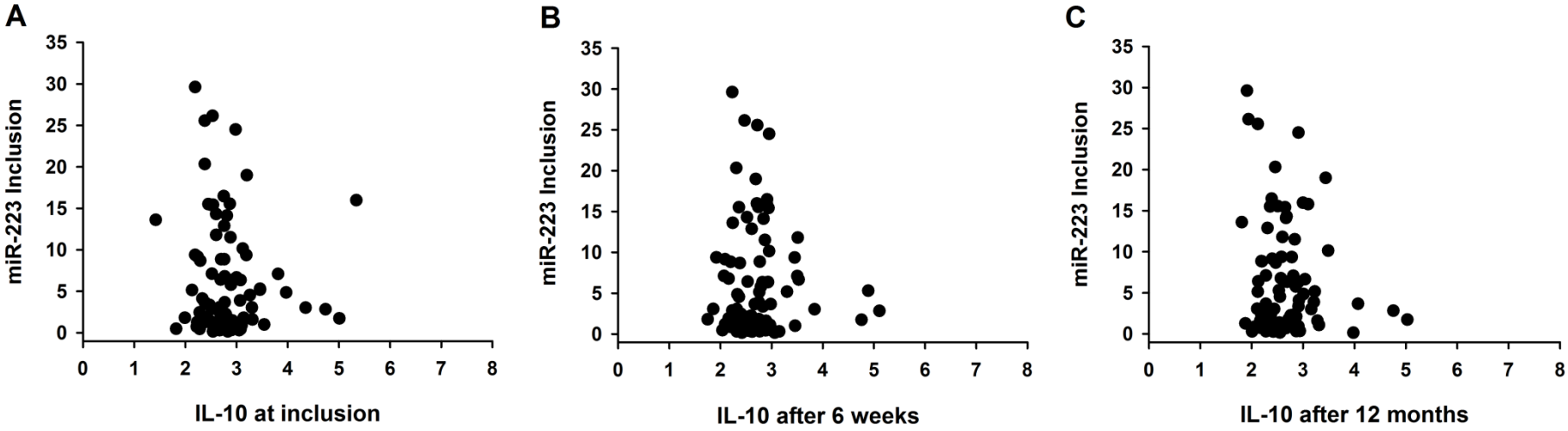

Figure 6.2 Expression of miR-223 at inclusion versus IL-10 expression in the serum from patients with lumbar radicular pain at different time points: A) at inclusion B) after 6 weeks C) after 12 months.

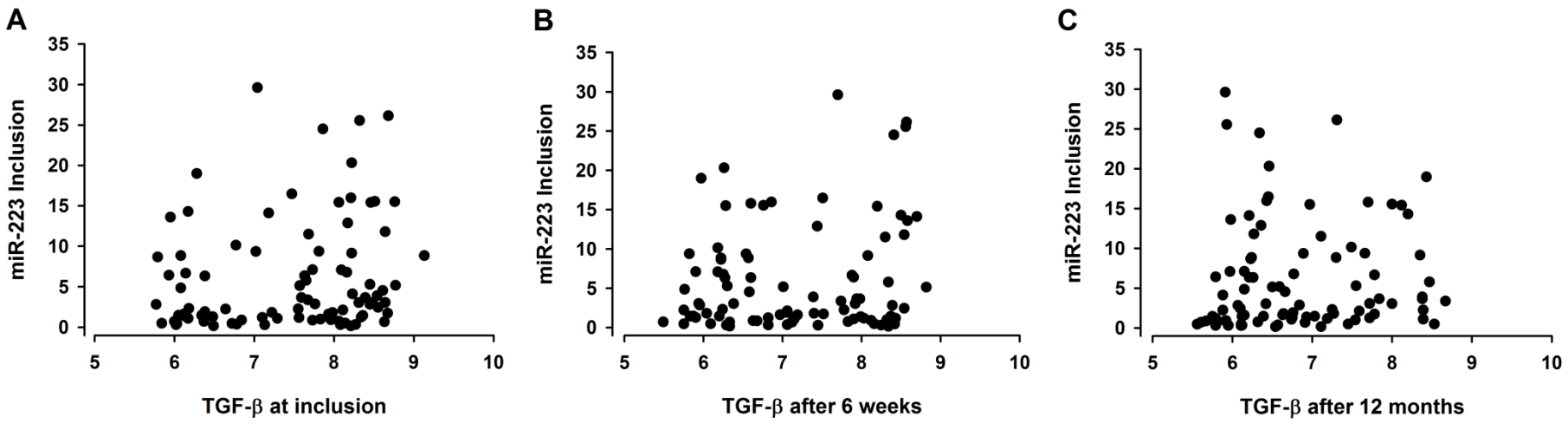

Figure 6.3 Expression of miR-223 at inclusion versus TGF-β expression in the serum from patients with lumbar radicular pain at different time points: A) at inclusion B) after 6 weeks C) after 12 months.