microRNAs in chronic pain

microRNAs bei chronischen Schmerzen

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“Only one excess is advisable in the world: the excess of gratitude!” - Jean de la Bruyère 1783

Abstract

Chronic pain is a common problem in clinical practice, not well understood clinically, and frequently tough to satisfactorily diagnose. Because the pathophysiology is so complex, finding effective treatments for people with chronic pain has been overall less than successful and typically reduced to an unsatisfactory trial-and-error process, all of which translates into a significant burden to society. Knowledge of the mechanisms underlying the development of chronic pain, and moreover why some patients experience pain and others not, may aid in developing specific treatment regimens. Although nerve injuries are major contributors to pain chronification, they cannot explain the entire phenomenon. Considerable research has underscored the importance of the immune system for the development and maintenance of chronic pain, albeit the exact factors regulating inflammatory reactions remain unclear. Understanding the putative molecular and cellular regulator switches of inflammatory reactions will open novel opportunities for immune modulatory analgesics with putatively higher specificity and less adverse effects. It has become clear that small, non-coding RNA molecules known as microRNAs are in fact potent regulators of many thousands of genes and possibly cross-communicate between cellular pathways in multiple systems acting as so-called “master-switches”. Aberrant expression of miRNAs is now implicated in numerous disorders, including nerve injuries as well as in inflammatory processes. Moreover, compelling evidence supports the idea that miRNAs also regulate pain, and in analogy to the oncology field aid in the differential diagnosis of disease subtypes. In fact, first reports describing characteristic miRNA expression profiles in blood or cerebrospinal fluid of patients with distinct pain conditions are starting to emerge, however evidence linking specific miRNA expression profiles to specific pain disorders is still insufficient. The present thesis aimed at first, identifying specific miRNA signatures in two distinct chronic pain conditions, namely peripheral neuropathies of different etiologies and fibromyalgia syndrome. Second, it aimed at identifying

miRNA profiles to better understand potential factors that differentiate painful from painless neuropathies and third, study the mechanistic role of miRNAs in the pathophysiology of pain, to pave the way for new druggable targets.

Three studies were conducted in order to identify miRNA expression signatures that are characteristic for the given chronic pain disorder. The first study measured expression of miR-21, miR-146a and miR-155 in white blood cells, skin and nerve biopsies of patients with peripheral neuropathies. It shows that peripheral neuropathies of different etiologies are associated with increased peripheral miR-21 and miR-146a, but decreased miR-155 expression. More importantly, it was shown that painful neuropathies have increased sural nerve miR-21 and miR-155 expression, but reduced miR-146a and miR-155 expression in distal skin of painful neuropathies. These results point towards the potential use of miRNAs profiles to stratify painful neuropathies. The seconds study extends these findings and first analyzed the role of miR-132-3p in patients and subsequently in an animal model of neuropathic pain. Interestingly, miR-132-3p was upregulated in white blood cells and sural nerve biopsies of patients with painful neuropathies and in animals after spared nerve injury. Pharmacologically modulating the expression of miR-132-3p dose-dependently reversed pain behavior and pain aversion, indicating the pro-nociceptive effect of miR-132-3p in chronic pain. This study thus demonstrates the potential analgesic impact by modulating miRNA expression. Fibromyalgia is associated with chronic widespread pain and, at least in a subgroup, impairment in small nerve fiber morphology and function. Interestingly, the disease probably comprises subgroups with different underlying pathomechanisms. In accordance with this notion, the third study shows that fibromyalgia is associated with both aberrant white blood cell and cutaneous miRNA expression. Being the first of its kind, this study identified miR-let-7d and its downstream target IGF-1R as potential culprit for impaired small nerve fiber homeostasis in a subset of patients with decreased intra-epidermal nerve fiber density. The work presented in this thesis is a substantial contribution towards the goal of better characterizing chronic pain

based on miRNA expression signatures and thus pave the way for new druggable targets.

Zusammenfassung

Chronische Schmerzen sind in der klinischen Praxis ein häufiges Problem, die Ätiologie und Pathogenese jedoch oftmals unklar. Aufgrund der Komplexität des pathophysiologischen Ursprunges chronischer Schmerzen, ist bei einem Teil der Patienten Schmerzfreiheit oder Schmerzreduktion mit gängigen Analgetika nur insuffizient zu erreichen. Dies führt zu einer enormen sozio-ökonomischen Belastung für die Gesellschaft. Daher können Kenntnisse über die Mechanismen, die der Entwicklung von chronischen Schmerzen zugrunde liegen, und darüber hinaus, warum einige Patienten Schmerzen entwickeln und andere nicht, bei der Entwicklung spezifischer und individueller Behandlungsschemata helfen. Eine Vielzahl an Studien belegen die Bedeutung des Immunsystems für die Entwicklung und Aufrechterhaltung chronischer Schmerzen, wenngleich die genauen Faktoren, die entzündliche Reaktionen regulieren, noch unklar bleiben. Rezente Entdeckungen der hochkonservierten, nicht-kodierenden RNA-Moleküle, sogenannten microRNAs, lassen in der Tat darauf schließen, dass diese eine wichtige Rolle im Netzwerk der Genregulation spielen. microRNAs regulieren die hochspezifische „cross-communication“ mehrerer simultaner Signaltransduktionsvorgänge zellulärer Prozesse, und werden daher auch "master-switches" genannt. Interessanterweise, wurden aberrante Expressionen spezifischer miRNAs in zahlreichen Krankheiten, einschließlich Nervenverletzungen, sowie in entzündlichen Prozessen nachgewiesen. Darüber hinaus belegen stichhaltige Beweise nicht nur die Idee, dass miRNAs auch bei der Regulierung von Schmerzen eine wichtige Rolle spielen, sondern auch hilfreich bei der Differentialdiagnose von Krankheits-Subtypen sein können. Dies wurde bei rezenten onkologischen Studien deutlich. Tatsächlich weisen erste Berichte auf ein charakteristisches miRNA-Expressionsprofil in Blut oder Zerebrospinalflüssigkeit von Patienten mit

verschiedenen Schmerztypen hin. Jedoch ist die Assoziation spezifischer miRNA-Expressionsprofile mit spezifischen Schmerzstörungen noch unzureichend. Die Zielvorgabe der vorliegenden Arbeit war daher zunächst, spezifische miRNA-Signaturen in zwei verschiedenen chronischen Schmerzzuständen zu identifizieren, nämlich peripheren Neuropathien verschiedener Ätiologien und dem Fibromyalgie-Syndrom. Zweitens wurden die erarbeiteten Ergebnisse dazu verwendet, bestimmte miRNA-Profile zu identifizieren, die schmerzhafte von schmerzlosen Neuropathien unterscheiden lassen und einen Hinweis auf die Pathologie der kleinkalibrigen Fasern bei der Fibromyalgie geben. Darüber hinaus wurde die mechanistische Rolle von miRNAs in der Pathophysiologie von Schmerzen Tierexperimentell untersucht, um künftig neuartige Therapien entwickeln zu können.

Die erste Studie untersuchte die Expression von miR-21, miR-146a und miR-155 in weißen Blutkörperchen, Haut- und Nervenbiopsien bei Patienten mit peripheren Neuropathien. Sie zeigt, dass periphere Neuropathien verschiedener Ätiologien mit erhöhten peripheren miR-21 und miR-146a und verminderter miR-155 Expression assoziiert sind. Wichtiger jedoch, dass Patienten mit schmerzhaften Neuropathien erhöhte miR-21 und miR-155-Expression im Suralis und verminderte miR-146a- und miR-155-Expression in distalen im Vergleich zu proximalen Hautbiopsien aufweisen. Diese Ergebnisse weisen auf die potenzielle Verwendung von miRNA-Profilen zur Stratifizierung schmerzhafter Neuropathien hin. Die zweite Studie baut dieses Ergebnis aus und untersuchte zunächst die Rolle von miR-132-3p im humanen und anschließend bei tierexperimentellen neuropathischen Schmerzen. Interessanterweise war miR-132-3p sowohl in weißen Blutkörperchen und Suralis-Nervenbiopsien von Patienten mit schmerzhaften Neuropathien als auch bei Tieren nach Läsion eines peripheren Nervens hochreguliert. Nach pharmakologischer Intervention gab es eine dosisabhängige Schmerzreduktion und Schmerzaversion, was somit auf den pro-nozizeptiven Effekt von miR-132-3p hinweist. Diese Studie zeigt somit die potenzielle analgetische Wirksamkeit microRNA-gerichteter pharmakologischer Interventionen. Das Fibromyalgie Syndrome ist eine chronische Erkrankung, die

von einem multilokulären Schmerzbild und Beeinträchtigungen in kleinen Nervenfasern dominiert wird. Es wird angenommen, dass die Erkrankung wahrscheinlich aus Subgruppen mit unterschiedlichen zugrunde liegenden Pathomechanismen besteht. Die hierzu durchgeführte Studie zeigt, dass Fibromyalgie-Patienten veränderte microRNA Expression sowohl in weißen Blutkörperchen als auch in der Haut aufweisen. Erstmals identifiziert diese Studie miR-let-7d und ihr „downstream-target“ IGF-1R als potentiellen Schädigungsmechanismus kleiner Nervenfaserfunktionen, in einer Subgruppe von Patienten mit verminderter intra-epidermalen Nervenfaserdichte. Die Ergebnisse, die in dieser Arbeit vorgestellt werden, liefern einen wesentlichen Beitrag, die Pathophysiologie chronischer Schmerzen, aufgrund von miRNA-Expressions-Signaturen zu charakterisieren.

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Abbreviations

	β	beta-
#	δ	delta-
	µ	micro-
	ACR	American College of Rheumatology
	ADS	Allgemeine Depressionsskala
A	AGO	Argonaute protein
	AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
	ANCA	anti-neutrophil cytoplasmic antibody
B	BDNF	brain-derived neurotrophic factor
	BH	Benjamini-Hochberg
	cDNA	complementary DNA
	CDT	cold detection threshold
	CIAP	chronic idiopathic axonal polyneuropathy
	CIDP	chronic inflammatory demyelinating neuropathy
	CCI	chronic constriction injury
	c-Maf	musculoponeurotic fibrosarcoma
C	CNS	central nervous system
	corr. coeff.	correlation coefficient
	CP	chronic pain
	CREB	cAMP response element binding protein
	CSF	cerebrospinal fluid
	Ct	cycle threshold
	CWP	chronic widespread pain

	DICER	endoribonuclease Dicer
D	DRG	dorsal root ganglia
	DROSHA	class 2 ribonuclease III enzyme
E	EDTA	ethylene-diamine-tetraacetic-acid
	ENA	anti-nuclear antigen
	FC	fold change
F	FIQ	Fibromyalgia Impact Questionnaire
	FM	fibromyalgia
	FMS	fibromyalgia syndrome
	GCPS	graded chronic pain scale
G	GFAP	glial fibrillary acidic protein
	GLT-1	glutamate transporter-1
	GluA	AMPA receptor subunit
	H&E	Hematoxylin and eosin stain
H	HDT	heat detection threshold
	HPA	hypothalamus-pituitary-adrenal
	HPLC	high performance liquid chromatography
	IASP	International Association for the Study of Pain
	Iba1	ionized calcium-binding adapter molecule 1
	IENF	intra-epidermal nerve fiber
I	IENFD	intra-epidermal nerve fiber density
	IGF-1R	insulin-like growth factor receptor 1
	IHC	immunohistochemistry
	IL	interleukin
	i.t.	intrathecal

	lat.	latin
L	LNA	locked nucleic acid
	LTP	long-term potentiation
	MDT	mechanical detection threshold
	mRNA	messenger RNA
M	miRNA	microRNA
	MOPS	3-(N-morpholino)propanesulfonic acid
	MPS	mechanical pain sensitivity
	MPT	mechanical pain threshold
	n	number of animals/patients/samples
	ncRNA	non-coding RNA
	NCV	motor nerve conduction velocity
N	NeuPSIG	Neuropathic Pain Special Interest Group
	NP	Neuropathic Pain
	NRS	numeric rating scale
	NPSI	neuropathic pain symptom inventory
O	ORF	open reading frame
	PEAP	place escape avoidance paradigm
	PGP9.5	protein gene product 9.5 antibody
	PHS	paradoxical heat sensation
P	PIAN	progressive idiopathic axonal neuropathy
	PNP	polyneuropathy
	PNS	peripheral nervous system
	PPT	pressure pain threshold
Q	qPCR	quantitative polymerase chain reaction
	QST	quantitative sensory testing

	RIN	RNA Integrity Number
R	RISC	RNA-induced silencing complex
	RNA	ribonucleic acid
	Scr	scrambled oligonucleotide
	SDS	sodium dodecyl sulfate
	SFN	small fiber neuropathy
	SNI	spared nerve injury
S	snorD	small nucleolar RNA, C/D box
	SNP	single nucleotide polymorphism
	SNRI	serotonin and norepinephrine reuptake inhibitors
	SSS	symptom severity score
	SSRI	selective serotonin reuptake inhibitors
T	TCA	tricyclic antidepressants
	TSL	thermal sensory limen
U	UTR	untranslated region
V	VDT	vibration detection threshold
	WBC	white blood cells
W	WPI	widespread pain index
	WT	wild type

Chapter 1

General Introduction

‘Ah, on what little things does happiness depend!’ —
Oscar Wilde, The Nightingale And The Rose, 1891

General Introduction

"Pain is as an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage," the International Association for the Study of Pain (IASP) (Merskey and Bogduk, 1994).

Figure 1: Schematic representation of Descartes' pain pathway. Based on Descartes' theory was pain a specific, straight-through sensory projection system. Adapted from Melzack&Katz 2004, The Gate Control Theory: Reaching for the Brain.

Historically, pain as a concept, reflects the parallel evolution of philosophical and physiological constructs that reflect upon the impact that injury and threats to survival have on behavior and thinking. In parallel with the evolution of the philosophical construct of pain, as a largely negative experience of suffering or punishment (i.e. a negative basic feeling or emotion that involves a subjective character of unpleasantness, aversion, harm or threat), thinkers such as Galen, Avicenna and Descartes noted that pain was an attribute of sensation. Especially Descartes's theory of the "human body machine" revolutionized the scientific construct of pain (Figure 1). He was among the main drive of scientific thinkers believing that the sensation of pain was a disturbance that traveled along specific paths to reach the brain. In the 19th and mid 20th century, notions of pain became thereupon influenced by the rapid evolution of physiological insights, such as specialized pain pathways and nociceptive reflexes (Gower, Sherrington), pain responses after injury (Lewis) and the classical "plastic" pain processing system (Melzack and Wall; the

Gate control theory) (Melzack and Wall, 1965). Perhaps one of the more exciting aspects of the physiological advances has been the unification of the concept that “perception” of pain is in the brain, and that the afferent information which initiates a given pain state can be driven (enhanced, i.e. hyperalgesia) and thus also be modified (reduced, i.e. analgesia) by intrinsic spinal mechanisms. Importantly, pioneering work by Melzack and Casey unveiled specialized regions in the brain that are associated with well defined and describable experiences (the sensory discriminative aspect of a painful stimulus), and that others are associated with the emotional component evoked by a painful stimulus (Melzack and Casey, 1968).

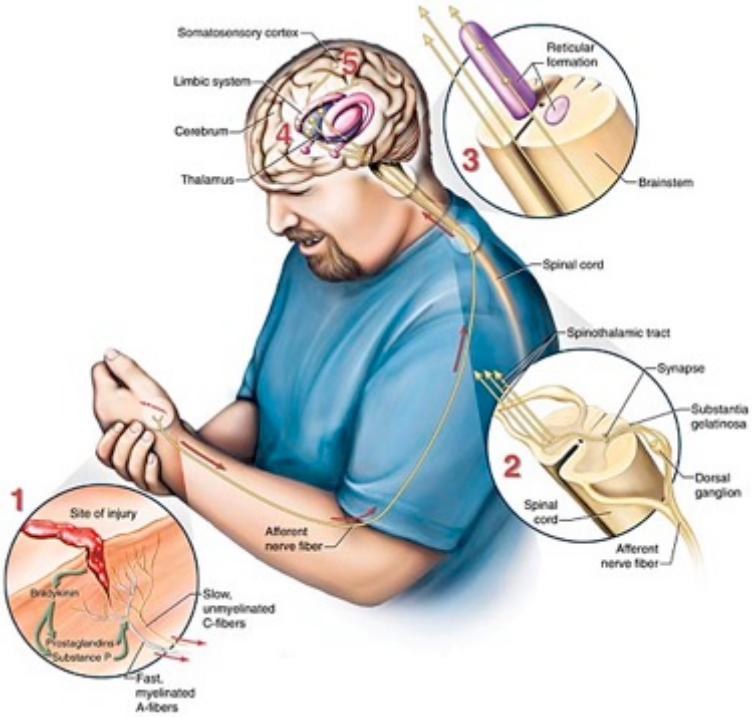

Figure 2: Neurologic transmission of pain stimuli. Pain stimuli (e.g. after tissue injury) travel via afferent neurons to the substantia gelatinosa in the dorsal horn of the spinal cord [1]. In the spinal cord the gating mechanism (according to Melzack and Wall’s theory) occurs and pain impulses travel via specialized tracts to the bain [2]. In the brain pain stimuli are processed, (interpretation and association of the stimuli) and modulated [3]. Published with permission: WildIris Medical Education, Inc. (Alexander, 2005).

The ability to detect potential threats (painful or noxious stimuli) to an organism’s well-being is essential for survival. Normally, pain is experienced when activity in primary afferent neurons (Aδ- and C-fibers afferents) convey sensory information such as thermal, mechanical and chemical stimuli from the periphery to the central nervous system (Willis and Westlund, 1997). This is nociceptive (or acute) pain, which is always evoked; examples of acute pain are pinprick, a stubbed toe or a cut in the skin (Figure 2). In the simplified graphical representation above, the transmission of pain stimuli are depicted. It starts with the site of injury at which the various

different sources of noxious stimuli are primarily sensed by nociceptors and conveyed via specialized afferent nerve fibers to the substantia gelatinosa in the spinal cord (i.e. the layer in which mainly C-fibers and to a lesser extent Aδ-fibers terminate). The spinal dorsal horn contains both neuroglia and nerve cells being involved in the modulation and thus output of pain signals, as first described in Melzack & Wall's gate control theory. With increasing knowledge about nociception, their theory became increasingly challenged. Today we know, that the interplay of excitatory and inhibitory interneurons, neuron-glia interactions, and the input of ascending and descending information, control the output of pain signals to higher brain centers via specialized tracts (e.g. spino-thalamic tract). Once the pain signals reach the cerebral cortex, the brain interprets the signal. It perceives the intensity and location of pain and associates it with experiences. Pain intensity is thus always subjective. After perception of the painful stimuli, the brain initiates the release of various substances that can modulate the painful signal (e.g. endorphins). It can also modulate pain signals via inhibitory descending fibers, and activate motor neurons and muscles to produce a reflexive retraction from the painful stimulus.

With resolution of damage or noxious stimulation, the pain state commonly resolves. If, however, alterations in the pain pathway occur that lead to hypersensitivity towards noxious stimuli, pain ceases its function as an acute warning system and instead becomes chronic and debilitating (Basbaum et al., 2009). Hypersensitivity can arise from alterations in the properties of peripheral nerves. The latter is commonly referred to as peripheral sensitization, in which e.g. inflammation-associated changes in the chemical environment of afferent fibers, often after tissue damage sensitize nociceptors (McMahon et al., 2008). Sensitization of pain transmitting fibers (Aδ and C-fibers) can induce ongoing activity, leading to the activation of specialized populations of neurons in the spinal cord, that then project heightened signals to higher cortical levels of the brain. This can lead to central sensitization, a hyperexcitable state of enhanced processing of painful messages (Woolf, 1983). Sensitization by inflammation or

neuropathy may thus result from peripheral and/or central processes and is the underlying concept for when acute pain becomes chronic.

Chronic pain resulting “as a direct consequence of a lesion or disease affecting the somatosensory system,” (Merskey and Bogduk, 1994) is classified as neuropathic pain (NP). Lesions or diseases that affect peripheral nerves such as in peripheral neuropathies lead to various patterns of sensorimotor and autonomic dysfunctions, depending on the type (motor, sensory, and autonomic) and complexity of impaired fibers. Interestingly, neuropathies are associated with obvious nerve-damage related “negative” symptoms, such as hypoesthesia (decreased feeling) and numbness, but paradoxically also with “positive” symptoms such as spontaneous paresthesias (burning and/or tingling sensations), dysesthesias (allodynia, hyperalgesia), and pain. Of note, most lesions of the peripheral nervous system do not cause chronic pain, implying that “the presence of pain after neuronal injury reflects qualitative changes in the neurobiological mechanisms encoding pain” (Koltzenburg, 2010). Taken together, acute pain is the sudden and sharp in sensation felt experience, after for example a pinprick, and serves as acute warning system to detect potential threats. When the painful sensation persists for an extended period beyond the resolution of the painful stimuli, chronic pain is present. Chronic pain, as after trauma or injury to the CNS is called neuropathic pain and encompasses multiple symptoms. The cause of NP is incompletely understood and not a complete diagnosis on its own. Hence the term NP rather refers to a condition that is composed of a variety of disorders (Baron et al., 2010; Jensen et al., 2011; Xu and Yaksh, 2011). In the following paragraph, the clinical relevance and challenges of NP are going to be discussed and the aims of the present thesis introduced.

Neuropathic pain: the clinical situation

NP is a major health issue, affecting 3.3% to 8.2% of adults (Bouhassira et al., 2008; Haanpää et al., 2011; Hughes, 2002; van Hecke et al., 2014), and is associated with a devastating decrease in the quality of life due to significant co-morbid conditions (Doth et al., 2010). In 2006 the total estimated healthcare costs in the United States were more than $150 billion (Gatchel and Okifuji, 2006), highlighting the immense socio-economic burden of chronic neuropathic pain (Berger et al., 2004). Since lesion and diseases of the somatosensory system can affect both, the CNS and PNS, NP can be separated into central and peripheral syndromes. Typical examples for peripheral pain syndromes are post-traumatic neuropathies, polyneuropathies, radiculopathies, or diabetic neuropathy. For central pain syndromes common cases include stroke, multiple sclerosis or spinal cord injury (Baron et al., 2010; Jensen et al., 2011). Interestingly, peripheral neuropathies can be either painful or painless, the reason for this is unknown (Üçeyler and Sommer, 2008). Painful neuropathies are commonly grouped into symmetrical polyneuropathies (PNP) (i.e. diseases affecting many nerves simultaneously) or asymmetrical neuropathies with a mono- or multiplex distribution. Despite the diverse array of reasons that may cause peripheral neuropathies, peripheral nerves exhibit only a few pathological reactions that span Wallerian degeneration, axonal degeneration, and segmental demyelination. As mentioned before, various symptoms and signs that reflect sensory, motor, and autonomic nerve fiber dysfunction, are the result of these pathological reactions in peripheral nerves and are correlated to neuroanatomical representations, providing differential diagnostic clues to possible underlying causes (Hughes, 2002). However, due to the complex nature and partially diffused distribution of symptoms and signs, thorough neurological examination is necessary for accurate diagnosis. In combination with detailed laboratory studies, the clinical diagnostic process of peripheral neuropathies encompasses a combination of different neurophysiological investigations such as nerve conduction studies. This allows the assessment of large fibers and thus the differentiation between demyelinating and axonal neuropathies and/or the

presence or absence of nerve conduction blocks (Kimura, 2001). In addition, morphological investigations in sural nerve and skin biopsy specimens or assessment of intra-epidermal nerve fibers (IENF) in the skin of patients provide an important diagnostic tool to establish the cause of a given peripheral neuropathy. Histological assessment can improve the limitations of nerve conduction studies (Dyck et al., 2005), and augment the diagnostic information of functional small fiber tests. However, likewise to date, no histological feature is currently able to accurately distinguish between painless and painful neuropathies (Kalliomaki et al., 2011).

Due to the complex heterogeneity and subjectivity, treatment of neuropathic pain is often unsatisfactory, despite major efforts being made to better describe and classify the signs and symptoms of neuropathies (Jensen and Baron, 2003). As a result, treatment strategies are based on symptoms rather than the initial cause. Pharmacological interventions are often the first choice of treatment, although other conservative management possibilities exist, such as physical therapy. Surgical interventions and electrical stimulation also count to the management possibilities if pain persists for an extended period of time. The Neuropathic Pain Special Interest Group (NeuPSIG) recently published evidence-based guidelines for the pharmacological treatment of neuropathic pain (Dworkin et al., 2007). These guidelines established specific first-line interventions, including tricyclic antidepressants often in combination with serotonin and norepinephrine reuptake inhibitors, anticonvulsants including Pregabalin and Gabapentin, and topical Lidocaine. Cases that do not respond to first-line medications are treated with opioids and tramadol, whereas third-line medications such as antiepileptic drugs are reserved for patients not tolerating, responding to, or being contraindicative to prior treatments (Attal et al., 2010). However, despite recent advances, epidemiological studies concluded that a large number of patients not only still remain diagnosed as idiopathic (i.e. a pathogenesis of unknown origin) but also receive suboptimal treatment (O'Connor and Dworkin, 2009). Particularly, one of the primary reasons behind inadequate treatment of patients with NP appears to be the diagnostic accuracy

(Martinez et al., 2014). Hence, these limitations request further investigations in the mechanisms underlying neuropathic pain, to not only optimize treatments, but also improve patient outcomes through forward-thinking research initiatives. One aim of the present thesis was thus identify potential biomarkers that would aid in the diagnostic make-up.

Fibromyalgia Syndrome: a subgroup of chronic widespread pain

Fibromyalgia (FM) from fibro (lat. = fiber), myos (greek = muscle) and, algos (greek = pain) is another chronic painful condition, but rather characterized by widespread pain mainly perceived in deep somatic tissues (i.e. muscles and joints). The definition was originally based on the American College of Rheumatology classification scheme (Wolfe et al., 1990), stating that individuals must have a history of widespread pain for at least 3 months and a painful sensitivity to pressure at 11 of 18 anatomically defined tender points. These diagnostic criteria have recently been updated and now incorporate the Widespread Pain Index (WPI) and the Symptom Severity Score (SSS), which also allows the assessment of comorbid conditions of FMS (Wolfe et al., 2010). With a prevalence of up to 3%, increasing with age, most dramatically in women, FMS greatly impairs the quality of life of patients and thus leaves a high socio-economic burden (Branco et al., 2010). There is still no cure for FMS, management is non-specific, multimodal and symptomatic, trying to improve sleep, restore physical function, re-establish the emotional balance and reduce pain (Ablin et al., 2013; Häuser et al., 2014; Sommer et al., 2012).

The clinical manifestations are usually more complex than widespread body pain alone. In a survey of 699 FMS patients, various discomforts were reported ranging from meteorosensitivity, disordered sleep, morning stiffness, fatigue, cognitive dysfunction and psychological distress to depression and anxiety (Clauw, 2015). But three cardinal symptoms - pain, fatigue and insomnia - are always present (Hauser et al., 2008; Mease, 2005). Importantly, the etiology and pathophysiology of pain in FMS still remains unclear. FMS was classically believed to be the cause of a chronic and generalized muscle stiffness, that lead

to mechanical overload of tender points, and hypoperfusion of muscles and tendons. Newer research however suggests that among multidimensional biological and psychological causes, genetic predispositions might also play a role (Buskila and Neumann, 2005). As summarized in Figure 3, the multidimensional pathogenesis is believed to be a complex cause of vegetative, endocrine and central nervous system changes. Individuals with an existent predisposition, such as familial aggregation or genetic polymorphisms in e.g. the serotonin transporter or catechol-O-methyltransferase gene (Ablin and Buskila, 2015), that are being exposed to multiple stressors (physical, biological or psychological), have a higher likelihood of developing FMS. Also chronification factors, such as impairment of central pain processing pathways, or more particularly a dysfunctional hypothalamic-pituitary-adrenal (HPA) axis have been suggested (Clauw, 2015). Moreover, impairments of the immune system have also been attributed to the FMS pathology, unfortunately with rather inconsistent overlap between studies (Üçeyler et al., 2011; Üçeyler et al., 2006; Wang et al., 2008).

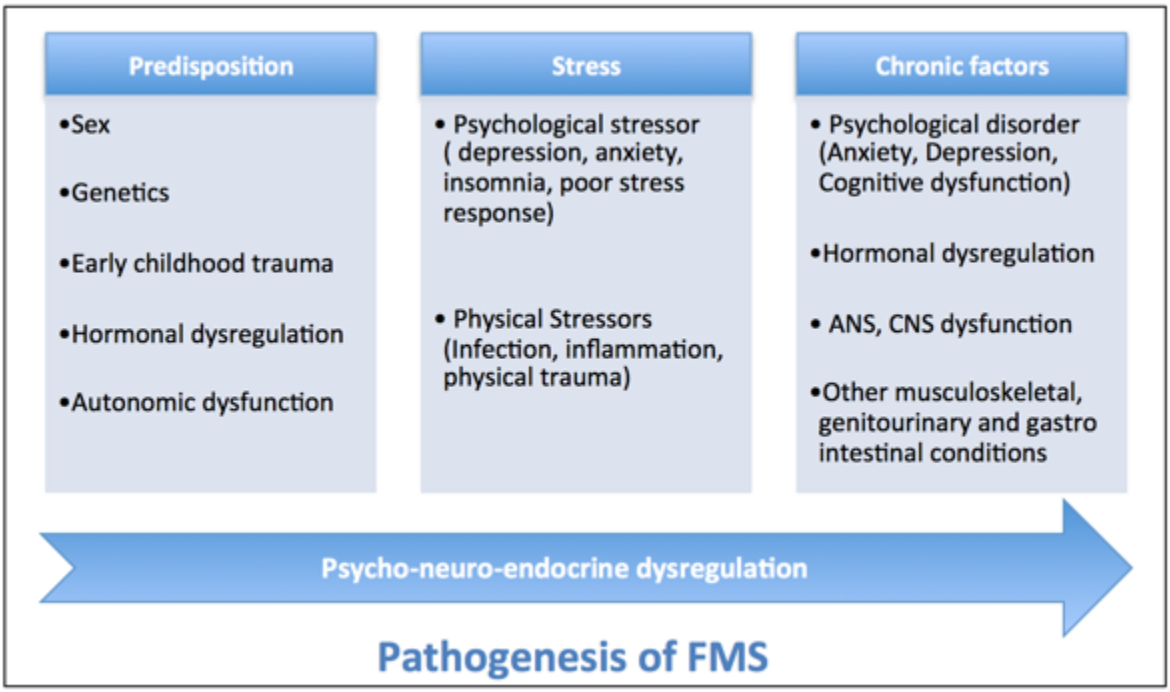

Figure 3: Pathogenesis of FMS. The pathogenesis of FMS is believed to be a complex multidimensional reciprocity of predisposing factors (e.g. sex (f), genetic polymorphisms, trauma), together with both physical and psychological lifetime stressors and various chronic factors (e.g. hormonal dysfunction), leading to a psycho-neuro-endocrine dysregulation possibly causing FMS. ANS, autonomic nervous system; CNS, central nervous system; f, female; FMS, Fibromyalgia Syndrome.

Several studies implicated impairments in small nerve fiber (Aδ- and C-fibers) properties of FM patients (Caro and Winter, 2014; de Tommaso et al., 2014; Oaklander et al., 2013; Serra et al., 2014; Üçeyler et al., 2013). But due to the heterogeneity and complexity of symptoms in FMS, not all patients displayed the same extent of impairments, and subgrouping of FM patients was proposed as a way to improve characterization, which could possibly optimize individual treatment regimens in the future (Müller et al., 2007; Thieme et al., 2004). In fact, the recently described small nerve fiber pathology in subsets of FMS cases not only further highlighted the diagnostic relevance of subgrouping FMS patients, but also symbolized a novel prospect in the pathophysiology of FMS (Giannoccaro et al., 2014; Leinders et al., 2016a; Üçeyler et al., 2013).

Taken together, the pathophysiology of pain in FMS is still unknown, but there is evidence that small nerve fiber impairment in FMS might play a key role. Based on these pioneering findings, the present thesis aimed to further investigate the role of small nerve fibers in the pathophysiology of pain in FMS. However, it is important to note that the observed abnormalities in small nerve fibers are not solely specific for FMS, and as mentioned earlier, peripheral neuropathies also display small fiber related signs and symptoms. Hence, comprehensive analogies have to be taken with great caution, which will be elaborated in the following paragraph.

Neuropathic Pain and Fibromyalgia Syndrome: differences and similarities

Interestingly, particular similarities exist especially in the attendant comorbidities and the perception of pain. In both cases patients frequently report heat hyperalgesia, burning or prickling sensations and pain attacks (Koroschetz et al., 2011). Usually these features are documented with tailored questionnaires that not only help to characterize the etiology of pain but also its comorbidities. There are furthermore specialized tests that measure the functional and morphological properties of small and large nerve fibers. Quantitative sensory testing (QST) for example assesses impairments in thermal, mechanical and pain perception

thresholds (Rolke et al., 2006), whereas staining of gene product 9.5 (PGP 9.5) in skin biopsies proved to be a reliable tool to immunhistochemically visualize and quantitatively assess small nerve fibers (Kennedy et al., 2000). Interestingly, both conditions show impairments in small nerve fiber function. In addition, FMS does not only present itself with impairments in thermal perception thresholds, but also a reduction in the density of intra-epidermal nerve fibers (IENFs) (Üçeyler et al., 2013). This is also observed in small-fiber neuropathy (SFN), a subgroup of peripheral neuropathies involving an impairment of only Aδ- and C-fibers (Devigili et al., 2008; Lacomis, 2002). There are thus seemingly striking similarities and one could assume that i) due to the frequently reported small fiber pathology, ii) the success of centrally acting treatments, and iii) the association of widespread body pain and tenderness with chronic sensitization of the CNS (summarized in (Woolf, 2011)), FMS fulfills an entity of the definition and thus belongs to the group of NP disorders. However, these notions have to be taken with great caution, as obvious differences in etiology and clearly in the spatial distribution of symptoms exist. Most importantly, FMS does not demonstrate nerve lesions in contrast to many NP syndromes, implying that abnormalities in neurophysiological assessments of large fibers should be a criterion for exclusion. Furthermore, muscle atrophies, numbness and paresis are not observed in FMS. Strikingly, direct comparison of SFN and FMS revealed significant differences in properties of small nerve fibers, thus rendering direct analogies invalid (Serra et al., 2014), and requesting future studies.

Taken together, while answering this question was not within the scope of the present thesis, a vast amount of data seems to support the role for central sensitization as the cause of symptoms in FMS and thus showing analogies with NP. However several important aspects remain unanswered. In addition, some FMS patients do show small fiber impairments, demanding further research to provide direct evidence for how small nerve fibers in FMS influence pain. In fact, improving diagnostic criteria for both FMS and NP could revolutionize the understanding of both diseases. Moreover, an objective biomarker that has the

power to improve diagnostics would greatly enhance understanding and open new possibilities for targeted treatment in both diseases.

microRNAs as biomarkers for chronic pain?

In recent years, non-coding RNAs emerged as important regulators of gene expression (i.e. the process of protein synthesis), of which microRNAs (miRNAs) are the best characterized to date. miRNAs are short (~22 nucleotide long) double-stranded, non-coding Ribonucleic-acid (RNA) molecules that post-transcriptionally regulate gene expression. Approximately 1500 miRNAs are encoded within the human genome (Kozomara and Griffiths-Jones, 2011). miRNAs have repeated binding motifs that potentially enable them to target several genes simultaneously. Rather than the total silencing or induction of targets, miRNAs are thought to perform rapid fine-tuning, so-called 'on' and 'off' switching of entire cellular pathways, designating them as 'master-switches' (Soreq and Wolf, 2011; Stefani and Slack, 2008). Indeed, miRNAs are powerful regulators of eukaryotic gene expression, regulating not only up to 50% of all mRNA transcripts (Filipowicz et al., 2008; Jonas and Izaurralde, 2015), but also showing highly regulated temporal and spatial expression patterns in specific regions (Krichevsky et al., 2003), crucial for homeostasis (Huttenhofer and Schattner, 2006), and pathological processes (Mattick, 2004). As described in Figure 3, miRNAs can exert their regulatory function in several different ways. The 5'-end strand usually binds to partially complementary ('seed') sequences found in the 3'-untranslated region (3' UTR) of messenger RNAs (mRNAs). The seed-region of miRNAs thereby determines target specificity. Partial binding causes target degradation or sterically hinders the translational machinery in cells to synthesize proteins, whereas mRNA cleavage is dependent on perfect complementarity between miRNA and target genes (Shin et al., 2010). Recently it has been asserted that miRNAs are also exchanged between cells via for example exosomes (Stoorvogel, 2012), and that some miRNAs present in the extracellular space may even directly interact with surface receptors (Fabbri et al., 2012; Park et al., 2014).

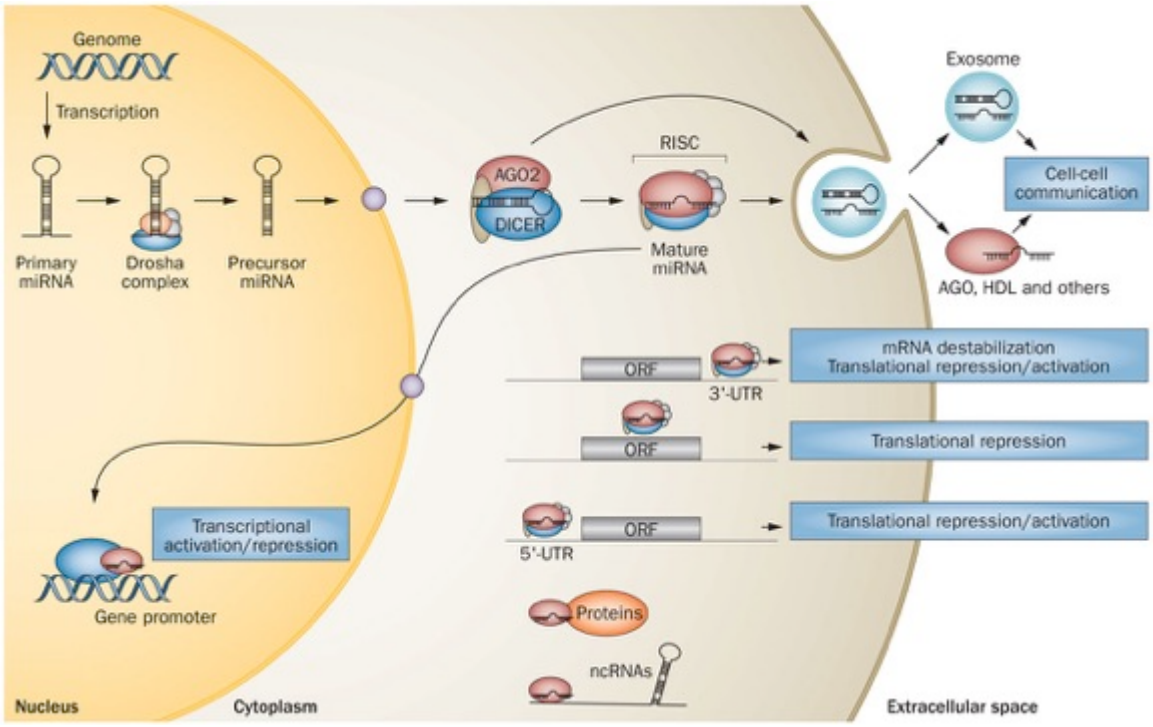

Figure 4: microRNA Biogenesis and function. The standard miRNA biogenesis pathway involves several steps. The primary miRNA transcripts (pri-miRNA) are transcribed from introns of the respective gene, by tRNA polymerase II. The pri-miRNA contains stem-loops (the so-called precursor (pre)-miRNA), in which the mature miRNA sequences are embedded. Pri-miRNAs are subsequently cleaved by the microprocessor RNAse III Drosha (Drosha complex) into 70-nt stem loop pre-miRNAs. Next, the pre-miRNA is exported to the cytoplasm and another RNAse III, Dicer, processes the pre-miRNA into 22nt long duplex of miRNAs (mature miRNA). Only one strand of this duplex gets incorporated into the RISC complex. The mature miRNAs predominantly bind to the 3-UTR of the target mRNA, leading to translational repression and mRNA destabilization. Specialized subsets of miRNAs can bind to the open reading frame (ORF) and the 5'-UTR, thereby repressing or activating translation. miRNAs can also be packaged into protein complexes (e.g. AGO) or exosomes and secreted into the extracellular space thereby engaging in cell to cell communication. Published with permission: Nature Publishing Group (Schwarzenbach et al., 2014). AGO, argonaute protein; HDL, high-density lipoprotein; ncRNAs, non-coding RNAs; ORF, open reading frame; 3'-UTR, 3 prime untranslated region, RISC, RNA-induced silencing complex

Several approaches exist to profile expression and analyze miRNA function. Genome-wide analysis of miRNA expression is achieved by employing micro-arrays, RNA sequencing and large-scale quantitative PCR (qPCR) platforms, that allow the simultaneous detection of multiple miRNAs (Cissell and Deo, 2009). Data obtained from the microarray platform usually requires further confirmation, which can be achieved by performing individual qPCR analysis. To gain insights into miRNA function, online databases for bioinformatically predicted and experimentally validated miRNA targets can be used once the miRNAs of interest have been identified (Hsu et al., 2011; Kozomara and Griffiths-Jones, 2011).

However, a major disadvantage is the great variety in online available algorithms that use different approaches to predict miR-mRNA interactions. Predicted targets might thus be dissimilar and non-overlapping throughout these platforms, bearing the potential danger to neglect many experimentally proven and validated targets. It is thus of utmost importance to study and verify the selected miRNAs' downstream-target interaction in order to translate findings into clinical practice. To confirm miRNA binding to its predicted target, reporter assays (e.g. luciferase assay) are helpful. Using miRNA-inhibitors and/or mimetics (e.g. locked nucleic acid, LNA oligonucleotides) in translational *in vitro* or *in vivo* experiments, the physiological role of a given miRNA can be demonstrated, and the biomarker potential tested. By definition, a biomarker is formally defined as a "proxy that allows remote and early detection of a given biological process (i.e. disease)," regardless of its mechanistic role in the condition being diagnosed" (Soreq, 2014). Of note, in neurological diseases researchers often need to rely on extra-cranial or peripheral sources of biomarkers to obtain live readouts of the disease state. The choice includes body fluids such as blood (serum, plasma, lymphocytes), cerebrospinal fluid (CSF) and biopsies (e.g. nerve and skin biopsy specimens) that can yield neuronal and/or non-neuronal cells.

The use of miRNAs as non-invasive biomarkers has gained significant attention since both their presence in all kinds of extra- and intracellular peripheral tissues (Chen et al., 2008; Gilad et al., 2008; Mitchell et al., 2008; Rao et al., 2013), and their particular advantage of having a robust stability and sensitivity (Jung et al., 2010). However, trivial questions arise when using miRNAs as biomarker for chronic pain: To what degree does the source resemble biological processes in the brain (if non-neuronal tissue was used)? And more importantly, does miRNA expression assessed in blood and/or peripheral tissue constitute a viable biomarker strategy for the purpose of diagnosis, prediction, and patient stratification of chronic pain? Moreover, can miRNAs be used as a therapeutic avenue for future analgesics? While the search for biomarkers in chronic pain syndromes is still in its infancy, these

questions have been addressed throughout the individual chapters of the present thesis.

Chronic pain, as mentioned earlier, encompasses peripheral and central mechanisms, rendering the use of various peripheral tissues an adequate readout to study its pathology. Indeed, chronic pain possess unique miRNA expression signatures important for pain circuitries, the cognitive, emotional, and behavioral components of pain (Kress et al., 2013). Furthermore, aberrant expression of several miRNAs has been reported throughout many loci of pre-clinical studies (Aldrich et al., 2009; Bai et al., 2007; Imai et al., 2011; Kusuda et al., 2011; Sakai and Suzuki, 2015; von Schack et al., 2011). Also in patients with distinct pain conditions, first reports hint towards characteristic miRNA expression profiles in blood or CSF (Andersen et al., 2016; Beyer et al., 2015; Bjersing et al., 2013; Heyn et al., 2016; Orlova et al., 2011; Pauley et al., 2008), however, evidence linking specific miRNA expression profiles to specific pain disorders is still insufficient. This is in particular related to standardization of quantification procedures, normalization, and appropriate reference controls throughout these studies. Furthermore, one of the hindrances in translating these findings into better therapy of chronic pain is the aforementioned pathophysiological complexity. Alterations in processes including ion channels, inflammatory mediators, neurotrophic factors, synaptic plasticity and many more are not only involved in acute circumstances but also change during the course of the disease (von Hehn et al., 2012). Another limitation lies in the use of animal models that mimic persistent pre-clinical pain states (Gregory et al., 2013; Mogil et al., 2010), but often lack the exact translational validity to bridge the gap between basic science and clinical practice. Furthermore, single time point measurements of miRNAs cannot adequately answer the question if alterations in their expression are the cause or consequence of the disease; for this longitudinal studies are necessary. Nonetheless, a minimally invasive pain biomarker ideally arising from translational research would dramatically advance the understanding and characterization of chronic pain. There are still many missing pieces of the puzzle, which warrants additional studies before the clinical

utilization, however the present thesis is aimed at establishing a fundamental and standardized basis for the use of miRNAs as diagnostic readout for chronic pain. Importantly, results throughout the different chapters of this thesis address the potential use of miRNAs as clinical biomarker for chronic NP (Leinders et al., 2016b) and FMS (Leinders et al., 2016a) and forms a promising basis for the future assessment and characterization of miRNAs in chronic pain.

Research questions and outline of the thesis

The research performed and described in this cumulative thesis is aimed at investigating the role of miRNAs as diagnostic biomarkers for chronic pain conditions. To study this research question, we i) investigated miRNA expression in different peripheral tissues of two distinct chronic pain conditions; ii) investigated the function of a particular candidate miRNA in a translational manner using an animal model for chronic neuropathic pain, and iii) analyzed the role of miRNAs in relation to small nerve fiber pathology.

In this thesis, the following research questions were addressed:

1. *What is the role of miRNAs detected in white blood cells, skin or nerve cells in the pathophysiology of chronic pain?*

2. *What is the pattern of miRNAs in peripheral neuropathies and are we able to translate findings from mice to men?*

3. *Are there any indications for miRNAs predisposing as biomarker for pain in neuropathies?*

4. *Is there a connection between miRNAs and small fiber pathology in FMS?*

A total of three studies were performed to answer the research questions described above. These three studies were conducted to establish pain-related miRNA research at the Department of Neurology, Würzburg and to pave the way for future miRNA studies of neurological diseases. In the following, an outline of each study is provided that are included as separate chapters in this thesis.

Chapter 2: "Aberrant microRNA expression in patients with painful peripheral neuropathies", published in *Neurobiology of Disease* *(Leinders et al., 2016)*

In Chapter 2 miRNA expression in three sources of peripheral tissue (WBC, skin and sural nerve) was investigated for the characterization of neuropathic pain in patients. The involvement of three immune-related miRNAs (miR-21, miR-146a, miR-155) in peripheral neuropathies of different etiologies as potential future biomarkers in the differentiation of painful versus painless neuropathies is outlined.

Chapter 3: "Increased miR-132-3p expression is associated with chronic neuropathic pain", published in *Experimental Neurology (Leinders et al., 2016)*

Using a similar study design in Chapter 3, the translational role of miR-132-3p in an animal model of chronic neuropathic pain was investigated. The results of this study provide important information regarding the role of miRNAs in the pathophysiology of neuropathic pain and substantiate the potential use of miRNAs in the diagnosis and characterization of neuropathic pain. It furthermore opens the possibility for future treatment possibilities in chronic neuropathic pain.

Chapter 4: "Increased cutaneous miR-let-7d expression correlates with small nerve fiber pathology in patients with fibromyalgia syndrome", published in *Pain (Leinders et al., 2016)*

In Chapter 4 the systemic and cutaneous expression of miRNAs were analyzed in FMS patients, in combination with quantitative assessment of small fiber function and density. miR-let-7d and its downstream target IGF-1R were identified as potential culprits for impaired small nerve fiber homeostasis. Results presented in this chapter provide a pathomechanistic role of miRNAs in subgroups of FMS patients with existing small nerve fiber pathology.

Finally, **Chapter 5** summarizes the findings from the preceding chapters and highlights different shortcomings with respect to the to-date use of miRNAs in chronic pain. Furthermore, research questions that came up in the general introduction are carefully addressed and related to the findings presented in this thesis, followed by an overall conclusion of this thesis.

While the methodological details of the following studies were not in the scope of this general introduction, the specific methods and hypothesis used to investigate miRNAs are given in the respective chapters.

Chapter 2

Aberrant microRNA expression in patients with painful peripheral neuropathies

Leinders Mathias, Üçeyler Nurcan, Thomann Anna, Sommer Claudia.

Neurobiology of Disease (2016)

Chapter 2: **Leinders M., Üçeyler N., Thomann A., Üçeyler N., Sommer C. (2016). Aberrant microRNA expression in patients with painful peripheral neuropathies. *submitted Neurobiology of Disease***

Abstract

Changes in the neuro-immune balance play a major role in the induction and maintenance of neuropathic pain. We recently reported pathophysiologically relevant alterations in skin and sural nerve cytokine expression in peripheral neuropathies of different etiologies. Immune processes and cytokine expression are under tight control of microRNAs (miRNAs). To extend previous findings we aimed at characterizing inflammation-regulating miRNA profiles in patients with peripheral neuropathies. In an unselected patient cohort with polyneuropathies of different etiologies seen at our neuromuscular center between 2014 and 2015, we determined the systemic and local relative expression of miR-21, miR-146a, and miR-155. In white blood cells we found higher miR-21 ($p<0.001$) and miR-146a ($p<0.001$) expression and lower miR-155 ($p<0.001$) expression when compared to healthy controls. In sural nerve, miR-21 ($p<0.001$) and miR-155 ($p<0.05$) expression were increased in painful compared to painless neuropathies. In painful neuropathies, skin biopsies from the lower leg had reduced miR-146a ($p<0.01$) and miR-155 ($p<0.01$) expression compared to the upper thigh. Thus, peripheral neuropathies are associated with aberrant miRNA expression in white blood cells, sural nerve, and skin. These miRNA patterns may help to identify factors that determine the painfulness of peripheral neuropathies and lead to druggable targets.

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Aberrant microRNA expression in patients with painful peripheral neuropathies

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Abstract

Changes in the neuro-immune balance play a major role in the induction and maintenance of neuropathic pain. We recently reported pathophysiologically relevant alterations in skin and sural nerve cytokine expression in peripheral neuropathies of different etiologies. Immune processes and cytokine expression are under tight control of microRNAs (miRNAs). To identify potential master switches in the neuro-immune balance, we aimed at characterizing inflammation-regulating miRNA profiles in patients with peripheral neuropathies. In an unselected patient cohort with polyneuropathies of different etiologies seen at our neuromuscular center between 2014 and 2015, we determined the systemic and local relative expression of miR-21, miR-146a, and miR-155. In white blood cells we found higher miR-21 ($p<0.001$) and miR-146a ($p<0.001$) expression and lower miR-155 ($p<0.001$) expression when compared to healthy controls. In sural nerve, miR-21 ($p<0.001$) and miR-155 ($p<0.05$) expression were increased in painful compared to painless neuropathies. In painful neuropathies, skin biopsies from the lower leg had reduced miR-146a ($p<0.01$) and miR-155 ($p<0.01$) expression compared to the thigh. Thus, peripheral neuropathies are associated with aberrant miRNA expression in white blood cells, sural nerve, and skin. These miRNA patterns may help to identify factors that determine the painfulness of peripheral neuropathies and lead to druggable targets.

Keywords

miRNA; miR-21; miR-146a; miR-155; pain; peripheral neuropathy; sural nerve biopsy; skin biopsy.

Introduction

Chronic pain accompanying peripheral neuropathies is a major health problem. It is still unknown why peripheral neuropathies with apparently similar etiology and pathology can be either painful or painless. Prominent Aδ- and C-fiber involvement, such as in amyloid neuropathies, is commonly regarded as a hallmark of painful neuropathies, however, neuropathies with non-selective fiber loss and those with apparent large fiber loss can be equally painful (von Hehn et al., 2012). Known factors involved in the pathophysiology of pain are cytokine-mediated neuro-immune interactions (Calvo et al., 2012). In particular, an immune dysbalance with increased expression of pro-inflammatory cytokines or a reduction of anti-inflammatory cytokines in blood, cerebrospinal fluid, and/or nerve tissue may promote pain and hyperalgesia (Backonja et al., 2008; Üçeyler et al., 2015; Üçeyler et al., 2007). However, due to their pleiotropic and redundant activity, interfering with cytokine function may not result in the desired analgesia. Therefore upstream “master switches” have been implied as potential targets of more efficient analgesics.

microRNAs (miRNA) are small non-coding RNA molecules that possess regulatory functions in multiple cellular systems (Mattick, 2004), enabling cross-

communication between cellular processes (Soreq and Wolf, 2011). Recent studies in preclinical neuropathic pain models indicate unique miRNA expression signatures that are characteristic for nerve-injury induced pain behavior (Aldrich et al., 2009; Bai et al., 2007; Imai et al., 2011; Kusuda et al., 2011; Leinders et al., 2016; von Schack et al., 2011). In addition, first reports hint towards aberrant miRNA expression in blood of patients with distinct chronic pain conditions (Andersen et al., 2016; Beyer et al., 2015; Ciccacci et al., 2014; Douglas et al., 2015; Leinders et al., 2016; Orlova et al., 2011). In our study we characterized systemic and local expression profiles of miR-21, miR-146a, and miR-155 in an unselected cohort of patients with polyneuropathies of different etiologies seen at our neuromuscular center within one year. These miRNAs are known to regulate multiple pathways in inflammation and pain (Faraoni et al., 2009; Li et al., 2010; Sheedy, 2015) and might thus be involved in the pathophysiology of painful conditions, as reviewed before (Rao et al., 2013; Sakai and Suzuki, 2015). miR-21 is associated with pain in experimental nerve injury models (Sakai and Suzuki, 2015). It was shown to promote neurite outgrowth (Strickland et al., 2011) and low expression was associated with decreased demyelination in autoimmune diseases (Murugaiyan et al., 2015). miR-146a, a regulator of the cell death inducers caspase-3 and Fas, was decreased after spinal cord injury (Yunta et al., 2012) and associated with painful conditions in mice (Andersen et al., 2014). In addition, miR-146 was associated with inflammatory degeneration (Enciu et al., 2012), nerve regeneration after axonal injury (Phay et al., 2015), and distal axonal outgrowth (Jia et al., 2016). miR-155 is ubiquitously expressed

in many cell types and tissues including the central nervous system (CNS) (Landgraf et al., 2007). It is critically involved in the regulation of inflammation-associated diseases (Vigorito et al., 2013), and was shown to be increased in synovial fibroblasts in patients with rheumatoid arthritis (Stanczyk et al., 2008) and the prefrontal cortex of mice with inflammatory pain (Poh et al., 2011). Furthermore, miR-155 was found to be up-regulated in active white matter lesions of patients with multiple sclerosis (Junker et al., 2009), whereas the absence of miR-155 induced resistance to experimental autoimmune encephalomyelitis in mice (Mycko et al., 2015). We thus characterized the expression of these miRNA candidates in white blood cells, sural nerve, and skin punch biopsy specimens in biomaterial of our patient cohort as available. We aimed at identifying the profiles of these miRNAs to better understand potential factors that differentiate painful from painless neuropathies and to potentially pave the way for new druggable targets.

Materials and Methods

Patient assessment and diagnostic classification

We included all available data and biomaterial collected within one year (2014-2015) from patients with neuropathies of different etiologies that were seen at the Department of Neurology, University of Würzburg for diagnostic work-up. This included blood withdrawal, sural nerve, and skin biopsy as needed. The study was approved by the Würzburg Medical Faculty Ethics Committee and written informed consent was obtained. The diagnosis of neuropathy was based on characteristic symptoms and signs in the neurological examination and typical

findings in the neurophysiological studies. For differential diagnosis detailed laboratory screening was performed in all patients as described before (Üçeyler et al., 2015). Moreover, all patients underwent complete electrophysiological assessment with standard nerve conduction studies in motor and sensory nerves of the upper and lower limbs, as part of the routine work-up as needed (Kimura, 2001). Table 1 summarizes the diagnostic criteria applied. Neuropathies were classified as painful if the patients reported neuropathic pain with an intensity of 3 or more on a numeric rating scale (NRS) ranging from 0 to 10 (0 meaning “no pain” and 10 “worst pain imaginable”). For standardized pain assessment the Graded Chronic Pain Scale (GCPS) (Von Korff et al., 1992) with 4 weeks recall and the Neuropathic Pain Symptom Inventory (NPSI) (Bouhassira et al., 2004; Sommer et al., 2011) with 24 hours recall were used.

Blood withdrawal

Venous blood withdrawn in EDTA-containing tubes between 8:00 - 9:00 AM after over-night fasting was used for the extraction of the total white blood cell fraction (WBC). WBCs were re-suspended in RNA-cell protective reagent (QIAGEN, Hilden, Germany) and stored at -80°C until further processing. Additionally, we collected blood samples from healthy, age- and gender-matched volunteers (see below) without neurologic disorders, infectious diseases or pain at study inclusion.

Sural nerve biopsy

Diagnostic sural nerve biopsy was performed if the etiology of the neuropathy remained unclear after thorough clinical, laboratory, and electrophysiological assessment following a standard procedure at the Department of Neurosurgery, University of Würzburg (Dyck and Thomas, 2005). For study purposes, 4 mm of the biopsy specimen was separated and stored in RNA-later (Qiagen, Hilden, Germany) over night at +4°C; on the following day RNA-later was removed and the specimen was frozen at -80°C before further processing.

Skin punch biopsy

Two 5-mm skin punch biopsies (lateral lower leg and upper thigh) were obtained from patients under local anesthesia. One third of the skin specimen that remained from routine work-up was used for qRT-PCR and was stored in RNA-later over night at +4°C; on the following day RNA-later was removed and the specimen was frozen at -80°C before further processing.

MiRNA expression analysis

For the generation of miRNA-specific first strand cDNA 5 ng of total RNA extracted from WBC, nerve, and skin was reverse transcribed using the Universal cDNA Synthesis kit II (Exiqon, Vedbaek, Denmark) following the manufacturer's recommendations. For each reaction, 4 µl of diluted (1:80) cDNA was PCR amplified applying the corresponding miRNA and reference primer sets, using the miCURY LNA™ Universal microRNA PCR (Exiqon, Vedbaek,

Denmark). To determine expression levels of the following miRNAs, specific miCURY LNA™ assays with the respective Assay-IDs were used: hsa-miR-21 (5'-3' UAGCUUAUCAGACUGAUGUUGA, MIMAT0000076), hsa-miR-146a (5'-3' UGAGAACUGAAUUCCAUGGGUU), and hsa-miR-155 (5'-3' UUAAUGCUAAUCGUGAUAGGGGU, ID: MIMAT0000646). Data were normalized to the expression of endogenous 5sRNA (5sRNA, V00589).

Evaluation of qRT-PCR results

For WBC, patients` data were compared with healthy controls using the delta-delta-Ct method. To evaluate fold changes in miRNA expression in nerve and skin samples we used the comparative deltaCt method among groups, i.e. relating each individual miRNA expression to the respective 5sRNA expression. For nerve and skin samples we illustrated the results as 1/deltaCt, since this will result in higher values for higher gene expression. In the case of nerve samples miRNA expression we further compared subgroups of neuropathies; in case of skin miRNA expression we compared skin from the lower leg (affected skin) to the thigh (unaffected skin) the of patients. To guarantee inter-plate comparability we chose one standard sample that was analyzed on each PCR plate. All miRNAs were measured as triplicates; 5sRNA values were tested for stability throughout the samples and measured as duplicates subsequently.

Statistical analysis

For statistical analysis SPSS Version 23 (IBM, Ehningen, Germany) was used. For graphical presentation of data we used GraphPad Prism 6.0 (GPP 6.0 GraphPad Software, Inc., San Diego, CA). Data distribution was tested with the Kolmogorov-Smirnov-test. The Mann-Whitney U-test was used for not normally distributed data. The Spearman correlation was calculated for miRNA expression of WBC, nerve, and skin for the individual subgroups. Statistical significance was accepted at $p<0.05$.

Results

Basic description of the patient cohort

We included 76 patients with peripheral neuropathies of different etiologies. Table 2 summarizes the clinical characteristics and diagnostic subgroups of the study cohort. The patients had a median age of 65 years (range 33-84) and consisted of 51 men (median age: 64 years, range 33-84) and 25 women (median age: 67 years, range 47-83). To dissect potential influences of inflammation from those of pain, we subdivided the patients into those with inflammatory and non-inflammatory neuropathies. 24/76 (32%) patients had an inflammatory neuropathy (chronic inflammatory demyelinating neuropathy [CIDP], vasculitic neuropathy, progressive idiopathic axonal polyneuropathy [PIAN], paraproteinemic neuropathy), 31/76 (40%) patients had a neuropathy of non-inflammatory origin (hereditary, chronic idiopathic axonal polyneuropathy [CIAP]), and in 21/76 cases (28%) the etiology of the neuropathy remained

unclear. 39/76 (51%) patients had a painful neuropathy (i.e. current pain intensity ≥3 NRS), compared to 37/76 (48%) patients with neuropathy without pain. We measured miRNA expression in the samples of those patients, where biomaterial had been obtained during routine diagnostics. WBC were available from 27/76 (36%) patients, sural nerve biopsy specimens from 63/76 (83%) patients, distal skin from 73/76 (96%), and proximal skin from 49/76 (65%) patients. Our control group for WBC experiments consisted of 26 men (median age: 60 years, range 38-69) and four women (median age range: 49, range: 41-53). Thus, the healthy controls were slightly younger than the patient group ($p < 0.05$). None of the controls reported any ongoing infectious disease, pain or neuropathy at the time of blood withdrawal.

miR-21, miR-146, and miR-155 are altered in WBC of patients with neuropathies

Sufficient material for miRNA analysis was available from 27 patients, of whom miR-21 was measurable in 25, miR-146 in 27, and miR-155 in 17 patients. We found that patients with polyneuropathies had higher miR-21 (2.2 fold, Figure 1A, $p < 0.001$) and miR-146a (10 fold, Figure 1B, $p < 0.001$) expression, and lower miR-155 expression (0.2 fold, Figure 1C, $p < 0.001$) when compared to 30 healthy controls. Furthermore, miR-21 correlated negatively with the NPSI sum score (corr. coeff.: -0.33, $p < 0.01$), and miR-146a correlated with the ADS sum score (corr. coeff.: 0.48, $p < 0.001$). There was no difference in WBC miRNA expression between patients with painful and painless neuropathies or between the subgroups of inflammatory and non-inflammatory polyneuropathies. Also,

disease duration (<3 years as group median and ≥3 years) did not influence miRNA expression.

miR-21 and miR-155 are increased in sural nerves of painful neuropathies

Sufficient material for miRNA analysis was available from 63 patients, of whom miR-21 was measurable in 15, miR-146 in 46, and miR-155 in 43 patients. We found that painful neuropathies were associated with higher miR-21 expression in the sural nerve when compared to painless neuropathies (1.4 fold, $p < 0.001$, Figure 2A). This was restricted to painful neuropathies of non-inflammatory origin (1.4 fold, $p < 0.01$, Figure 2B). Interestingly, we found that miR-21 correlated with the patients' current pain intensity (corr. coeff.: 0.72, $p < 0.0001$), the ADS sum score (corr. coeff.: 0.53, $p < 0.001$), several items of the NPSI (e.g. NPSI sum score, corr. coeff.: 0.69, $p < 0.0001$) and several GCPS items (e.g. mean pain intensity, corr. coeff.: 0.4, $p < 0.01$). Also, miR-155 was higher in painful neuropathies ($p < 0.05$, Figure 2C), without difference between those of inflammatory and non-inflammatory etiology. miR-146a expression did not differ for any of the analyzed subgroups. Also, disease duration had no influence on any of the investigated markers in the sural nerve (cases with <3 versus ≥3 years).

miR-146a and miR-155 expression are reduced in lower leg skin in painful neuropathies

miR-21 was measurable in 55 distal and in 34 proximal skin biopsies. miR-146a was detectable in 52 distal and in 36 proximal biopsies, and miR-155 in 50 distal and in 39 proximal biopsies. We compared distal (affected) to proximal (non affected) skin. miR-21 expression did not differ between distal and proximal skin. The major finding was a lower miR-146a (0.90 fold, $p < 0.001$, Figure 3A) and miR-155 (0.93 fold, $p < 0.001$, Figure 3B) expression in distal skin of patients with neuropathies compared to proximal skin, which was restricted to the subgroup of patients with painful neuropathies (miR-146a: 0.83 fold, $p < 0.001$ Figure 3C; miR-155: 0.93 fold, $p < 0.01$; Figure 3D). No such difference was found in patients with painless neuropathies ($p > 0.05$; data not shown). Of note, miR-146a expression was lower in distal skin of patients with painful inflammatory (0.9 fold, $p < 0.01$, data not shown) and painful non-inflammatory neuropathies (0.87 fold, $p < 0.001$, data not shown), compared to proximal skin. Also, miR-155 was lower in distal skin of patients with painful inflammatory neuropathies (0.89 fold, $p < 0.01$, data not shown). We found that proximal skin expression of miR-146 weakly correlated with the GCPS current pain intensity (corr. coeff.: 0.3, $p < 0.01$). No correlation was found for any other of the investigated markers and biopsy sites. Also, no influence of disease duration was observed.

Discussion

We investigated the expression of miR-21, miR-146a, and miR-155 in patients with neuropathies of different etiologies, including WBC, sural nerve and skin biopsy specimens. Table 3 gives an overview of the results based on the analyzed diagnostic subgroups. We show that peripheral neuropathies are associated with increased WBC miR-21 and miR-146a, but lower miR-155, expression. Comparing painful to painless neuropathies, miR-21 and miR-155 were increased in the biopsied sural nerve. In addition, miR-146a and miR-155 expression was reduced in distal versus proximal skin of painful neuropathies only. None of these miRNAs have been directly studied in patients with neuropathies to date; however, there is emerging evidence for their involvement in the development and homeostasis of the nervous system (Bhalala et al., 2013; Follert et al., 2014), including chronic pain (Bali and Kuner, 2014).

MiR-21 has a wide range of physiological and pathophysiological functions, and was denoted as key regulator of immunological processes (Sheedy, 2015). MiR-21 was reportedly upregulated after various nerve injuries (Sakai and Suzuki, 2013; Wu et al., 2011; Yu et al., 2011), and continued up-regulation was observed in entrapment injury, even at 6 months after the lesion (Rau et al., 2010). Thus our findings in WBC and sural nerve are along those lines. Painful neuropathies are associated with increased systemic (Backonja et al., 2008; Üçeyler et al., 2007) and local (sural nerve- and skin biopsy) (Empl et al., 2001; Lindenlaub and Sommer, 2003; Üçeyler et al., 2015) expression levels of pro-inflammatory mediators, suggesting a reciprocal relationship with miRNAs.

We have shown before that gene expression levels of IL-10 were increased in sural nerve specimens of patients with painful neuropathies (Üçeyler et al., 2015), which might be under influence of miR-21, a known inducer of IL-10 expression (Roy and Sen, 2011). Of note, we also reported increased IL-6 levels, which in turn is a potent inducer of miR-21 in immune cells (Loffler et al., 2007) and DRG neurons (Zhou et al., 2015) further underpinning the reciprocal relationship of inflammatory mediators and miRNAs.

miR-146a is one of the most intensively studied miRNAs known to negatively regulate the acute and innate immune response (Li et al., 2010; Taganov et al., 2006; Williams et al., 2008). Increased miR-146a expression was observed in various immune cell types in patients with rheumatoid and osteoarthritis (Ceribelli et al., 2011; Li et al., 2011; Pauley et al., 2008). Aberrant expression profiles have also been reported in systemic lupus erythematosus (Shen et al., 2012), and conditions such as visceral pain (Zhang and Banerjee, 2015), irritable bowel syndrome (Fourie et al., 2014), and migraine (Andersen et al., 2016). Also in pre-clinical pain models (Pauley et al., 2008; Strickland et al., 2014a; Strickland et al., 2014b; Yunta et al., 2012) miR-146a was shown to be involved. Interestingly, polymorphisms in the miR-146a gene have recently been shown to contribute to neuropathy in type II diabetes, indicating that also human neuropathies are associated with aberrant miRNA expression levels (Moura et al., 2014). Despite the frequently reported upregulation in white blood cells, as described above, Li and colleagues (Li et al., 2011) reported a marked decrease in combination with lower inflammatory transcripts in neuronal cells, highlighting

the ambiguous effect of miR-146. In skin, we have previously shown that cytokine levels are increased in painful distal skin of length dependent small fiber neuropathies. Concomitantly, we found reductions in miR146a (and miR155), indicating the involvement in inflammatory skin reactions. Intriguingly, it was previously shown, that in inflamed skin of patients with atopic dermatitis miR-146a was increased, inhibiting the expression of numerous pro-inflammatory factors (Rebane et al., 2014). The same group furthermore showed that knocking out miR-146a resulted in a stronger dermal inflammatory reaction and increased accumulation of infiltrating immune cells (Rebane et al., 2014). Our results are also indicative of a different peripheral (WBC) versus local effect (distal, affected skin) of miR-146. However, given that miR-146a inhibits various components of the toll-like receptor (TLR) and NF-κB pathway in immune cells and thus decreases the release of inflammatory mediators (Lindsay, 2008), one could speculate that locally down-regulated miR-146 might result in elevated pro-inflammatory cascades, thus ultimately promoting pain. In fact, we show that only painful neuropathies have lower miR-146a expression in the affected skin of the distal leg.

miR-155 represents a typical multi-functional miRNA that regulates multiple biological pathways simultaneously and exerts pro-inflammatory actions (Faraoni et al., 2009; O'Connell et al., 2012). In the rodent CNS, miR-155 was shown to be increased during carrageenan-induced inflammation (Poh et al., 2011), following chronic constriction injury (CCI), and in an osteoarthritis model (Li et al., 2013; Tan et al., 2015). Also, in patients with rheumatoid arthritis it was

increased (Stanczyk et al., 2008), and in a subset of CRPS patients it correlated with expression of inflammatory and immune-related markers (Orlova et al., 2011). Interestingly, we found an increased miR-155 expression in sural nerve biopsies of patients with painful neuropathies, which is in agreement with the literature. However, we also found a concomitant decrease in WBC miR-155 expression and in distal skin specimens, especially in patients with painful inflammatory neuropathies. This might seem unexpected at first and contradict the general notion that miR-155 is pro-inflammatory. However, the skin is composed of various different cell types and miR-155 might have cell-specific functions. Furthermore, peripheral neuropathies usually do not present themselves with overt inflammatory skin reactions, but rather neuropathic small nerve fiber related symptoms and signs in which miR-155 might play a different role. The decrease is thus as yet unexplained and may indicate a physiologic response towards resolution of inflammation. Of note, miR-155 was shown to be involved in T-cell differentiation and production of interleukin-4 (IL-4) and IL-10 via c-Maf (musculoaponeurotic fibrosarcoma) (Rodriguez et al., 2007) – two major anti-inflammatory and analgesic cytokines shown to be upregulated in painless peripheral neuropathies before (Üçeyler et al., 2007). This might be related to our findings, as reduced miR-155 expression is associated with T-cell differentiation towards IL-4 producing Th2-cells (Thai et al., 2007). However, opposing effects of miR-146 and miR-155, counterbalancing their regulation are more likely, as previously described (So et al., 2013), which might thus account for the present findings.

The major limitation of our study is the absence of skin biopsies from a healthy control group for the comparison of cutaneous miRNA expression. Also, the healthy controls do not exactly match with the patient group with regard to age and gender distribution. Another limitation is that we only assessed miRNA expression, but did not analyze downstream-target or protein expression nor did we characterize the distribution of immune cells in our WBC samples. This was mainly due to the limited amount of biosamples that did not allow quantitative measurements and furthermore resulting in low number of investigated markers in individual specimens. Our study can thus not answer if aberrant miRNA expression is the cause or consequence of peripheral neuropathies as profiles are highly distinctive depending on the cause. It does however address the fact that neuropathies are not solely restricted to peripheral nerves but involve skin and systemic expression, presumably via the interaction of the latter. We thus postulate that miRNA expression patterns derived from simple blood and/or skin punch biopsies may in the future be used to better understand the pathophysiology of painful peripheral neuropathies and lead the way to targeted therapy.

Conclusion

Taken together, there is evidence for aberrant miRNA expression in peripheral neuropathies of different etiologies, suggesting that neuropathies not only affect peripheral nerves, but also skin and skin homeostasis. We speculate that deregulated skin miRNA expression is under reciprocal control of pro-inflammatory mediators. Furthermore, the correlation of increased sural miRNA

expression and pain in peripheral neuropathies is striking, and these findings need to be studied in more detail in the future and might ultimately lead to improved diagnostics and characterization of painful neuropathies.

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Conflicts of interest

The authors declare no conflicts of interest.

Abbreviations

ADS, Allgemeine Depressionsskala; c-Maf, musculoaponeurotic fibrosarcoma; CCI, chronic constriction injury; CIAP, chronic idiopathic axonal polyneuropathy; CIDP, chronic inflammatory demyelinating neuropathy; CNS, central nervous system; corr. coeff., correlation coefficient; GCPS, graded chronic pain scale, miRNA, microRNA; IL-, Interleukin; NPSI, neuropathic pain symptom inventory; numeric rating scale, NRS; PIAN, progressive idiopathic axonal neuropathy; TLR-, toll-like receptor; WBC, white blood cells.

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Figure Legends

Figure 1: Scatter plots showing expression of miR-21, miR-146a, and miR-155 in white blood cells of patients with peripheral neuropathies. A) miR-21 expression was higher in patients with neuropathy compared to controls; B) miR-146a expression was higher and C) miR-155 expression was lower in patients with polyneuropathy. Data represent relative miRNA expression normalized to endogenous reference control 5Srna. Mann Whitney *U*-comparisons; ***P < 0.001. N of investigated WBC miRNA expression: miR-21: painful neuropathy: 11, painless neuropathy: 12, inflammatory painful: 5, inflammatory painless: 3, non-inflammatory painful: 6, non-inflammatory painless: 7. For miR-146a: painful neuropathy: 14, painless neuropathy: 12, inflammatory painful: 7, inflammatory painless: 3, non-inflammatory painful: 6, non-inflammatory painless: 7. For miR-155: painful neuropathy: 14, painless neuropathy: 19, inflammatory painful: 8, inflammatory painless: 2, non-inflammatory painful: 5, non-inflammatory painless: 6.

Figure 2: Altered expression of miR-21 in sural nerve biopsies. Scatterplots indicate 1/deltaCt values, i.e. reciprocal relation of the Ct value of miR-21 normalized to the reference control 5sRNA (A and B; ***p < 0.001, **p < 0.01). miR-21 showed higher sural nerve expression levels in painful neuropathies compared to painless neuropathies of different etiologies. miR-21 expression was higher in painful compared to painless neuropathies without the diagnosis of inflammation. Data are expressed as mean ± S.E.M; Mann Whitney *U*-comparisons; *N* of investigated sural nerve biopsies: painful neuropathy: 8, painless neuropathy: 7, inflammatory painful: 3, inflammatory painless: 2, non-inflammatory painful: 5, non-inflammatory painless: 5.

Figure 3: Altered expression of miRNAs in lower leg and thigh biopsies. Boxplots show 1/deltaCt values, i.e. reciprocal relation of the Ct value of the respective miRNA normalized to the reference control 5sRNA A) Distal skin miR-146a, and B) distal miR-155 expression was lower than in proximal biopsies when all neuropathies were compared (***$p < 0.001$). C) When distal skin of painful neuropathies was compared to proximal skin, miR-146a expression was lower in distal skin. (***$p < 0.001$). D) Likewise, distal miR-155 skin expression was lower than proximal in painful neuropathies (**$p < 0.01$). Data are expressed as mean ± S.E.M; Mann Whitney *U*-comparisons; *N* of investigated distal skin biopsies: painful neuropathy: 26, painless neuropathy: 26, inflammatory painful: 11, inflammatory painless: 9, non-inflammatory painful: 9, non-inflammatory painless: 14. *N* of investigated proximal skin biopsies: painful neuropathy: 21, painless neuropathy: 18, inflammatory painful: 6, inflammatory painless: 6, non-inflammatory painful: 10, non-inflammatory painless: 10.

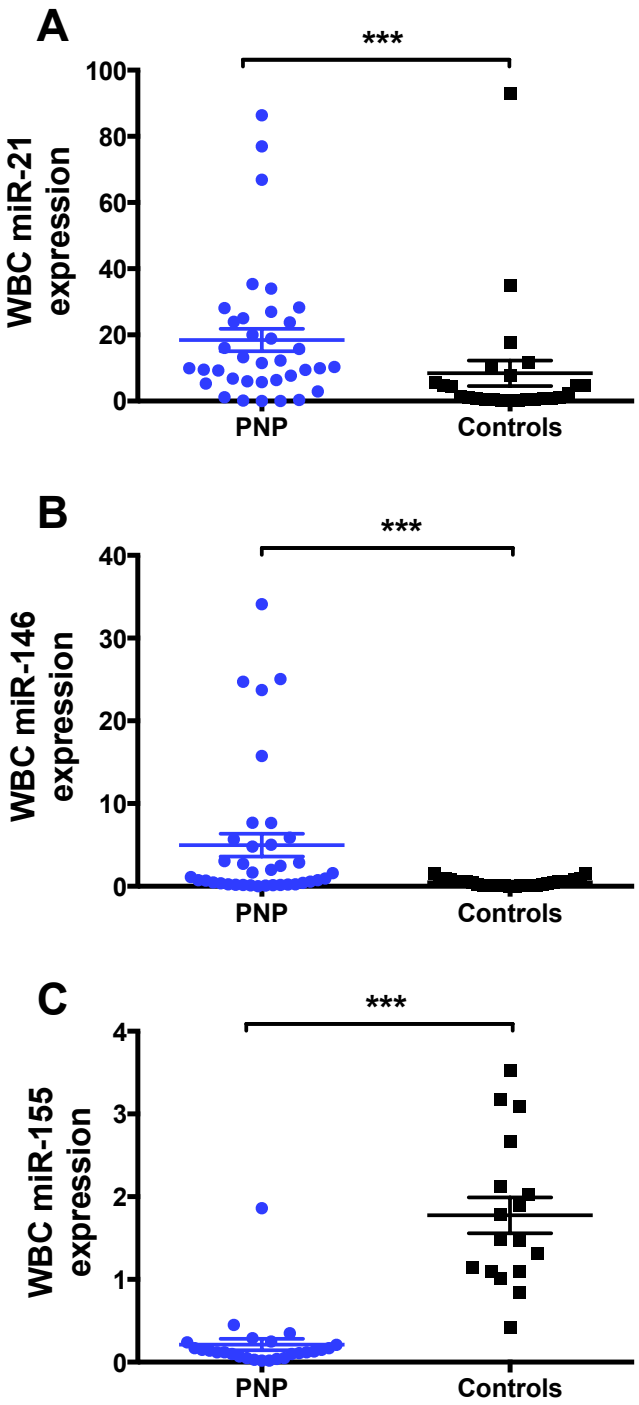

Figure 1: Scatter plots showing expression of miR-21, miR-146a, and miR-155 in white blood cells of patients with peripheral neuropathies

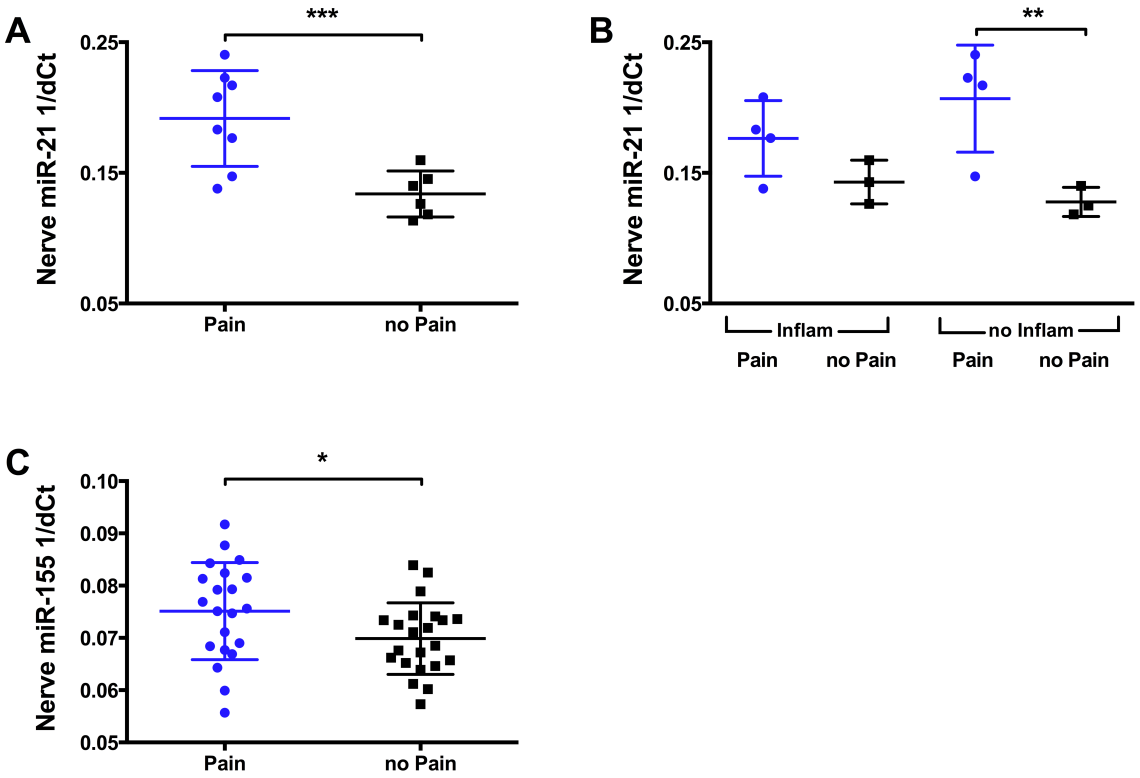

Figure 2: Altered expression of miR-21 in sural nerve biopsies.

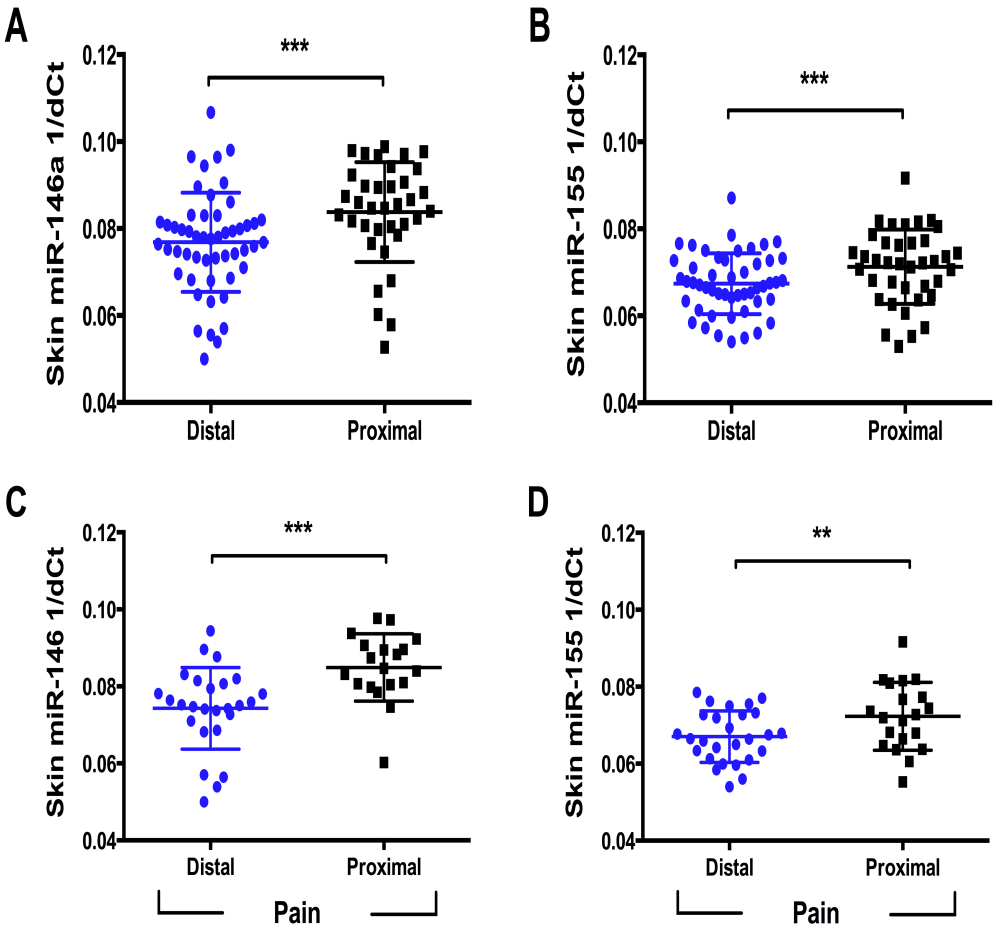

Figure 3: Altered expression of miRNAs in lower leg and thigh biopsies.

Table 1: Clinical characteristics and diagnostic subgroups of the entire study cohort

Item	Number (% of entire group)
M, F, (N)	51, 25
Median age (range)	65 years (33 - 84)
Median disease duration (range in years)	4 years (0.1 – 27)
Diagnostic subgroups (N and % of entire group):	
Unknown etiology: **i)** electrophysiologically axonal **ii)** electrophysiologically demyelinating **iii)** electrophysiologically mixed	21 (28%) 10 (14%) 3 (4%) 8 (10%)
CIAP	12 (16%)
PIAN	9 (12%)
Hereditary neuropathy	8 (10%)
NSVN	7 (9%)
CIDP	6 (8%)
Other non-inflammatory neuropathy	6 (8%)
Paraproteinemic neuropathy (IgM) MADSAM AMSAN SFN	2 (3%) 2 (3%) 1 (1%) 2 (3%)
Painful, painless (N)	39, 37

AMSAN acute motor and sensory axonal neuropathy, CIAP chronic idiopathic axonal polyneuropathy, CIDP chronic inflammatory demyelinating polyneuropathy, F female, INCAT Inflammatory Neuropathy Cause and Treatment Group, M male, MADSAM multifocal acquired demyelinating sensory and motor neuropathy, MMN multifocal motor neuropathy, N number, NSVN non-systemic vasculitic neuropathy, PIAN progressive idiopathic axonal neuropathy, SFN small fiber neuropathy.

Table 2: Overview of the miRNA expression changes based on tissue and diagnostic subgroups.

Tissue / Diagnostic subgroups	Blood	Nerve		Skin (distal vs. proximal)				
	NP vs. HC	Pain (+) NP	Non-inflam. NP	Pain (+) NP	Inflam. NP	Non-inflam. NP	Pain (+) Inflam. NP	Pain (+) Non-inflam. NP
miR-21	↑	↑	↑	-	-	-	-	-
miR-146a	↑	-	-	↓	↓	↓	↓	↓
miR-155	↓	↑	-	↓	↓	-	↓	-

Abbreviations: ↑ upregulation; ↓ downregulation; HC: healthy controls; inflam: inflammatory; miR: miRNA; Non-inflam: non-inflammatory NP: neuropathy; Pain (+): painful.

Supplementary Table 1: Overview of the diagnostic criteria

Diagnosis	Additional information	Reference
Chronic inflammatory demyelinating neuropathy (CIDP)	Based on the INCAT criteria (inflammatory neuropathy cause and treatment)	(Hughes et al., 2001)
Chronic idiopathic axonal polyneuropathy (CIAP)	Sensory-motor, neurophysiologically axonal neuropathy, slow onset and progress; axonal histology without inflammation; normal CSF, no effect of steroid treatment	(Vrancken et al., 2004)
Progressive idiopathic axonal neuropathy (PIAN)	Acute or subacute presentation with slow progression of sensory-motor symptoms; axonal histology with inflammation and neurophysiology; increased CSF and positive response to steroid treatment	(Vrancken et al., 2004)
Hereditary neuropathy	According to a combination of positive family history, genetic and neurophysiological data	
Neuropathy in systemic vasculitic and non-systemic vasculitis (NSVN)		(Collins et al., 2000)
Other origin	Cases of definite other etiology, i.e. neuropathy due to amyloidosis, paraneoplastic neuropathy or vitamin deficiency	
Unknown etiology	Cases in whom a definitive diagnosis as detailed as above was not possible at the time point of examination	
Idiopathic small fiber neuropathy (SFN)		(Lacomis, 2002)
Multifocal acquired demyelinating sensory and motor neuropathy (MADSAM)		(Lewis et al., 1982)

Abbreviations: *CIAP* chronic idiopathic axonal polyneuropathy, *CIDP* chronic inflammatory demyelinating polyneuropathy, *CSF* cerebrospinal fluid, INCAT Inflammatory Neuropathy Cause and Treatment Group, *MADSAM* multifocal acquired demyelinating sensory and motor neuropathy, *NSVN* non- systemic vasculitic neuropathy, *PIAN* progressive idiopathic axonal neuropathy, SFN small fiber neuropathy.

Chapter 3

Increased miR-132-3p expression is associated with chronic neuropathic pain

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Experimental Neurology (2016)

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Abstract

Alterations in the neuro-immune balance play a major role in the pathophysiology of chronic neuropathic pain. MicroRNAs (miRNA) can regulate both immune and neuronal processes and may function as master switches in chronic pain development and maintenance. We set out to analyze the role of miR-132-3p, first in patients with peripheral neuropathies and second in an animal model of neuropathic pain. We initially determined miR-132- 3p expression by measuring its levels in white blood cells (WBC) of 30 patients and 30 healthy controls and next in sural nerve biopsies of 81 patients with painful or painless inflammatory or non-inflammatory neuropathies based on clinical diagnosis. We found a 2.6 fold increase in miR-132-3p expression in WBC of neuropathy patients compared to healthy controls ($p < 0.001$). MiR-132-3p expression was also slightly up-regulated in sural nerve biopsies from neuropathy patients suffering from neuropathic pain compared to those without pain (1.2 fold; $p < 0.001$).

These promising findings were investigated further in an animal model of neuropathic pain, the spared nerve injury model (SNI). For this purpose miR-132-3p expression levels were measured in dorsal root ganglia and spinal cord of rats. Subsequently, miR-132-3p expression was pharmacologically modulated with miRNA antagonists or mimetics, and evoked pain and pain aversion were assessed.

Spinal miR-132-3p levels were highest 10 days after SNI, a time when persistent allodynia was established ($p < 0.05$). Spinal administration of miR-132-3p antagonists via intrathecal (i.t.) catheters dose dependently reversed mechanical allodyina ($p < 0.001$) and eliminated pain behavior in the place escape avoidance paradigm ($p < 0.001$). Intrathecal administration of miR-132-3p mimetic dose-dependently induced pain behavior in naïve rats ($p < 0.001$). Taken together these results indicate a pro-nociceptive effect of miR-132-3p in chronic neuropathic pain.

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Increased miR-132-3p expression is associated with chronic neuropathic pain

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ABSTRACT

Alterations in the neuro-immune balance play a major role in the pathophysiology of chronic neuropathic pain. MicroRNAs (miRNA) can regulate both immune and neuronal processes and may function as master switches in chronic pain development and maintenance. We set out to analyze the role of miR-132-3p, first in patients with peripheral neuropathies and second in an animal model of neuropathic pain. We initially determined miR-132-3p expression by measuring its levels in white blood cells (WBC) of 30 patients and 30 healthy controls and next in sural nerve biopsies of 81 patients with painful or painless inflammatory or non-inflammatory neuropathies based on clinical diagnosis. We found a 2.6 fold increase in miR-132-3p expression in WBC of neuropathy patients compared to healthy controls ($p < 0.001$). MiR-132-3p expression was also slightly up-regulated in sural nerve biopsies from neuropathy patients suffering from neuropathic pain compared to those without pain (1.2 fold; $p < 0.001$).

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1. Introduction

Neuropathic pain is characteristically severe and persistent and may greatly impair health related quality of life by additionally inducing anxiety, depression, and cognitive impairment (Breivik et al., 2006). There is ample evidence for a potential role of the immune system and particularly of pro- and anti-inflammatory mediators in the pathophysiology of neuropathic pain (Kuner, 2010; McMahon and Malcangio, 2009). Peripheral neuropathies of the same etiology can either be painful or painless (Üçeyler et al., 2007). The mechanism for this discrepancy is unknown.

In recent years, non-coding RNAs have been studied in normal cellular functioning as well as in pathological processes (Huttenhofer and Schattner, 2006; Mattick, 2004). Micro-RNAs (miRNAs) are a family of non-coding RNAs that post-transcriptionally regulate gene expression by inhibiting mRNA translation or inhibiting mRNA and protein degradation (Mattick, 2004). Various diseases, including neuropathic pain

Abbreviation: AMPAR, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor; ANCA, *anti*-neutrophil cytoplasmic antibody; BDNF, brain-derived neurotrophic factor; CIAP, chronic idiopathic axonal polyneuropathy; CIDP, chronic inflammatory demyelinating neuropathy; CCI, chronic constriction injury; CREB, cAMP response element-binding protein; DRG, dorsal root ganglia; EDTA, ethylene-diamine-tetraacetic-acid; ENA, *anti*-nuclear antigen; GCPS, graded chronic pain scale; GFAP, glial fibrillary acidic protein; GLT, 1 glutamate transporter; GluA, AMPA receptor subunit; H&E, Hematoxylin and eosin stain; HPLC, high performance liquid chromatography; Iba1, ionized calcium-binding adapter molecule 1; i.t., intrathecal; LNA, locked nucleic acid; LTP, long-term potentiation; miRNA, microRNA; MOPS, 3-(*N*-morpholino)propanesulfonic acid; NCV, motor nerve conduction velocity; NRS, numeric rating scale; NPSI, neuropathic pain symptom inventory; PEAP, place escape avoidance paradigm; PIAN, progressive idiopathic axonal neuropathy; PNP, polyneuropathy; RNA, ribonucleic acid; Scr, scrambled oligonucleotide; SDS, sodium dodecyl sulfate; SNI, spared nerve injury; WBC, white blood cells.

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disorders, appear to possess unique miRNA expression signatures. Recent reports on modulation of miRNA function in both neuronal and immune processes predict the therapeutic potential of manipulating miRNAs in diseases affecting the immune system and the brain (O'Connor et al., 2012; Soreq and Wolf, 2011). miRNAs that communicate between the nervous and immune system have been termed "neurimmiRs" and primarily target transcription factors or other regulatory genes, which enable simultaneous cross-communication between neural and immune compartments (Soreq and Wolf, 2011). Thus, miRNAs possibly control cellular pathways in multiple systems and act as "master-switches" (Soreq and Wolf, 2011).

Aberrant expression of several miRNAs has been reported throughout many peripheral and central nervous system loci associated with pain perception (Aldrich et al., 2009; Bai et al., 2007; Imai et al., 2011; Kusuda et al., 2011; von Schack et al., 2011). First reports describing characteristic miRNA expression profiles in blood or cerebrospinal fluid of patients with distinct pain conditions are starting to emerge (Andersen et al., 2016; Beyer et al., 2015; Bjersing et al., 2013; Orlova et al., 2011), however evidence linking specific miRNA expression profiles to specific pain disorders is still insufficient.

miR-132 is abundantly expressed in the brain and is emerging as a regulator of cognition, neuronal plasticity, and memory. It can regulate synapse structure and function (Bredy et al., 2011; Miller et al., 2012; Schratt, 2009; Soreq and Wolf, 2011). Hippocampal miR-132 mediates stress-induced cognitive deficits through suppression of acetylcholinesterase (Haramati et al., 2011) and miR-132 has recently been implicated in neuropathic pain after chronic constriction injury (CCI) (Arai et al., 2013). Similarly, spinal cord miR-132 is now proposed as a mediator of neuropathic pain following spared nerve injury (SNI) (Zhang et al., 2015). However, direct links between pain and miR-132 expression levels in human and/or animal models of neuropathic pain still remain elusive.

The current studies evaluated blood and sural nerve miR-132-3p, a splice variant of miR-132, expression in patients suffering from chronic neuropathic pain accompanying peripheral neuropathy and analyzed the role of miR-132-3p in pain behavior in an animal model of neuropathic pain.

2. Materials and Methods

2.1. Subjects

2.1.1. Patient assessment and diagnostic classification

Patients with neuropathies of different etiologies were recruited at the Department of Neurology, University of Würzburg between 2014 and 2015, where they underwent diagnostic work-up, including sural nerve biopsy. The study was approved by the Würzburg Medical Faculty Ethics Committee and written informed consent was obtained from every study participant before recruitment. The diagnosis of neuropathy was based on characteristic symptoms and signs in the neurological examination and typical findings in the electrophysiological assessment with standard nerve conduction studies in motor and sensory nerves of the upper and lower limbs (Kimura, 2001). Motor nerve conduction velocity (NCV) and evoked compound muscle action potential of the median, tibial and peroneal nerves were measured orthodromically. Sensory conduction velocity and amplitude of the nerve action potential were measured antidromically in the median and sural nerves. Skin temperature in both upper and lower extremities was controlled (> 32 °C) during the examinations. For differential diagnosis detailed laboratory studies included: glucose metabolism (HbA1c, oral glucose tolerance test), whole blood and differential cell counts, erythrocyte sedimentation rate, *C*-reactive protein, serum electrolytes, monoclonal immunoglobulins, vitamin levels (B6, B12), folic acid, renal and liver function tests, thyroid function tests, *anti*-nuclear antigen (ENA), *anti*-neutrophil cytoplasmic autoantibody (ANCA), rheumatoid factor, serology of borreliosis, immunofixation, and serum electrophoresis. In addition, all patients underwent a diagnostic lumbar puncture and cerebrospinal fluid was checked for pathological cell counts and protein levels. Diagnostic subgroups and definition of neuropathies are summarized in supplemental patient diagnostic criteria.

All patients were specifically asked for details regarding symptoms and signs that may have been associated with other sources of pain, any patient reporting other sources of pain or ongoing infection was excluded. Neuropathies were classified as painful if the patients reported pain with an intensity of 3 or more on a numeric rating scale (NRS) ranging from 0 to 10 (0 meaning "no pain" and 10 "worst pain imaginable"), as previously reported (Üçeyler et al., 2015; Üçeyler et al., 2007). The Graded Chronic Pain Scale (GCPS) (Von Korff et al., 1992) for 4 week recall and the Neuropathic Pain Symptom Inventory (NPSI) (Bouhassira et al., 2004) for the last 24 h recall were also used to assess pain. The control group consisted of healthy and age-and sex-matched (to the neuropathy patients shown in Fig. 1A) volunteers without infectious disease or pain at study inclusion.

2.1.2. Blood withdrawal for miRNA expression analysis

To reduce circadian variations, venous blood was collected from 30/81 patients and 30 healthy controls between 8:00 and 9:00 AM. For quantitative real-time PCR (RT-PCR), 9 ml whole blood was withdrawn in EDTA-containing tubes and the total white blood cell fraction (WBC) isolated. Isolated WBCs were re-suspended in RNA-cell protective reagent (QIAGEN, Hilden, Germany) and stored at −80 °C until further processing.

2.1.3. Sural nerve biopsy

Diagnostic sural nerve biopsy was performed in 67/81 patients at the Department of Neurosurgery, University of Würzburg (Dyck et al., 2005). For miRNA expression analysis, approximately 4 mm of the biopsy specimen was separated and stored in RNA-later overnight at 4 °C; on the following day RNA-later was removed and the specimen was frozen at −80 °C.

2.1.4. PCR amplification of miRNA

Peripheral nerve specimens as the basis of major pathology were obtained from patients and rats. Isolation of miRNAs was performed on all samples (WBCs, nerve, DRGs and spinal cord) using the miRNEASY kit (Qiagen, Hilden, Germany), following the manufacturer's protocol. For the generation of miRNA-specific first strand cDNA, 5 ng of total RNA was reverse transcribed using the Universal cDNA Synthesis kit II (Exiqon, Vedbaek, Denmark) following manufacturer's recommendations. For each reaction, 4 μl of diluted (1:80) cDNA was PCR amplified applying the corresponding miRNA and reference primer sets, using the miCURY LNA™ Universal microRNA PCR (Exiqon). The expression levels of miR-132-3p (5′-3′ UAACAGUCUACAGCCAUGGUCG, MIMAT0000426) and its splice variant miR-132-5p (5′-3′ ACCGUGGCUUUCGAUUGUUACU, MIMAT0004594) were normalized to the expression of endogenous 5 s RNA (5 s RNA, V00589). For individual target normalization, we tested different endogenous controls (U6, snord48, snord44, and 5sRNA) of which 5sRNA (housekeeping gene) was the most stable and thus, was used for both human and rat tissue. Each miRNA was amplified in triplicate and threshold cycle (Ct) values were obtained. Fold changes in miRNA expression among groups were calculated using interplate calibrators (a standard sample that was run on each PCR plate) by means of the delta-delta Ct method.

2.1.5. miRNA target validation

We set out to analyze the role of AMPA-receptor subunit GluA1, in an animal model of neuropathic pain. We performed a comprehensive target prediction analysis of miR-132-3p by employing four databases: TargetScan (Friedman et al., 2009), microRNA.org (Betel et al., 2008), miRTarBase (Hsu et al., 2011), and DIANAmicroT (Paraskevopoulou et al., 2013). GluA1 was identified as a potential downstream target by at least two of the four prediction algorithms. To further narrow down

the numerous miRNA downstream candidates we performed an additional Blastn alignment search (Altschul et al., 1990) of GluA1 (NM_031608.1) to specifically check for putative binding sites of miR-132-3p in the GluA1 gene.

2.1.6. Animals

Adult male Holtzman rats (250–300 g, Harlan Industries, Indianapolis, USA) were used. Animals were fed *ad libitum* and maintained in a controlled temperature and humidity environment, under a 12 h light/dark cycle. Animals recovered from shipping for a minimum of 2 days before entering the study; on the day of the experiment, animals were allowed to acclimate to the laboratory and test equipment for at least 1 h. All procedures were performed during the light cycle. Experiments were in compliance with the National Institute of Health Guide for the Care and Use of Laboratory Animals, and the Institutional Animal Care and Use Committee of the University of California, San Diego, approved all animal protocols.

2.1.7. Intrathecal catheterization and spared nerve injury (SNI)

Naïve rats were anesthetized with isoflurane (5% induction-, 2.5% maintenance) in a 50% O_2/room air mixture. Polyethylene (PE-5, 8.5 cm, Scientific Commodities Inc., AZ, USA) catheters were implanted intrathecally under aseptic conditions, as described elsewhere (Malkmus and Yaksh, 2004). The catheter tip ended over the L4 spinal segment. Immediately after implantation, SNI or a sham surgery was performed, according to the method of Decosterd and Woolf (Decosterd and Woolf, 2000). Briefly, skin on the lateral surface of the left thigh was incised and a blunt dissection made directly through the biceps femoris muscle, thereby exposing the sciatic nerve. Distal to the trifurcation of its branches, the common peroneal and the tibial nerves were tightly ligated, using 5.0 silk and a 2–4 mm piece of each distal nerve stump was removed; the sural nerve was untouched. Incisions were closed with muscle and skin sutures. In sham surgery, the sciatic nerve branches were exposed, but not otherwise harmed. All rats received subcutaneous lactated Ringer's solution (1 cm^3/50 g body weight, Baxter HealthCare Corporation, Deerfield, IL, USA) with carprofen (5 mg/kg, Rimadyl Pfizer Inc., New York, NY, USA) immediately post-surgery and were housed individually. Behavioral experiments were conducted 5–21 days after surgery. Rats that displayed behavioral or motor deficits or loss of cannula patency were excluded from the study (<1%).

2.1.8. Behavioral paradigm and testing

Behavioral testing was conducted between 9:00 AM and 4:00 PM by an experimenter blinded to treatment group. Mechanical paw withdrawal thresholds were determined prior to i.t. drug delivery (baseline, day 0) and at designated time points after unilateral SNI of the left hind limb (days 5, 10, 14, 18, 20). In one small subset of animals (3–4/group), days 11, 12, 13 and 21 were added.

2.1.9. Nociceptive threshold testing

Rats were placed on an elevated mesh floor in individual test chambers. Withdrawal thresholds to punctate mechanical stimuli were determined using calibrated von Frey filaments (Stoelting, Wood Dale, IL, USA) and the "up-down" method as described previously (Chaplan et al., 1994). Latencies to thermal stimulation were determined using a modified Hargreaves system (UCSD University Anesthesia Research and Development Group, CA, USA) (Dirig et al., 1997). After 45 min of adaptation on a glass plate, a radiant heat stimulus was delivered to each individual paw and withdrawal latency was recorded. Each hindpaw was tested 3 times and the average of all 6 tests was used as the animal mean. To prevent tissue damage by the heat, we used a stimulus cut-off time of 20 s.

2.1.10. Place escape avoidance paradigm (PEAP)

To test the aversive aspect of pain the place escape avoidance paradigm (PEAP) was employed (Fuchs and McNabb, 2012). Rats were allowed unrestricted movement within a box (40.6 × 15.9 × 30.5 cm Plexiglas) one end of which was opaque black (sides and the top of the dark area) and the other end semi-translucent white (sides and top-light area). The animals were placed in the light area at the start of each test session. Mechanical stimulation with a suprathreshold von Frey monofilament (255 mN; 26 g) was applied at 15 s intervals to the sural nerve receptive field on the plantar paw. When the animal was on the dark side of the box, the left (i.e. injured) paw was stimulated and when the animal was in the light area, the right (i.e. uninjured) paw was stimulated. The location of the animal at the time of stimulus presentation was noted over the entire test period of 35 min. The percentage of stimuli applied to each foot was calculated and interpreted as time spent on each side of the test chamber.

2.1.11. MiRNA inhibitor and mimetic

A locked-nucleic acid (LNA) based *in vivo* inhibitor for miR-132-3p and a mismatch control (scrambled oligonucleotide, Scr) were purchased from Exiqon (Vedbaek, Denmark). Sequences of the oligonucleotides were as follows: miR-132-3p (5′-3′ ATGGCTGTAGACTGTT) and Scr (5′-3′ ACGTCTATACGCCCA). A miRIDIAN microRNA mimetic for miR-132-3p (C-320,363-03-0002) (Dharmacon, GE Healthcare Europe, Freiburg, Germany) was custom-modified with a 3′-cholesterol conjugation on the passenger strand to facilitate its *in vivo* uptake. Both inhibitor and mimetics were synthesized from ribonucleotides and their functional efficacy was tested *in vivo*. Both oligonucleotides were purified via HPLC and the lyophilized powder was reconstituted in 1× PBS at pH 7.4 at concentrations of 1 mM and stored in aliquots at −20 °C to avoid freeze-thaw cycles.

The inhibitor and mimetic were administered to awake rats via the i.t. catheters. Prior to injection, active or mismatch inhibitors were mixed with (1:5 *w*/*v*) i-Fect™ in vivo transfection reagent (Neuromics, Edina, USA) to final doses of 5, 2 and 1 μg in 10 μl. Bolus injections were performed every 24 h for 3 consecutive days starting on day 10 after SNI or sham surgery. The mimetic was mixed with i-Fect™ (1:5 *w*/*v*) to final doses of 8, 5, 3 and 1 μg and the injection sequence to naïve animals followed the same pattern as that for the antagonist. Each drug was mixed with sterile saline at pH 7.4 to achieve the desired dilution. Each injection was followed by a 10 μl saline flush.

2.1.12. Immunohistochemistry

Rats, that had been injected with mimetic or the Scr control, were deeply anesthetized with 5% isoflurane and perfused with room temperature saline, followed by cold (4 °C) 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. The lumbar enlargement, and L4 and L5 DRGs were harvested and post-fixed for 4 h; tissue was cryoprotected in 30% sucrose in 0.1 M PBS. Fixed tissue was embedded in O.C.T. compound (Tissue-Tek, Torrance, CA, USA) and stored at −20 °C. Transverse DRG and spinal cord sections (10 μm) were cut on a Leica CM 1800 cryostat. Spinal cord sections were mounted and labeled with goat *anti*-Iba1 (microglia, 1:750, Abcam, Cambridge, MA, USA), mouse anti-glial fibrillary acidic protein (GFAP, astrocytes, 1:500; Millipore Temecula, CA, USA) and goat-anti MAC387 (MAC387, macrophages, 1:250, Thermo Fischer, Rockford, IL, USA), to check for signs of glial activation in the spinal cord dorsal horn and infiltration of peripheral macrophages at the injection site (catheter tip). The DRGs were stained with rabbit anti-activation transcription factor 3 (ATF3, 1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Binding sites were visualized with species matched goat anti-rabbit secondary antibody conjugated with Alexa Fluor 488 or goat anti-mouse antibody conjugated with Alexa Fluor 594 (both at 1:500, Invitrogen, Carlsbad, CA, USA). Control sections had the primary antibody omitted. Images were acquired with a Leica TCS SP5 confocal system (Leica Microsystems GmbH, Wetzlar, Germany); *z*-stacks were obtained and images processed with LAS AF software

(Leica Microsystems GmbH, Wetzlar, Germany). All reported findings were observed in multiple sections in at least 3 animals per condition.

2.1.13. Western blot analysis for AMPA receptor subunits

Rats were deeply anesthetized and perfused with room temperature saline. Spinal cords were harvested and the dorsal half of the lumbar enlargement dissected. Tissue was immediately frozen in extraction lysis buffer (0.5% Triton X-100, 50 mM Tris-HCl, 150 nM NaCl, 1 mM EDTA and 3% SDS). Protein concentrations were determined and Western blot analysis performed. Samples were loaded on a Nu-Page 4–12% Bis-Tris Gel (Invitrogen, Carlsbad, CA, USA) in MOPS running buffer and transferred onto a single nitrocellulose membrane. Antibodies against the pAKT (ser473, 1:1000; Cell Signaling Technology, Beverly, MA, USA) and GluA1- and GluA2- AMPA receptor subunits were from rabbit (1:1000, Millipore, Temecula, CA, USA). Beta-actin was used as a loading control (Sigma, St. Louis, USA). The membrane was stripped following each analysis. Immunoblots were scanned and a densitometric analysis performed using ImageJ software (U. S. National Institutes of Health, Bethesda, Maryland, USA).

2.2. Statistical analysis

Graph Pad Prism 4.0 (GPP 4.0, GraphPad Software, Inc., San Diego, CA) was used for statistical analyses and graphs. Data distribution was tested with the Kolmogorov-Smirnov-test and by visual inspection of data histograms. For non-normally distributed values of the human qRT-PCR results, data were expressed as medians and quartiles and the non-parametric Mann-Whitney *U* test Spearman's rho were used for analysis. Descriptive patient data were reported as median and range (minimum to maximum value). All other data were expressed as mean ± standard error of the mean (SEM). Two-way repeated measures ANOVA followed by Bonferroni post hoc testing were used to assess statistical significance in behavioral experiments. One-way ANOVA followed by Tukey's post hoc testing was performed for rat qRT-PCR data, place-escape avoidance and the Hargreaves test. Student's *t*-tests were performed for reporter assays and Western-blot data. Statistical significance was accepted with $p < 0.05$.

3. Results

3.1. Basic description of the patient cohort

We included 81 patients with neuropathies of different etiology. The study cohort had a median age of 66 years (range 33–84 years) and consisted of 55 men (median age: 66 years, range 33–84 years) and 26 women (median age: 67 years, range 47–84 years). Clinical characteristics of the cohort and diagnostic subgroups are summarized in Table 1. In 23/81 (28%) patients an inflammatory neuropathy was diagnosed (CIDP, vasculitic neuropathy, PIAN, paraproteinemic neuropathy), while in 24/81 patients (30%) neuropathy was of non-inflammatory origin (diabetic, hereditary, CIAP). In 34/81 cases (42%) the etiology of the neuropathy was unclear. Forty-two (51%) patients had a painful neuropathy (i.e. current pain intensity ≥3, NRS) compared to 39 patients (49%) with neuropathy without pain.

3.2. Healthy controls

The control group for WBC analysis of miR-132-3p consisted of 26 men and 4 women, with a median age of 56.5 years (range 38 to 69 years). None of the controls reported any ongoing infectious disease, pain or neuropathy.

3.3. Expression of miR-132-3p in polyneuropathy patients

We found that patients with polyneuropathies collectively had higher miR-132-3p levels than healthy controls ($p < 0.001$, Fig. 1A). There was no difference in miR-132-3p expression in WBCs when comparing between subsets of inflammatory versus non-inflammatory or painful versus non-painful polyneuropathies.

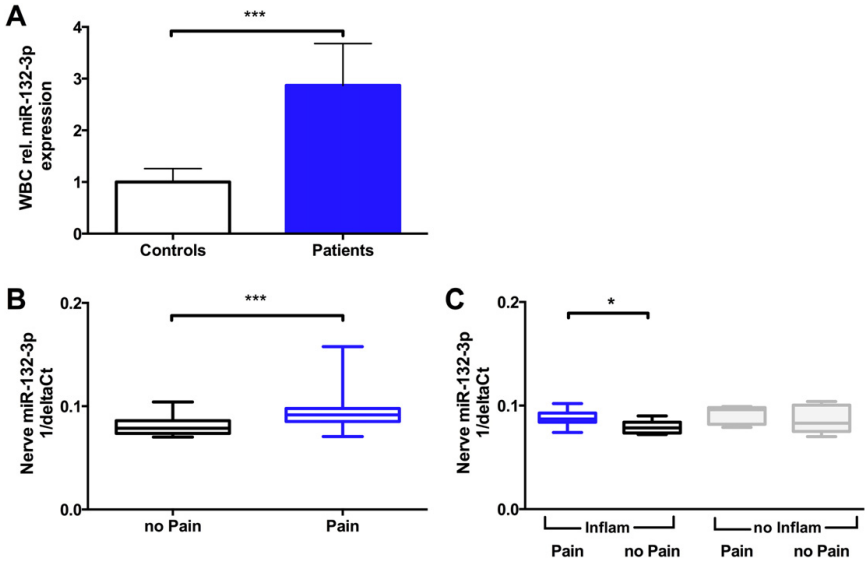

Fig. 1. miR-132-3p gene expression in patients with polyneuropathies. A) Bar graph indicates that miR-132-3p expression was higher in white blood cells of patients with polyneuropathy of different etiologies compared to controls ($n = 30$/group). Data represents relative miR-132-3p expression normalized to controls. ***$p < 0.001$ B) Gene expression of miR-132-3p was also higher in patients with painful neuropathies ($n = 25$) compared to patients with painless neuropathies ($n = 23$). C) Pain was associated with higher miR-132-3p expression in inflammatory and non-inflammatory neuropathies, when sub-grouped based on the diagnosis. Y-axis in panels B & C are in arbitrary units. Data are expressed as median values with upper and lower 25% percentiles indicated; Mann Whitney U-comparisons; ***$p < 0.001$, *$p < 0.05$.

Table 1
Clinical characteristics and diagnostic subgroups of the study cohort.

Item	Number (% of entire group)
M, F, (N)	55, 26
Median age (range)	66 years (33–84)
Median disease duration (range in years)	4 years (0.1–27)
Diagnostic subgroups (N and % of entire group):	
Unknown etiology:	34 (42%)
i) electrophysiologically axonal	17 (21%)
ii) electrophysiologically demyelinating	4 (5%)
iii) electrophysiologically mixed	13 (16%)
CIAP	9 (11%)
PIAN	9 (11%)
Vasculitic neuropathy: NSVN	6 (7%)
Diabetic neuropathy	5 (6%)
Hereditary neuropathy	5 (6%)
CIDP	4 (5%)
Other non-inflammatory neuropathy	3 (4%)
Paraproteinemic neuropathy (IgM)	2 (2%)
MADSAM	1 (1%)
AMSAN	1 (1%)
SFN	2 (2%)
Painful, painless (N)	42, 39

CIAP chronic idiopathic axonal polyneuropathy, *CIDP* chronic inflammatory demyelinating polyneuropathy, F female, INCAT Inflammatory Neuropathy Cause and Treatment Group, M male, *MADSAM* multifocal acquired demyelinating sensory and motor neuropathy, M male, *MMN* multifocal motor neuropathy, N number, *NSVN* non- systemic vasculitic neuropathy, *PIAN* progressive idiopathic axonal neuropathy, SFN small fiber neuropathy.

In contrast, expression of miR-132-3p was higher in sural nerve specimens of patients with painful neuropathies, i.e. NRS ≥ 3 compared to patients with painless neuropathies, NRS < 3 ($p < 0.001$, Fig. 1B). We additionally tested the group of patients with NRS = 0 (i.e. painless) and NRS > 0 (i.e. painful) and observed that patients with pain had higher miR-132-3p expression than patients without pain ($p = 0.0134$). We then performed a correlation analysis between NRS values and miR-132-3p levels and found a correlation between NRS values and miR-132-3p levels (Spearman's rho; $r = 0.3274$, $p = 0.02$).

Interestingly, pain was associated with higher miR-132-3p expression in inflammatory, but not in non-inflammatory neuropathies (1.1 Fold change, Fig. 1C). Disease duration (<3 versus ≥3 years) had no influence on miR-132-3p expression in sural nerve. There was no sex related difference in miR-132-3p expression in sural nerve biopsy specimens with neuropathies ($p = 0.08$). In addition, stratification for painful and painless neuropathies did not reveal sex specific expression differences ($p = 0.4$; data not shown).

Of note, no difference in miR-132-5p, a second splice variant of miR-132, was observed in either WBC or sural nerve specimens ($p > 0.05$; data not shown). We therefore focused exclusively on miR-132-3p.

3.4. SNI increases miR-132-3p expression in rat spinal cord and DRG

We measured miR-132-3p expression in dorsal spinal cord, DRG, and sural nerve specimens after SNI or sham surgery in rats without catheters on post-surgical days 3 and 10 (Fig. 2). On day 3, dorsal spinal cord tissue ipsilateral to the SNI was no different than contralateral miR-132-3p levels ($p = 0.7561$), but tended to have lower levels following sham surgery ($p = 0.06$). At this time, rats did not exhibit consistent pain behavior. However, on day 10 post-surgery when a profound and persistent decrease in mechanical withdrawal threshold was present, spinal miR-132-3p expression was highest in ipsilateral SNI tissue. At both time points, expression of miR-132-3p in DRGs ipsilateral to the SNI was higher than DRGs ipsilateral to sham surgery or naïve tissues. Despite the fact that the sensory nerve cell bodies of the injured fibers showed a 6–7 fold increase in miR-132-3p compared to naive, sural nerves displayed no difference among ipsilateral SNI, contralateral SNI and sham-operated tissue at either time point ($p > 0.9$). Thus the nerve injury elicited an increase of miR-132-3p in sensory nerves in both neuropathy patients and SNI rats, but the distribution was different.

Gene expression of miR-132-3p in DRGs and spinal cord of naïve animals was not different than that seen in sham operated rats 10 days post-surgery. There was no difference in miR-132-3p expression between tissue ipsilateral to SNI in rats with (13 days-post surgery) or without (10 days) catheters (Fig. 2 B,C and D,E respectively). Daily i.t. administration of a miR-132-3p antagonist to SNI animals, starting on day 10, reversed the ipsilateral spinal up-regulation of gene expression by day 13, this was not observed following Scr injections. In the same animals, antagonist injections also reversed the ipsilateral DRG up-regulation of miR-132-3p 13 days post SNI ($p = 0.02$), however, unlike the spinal tissue, Scr injection caused a down-regulation in the DRG. This was an unexpected observation and could have multiple explanations in addition to direct off-target effects, such as unwanted protein binding due to length and polarity of the Scr, as previously reported (Stein, 1996, 2001).

3.5. SNI-induced mechanical allodynia is reversed by miR-132-3p antagonism

Baseline mechanical paw withdrawal thresholds did not differ among the various treatment groups. We started with a high dose (5 μg) of a miR-132-3p inhibitor, Scr or vehicle (10 μl, i-Fect™ transfection agent) to determine if these oligonucleotides produced toxic effects. Neither of the drugs led to any observable impairment or discomfort in naive animals. We then proceeded with the i.t. treatment paradigm of 5-, 2- or 1 μg *anti*-miR-132-3p or 5 μg Scr given as bolus injections on three consecutive days, starting on day 10 after SNI. At this time, all animals exhibited a pronounced allodynia with withdrawal thresholds of <9.80 mN (1 g). Only the 5 μg dose reversed the SNI-induced allodynia ($p < 0.001$, Fig. 3A), which lasted until at least day 18 ($p < 0.001$, Fig. 3A). Fig. 3B represents the efficacy of miR-132-3p antagonism in a small subset of animals that were tested more frequently ($n = 3$–4/group). Animals showed a gradual increase in mechanical withdrawal thresholds after each injection (day 11, 12, 13) lasting for up to 7 days after the last injection, after which, pain behavior began to reemerge ($p < 0.001$, Fig. 3B). Treatment with the control Scr oligo was without effect ($p > 0.9$; Fig. 3A and B).

3.6. Spinal miR-132-3p antagonism causes loss of pain aversion in the PEAP test

Fig. 3C depicts the PEAP chamber with its two testing areas. On average, naïve rats spent 20–30% of the time in the light side of the chamber, regardless if they were stimulated with the von Frey hair filament or not (Fig. 3D). Following SNI, this pattern changed and animals spent 60–70% of the time on the light side. This reflects the increased aversion produced by left paw stimulation after SNI. In contrast, following three days of *anti*-miR-132-3p treatment, time spent in the light area decreased to approximately 20%. This is similar to what is seen for sham-operated rats, i.e. the antagonist reversed the aversive nature of the left paw stimulus ($p < 0.001$, Fig. 3D). Behavior of SNI animals treated with the Scr control did not differ from SNI/vehicle animals ($p > 0.9$).

3.7. Intrathecal injection of miR-132-3p mimetic causes mechanical and thermal hyperalgesia

Baseline mechanical paw withdrawal thresholds did not differ among the various treatment groups. A synthetic miR-132-3p mimetic was administered i.t. for three consecutive days and mechanical paw withdrawal thresholds (Fig. 4A) were determined. A dose of 8 μg miR-132-3p mimetic induced vocalization after injection, motor weakness of one or both hindlimbs, and limping that was readily apparent before the third injection. Animals still exhibited sensitivity to paw stimulation with von Frey hair filaments. Mean mechanical thresholds dropped to

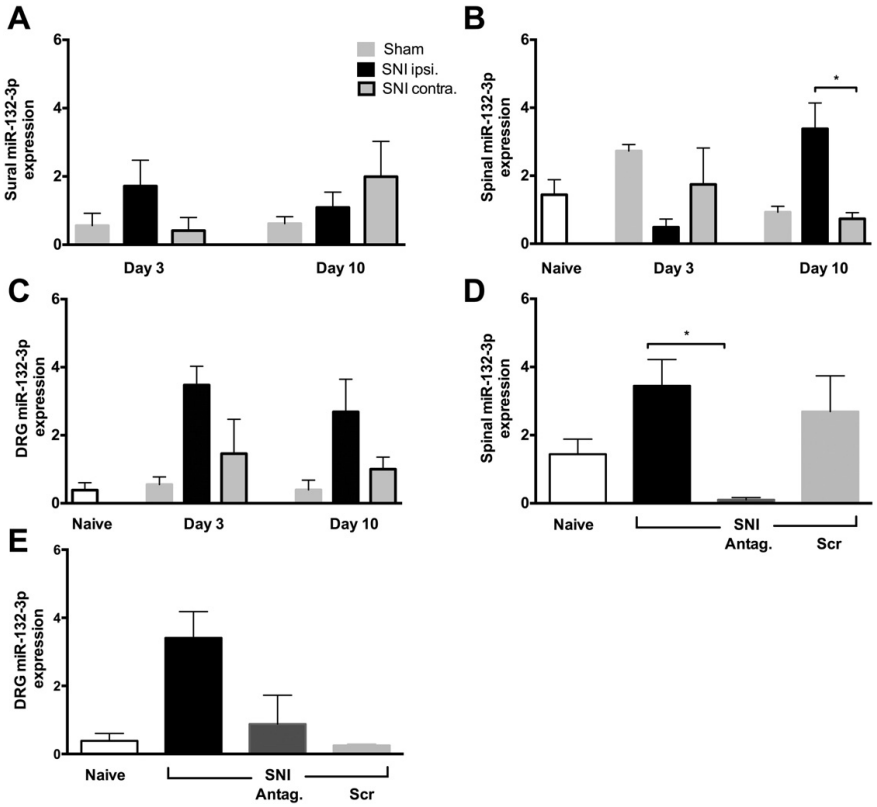

Fig. 2. miR-132-3p gene expression analysis after SNI. panels A-C: Expression of miR-132-3p on day 3 and 10 after SNI or sham surgery. A) Sural nerve expression did not change at either time point. B) On post-operative day 3, spinal cord miR-132-3p expression levels did not change in SNI-operated animals, but increased on day 10 compared to the contralateral side and sham C) expression of miR-132-3p was higher in DRGs of SNI animals compared to naïve and sham operated rats at both times. Panels D & E: Relative gene expression of endogenous miR-132-3p in spinal cord and DRG after i.t. administration of antagonist or Scr. D) Spinal expression of miR-132-3p tended to increase 13 days after SNI when compared to naïve. Injection of 5 μg antagonist (Antag.) reversed this tendency and resulted in a significant decline in miR-132-3p levels, while injection of 5 μg Scr had no effect. E) In DRGs of the same animals, miR-132-3p was up-regulated after SNI and decreased to naïve levels after i.t. antagonism. Injection of Scr also reversed the SNI-induced increase. Y-axis is in arbitrary units. Insert refers to panels A-C only. Data are expressed as mean ± SEM 1-way ANOVA followed by Tukey's post-hoc tests; $^{**}p < 0.01$, $^{*}p < 0.05$; $n = 3$–6.

below 49 mN (5 g) for treatment groups given 5 and 8 μg mimetic and remained depressed through day 7, there was however a transient increase in threshold on day 3. Lower doses of 3 and 1 μg did not result in behavioral changes after three injections and testing was discontinued. We also examined thermal latencies in animals given mimetic on the day after their last injection. These results mirrored those seen for mechanical thresholds on day 1 in that only the higher doses of 8 μg and 5 μg resulted in thermal withdrawal latency different from Scr-treated animals (Fig. 4B).

3.8. Exogenous miR-132-3p administration leads to spinal microglia activation

Injection of 8 μg miR-132-3p mimetic led to increased Iba1 expression at the site of the catheter tip and the entire spinal cord section compared to the injection of Scr-control (Fig. 5A, B). In parallel, we observed staining for MAC387 positive cells indicative of peripheral macrophage infiltration exclusively at the catheter site. No such infiltration was found in Scr-injected rats (see supplemental Methods and Fig. 1). Staining for GFAP did not reveal any obvious difference from control animals (data not shown). Interestingly, we also did not observe an appreciable number of ATF3 positive DRG neurons in animals injected with mimetic (data not shown).

3.9. MiR-132-3p targets and decreases spinal AMPA receptor subunit GluA1

We verified that miR-132-3p targets GluA1 mRNA in a luciferase assay (see Supplemental Fig. 2). To confirm our hypothesis that miR-132-3p not only targets GluA1 mRNA, but also decreases its protein expression, we performed Western blots for GluA1 in whole tissue homogenates of dorsal spinal cord from rats that had received three daily bolus injections of 5 μg miR-132-3p mimetic, tissue was collected the day after the last injection (Fig. 6A and B). Western blots for GluA2 and phosphorylated protein kinase B (pAKT) were also performed to look at the specificity of the mimetic effect. Neither GluA2 nor AKT was predicted by our algorithms to be a direct target of miR-132-3p. Injection of synthetic miR-132-3p decreased the expression of GluA1 when compared to naïve animals as predicted (GluA1; $p < 0.05$). Interestingly, in the same samples, GluA2 levels increased in comparison to naïve animals (GluA2; $p < 0.05$). No changes in spinal cord dorsal horn pAKT levels were observed (data not shown), despite the presence of pain behavior.

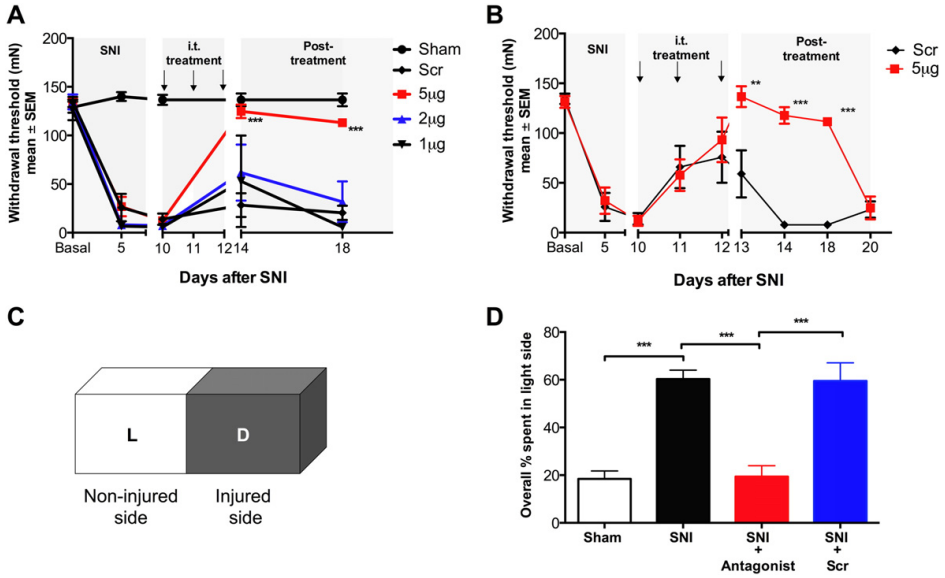

Fig. 3. miR-132-3p antagonism reverses pain behavior. A) Mechanical paw withdrawal thresholds in SNI animals before and after i.t. injection of miR-132-3p antagonist. The high dose (5 μg) reverses pain behavior in SNI rats for at least 7 days ($n = 6$–12). B) Each successive 5 μg miR-132-3p injection of inhibitor resulted in progressively more *anti*-allodynia on the following days, this lasted for at least 5 days after the last injection ($n = 3$–4). C) Schematic of the PEAP testing chamber. D) Percent of time animals spend within the light side of the test chamber 13 days after SNI. SNI increases the amount of time spent in the light side, compared to sham-operated animals, this was reversed by the antagonist, but not by Scr ($n = 8$–12). 2-way ANOVA comparisons with post-hoc Bonferroni corrections (A&B), and 1-way ANOVA with Tukey's post-hoc tests (D); $^{***}p < 0.001$, $^{**}p < 0.01$.

3.10. SNI causes spinal up-regulation of AMPA receptor subunits GluA1 and GluA2

We next measured the expression of spinal AMPA receptor subunits after SNI. We performed Western blots for GluA1 and GluA2 in whole tissue homogenates of the dorsal spinal cord harvested 10 days after surgery. Fig. 6C shows the up-regulation of GluA1 after SNI (3.5 fold) compared to sham animals (GluA1, $p < 0.05$). In the same samples, GluA2 levels also increased by 2.5 fold over sham ($p < 0.05$, Fig. 6C and D).

4. Discussion

miRNAs have a vital role in post-transcriptional regulation, are widely expressed throughout the brain, are regulated by neuronal activity and some, including miR-132 are thought to be necessary for neuronal plasticity required for memory consolidation and pathological pain (Elramah et al., 2014; Soreq and Wolf, 2011). For example, overexpression of hippocampal miR-132 increased local excitatory postsynaptic currents and impaired learning and memory processes (Edbauer et al., 2010), and induction of long-term potentiation (LTP) resulted in a delayed up-regulation of miR-132 (Wibrand et al., 2012; Wibrand et al., 2010). In contrast, low levels of miR-132 enhanced cognitive capacity (Hansen et al., 2013; Hansen et al., 2010). Thus, given the many parallels between memory consolidation and spinal sensitization (in the form of spinal LTP) leading to enhanced pain (Basbaum et al., 2009), pathological regulation of miR-132 might modulate the development of chronic pain.

Despite recent advances, chronic neuropathic pain remains a major challenge for clinicians and pre-clinical scientists. There is an urgent demand for the development of specific mechanism-based therapies.

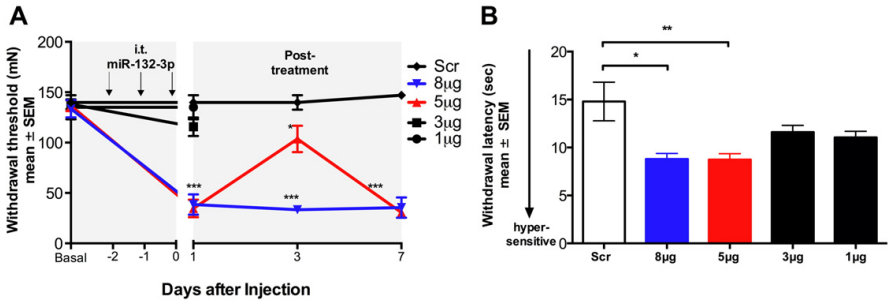

Fig. 4. Exogenous miR-132-3p induces pain behavior. Pain behavior in i.t. catheter implanted rats after 3-day i.t. bolus administration of a miR-132-3p mimetic. A) Administration increases mechanical allodynia in rats for at least 7 days after the last treatment. Control oligo (Scr) administration had no effect ($n = 3$–8 each). B) miR-132-3p mimetic dose dependently increases heat hypersensitivity in rats. Control oligo Scr had no effect ($n = 3$–12 each). 2-way ANOVA comparisons with post-hoc Bonferroni corrections (A), and 1-way ANOVA comparisons with post-hoc Tukey's corrections (B); $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$.

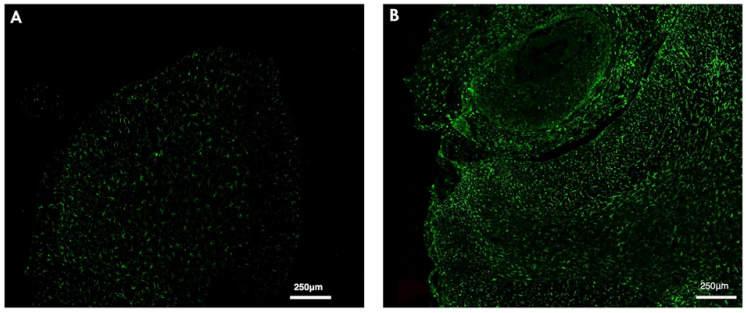

Fig. 5. Exogenous miR-132-3p activates microglial cells. L5 spinal cord below level of catheter tip stained for activated microglia and macrophages A) Scr-injected rats show less activation of microglia (Iba1, green) compared to B) 8 μg miR-132-3p injected rats.

Using a comprehensive approach combining patient data with behavioral and molecular methods in a clinically relevant animal model of neuropathic pain, this study showed for the first time that chronic neuropathic pain is associated with dysregulation of miR-132-3p. Disease specific aberrant miRNA expression signatures have previously been reported, e.g. in blood of cancer patients (Calin and Croce, 2006) and other disorders (Cogswell et al., 2008; Gillardon et al., 2008; Lee et al., 2011; Wang et al., 2011; Zhang et al., 2010). Furthermore, several recent studies associated circulating miRNAs with chronic pain conditions such as osteoarthritis (Beyer et al., 2015), rheumatoid arthritis (Pauley et al., 2008), CRPS (Orlova et al., 2011), fibromyalgia syndrome (Bjersing et al., 2013), and migraine attacks (Andersen et al., 2016), thus potentially enabling patient stratification and disease characterization. Importantly, MiR-132 found in a subset of CRPS patients correlated with expression of inflammatory and immune-related markers (Orlova et al., 2011). We presently show that peripheral neuropathies are associated with an increase of miR-132-3p in WBCs and that painful inflammatory neuropathies in particular are associated with increased miR-132-3p in patient sural nerves. Although all fold-changes found for miR-132-3p expression were comparatively small, Joilin et al. reported that two-thirds of hippocampal miRNAs in the rat differentially expressed following LTP-inducing stimulation, including miR-132-3p, showed less than a 1 fold-change and only 4/65 showed more than a 2-fold change (Joilin et al., 2014). These modest changes appear to be typical of studies examining LTP-induced changes in miRNA (Ryan et al., 2011; Ryan et al., 2012; Wibrand et al., 2010). Given the parallels between spinal and hippocampal LTP, this level of spinal change is not unexpected. These findings suggest that neuropathies may either alter systemic and peripheral nerve miR-132-3p levels, or that specific changes in miR-132-3p expression may actively contribute to pain. We thus set out to study this phenomenon in a well-established animal model of chronic neuropathic pain.

Our data indicate that spinal miR-132-3p expression has a biphasic response after SNI, a semi-acute (day 3) tendency to decrease followed by a much later (day 10) significant increase at a time when pain behavior was well established. This resembles the pattern of hippocampal

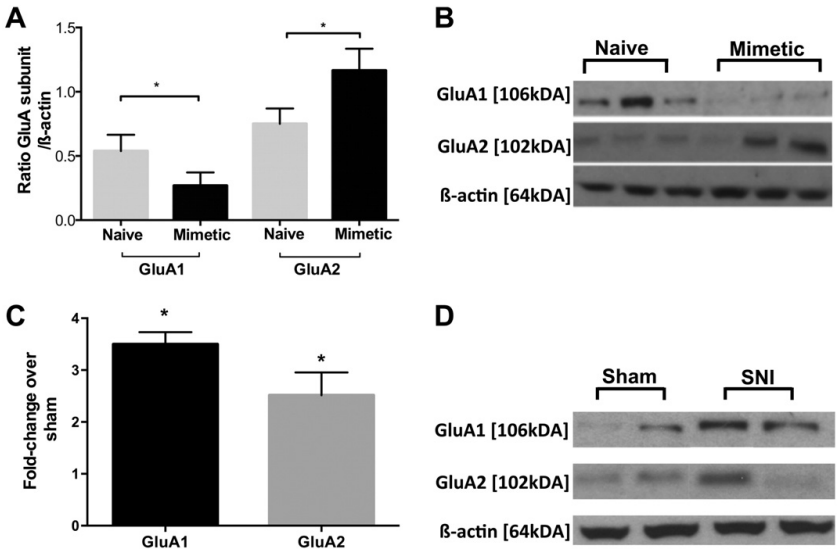

Fig. 6. Exogenous miR-132-3p changes levels of AMPAR subunits. Western blot results after A) spinal administration of miR-132-3p and B) SNI. A) Levels of GluA1 in whole cell homogenates of spinal dorsal horn decrease after injection of miR-132-3p mimetic, GluA2 levels simultaneously increased in the same tissue (*t*-test, $n = 3$/group). B) Spinal levels of GluA1 and GluA2 increase after SNI ($n = 3$/group) values are expressed as fold change compared to sham. C) and D) Western blots illustrating protein expression under each condition Student's t-test $^{*}p < 0.05$.

miR-132 expression following induction of LTP (Joilin et al., 2014; Wibrand et al., 2012; Wibrand et al., 2010). This early decrease was also observed for miR-132 following SNI in mice (Zhang et al., 2015), however in these experiments levels of the miR were still below basal on day 7, albeit higher than they were on day 3. This discrepancy could be due to differences in species, time point on the biphasic curve or other technical factors. In our experiments, miR-132-3p expression in the DRG was up-regulated at both time points implying an ongoing role for miR-132-3p in the regulation of SNI-induced pain-related signals from the periphery to the spinal cord. Having demonstrated that spinal miR-132-3p was elevated in SNI animals with well-established pain behavior, the questions became, did miR-132-3p alter endogenous expression of the miRNA? and, Was it necessary for the expression of the pain behavior? Intrathecal administration of the miR-132-3p inhibitor 13 days after SNI, reduces its endogenous expression levels. Administration of the Scr control was without effect on both the pain behavior and the SNI-induced increased miR-132-3p expression in the spinal cord as expected, but surprisingly inhibited up-regulation of miR-132-3p in the DRG. This latter effect could be due to off-target effects. Importantly, the antagonist reversed mechanical allodynia and pain aversion in SNI animals, thus linking spinal expression levels of miR-132-3p with pain behavior. Intrathecal administration of miR-132-3p mimetic probably resulting in spinal levels above the "physiological values" produced by SNI induced long lasting mechanical and thermal hyperalgesia. Taken together, these data provide strong evidence that increased miR-132-3p expression is not only associated with human neuropathic pain but is also necessary for maintenance of rodent neuropathic pain behavior and aversion, via as yet unknown mechanisms.

In chronic pain, spinal neuroinflammation is often characterized by activation of glia cells, infiltration of leukocytes and increased production of inflammatory mediators (Calvo et al., 2012; Taves et al., 2013). Interestingly, spinal administration of exogenous miR-132-3p led to pronounced microglial (Iba1) activation throughout the dorsal horn. Of particular interest, the macrophage marker MAC387 was positive in the mass at the catheter tip, but not in the remainder of the dorsal horn, suggesting a role for miR-132-3p in the recruitment of peripheral macrophages (see Supplemental Fig. 1). Indeed, miR-132-3p has been shown to be involved in both inflammatory cascades and immune cell infiltration (Marques-Rocha et al., 2015; Taganov et al., 2006). At present we cannot differentiate between the relative contributions to the pain response of miR-132-3p activation of pro-nociceptive signal transduction cascades and an artifact caused by the mass-induced spinal cord compression.

"Single NeurimmiRs affect a pathway of inter-related transcripts, all involved in a cellular process, rather than single proteins (Soreq and Wolf, 2011)". As stated above, miRNAs directly induce down-regulation of target molecules (Huttenhofer and Schattner, 2006). After reviewing several target prediction algorithms for downstream molecules involved in spinal sensitization we chose to measure GluA1. This was probably a mis-step on our part, as down-regulation of GluA1 would be expected to reduce pain behavior (Hartmann et al., 2004). We found that i.t. injection of the miR-132-3p mimetic, decreased levels of GluA1, but resulted in increased spinal levels of GluA2. In contrast, SNI, the more physiologic pain model, up-regulated miR-132-3p, GluA1 and GluA2. At first glance these effects on pain and GluA1 are contradictory. However, Cheng et al. reported that miR-132 also alters cellular activity by targeting, presumably down-regulating, K^+ channels (Cheng et al., 2007). Moreover, miR-132 is predicted to be upstream of the glutamate transporter GLT-1. Neuropathic pain is associated with downregulation of GLT-1, a subsequent deficit of glutamate clearance, and enhanced glutamatergic transmission (Ji et al., 2013). Thus, it is

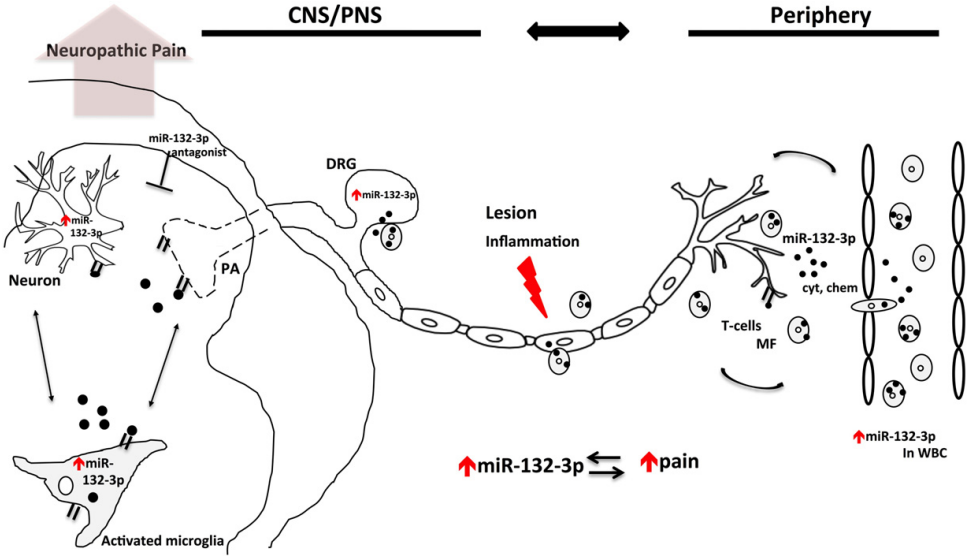

Fig. 7. miR-132-3p as a potential mediator of CNS-immune modulation involved in neuropathic pain. Following peripheral or central immune insults, immune cell (white blood cells; macrophages) changes in miR-132-3p levels might influence neuronal functions, whether directly by suppressing genes within neurons or indirectly by influencing the functioning of immune or supporting glial cells (i.e. microglia) or vice versa. Selective high-miR-132-3p expressing immune cells (e.g. macrophages) presumably infiltrate the DRG and sensitize peripheral neurons in addition to releasing pro-inflammatory mediators (e.g. cytokines and chemokines). Consequent changes in the expression of miR-132-3p alter both neuron–glia interactions and neuronal functions, affecting plasticity, neurotransmission, and possibly leading to neuropathic pain. Spinal administration of miR-132-3p antagonists alleviates pain behavior following peripheral insults. Pathological events and processes are shown in red, black dots represent miR-132-3p. Abbreviations: MF- macrophages; Chem- chemokine; CNS- central nervous system; Cyt- cytokine; PA- primary afferent; PNS- peripheral nervous system; WBC- white blood cells. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

plausible that injection of mimetic could decrease GluA1 while still producing pain behavior.

Ten days post-SNI, ipsilateral spinal tissue has up-regulated levels of miR-132-3p. However, SNI induces multiple effects beyond miR-132-3p, including induction of other miRNAs and of pro-nociceptive factors some of which may induce increased GluA1. The algorithm by which SNI results in up-regulation of GluA1 is complex as many neuronal miRNAs are postulated to participate in both feed-forward and feedback homeostatic loops (Tsang et al., 2007). The final outcome of GluA1 expression changes is dependent on the sum total of all of these factors. While the exact mechanisms remain elusive, the observation is solid and will hopefully lead to further experiments.

Our study has several limitations, miRNA expression in WBC was only assessed in patients and not in rats and we did not test downstream target expression in patient samples. It could have been more illuminating if we had examined other downstream targets in the rats such as GLT-1, which were more likely to have shown the same changes following mimetic administration and SNI. Our findings furthermore raise the question as to whether the increased peripheral miR-132-3p in WBC of patients is directly involved in the pathophysiology of pain, possibly via release of sensitizing agents, e.g. pro-inflammatory cytokines, infiltration of macrophages into the central nervous system or some combination of the two (Fig. 7). Our study cannot explain whether increased miR-132-3p expression in WBC is the cause or consequence of peripheral neuropathies and if peripheral nerve injury induces the same increase in WBC miRNA expression in rats as it does in neuropathy patients. It does however address the fact that neuropathies are not solely restricted to peripheral nerves, but involve changes in systemic miRNA expression, possibly via feedback loops between the immune- and central nervous systems. Furthermore, it provides interesting insights into the translational aspect of miR-132-3p in the pathophysiology of chronic neuropathic pain.

5. Conclusion

Taken together these findings imply that aberrant neural miR-132-3p expression is associated with human neuropathic pain, and that in animals targeted antagonism of miR-132-3p results in dose-dependent reversal of pain behavior while miR-132-3p mimicry results in a dose-dependent induction of pain behavior allowing us to designate miR-132-3p as a pro-nociceptive miRNA in our study. Importantly, alterations in miRNA levels may be indicative of their functional involvement in pain pathophysiology, and miRNA-based diagnostics and therapeutics might have the advantage of targeting multiple pain-associated genes simultaneously. However, administration of miR-132-3p elsewhere to different tissues or by different routes could have different effects and thus more detailed work is necessary to better understand the molecular pathways in neuropathic pain.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.expneurol.2016.06.025.

Conflicts of interest

The authors have no conflicts of interest.

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Supplemental patient diagnostic criteria

The following diagnostic subgroups were distinguished: 1) Chronic inflammatory demyelinating neuropathy (CIDP), based on the INCAT (inflammatory neuropathy cause and treatment) criteria (Hughes et al., 2001); 2) Chronic idiopathic axonal polyneuropathy (CIAP) was diagnosed based on the clinical presentation of a sensory-motor and neurophysiologically determined axonal neuropathy, with slow onset and progression. Histology in these cases was axonal but without signs of inflammation, cerebrospinal fluid (CSF) was normal and steroid treatment had no effect (Vrancken et al., 2004). 3) Progressive idiopathic axonal neuropathy (PIAN), based on the acute or subacute presentation with slow progression of sensory-motor symptoms. Patients had axonal histology with inflammation and neurophysiology. CSF protein was increased and patients had a positive response upon steroid treatment (Vrancken et al., 2004). 4) Diabetic neuropathy, was diagnosed when the patient had diabetes mellitus type I or II and if typical clinical, laboratory, and electrophysiological findings were present. 5) Hereditary neuropathy was based on the combination of typical clinical presentation, positive family history, genetic and neurophysiological data. 6) Vasculitic neuropathy was diagnosed according to published criteria and patients were divided into systemic and non-systemic vasculitic neuropathy (Collins et al., 2000). 7) All cases of other known etiology, such as amyloidosis, paraneoplastic neuropathy or neuropathy due to vitamin deficiency were collectively designated being of “other origin”. 8) In all other cases neuropathies were classified of “unknown origin”.

Supplemental Material and Methods

miRNA target validation

For the luciferase assay HEK cells were cultured in Dulbecco's modified Eagle's medium (Gibco®, Thermo Fischer) containing 10% inactive FBS (Gibco®), 200 units/mL of penicillin and 200 µg/mL of streptomycin (Gibco® Pen-Strep, 15140) and 1X L-glutamine (Gibco®). Approximately $1x10^6$ cells were plated in 6-wells plates 24h before transfection and cultured in antibiotic free medium. Cells were then co-transfected with 2 µg of GluA1 reporter vector (pEZX-MT06, Genecopoeia™, Rockville, MD, USA) and a dual-luciferase assay performed employing 50 nM of either miR-132-3p antagonist, miR-132 mimic or scrambled oligonucleotide, using Lipofectamine® 2000 Reagent (5 µl/well, Invitrogen, formerly Life Technologies, Thermo Fischer) following manufacturer`s instructions. Forty eight h later, luciferase activities were quantified using a Luc-Pair miR Duo-Luciferase Assay kit (Genecopoeia™) and normalized to their respective control experiment. Data is expressed as ratio of Firefly activity/renilla activity for each condition in comparison to the respective mismatch control vector expression.

Immunohistochemistry

Rats, that had been injected with mimetic or the Scr control, were deeply anesthetized with 5% isoflurane and perfused with room temperature saline, followed by cold (4°C) 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. The lumbar enlargement, were harvested and post-fixed for 4h; tissue was cryoprotected in 30% sucrose in 0.1 M PBS. Fixed tissue was embedded in O.C.T. compound (Tissue-Tek, Torrance, CA, USA) and stored at -20C°. Transverse DRG and spinal cord sections (10 µm) were cut on a Leica CM 1800 cryostat. Spinal cord sections were mounted and labeled with goat-anti MAC387 (MAC387, macrophages, 1:250, Thermo Fischer, Rockford, IL, USA) to check for signs of activation in the spinal cord dorsal horn and infiltration of peripheral macrophages at the injection site (catheter tip).

To check for possible cytotoxicity of the mimetic, 10µm spinal cord sections were stained with hematoxylin-eosin (H&E). Particular attention was paid to the presence or absence of morphological changes or signs of spinal cord compression. Brightfield images were taken with a BX51 microscope (Olympus, Melville, NY, USA). All reported findings were observed in multiple sections in at least 3 animals per condition

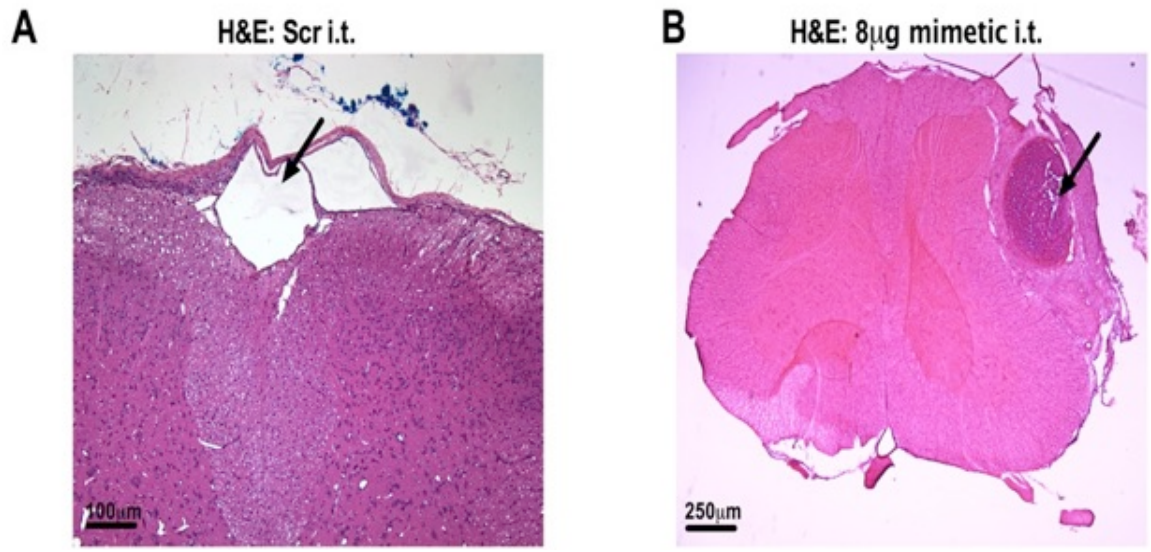
Supplemental Results

Intrathecal administration of miR-132 *induces recruitment of immune cells*

I.t. administration of 8µg of the miR-132 mimetic caused recruitment of inflammatory cells to the site of the catheter tip resulting in a dense cellular infiltration compressing the spinal cord and likely caused the observed motor deficits (supplemental Figure 1). We observed staining for MAC387 positive cells indicative of peripheral macrophage infiltration only at the catheter site (supplemental Figure 2). No such infiltration was found in Scr-injected rats.

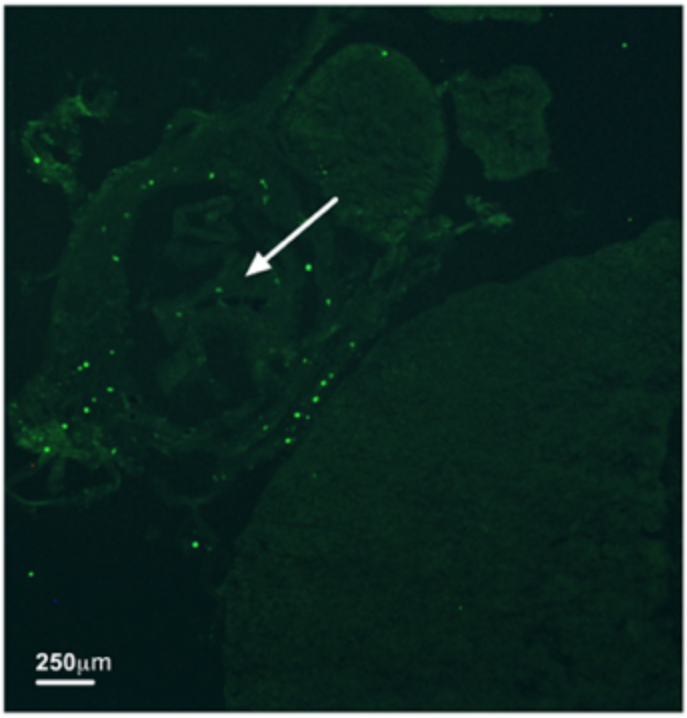
MiR-132 targets GluA1

To confirm validity of GluA1 as a functional target of miRNA-132-3p and to modulate its expression, we constructed a luciferase reporter vector in which the 3'UTR of GluA1 mRNA was fused to the luciferase coding sequence under a SV40 promoter in a dual luciferase vector (pEZX-MT06 vector, Genecopoeia®). In a HEK293 cell-based heterologous expression system, either a miR-132-3p antagonist or mismatch inhibitor (Scr) was co-transfected with the GluA1 reporter vector. In a parallel experiment, either miR-132-3p mimic or non-targeting mimic (Scr) was co-transfected with the GluA1 reporter vector and luciferase expression was measured 48h thereafter. Administration of miR-132-3p antagonist increased the translation of luciferase protein from the GLuA1 reporter construct in comparison to Scr, whereas expression of the miR-132-3p mimetic exerted the opposite effect (supplemental Figure 3, $p<0.05$). These results demonstrate that miR-132-3p directly targets the GLUA1 3'UTR region to modulate its expression.



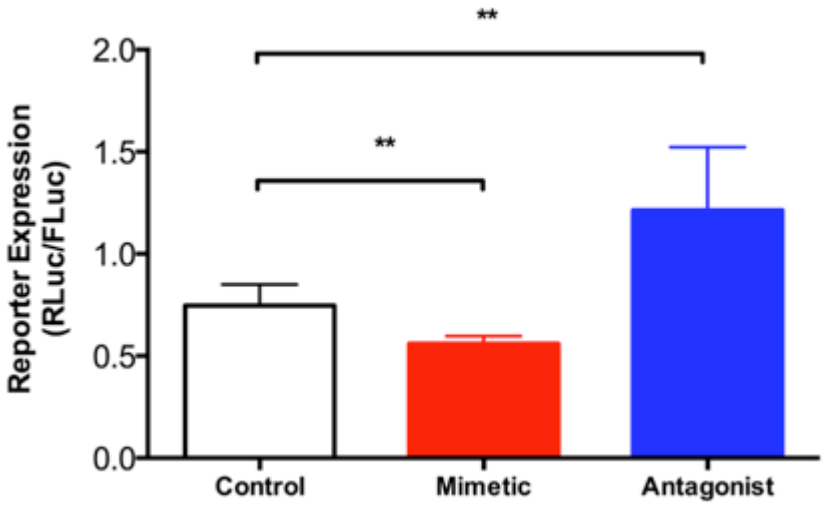
Supplemental Figure 1

L5 spinal cord below level of catheter tip, H&E stained A) Scr-oligo injection. B) Injection of 8μg miR-132 mimetic causes cellular infiltration just below catheter tip (arrow) and compression (higher power magnification, 8μg) of spinal cord, leading to overt motor deficits. H&E staining revealed unknown mass formation in drug injected animals, likely to be involved in the observed behavioral effects. Scale bars represent 100μm and 250μm, respectively.



Supplemental Figure 2

Spinal infiltration of immune cells after exogenous administration of miR-132 at the site of the catheter tip. Macrophage marker MAC387 was indicative of infiltration of peripheral macrophages. Increased signal is observed at the catheter tip (arrow). Scale bar represents 250 µm.



Supplemental Figure 3

Luciferase-reporter based assay in HEK293 cells was performed to confirm binding of miR-132 to the 3'UTR of GluA1. A) The assay demonstrates changes in translation of the GluA1 gene following suppression or induction of miR-132-3p expression via graded delivery of the specific mimetic or inhibitor as compared to control (t-test, n=6/gp); values are expressed as Reporter expression Rluc/Fluc.

Chapter 4

Increased cutaneous miR-let-7d expression correlates with small nerve fiber pathology in patients with fibromyalgia syndrome

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Chapter 4: Leinders M., Doppler K., Klein T., Deckart M., Rittner H., Sommer C., Üçeyler N. (2016). Increased cutaneous miR-let-7d expression correlates with small nerve fiber pathology in patients with fibromyalgia syndrome. Pain Journal

Abstract

Fibromyalgia syndrome (FMS) is a chronic widespread pain condition probably comprising subgroups with different underlying pathomechanisms. There is increasing evidence for small nerve fiber impairment in subgroups of patients with FMS. MicroRNAs (miRNA) regulate molecular factors determining nerve de- and regeneration. We investigated whether systemic and cutaneous miRNA expression in FMS patients is related to small nerve fiber pathology. We confirmed previous findings of disturbed small fiber function and of reduced intraepidermal nerve fiber density in subgroups of patients with FMS. We found 51 aberrantly expressed miRNAs in white blood cells (WBC) of FMS patients, of which miR-let-7d correlated with reduced small nerve fiber density in FMS patients. Furthermore, we demonstrated miR-let-7d and its downstream target insulin-like growth factor-1 (IGF-1R) as being aberrantly expressed in skin of FMS patients with small nerve fiber impairment. Our study gives further evidence of small nerve fiber pathology in FMS subgroups and provides a missing link in the pathomechanism that may lead to small fiber loss in subgroups of FMS patients.

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Research Paper

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Increased cutaneous miR-let-7d expression correlates with small nerve fiber pathology in patients with fibromyalgia syndrome

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Abstract

Fibromyalgia syndrome (FMS) is a chronic widespread pain condition probably comprising subgroups with different underlying pathomechanisms. There is increasing evidence for small nerve fiber impairment in subgroups of patients with FMS. MicroRNAs (miRNAs) regulate molecular factors determining nerve de- and re-generation. We investigated whether systemic and cutaneous miRNA expression in patients with FMS is related to small nerve fiber pathology. We confirmed previous findings of disturbed small fiber function and reduced intraepidermal nerve fiber density in subgroups of patients with FMS. We found 51 aberrantly expressed miRNAs in white blood cells of patients with FMS, of which miR-let-7d correlated with reduced small nerve fiber density in patients with FMS. Furthermore, we demonstrated miR-let-7d and its downstream target insulin-like growth factor-1 receptor as being aberrantly expressed in skin of patients with FMS with small nerve fiber impairment. Our study gives further evidence of small nerve fiber pathology in FMS subgroups and provides a missing link in the pathomechanism that may lead to small fiber loss in subgroups of patients with FMS.

Keywords: Fibromyalgia syndrome, MicroRNA, miR, Microarray, Skin biopsy, miRNAlet-7d, IGF-1R

1. Introduction

Fibromyalgia syndrome (FMS) is a clinically well-characterized chronic widespread pain condition, regularly accompanied by symptoms such as chronic fatigue, sleep disturbances, and depressive episodes.[35] However the pathophysiology of pain in FMS is still incompletely understood. Factors that are possibly involved in the pathophysiology of pain in FMS are impairment of central pain processing pathways, a dysfunctional hypothalamic–pituitary–adrenal axis,[77] an imbalance of the immune system,[11,83,89] and the recently described alterations in small nerve fibers (A-δ and C-fibers). So far, no differences in clinical or electrophysiological aspects have been found between patients with FMS with normal and reduced intraepidermal nerve fiber density (IENFD). This may be due to the low numbers of patients with FMS who have been investigated with regard to IENFD in the individual studies, hindering adequate statistical analysis. Indeed, several recent studies focused on small fiber impairment in patients with FMS and found a reduction of the IENFD in a subgroup of about 50% of patients.[14,21,22,49,60,84] The exact mechanism behind this observation still remains elusive, and the question is which systemic or local factors may contribute to nerve fiber damage.

Noncoding RNAs, including microRNAs (miRNAs, miR-) have been implicated in normal cellular functioning as well as pathological processes.[39,58] MiRNAs are small noncoding RNAs that posttranscriptionally regulate gene expression. Various diseases, including neuropathic pain disorders, appear to posses unique miRNA expression signatures.[50] MiRNAs control multiple cellular pathways and act as "master switches," including the control of genes that encode cellular enzymes, receptor proteins and ion channels, all being involved in the pathophysiology of chronic pain. Several pain conditions have recently been shown to be associated with deregulated expression levels of distinct miRNAs in specific pain pathways, from primary afferent nociceptors in the periphery to brain areas associated with the emotional components of pain perception.[1,2,42,52,88] Furthermore, aberrant systemic miRNA expression patterns in patients with FMS have been reported and shown to correlate with the various comorbidities associated with FMS.[8,9] However, so far no study focused on the connection between miRNA expression and small fiber pathology in FMS. Here, we investigated systemic and cutaneous miRNA expression in blood and skin samples of clinically well-characterized patients with FMS and compared our results with age- and gender-matched healthy controls. We hypothesized that patients with FMS show a unique systemic miRNA expression profile, which in turn might translate to impaired skin miRNA homeostasis as the basis of peripheral nerve fiber pathology in FMS.

2. Materials and methods

2.1. Patients and controls

Thirty patients (28 women, and 2 men; **Table 1**) with a diagnosis of FMS attending the pain center "Klinik Am Arkauwald," Bad

Sponsorships or competing interests that may be relevant to content are disclosed at the end of this article.

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Table 1

Demographics and clinical data of patients with fibromyalgia syndrome (FMS) and healthy controls.

	FMS (n = 30)	Healthy controls (n = 34)
Male, female	2, 28	4, 30
Median age (range), y	51 (39-74)	53.5 (24-67)
Median disease duration (range), y	12 (2-40)	NA
Antidepressants, yes	20 (66%)	0 (0%)
Analgesics, yes	28 (93%)	0%
Questionnaires		
Median NPSI-G sum score (range)	0.3 (0.05-0.78)	0
NPSI-G discriminative score (range)	57 (39-80.4)	42
Median GCPS score (average recall 4 wk) (range)	6.5 (3-9)	0
Median FIQ score (range)	65.4 (36.0-84.3)	0
Median ADS score (range)	25 (7-51)	0
Nerve conduction studies		
Sural nerve		
Sensory nerve action potential, μV	17 (9-35)	NA
Nerve conduction velocity, m/s	48.2 (43.4-72.3)	NA
Tibial nerve		
Distal compound motor action potential, mV	18 (10.0-33.4)	NA
Distal motor latency, ms	3.5 (2.8-4.7)	NA
Nerve conduction velocity, m/s	47 (40.6-55.0)	NA
Morphology		
Median distal IENFD	6.3 (1.8-15.9)	
Median proximal IENFD (range), fibers/mm	10.4 (3.7-20.6)	

ADS = "Allgemeine Depressionsskala," ie, German version of the Center for Epidemiologic Studies Depression Scale; FIQ, Fibromyalgia Impact Questionnaire; GCPS, Graded Chronic Pain Scale; IENFD, intraepidermal nerve fiber density (fibers/mm; normal values lower leg Würzburg laboratory: 9 ± 3 fibers/mm; normal IENFD ≥6 fibers/mm: 14/28, reduced IENFD <6 fibers/mm: 14/28); NPSI-G, german version of the neuropathic pain symptom inventory.

Mergentheim, Germany (16/30) or the day care pain clinic of the Department of Anesthesiology, University of Würzburg (14/30) were prospectively recruited between 2013 and 2014. Diagnosis was based on the 1990 American College of Rheumatology diagnostic criteria.[91] Data from 26 of these patients were included in a previous study on the dermal nerve fiber ultrastructure.[22] In addition, 34 age- and gender-matched healthy controls (30 women, and 4 men; **Table 1**) were recruited between 2013 and 2014 for blood miRNA analysis at the Department of Neurology, University of Würzburg (supplemental Fig. 1, available online at http://links.lww.com/PAIN/A315). Our study was approved by the Ethics Committee of the University of Würzburg Medical Faculty. All patients and controls gave written informed consent to take part in the study.

2.2. Clinical examination, questionnaire assessment, and laboratory tests

All patients underwent neurological examination and were assessed with questionnaires for pain and impairment due to FMS symptoms. These questionnaires included the German versions of the following: the Neuropathic Pain Symptom Inventory (NPSI-G),[12,78] the Graded Chronic Pain Scale (GCPS),[87] and the Fibromyalgia Impact Questionnaire (FIQ).[13,61] The NPSI-G analyses neuropathic pain intensity and quality resulting in a sum score between 0 (no pain) and 1 (maximum pain) in combination with subscores for different pain characteristics. The NPSI-G discriminative score was used to distinguish neuropathic from nonneuropathic pain, resulting in a score ranging from 42.4 to 80, with a cutoff value at 53.5 for neuropathic pain.[78] The GCPS rates pain intensity on a scale from 0 to 10 and grades the pain disability from 0 to 4. The FIQ rates the impact of FMS symptoms on the health status of a patient, scores range from 50 (moderately affected patient with FMS) to ≥70 (severely affected patient with FMS). In addition, all patients were examined for depressive symptoms using the German translation of the Center for Epidemiologic Studies Depression Scale ("Allgemeine Depressionsskala," ADS).[69] The ADS ranges from 0 to 60 with a cutoff score of ≥16, implying clinically significant depressive symptoms. Questionnaire data were compared with data of the n = 34 healthy controls recruited for blood miRNA analysis. To exclude large fiber involvement and other peripheral nerve pathology, nerve conduction studies of the right tibial (motor) and sural (sensory) nerve were performed in patients with FMS according to standard procedures.[47] For evaluation of small fiber involvement, patients underwent quantitative sensory testing (QST) and a 5-mm skin punch biopsy was obtained for IENFD assessment and cutaneous miRNA expression analysis from the lateral lower leg and the lateral upper thigh. In addition, an oral glucose tolerance test was conducted in all study participants to exclude pathological glucose tolerance or diabetes mellitus.

2.3. Quantitative sensory testing

The quantitative sensory testing was performed following a standardized procedure in all patients (Somedic, Hörby, Sweden).[71] Test values of all patients were compared with separately investigated age- and gender-matched healthy controls of our laboratory by transforming the obtained raw values for each QST item into a *z*-score sensory profile. This control group consisted of 56 age- and gender-matched healthy volunteers from our database (52 women, and 4 men; median age range 52 years, 23-78 years). *Z*-scores were calculated as follows: *z*-score = (value of the subject − mean value of controls)/SD of controls. Negative *z*-scores represent loss of function; positive *z*-scores indicate gain of function. The following parameters were assessed: cold and heat detection thresholds (CDT, and HDT), the ability to detect temperature changes (thermal sensory limen, TSL), mechanical detection and pain thresholds (MDT and MPT), mechanical pain sensitivity, pressure pain threshold (PPT), paradoxical heat sensation, and vibration detection threshold.

2.4. Blood withdrawal and miRNA isolation

To reduce circadian variation, venous blood was collected from all subjects between 8:00 AM and 9:00 AM after overnight fasting. None of the tested subjects had any clinical signs of ongoing infection. For quantitative real-time PCR (qRT-PCR), 9 mL of whole blood was withdrawn in EDTA-containing tubes, and the total white blood cell (WBC) fraction was extracted. Isolated WBCs were resuspended in the RNAProtect Cell Reagent (Qiagen, Hilden, Germany) and stored at −80°C until further processing. MiRNAs were isolated from all WBC samples using the miRNeasy kit (Qiagen) and following the manufacturer's protocol.

2.5. Skin biopsies and cutaneous miRNA isolation

For the assessment of IENFD, skin punch biopsies of 5-mm diameter (distal lateral lower leg and proximal lateral upper thigh) were obtained in 27 of 30 patients and processed as described earlier[81]; 2 patients refused to undergo biopsy. To determine IENFD, 50-μm skin sections were prepared and immunostained

with the antibodies against the pan-axonal marker protein-gene product 9.5 (1:1000; Ultraclone, Isle of Wight, United Kingdom) and reacted with an appropriate fluorescent secondary antibody (Cy3, 1:100; Dianova, Hamburg, Germany). The intraepidermal nerve fiber density was quantified by an observer blinded to subject's diagnosis and following published rules,[53] using a fluorescence microscope (Axiophot 2; Zeiss, Oberkochen, Germany) with an Axiocam MRm camera (Zeiss), and SPOT software (Diagnostic Instruments, Inc, Sterming Heights, MI). As reference values, we used our laboratory normative values obtained from 54 control subjects for the lower leg (50 women, and 4 men; median age: 43 years, range 16-70 years, IENFD 8.5 fibers/mm, range 6.1-15.3) and 34 control subjects for the upper thigh (30 women, and 4 men; median age 44 years, range 22-79 years, IENFD 12.5 fibers/mm, range 9.0-19.9).

For the analysis of both cutaneous miRNA and target gene expression, 10 cryosections of 10 μm, were obtained and frozen at −80°C until further processing. For the extraction of total mRNA, frozen cryosections were processed using the RNeasy Micro kit (Qiagen) and on-column miRNA fraction enrichment following the manufacturer's recommendation with modifications.

2.6. MicroRNA array profiling

We used the Exiqon miRCURY LNA miRNA array profiling service (Exiqon Services, Vedbaek, Denmark) to obtain WBC miRNA expression profiles of 12 patients with FMS and 12 healthy age-matched controls. Selection of 12 patients with FMS for miRNA array profiling was based on age (median age 48 years, range: 39-60), disease duration (median disease duration 18 years, 10-27), current pain intensity (GCPS; median pain intensity 6, 3-8), and IENFD status (lower leg IENFD <6 fibers/mm, ie, reduced IENFD). Exiqon verified the quality of total RNA with an Agilent 2100 Bionanalyzer profile. Total RNA of 750 ng was labeled with Hy3 and Hy5 fluorescent labels, using the miRCURY LNA miRNA Hi-Power Labeling Kit, Hy3/Hy5 (Exiqon). The labeled samples were hybridized to the miRCURY LNA miRNA Array seventh gen (Exiqon), registered in the miRBase 19.0 version that covered 2042 human miRNA probes. The miRNA array slides were scanned using the Agilent G2565BA Microarray Scanner System (Agilent Technologies, Inc, Santa Clara, CA), and the image analysis was performed using the ImaGene 9 (miRCURY LNA miRNA Array Analysis Software; Exiqon). The quantified signals were background corrected (Normexp with offset value 10) and normalized using the global Lowess (LOcally WEighted Scatterplot Smoothing) regression algorithm. The criteria for candidate selection and individual validation were miRNA fold change (FC; ±1 log-fold change), statistical significance after Benjamini–Hochberg correction, and sufficient array signal intensity (7.5-14.5). For individual target analysis of differentially expressed miRNAs and to identify genes that represent putative targets, a prediction analysis was performed by comprehensively using 4 state-of-art algorithms, namely TargetScan,[29] miRNA.org,[6] miRTarBase,[38] and DIANAmicroT.[64] We chose miR candidates who showed the highest significance ($P < 0.05$) for intergroup differences after Benjamini–Hochberg correction and were found to be associated with pain and nerve fiber degeneration in the literature.

2.7. MicroRNA expression analysis

For the generation of miRNA-specific first-strand cDNA of RNA extracted from WBC and skin, 5 ng of total RNA was reverse transcribed using the Universal cDNA Synthesis kit II (Exiqon) following manufacturer's recommendations. For each reaction, 4 μL of diluted (1:80) cDNA was PCR amplified using the corresponding miRNA and reference primer sets, using the miCURY LNA Universal miRNA PCR (Exiqon). To determine expression level of the following miRNAs, specific miCURY LNA assays with the respective assay IDs were used: hsa-miR-let-7d (5′-3′AGAGGUAGUAGGUUGCAUAGUU, MIMAT0000065), hsa-miR-103 (5′-3′AGCAGCAUUGUACAGGGCUAUGA, MIMAT 0000101), hsa-miR-151 (5′-3′CUAGACUGAAGCUCCUUGAGG, MIMAT0000757), and hsa-miR-199a (MIMAT0000232, ACAGUA GUCUGCACAUUGGUUA), and normalized to the expression of endogenous 5 seconds RNA (5 seconds RNA, V00589). Each miRNA was amplified in triplicate and Ct values were obtained. Fold changes in miRNA expression among groups were calculated using interplate calibrators by means of the delta-delta Ct method. For individual target verification, we tested different endogenous controls (U6, snord48, snord44, and 5sRNA) of which 5sRNA was the most stable in both groups and was further used.

2.8. Gene expression analysis and downstream target verification

For gene expression analysis of miRNA downstream targets, TaqMan qRT-PCR (Applied Biosystems, Darmstadt, Germany) was used. All PCR reagents and cyclers were used from Life Technologies (Carlsbad, CA). One hundred ng of RNA was reverse transcribed to cDNA using TaqMan Reverse Transcription Reagents, following manufacturer's protocol. For each sample, 5-μL cDNA was used in qRT-PCR, which was performed using a StepOnePlus cycler (Applied Biosystems). Gene expression of insulin-like growth factor-1 receptor (IGF-1R, Assay ID: Hs00609566_m1) was investigated in skin biopsies of all patients with FMS and healthy controls. As an endogenous control, 18sRNA (Assay ID: Hs99999901_s1) was used.

To visualize cutaneous IGF-1R expression, we applied an antibody against IGF-1R (IGFR-I, 1:200; Abcam, Cambridge, United Kingdom). Frozen sections of 16 μm were stained using standard immunohistochemistry (ABC kit; Vector, Westheim, Germany). The amount of epidermal IGF-1R expressed as mean intensity per area was measured using ImageJ 1.49 software (http://imagej.nih.gov/ij/) in accordance with a previously described method.[86] The expression of IGF-1R in distal skin of all patients with FMS was compared with those of healthy age- and gender-matched controls.

2.9. Statistical analysis

SPSS Statistics 22 software (IBM, Ehningen, Germany) was used for statistical analysis. The nonparametric Mann–Whitney *U* test was applied when data were not normally distributed, which was the case for IENFD and qRT-PCR measurements. A *t* test was used for comparison of *z*-scores of QST data. Correlations were assessed, using the bivariate Spearman correlation. After quantile background correction and global Lowess normalization to correct for systematic differences between arrays and experimental groups,[10,17] Benjamini–Hochberg correction was performed to obtain significantly differentially expressed miRNAs in the miRNA array. Data were expressed as FC (2^logFC). Statistical significance was accepted with $P < 0.05$.

3. Results

3.1. Clinical and laboratory findings

Table 1 gives demographic and baseline data of the patients with FMS cohort and healthy controls. No study participant had any indicator of ongoing infection, and no patient had diabetes or

prediabetes. Neurological examination was normal in all cases. Patients with FMS had higher NPSI-G median sum scores (0.3; range 0.05-0.78; $P < 0.0001$) and NPSI-G discriminative scores (57; range 39-80.4; $P < 0.0001$) than healthy controls (NPSI-G sum score: 0; NPSI-G discriminative score: 42), higher median pain ratings as averaged over 4 weeks (6.5, 3-9 of 10 on a numeric rating scale; $P < 0.0001$) and higher FIQ scores (65, range 36-84; $P < 0.0001$) than healthy controls (GCPS and FIQ: 0). In addition, patients with FMS revealed higher ADS scores (25; range 7-51; $P < 0.0001$) than healthy controls (ADS: 0). Nerve conduction studies showed normal values for all parameters analyzed; in 1 patient (#16), tibial nerve conduction studies were technically hampered by patient's constitution: because of a high body mass index of 42 kg/m^2, supramaximal stimulation of the tibial nerve was not possible; however, sural nerve conduction studies were normal, and the patient had no clinical signs of large fiber neuropathy.

3.2. Patients with FMS have impaired small fiber function

Detailed sensory examination revealed thermal hypesthesia in 12 of 30 patients (supplemental Table 1, available online at http://links.lww.com/PAIN/A315) as measured with QST. Patients with FMS had elevated CDT ($P < 0.01$), warm (WDT; $P < 0.05$), and MDT (**Fig. 1A**, $P < 0.01$) at the dorsal foot when compared with healthy controls. As expected,[22,70,84] PPTs were decreased when compared with healthy controls (**Fig. 1A**, PPT; $P < 0.001$). To control for an influence of opioid intake on QST results, we compared QST data of patients who were treated with opioids at the time of the investigation (n = 11) with those who were not (n = 19). No intergroup difference was found except for TSL, which was lower in patients using opioids compared with those who did not ($P = 0.036$), ie, patients using opioids detected temperature differences faster. Since both subgroups individually were not different from controls, this intergroup difference may not be clinically relevant.

3.3. Skin innervation is reduced in patients with FMS

We previously reported a reduction of epidermal innervation in approximately 50% of our FMS study cohort.[84] Here, we confirm this finding: in 50% of the patients with FMS, we found a reduction of lower leg skin innervation (<6 fibers/mm, 14/28 patients with FMS, median 4.7 fibers/mm, range 1.8-6.0) compared with a normal (≥6 fibers/mm, 14/28, median 9.3 fibers/mm, range 6.6-15.9) IENFD in the other subgroup. At the lower leg, IENFD of patients with FMS was reduced compared with healthy controls (FMS: 6.3 fibers/mm, range 1.8-15.9, control subjects: 8.5 fibers/mm, range 6.1-15.3, $P < 0.01$). Also at the upper thigh, IENFD of patients with FMS was lower than that in healthy controls (FMS: 10.4 fibers/mm, range 3.7-20.5, control subjects: 12.5 fibers/mm, range 9.0-19.9; $P < 0.01$).

3.4. Impaired small fiber function in patients with FMS is related to reduced intraepidermal nerve fiber density

Patients were not different for any questionnaire parameter analyzed when subgrouped into those with reduced (14/28) and those with normal IENFD (14/28). However, when subgrouped, CDT and MDT were elevated only in patients with reduced IENFD (**Fig. 1B**, $P < 0.01$). Moreover, PPT was reduced in patients with FMS with reduced IENFD when compared with patients with normal IENFD (**Fig. 1B**, $P < 0.05$). Interestingly, WDT was not different between patients with FMS with normal and reduced IENFD (**Fig. 1B**); 10/14 patients with FMS with reduced IENFD and 10/14 patients with FMS with normal IENFD showed increased WDT.

3.5. Patients with FMS show aberrant genome-wide miRNA expression in white blood cell

Next, we measured the genome-wide expression of miRNAs in WBC of 12 patients with reduced IENFD (<6 IENFD) and 12 age- and gender-matched healthy controls (see miRNA array profiling). Supplemental Table 2 provides an overview of the individual data on RNA quality (available online at http://links.lww.com/PAIN/A315). The overall RNA integrity number values were ≥7, indicating good quality and low fragmentation of total RNA. Mean signal intensities for each miRNA were compared across WBCs obtained from patients with FMS with the group of healthy controls. Since 114 miRNAs showed differential expression with a P-value <0.05, we sought to identify the most prominent changes by focusing on miRNAs which showed the highest FC (upregulation or downregulation) in expression and with most stringent array and biological replicate standards. Fifty-one miRNAs survived these standards and are depicted by a heat plot (supplemental Table 3, available online at http://links.lww.com/PAIN/A315; **Fig. 2**) comparing patients with FMS with healthy controls. Four miRNAs were prominently downregulated and 47

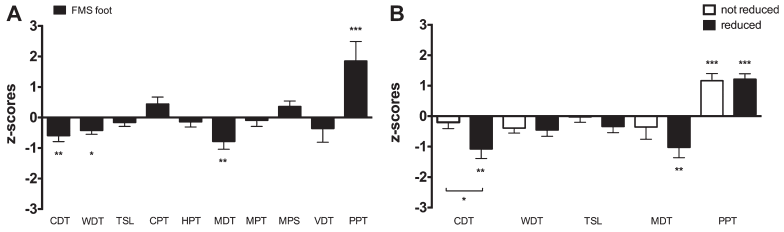

Figure 1. (A) Quantitative sensory testing profile of patients with fibromyalgia syndrome (FMS, n = 30) and (B) selected parameters subgrouped into patients with reduced (n = 14) and normal (n = 14) intraepidermal nerve fiber density compared with age- and gender-matched healthy control subjects (black zero-line). (A) Patients with FMS have elevated thermal detection thresholds (CDT and WDT). Also, the mechanical detection threshold (MDT) is elevated, whereas pressure pain thresholds (PPTs) are decreased. (B) Patients with reduced intraepidermal nerve fiber density have lower CDT and MDT compared with patients with normal nerve fiber density, while PPT is decreased in both groups when compared with controls (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$); n = 30 patients and n = 56 healthy controls. CDT, cold detection threshold; CPT, cold pain threshold; HPT, heat pain threshold; MPS, mechanical pain sensitivity; MPT, mechanical pain threshold; PPT, pressure pain threshold; TSL, thermal sensory limen; VDT, vibration detection threshold; WDT, warm detection threshold.

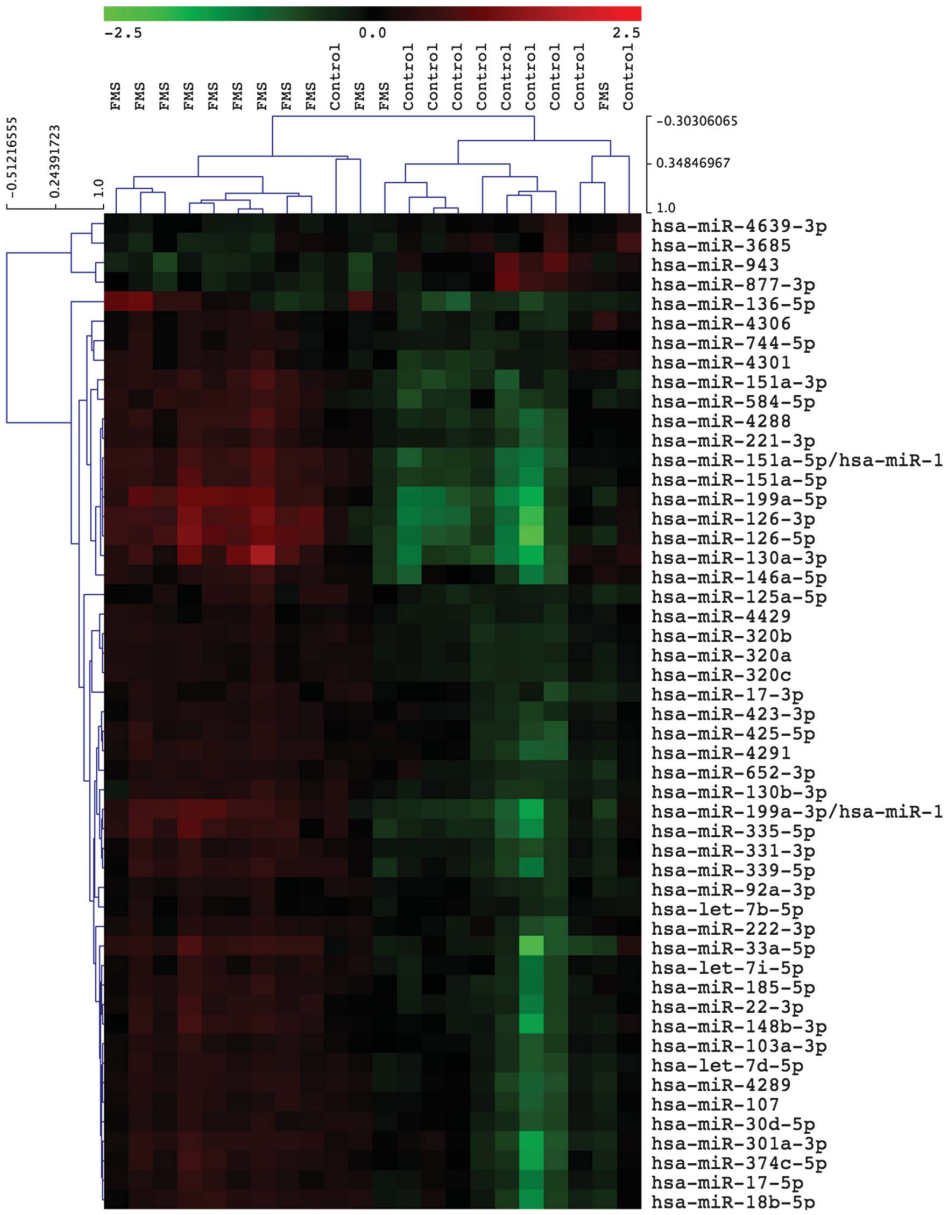

Figure 2. Heat map of miRNAs found to be significantly upregulated or downregulated using microarray analysis in patients with fibromyalgia syndrome (FMS) compared with healthy controls. Scale indicates expression intensities obtained from the microarray experiment.

were upregulated in WBC of patients with FMS compared with healthy controls. Microarray profiling data were validated with independent qRT-PCR for those targets (**Table 2** and **Fig. 3**) that showed promising literature-based association with pathways in the pathophysiology of pain. We tested the potential impact of poor RNA quality on the observed differences and did not find any correlation between individual RNA integrity number data and FCs of the selected candidates.

3.6. MiR-let-7d in white blood cell correlates with pain and impairment of small fiber function in patients with FMS

Figure 3 depicts individual miRNA validation of the predicted targets in WBC of patients with FMS when compared with healthy controls. All the following analyzed targets: miR-103 (**Fig. 3A**, −0.52 FC, $P < 0.001$), miR-146a (**Fig. 3B**, −0.5 FC, $P < 0.01$), and miR-let-7d (**Fig. 3C**, −0.7 FC, $P < 0.001$) were downregulated when measured in the entire FMS cohort. MiR-151 did not show any differential expression (data not shown). Although the microarray data (n = 12

Table 2

Summary of differentially expressed miRNAs in the microarray that were chosen for validation.

miRNA	Fc	BH-corrected *P*
hsa-miR-199a	2.3	0.004
hsa-miR-151	1.8	0.004
hsa-miR-103	1.3	0.02
hsa-miR-let-7d	1.3	0.02
hsa-miR-146a	1.4	0.03

BH, Benjamini–Hochberg correction; Fc, fold change.

patients with FMS with reduced IENFD vs n = 12 controls) suggested an upregulation of miR-let-7d, the individual validation using the same patient group indicated lower expression. Interestingly, in patients with FMS, miR-let-7d gene expression in WBC positively correlated with IENFD ($P < 0.05$, n = 30, $r^2 = 0.2$) and the mean pain intensity over the last 4 weeks (GCPS, $P < 0.05$, n = 30, $r^2 = 0.23$), the latter in particular among patients with reduced IENFD ($P < 0.05$, n = 14, $r^2 = 0.4$).

3.7. MiR-let-7d expression is higher in skin biopsies of patients with FMS with reduced intraepidermal nerve fiber density

To assess a potential influence of miR-let-7d on IENFD in patients with FMS, we set out to measure miR-let-7d expression directly in skin biopsies of patients with FMS and healthy controls. MiR-let-7d expression was higher in patients with FMS than that in healthy controls (**Fig. 4A**, 1.72 FC, $P < 0.001$), in particular in the subgroup with reduced distal IENFD (**Fig. 4B**, 2.64 FC, $P < 0.05$). No difference was observed in miR-let-7d expression when compared with normal IENFD (data not shown). In addition, no differential expression was observed for miR-103 when comparing patients with FMS and healthy controls. Next, we followed an unbiased and comprehensive approach to predict the mRNA targets for miR-let-7d by adapting different state-of-art algorithms.[16,33,64,85] The respective results of the individual prediction algorithms are provided by the following links:

TargetScan (http://www.targetscan.org/cgi-bin/targetscan/vert_71/view_gene.cgi?rs=ENST00000268035.6&taxid=9606&showcnc=0&shownc=0&shownc_nc=&showncf1=&showncf2=&subset=1), microRNA.org (http://www.microrna.org/microrna/getMrna.do?gene=3480&utr=31402&organism=9606#hd) and miRTarBase (http://mirtarbase.mbc.nctu.edu.tw/php/detail.php?mirtid=MIRT005364#target). Among the different targets, IGF-1R presented an interesting protein that is known to be regulated by miR-let-7d.[44,95] Indeed, when comparing the expression of IGF-1R in patients with FMS, IGF-1R mRNA levels were lower in skin biopsies from the lower leg of patients with FMS compared with skin biopsies from the upper thigh (**Fig. 4C**, $P < 0.001$). When comparing distal to proximal IGF-1R expression in healthy controls, no difference was observed. Moreover, patients with FMS with reduced IENFD (n = 14) had lower IGF-1R expression in the skin of the distal leg compared with patients with normal IENFD (n = 13, **Fig. 4D**, $P < 0.01$) and skin from the distal leg of healthy controls (**Fig. 4D**, n = 19 controls, $P < 0.05$). No difference in IGF-1R expression was observed between distal and proximal skin biopsies of healthy controls (data not shown).

3.8. Patients with FMS with reduced IENFD have lower skin insulin-like growth factor-1 receptor immunoreactivity than healthy controls

Figure 5A shows a representative image of IGF-1R immunofluorescence in the epidermal skin from the lower leg of a patient with FMS. Epidermal IGF-1R immunoreactivity appears to be membranous, mainly staining keratinocytes. The quantification of IGF-1R revealed lower IGF-1R immunoreactivity in skin samples of patients with FMS with reduced IENFD compared with healthy controls (**Fig. 5B**, $P < 0.05$), which might be linked to increased distal miR-let-7d expression. In addition, IGF-1R was lower in patients with FMS with reduced IENFD when compared with patients with FMS with normal IENFD (**Fig. 5B**, $P < 0.05$).

4. Discussion

Despite recent advances, understanding and treating chronic pain remains a major challenge for clinicians and preclinical scientists. Using a comprehensive method of combining patient data with molecular approaches in a clinically well-characterized patient cohort, this study showed that aberrant systemic miRNA expression profiles allow stratifying patients with FMS on the basis of specific regulatory miRNAs. We furthermore demonstrated the possible role of miRNAs in the reduction of intraepidermal innervation and regeneration of nerve fibers in patients with FMS.

In this study, we used a standardized and validated QST protocol[71] allowing the assessment of small fiber function, and found increased perception thresholds for thermal and

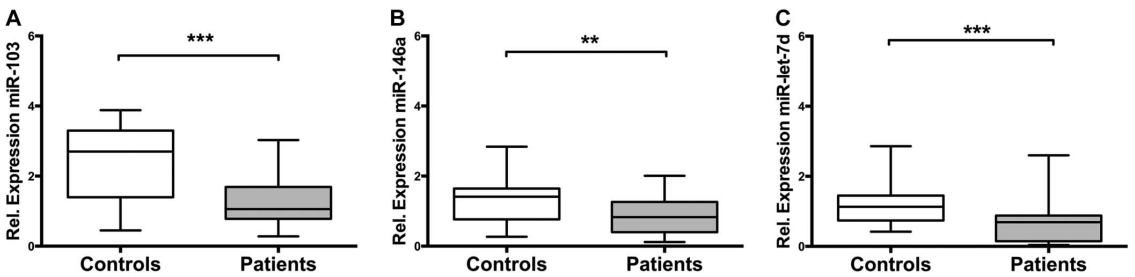

Figure 3. miRNA expression of (A) miR-103, (B) miR-146a, and (C) miR-let-7d in white blood cells of patients with fibromyalgia syndrome (FMS) and healthy controls. Expression of miR-103, miR-146a, and miR-let-7d was lower in patients with FMS compared with healthy controls, when measured over the entire cohort (**$P < 0.01$, ***$P < 0.001$); n = 30 patients and n = 30 healthy controls. Ct values for WBC expression of miR-103 (patients: median 19.30 [range 13.05-23.26]; controls: median 19.93 [range 19.23-24.69]), miR-146a (patients: median 22.87 [range 18.49-25.80] controls: median 22.54 [range 21.74-24.66]), and miR-let-7d (patients: median 25.51 [range 21.22-29.46]; controls: median 24.34 [range 20.55-27.26]).

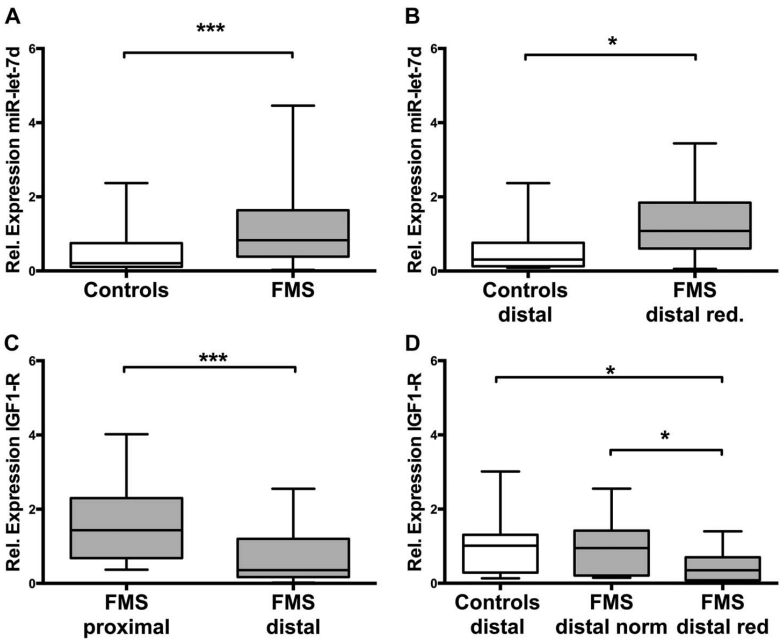

Figure 4. Gene expression of (A and B) miR-let-7d and (C and D) IGF-1R in skin biopsies of patients with fibromyalgia syndrome (FMS) and healthy controls. (A) miR-let-7d was higher in patients with FMS compared with healthy controls and especially in patients with reduced intraepidermal nerve fiber density (B). (C) Gene expression of the downstream target of miR-let-7d, IGF-1R was lower in the distal skin of the leg of FMS patients, when compared with the proximal skin. (D) IGF-1R gene expression was lower in patients with FMS with reduced intraepidermal nerve fiber density, when compared with healthy controls and patients with FMS with normal nerve fiber density (*$P < 0.05$, ***$P < 0.001$). For miR-let-7d in patients with FMS: skin from upper thigh n = 27, skin from lower leg n = 28; in controls, skin from upper thigh n = 19, skin from lower leg n = 21. For IGF-1R, n = 25 patients (upper thigh), n = 27 patients (lower leg), and n = 21 controls (upper thigh), n = 21 controls (lower leg). Distal = skin of the lower leg. Ct values for skin biopsy expression of distal miR-let-7d (patients: median 29.34 [range 23.91-34.30]; controls: median 29.22 [range 22.15-35.74]) and proximal miR-let-7d (patients: median 30.03 [range 26.52-34.87]; controls: median 28.87 [range 23.52-34.87]). IGF-1R, insulin-like growth factor-1 receptor, red, reduced intraepidermal nerve fiber density.

mechanical stimuli indicating small nerve fiber malfunction. As expected, we also found reduced PPT in patients with FMS, without differences between the subgroups with and without reduced IENFD. One caveat is that QST was performed under regular analgesic medication in the patient cohort, and although statistical analysis only revealed a difference in QST results for TSL between patients with and without opioids, data need to be interpreted with caution. Skin innervation as quantified in skin punch biopsies was reduced in 50% of the patients with FMS, and in line with previous reports,[22,31,60,74,84] this reduction was observed in the distal and proximal skin biopsies.

The elevated MDTs in our patients with FMS might be caused by functional changes in or loss of mechanosensitive C-fiber afferents. What is more difficult to understand are decreased pain thresholds in patients with a reduced density of nociceptive endings. Although some studies suggest central mechanisms to

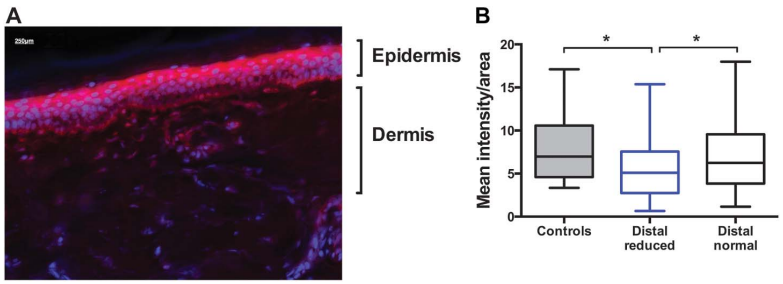

Figure 5. Immunoreactivity of IGF-1R in skin biopsies of patients with fibromyalgia syndrome (FMS) and healthy controls. (A) Representative photomicrograph of IGF-1R immunoreacted distal skin of a patient with FMS. IGF-1R (red) was mainly found in the epidermal and to a lesser extent in subepidermal layers. Nuclei are visualized with DAPI staining. (B) IGF-1R was lower in the distal skin of patients with FMS when compared with healthy controls and lower in patients with FMS with reduced small nerve fiber density compared with patients with normal nerve fiber density (*$P < 0.05$). n = 28 patients with FMS (skin from lower leg) and n = 25 controls (skin from lower leg). IGF-1R = insulin-like growth factor-1 receptor. Scale bar = 250 μm.

allow for this apparent discrepancy,[19,59] others suggest local factors such as proinflammatory and algesic cytokines[82] or reduced small fiber diameters as a correlate of nerve fiber lesion[22] that may influence peripheral hyperalgesia. In this study, we pursued the hypothesis of local factors influencing peripheral nerve homeostasis and proposed altered local and systemic miRNA expression as rationale for reduced nerve fiber density and associated symptoms in FMS.

Indeed, in a first attempt to characterize FMS based on altered miRNA expression profiles, others reported the dysregulation of several miRNAs in cerebrospinal fluid, serum, and peripheral blood mononuclear cells and surprisingly found individual correlations of selected miRNAs with FMS symptoms.[8,9,15] We took the screening of miRNAs in FMS to the next level, and first screened the expression of WBC miRNAs in a well-characterized group of patients with FMS. We show that FMS is associated with dysregulation of 55 miRNAs in WBC. Of these, 51 aberrantly expressed miRNAs, interestingly several miRNAs (miR-let-7d, miR-103, and miR-151) were in common with those reported previously.[8,9,15] Second, we show that 3 of our 5 selected candidates are differentially expressed when compared over the entire FMS cohort. All selected candidates have been shown to be associated with a variety of human diseases such as cancer,[26] neurodegenerative diseases,[51,54,55] stroke,[57] cardiovascular diseases,[3] and chronic pain.[27] Especially, the association with preclinical and clinical chronic pain is intriguing.[7,50,62,65] Of particular interest is miR-let-7d which was not only found to be dysregulated in FMS but also correlated with the mean pain intensity (GCPS) over the last 4 weeks and with a reduction of IENFD in patients with FMS. We thus measured the expression of miR-let-7d in distal and proximal skin biopsies of patients with FMS and found that miR-let-7d expression was higher in the distal skin of patients with FMS when IENFD was reduced.

Let-7d miRNAs are among the first miRNAs described to play important roles in cell proliferation, differentiation, and brain development and have recently been shown to be prognostic markers in several malignancies.[72] Aberrant miR-let-7d expression has been associated with chronic pain before[62] and shown to affect the endogenous opioid system and opioid tolerance.[37] We hypothesized that downregulated miR-let-7d in distal skin in FMS may account for reduced nerve fiber innervation in patients with FMS. Nerve regeneration is a complex spatiotemporal sequence of specific events involving the IGF-1/IGF-1R signaling pathway.[40,92] Insulin-like growth factor-1 is involved in muscle regeneration,[36,41] and also plays a key role in the peripheral nervous system,[25,34,43] controlling Schwann cell viability,[79] promoting motor neuron neurite outgrowth,[56,63] and regulating peripheral nerve regeneration[46,48,67,93] and inflammation.[76] Indeed, FMS has been associated with impaired growth hormone responses leading to reduced IGF-1 production,[4,5,20] but supplemental therapies have only been partially successful. Several miRNAs have been reported to regulate different components of the IGF pathway,[45] in which miR-let-7 arguably plays a key role. Indeed, IGFs-1/IGF-1Rs have been validated as downstream targets of miR-let-7d by others before. We thus measured miR-let-7d and IGF-1R mRNA in skin biopsies of patients with FMS and showed an increased miR-let-7d expression and a reciprocal decrease in IGF-1R mRNA, selectively in patients with reduced IENFD. Coherently, we found a reduction in IGF-1R immunoreactivity when IENFD was reduced. Furthermore, the downstream targets of miR-let-7d, IGFs-1/IGF-1Rs are major players in muscle regeneration.[23,66,68,90] Since pain in FMS is mostly described as deep muscle pain, muscle in patients with FMS has been investigated with various methods (histology, electromyography, metabolism, and imaging), however, so far without specific findings.[19] Our data might open a new avenue of studying the role of muscle in patients with FMS.

Taken together, there is strong evidence for small nerve fiber impairment in a subgroup of patients suffering from FMS, which is possibly linked to altered miRNA expression and concurrent decreased cutaneous IGF-1R signaling. However, since miR-let-7d is involved in a variety of physiological and pathophysiological processes, we cannot exclude actions on alternative pathways independent of IGF-1R. Interestingly, reduced axon diameters were recently reported in patients with FMS,[22] similarly to what had been observed in IGF-1–knockout mice.[30] Another intriguing possibility of individual variability in the severity of FMS symptoms and/or associated comorbidities may be explained by miRNA-related single nucleotide polymorphisms (SNPs). As for other diseases, SNPs could significantly alter the biogenesis and thus function of a given miRNA.[75] Indeed, SNPs in the let-7 gene have been shown to alter individual susceptibility to type 2 diabetes,[94] and SNPs in the miR-146a gene were shown to contribute to susceptibility to peripheral neuropathy.[18] Moreover, SNPs in the miRNA binding site of IGF-1R have been shown for other miRNAs before,[32] however, not for let-7 yet. Nonetheless, there is thus increasing evidence that genetic variants altering miRNA function point toward individual susceptibility in symptom-related diseases, which thus raises the interest in future studies on SNP-related miRNA changes in FMS.

Our study has several limitations, such as small sample size and different treatment regimens of patients with FMS. The fact that IENFD does not correlate with all QST parameters of small nerve fiber function is not surprising, since the pan-axonal marker protein-gene product 9.5 does not differentiate for nerve fiber function but gives a mere information on nerve fiber quantity. Thus, a "normal" IENFD may be associated with elevated perception thresholds if these fibers are functionally impaired, and a few but intact epidermal nerve fibers may be sufficient to maintain normal perception thresholds.[24,73,84] The currently applied techniques for bioinformatical prediction analyses using commercially available algorithms may lead to an overestimation and to the identification of nonfunctional targets. Interestingly, we observed many more increases than decreases of miRNA expression in our microarray analysis. One possibility to explain this finding is that miR-let-7d may be causally involved in miRNA metabolism and that the impact of its increase extends to many other miRNAs.[28,80] It furthermore remains elusive if and how reduced epidermal IGF-1R leads to decreased nerve fiber terminals in the distal skin of patients with FMS, and one can only speculate that decreased receptor expression leads to decreased peripheral nerve regeneration as has been shown before.[43] Further research is needed to provide direct evidence for if and how cells in the epidermal layer, such as keratinocytes may influence the regeneration of intraepidermal nerve fibers. Nonetheless, given the data detailed above, we assume a pathophysiologic role of miRNAs in a subgroup of patients with FMS. Moreover, our study together with previous findings[22,74,83,84] also may have important implications in improving diagnostic criteria of FMS. So far, FMS diagnosis is based on the subjective report of patients on painful areas and pain intensity. An objective biomarker would dramatically improve FMS diagnostics and also open new possibilities for targeted treatment. At present, it is too early to either use IENFD or miRNA measurements as a diagnosis for FMS, and more research is needed.

Conflicts of interest statement

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Appendix A. Supplemental Digital Content

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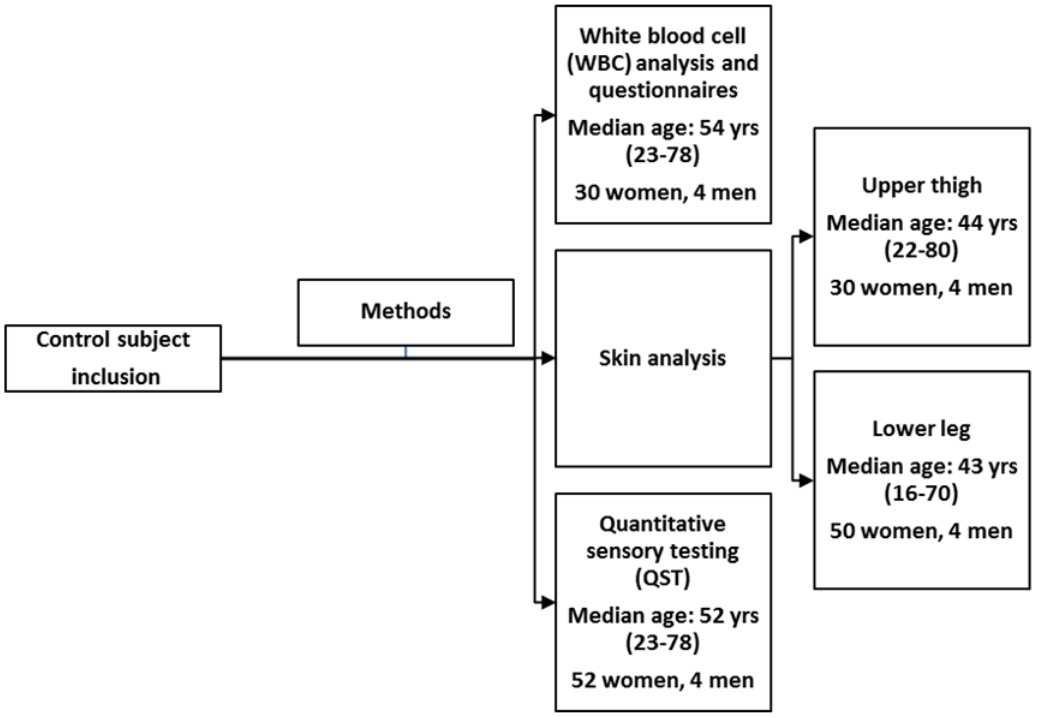
Supplemental Figures and Tables

Supplemental Figure 1



Supplemental Figure 1

Supplemental Table 1 (S1): Clinical profile of patients with fibromyalgia syndrome.

No.	Age	Sex	Disease Duration (years)	Abnormal QST	Distal IENFD (fibers/mm)	Proximal IENFD (fibers/mm)	Treatment	Relevant Comorbidity
1	42	F	10	-	9.3	13.2	NSAID	Hypothyroidism
2	50	F	27	CDT WDT	-	-	Pregabalin, Opioids,	None
3	52	F	7	CDT, WDT, TSL, PHS, HPT	3.9	5.3	NSAID, Lidocaine	Migraine
4	43	F	18	PPT	13.2	11.2	NSAID	Hypothyroidism
5	54	F	7	TSL	5.3	12.5	TCA, SSNRI	None
6	39	F	13	WDT, MPS	2.4	7.1	NSAID, TCA	Depression
7	48	F	23	PPT	15.9	6.2	NSAID, SSNRI	None
8	53	F	3	WDT, PHS	10	5.2	SSNRI, Opioids	Hypothyroidism Crohn's disease
9	44	F	2	PHS	14.0	19.4	SSNRI	None

10	54	F	10	CDT, WDT, TSL	8.3	6.9	NSAID, SSNRI	Hypothyroidism
11	52	F	9	-	4.7	10.4	NSAID, Opioids	Hypothyroidism
12	40	F	15	-	2.4	3.7	NSAID, Opioids, SSNRI, Gabapentin, TCA	None
13	48	F	26	PPT	8.5	-	NSAID, Opioids, Pregabalin	None
14	60	M	16	CDT, TSL	4.9	7.9	NSAID, TCA, Pregabalin	None
15	58	F	40	-	8.2	8.7	TCA, SSNRI	None
16	50	F	18	PHS	6.6	12.5	TCA, Gabapentin	Hypothyroidism
17	48	F	7	-	10.6	19.0	SSNRI, Opioids, Pregabalin	None
18	42	F	4	-	6.0	3.8	SSNRI, Opioids	Hypothyroidism
19	62	F	32	CDT	1.8	6.8	NSAID	None
20	74	F	30	PPT	4	12.3	TCA, Opioids	None

21	56	F	3	-	5.38	10.7	TCA	None
22	54	M	5	TSL, PHS	4.7	10.5	TCA	None
23	49	F	20	PPT	4.6	12.5	Opioids, TCA, Pregabalin	Spondylarthritis
24	72	F	8	PPT	14.5	20.6	NSAID, Opioids	None
25	41	F	15	PPT	5.3	9.3	NSAID	None
26	60	F	32	PPT, CDT	-	-	Pregabalin, TCA, Opioids	None
27	49	F	32	-	6.9	7.8	NSAID, SSNRI	None
28	54	F	5	PHS	7.4	10.8	TCA, pregabalin	None
29	55	F	11	TSL, PHS	5.4	11.56	None	None
30	48	F	6	TSL, PHS, PPT	12.2	7.9	SSNRI	Hypothyroidism

Abbreviations: CDT = cold detection threshold; CPT = cold pain threshold; FMS = fibromyalgia syndrome; HPT = heat pain threshold; MDT = mechanical detection threshold; MPS = mechanical pain sensitivity; MPT = mechanical pain threshold; NSAID = non steroidal anti-inflammatory drug; PPT = pressure pain threshold; SSNRI = selective serotonin reuptake inhibitor; TCA = tricyclic antidepressants; TSL = thermal sensory limen; VDT = vibration detection threshold; WDT = warm detection threshold

Supplemental Table 2 (S2): Individual Agilent Bioanalyzer results.

Sample	RNA concentration [ng/ul]	rRNA Ratio [28s/18s]	RNA Integrity Number (RIN)
FMS			
1	137	1.5	7.9
2	113	1.5	9.2
3	116	1.6	8.8
4	93	1.6	8.9
5	93	1.3	7.2
6	82	1.2	8.1
7	198	1.6	7.8
8	155	1.4	7.4
9	44	1.2	7
10	269	1.6	8.1
11	152	3.8	5.1
12	144	1.2	8.1
Controls			
1	91	1.4	7.4
2	116	1.6	8.9
3	154	1.6	9.1
4	43	1.6	9.3
5	81	1.6	8.8
6	154	1.6	9.1
7	185	1.4	6.8
8	162	1.7	7.9
9	203	1.8	8.2
10	161	1.4	8.7
11	201	1.7	8.2
12	161	1.6	7.9

Abbreviations: FMS = Fibromyalgia Syndrome; RIN = RNA integrity number; RNA = ribonucleic acid; rRNA = ribosomal ribonucleic acid.

Supplemental Table 3 (S3): Overview of the 51 differentially expressed miRNAs

miRNA	Average disease	Average Control	logFc	p-value	Rank	BH-corrected p-value
hsa-miR-4288	-0.383	0.329	0.713	0.0000225	1	0.0036
hsa-miR-151a-3p	-0.390	0.306	0.696	0.0000270	2	0.0036
hsa-miR-151a-5p/hsa-miR-151b	-0.522	0.406	0.928	0.0000348	3	0.0036
hsa-miR-151a-5p	-0.482	0.386	0.868	0.0000575	4	0.0038
hsa-miR-199a-5p	-0.688	0.528	1.215	0.0000722	5	0.0038
hsa-miR-943	0.301	-0.276	-0.577	0.0000898	6	0.0038
hsa-miR-126-3p	-0.696	0.545	1.241	0.0001033	7	0.0038
hsa-miR-221-3p	-0.296	0.253	0.549	0.0001143	8	0.0038
hsa-miR-185-5p	-0.290	0.256	0.546	0.0001230	9	0.0038
hsa-miR-584-5p	-0.359	0.253	0.612	0.0001237	10	0.0038
hsa-miR-320b	-0.197	0.172	0.369	0.0001423	11	0.0040
hsa-miR-4306	-0.170	0.178	0.348	0.0001700	12	0.0043
hsa-miR-425-5p	-0.237	0.206	0.442	0.0003483	13	0.0063
hsa-miR-126-5p	-0.696	0.521	1.217	0.0003498	14	0.0063
hsa-miR-320a	-0.175	0.130	0.305	0.0003526	15	0.0063
hsa-miR-130a-3p	-0.603	0.507	1.110	0.0003687	16	0.0063
hsa-miR-4429	-0.155	0.117	0.273	0.0003816	17	0.0063
hsa-miR-22-3p	-0.293	0.249	0.542	0.0003862	18	0.0063
hsa-miR-320c	-0.190	0.139	0.330	0.0003927	19	0.0063
hsa-miR-744-5p	-0.140	0.132	0.272	0.0005008	20	0.0077
hsa-miR-4301	-0.223	0.189	0.413	0.0005723	21	0.0083
hsa-miR-423-3p	-0.179	0.159	0.339	0.0007469	22	0.0104
hsa-miR-4291	-0.267	0.235	0.502	0.0007891	23	0.0105
hsa-miR-331-3p	-0.241	0.185	0.426	0.0009177	24	0.0117
hsa-miR-199a-3p/hsa-miR-199b-3p	-0.465	0.356	0.821	0.0011561	25	0.0139
hsa-miR-103a-3p	-0.207	0.173	0.380	0.0011842	26	0.0139
hsa-miR-136-5p	-0.450	0.222	0.672	0.0012974	27	0.0147
hsa-miR-130b-3p	-0.214	0.160	0.374	0.0016740	28	0.0178
hsa-let-7i-5p	-0.245	0.185	0.430	0.0018989	29	0.0192
hsa-miR-335-5p	-0.386	0.298	0.684	0.0019475	30	0.0192
hsa-miR-222-3p	-0.190	0.186	0.376	0.0020214	31	0.0193
hsa-let-7d-5p	-0.227	0.181	0.408	0.0021012	32	0.0195
hsa-miR-4289	-0.265	0.203	0.468	0.0024911	33	0.0220
hsa-miR-107	-0.231	0.181	0.412	0.0025162	34	0.0220
hsa-miR-148b-3p	-0.301	0.262	0.563	0.0035485	35	0.0299
hsa-miR-92a-3p	-0.146	0.119	0.265	0.0036785	36	0.0299
hsa-miR-4639-3p	0.102	-0.088	-0.190	0.0037176	37	0.0299

hsa-miR-30d-5p	-0.195	0.167	0.362	0.0040781	38	0.0320
hsa-miR-301a-3p	-0.311	0.270	0.581	0.0044048	39	0.0337
hsa-miR-33a-5p	-0.414	0.325	0.739	0.0045935	40	0.0343
hsa-miR-652-3p	-0.166	0.136	0.302	0.0053254	41	0.0370
hsa-miR-339-5p	-0.270	0.217	0.487	0.0053433	42	0.0370
hsa-miR-3685	0.176	-0.141	-0.318	0.0054427	43	0.0370
hsa-miR-17-3p	-0.185	0.125	0.310	0.0059137	44	0.0389
hsa-miR-146a-5p	-0.301	0.227	0.528	0.0059722	45	0.0389
hsa-miR-125a-5p	-0.161	0.120	0.281	0.0070699	46	0.0422
hsa-miR-374c-5p	-0.286	0.230	0.517	0.0071171	47	0.0422
hsa-miR-17-5p	-0.226	0.176	0.402	0.0072663	48	0.0422
hsa-miR-877-3p	0.194	-0.186	-0.380	0.0073088	49	0.0422
hsa-let-7b-5p	-0.103	0.086	0.189	0.0076141	50	0.0431
hsa-miR-18b-5p	-0.270	0.202	0.472	0.0078448	51	0.0436

Abbreviations: BH, Benjamini-Hochberg; logFc, logarithmic fold change; hsa, homo sapiens; miR, microRNA

Chapter 5

General Discussion

Mathias Leinders

General discussion

The present cumulative dissertation comprises three human studies investigating the mechanistic role of miRNAs in chronic pain and translates pre-clinical findings from bench to bedside. Using different techniques, functional paradigms and diverse individual aspects of molecular biology, morphology and pre-clinical behavior, specific scientific hypotheses regarding the expression and functional relevance of miRNAs in chronic pain were addressed. The findings and implications of each study are critically discussed in a general context and highlights carefully translated into future prospects.

Chapter 2: Aberrant microRNA expression in patients with painful peripheral neuropathies.

In this chapter we characterized miRNA expression profiles in patients with peripheral neuropathies of different etiologies. We show that in WBC, neuropathies are associated with increased miR-21, miR-146a and decreased miR-155 expression levels. In sural nerve we found that miR-21 and miR-155 were increased, in particular in painful neuropathies, which correlated with individual pain questionnaire ratings. In addition, miR-146a and miR-155 expression was reduced in the skin of the distal leg, only in patients with painful neuropathies. To date these unique results provide promising support for the aim of the present thesis, and show that miRNA signatures can differentiate painful from painless neuropathies, but cannot yet explain the underlying cause.

As mentioned before, it still remains unclear why neuropathies with apparently similar pathology and etiology can be either painful or painless. It is commonly accepted that neuropathies with prominent Aδ- and C-fiber involvement are painful, however those with non-selective and apparent large fiber loss can be equally painful (von Hehn et al., 2012), highlighting the

ambiguity of pain in peripheral neuropathies. Known factors involved in the pathophysiology of pain are for example cytokine-mediated neuro-immune interactions (Calvo et al., 2012), but also the recently discovered miRNAs. Indeed, Zhao and colleagues (2012) published the first groundbreaking findings demonstrating a role of miRNAs in the pathophysiology of pain. They were able to show that conditionally knocking out Dicer in sensory neurons attenuated inflammatory pain (Zhao et al., 2010). Numerous studies have since highlighted the importance of miRNAs in experimental pain conditions, as reviewed elsewhere (Andersen et al., 2014; Elramah et al., 2014; Kress et al., 2013; Sakai and Suzuki, 2015). Also, several reports of distinct human chronic pain conditions proposed the involvement of systemic miRNAs (Andersen et al., 2016; Beyer et al., 2015; Leinders et al., 2016a; Leinders et al., 2016b; McDonald et al., 2016; Orlova et al., 2011), highlighting their novel potential as biomarkers for clinical diagnosis and possibly personalized pain medicine. This created a lot of enthusiasm among scientist, and in analogy to developments in the oncology field, experts proposed that specific miRNA expression signatures might be highly useful for the diagnosis, treatment prognosis and outcome of chronic pain and predispose as biomarkers. For example, selective miRNA expression signatures have been associated with specific tumor types, and furthermore predicted treatment outcome (Hummel et al., 2010; Nair et al., 2012). Thus also miRNA profiles were suggested to be associated with increased risk of pain chronification and responsiveness to analgesic drugs (Kress et al., 2013). However, at present no published study was able to support this hypothesis.
The novelty of the present study is the combination of patient samples, derived from the routine clinical diagnostic process, with miRNA expression profiles that differentiate painful from painless neuropathies.

We choose to study the expression of miR-21, miR-146a and miR-155, because all three miRNAs are known to regulate multiple pathways in inflammation and pain. miR-21 is known to have a wide range of physiological and pathophysiological functions, including experimental pain (Rau et al., 2010; Sakai and Suzuki, 2013; Wu et al., 2011; Yu et al., 2011). Moreover, miR-21 was

shown to promote neurite outgrowth (Strickland et al., 2011) and low expression was associated with decreased demyelination in experimental autoimmune diseases (Murugaiyan et al., 2015). Interestingly, we observed a strong upregulation in WBC and sural nerve of patients with painful non-inflammatory neuropathies. More importantly, sural miR-21 expression correlated with several items of the pain questionnaires, such as the NPSI, GCPS, ADS, and the patient's current pain intensity. At first glance these findings sound promising, but important aspects have to be kept in mind. First, it is currently the first and only study analyzing human sural miR-21 expression, and in terms of the invasive nature of sural biopsies less likely to find routine clinical practice. Second, the individually analyzed quantity of biopsy samples were relatively low compared to the two other investigated markers, limiting the significance of these findings. Third, we did not observe a difference in skin miR-21 expression the reason for this is unknown. Whereas increases of miR-21 in for example human psoriatic skin lesions have been shown before (Thieme et al., 2004), might our results point towards limitations of our study design or, and more likely, indicate that cutaneous miR-21 expression is not involved the pathophysiology of neuropathies. The latter might be supported by the clinical presentation of peripheral neuropathies, in which overt inflammatory skin reactions are usually not observed, and furthermore by the relatively low abundance of neuronal cells in the skin. Lastly, we merely investigated miR-21 expression and thus cannot make any conclusion about down-stream targets and hence mechanistic functions of miR-21 in human peripheral neuropathies. Nonetheless, the systemic and sural increases in miR-21 are striking and in agreement with the literature and need to be addressed in future experiments. With respect to the cutaneous role of miR-21, it needs to be elucidated if expression changes and location of miR-21 are solely restricted to bigger nerves, or also occur at the smaller epidermal nerve fibers, and if neuronal miR-21 is also associated with demyelination in human peripheral neuropathies.

miR-146a and miR-155 have both been extensively studied throughout immunological processes, as reviewed elsewhere (Faraoni et al., 2009; Li et al.,

2010). Also their role in experimental and human pain conditions is increasingly recognized (Andersen et al., 2014). Whereas, increased miR-146a expression was involved in inflammatory cascades promoting various degenerative diseases (Enciu et al., 2012), regulated distal axonal outgrowth in an experimental model of diabetic neuropathy (Jia et al., 2016), was increased miR-155 detected in white matter lesions of patients with multiple sclerosis (Junker et al., 2009), and shown to be protective against experimental autoimmune encephalomyelitis in mice (Mycko et al., 2015). Interestingly, the increased systemic pattern of miR-146 (and miR-21) but decreased miR-155 in our cohort might indicate differential roles of these miRNAs in immune cells. Whereas increased miR-146a expression is in agreement with the literature, remains the marked down-regulation of miR-155 puzzling. In general, miR-155 is known to suppress negative regulators of the TLR-4 pathway, such as SHIP1 and SOCS1, thereby exerting pro-inflammatory actions (O'Connell et al., 2012). It is thus likely that decreased WBC miR-155 expression might point towards a resolution of inflammation, although we did not assess any parameters of inflammation in our cohort. Of note, Üçeyler and colleagues (2007) have previously shown that painless neuropathies are associated with higher systemic IL-4 and IL-10 expression levels (Üçeyler et al., 2007), which could in part explain our findings as T-cells low in miR-155 levels differentiate into IL-4 producing Th2-cells in-vitro (Thai et al., 2007). However, at present the results of our study cannot support this hypothesis, and the recently described opposing effects of miR-146a and miR-155 downright counterbalancing their regulation are more likely (So et al., 2013). Interestingly both, miR-146a and miR-155 were downregulated in distal skin of patients with painful neuropathies of different etiologies and weakly correlated with the GCPS's current pain intensity. Given the inhibitory role of miR-146a (Lindsay, 2008) in the release of pro-inflammatory cytokines and the role in infiltration of immune cells in atopic dermatitis (Rebane et al., 2014), in combination with previous findings of increased cytokine levels in distal skin of painful length dependent small fiber neuropathies (Üçeyler et al., 2015), it is likely that miR-146a locally promotes immune-related cascades causing pain. miR-155

on the other hand displayed the same marked down-regulation in distal skin of painful neuropathies as was observed in WBC. As mentioned before, these findings remain inconclusive and might point towards a resolution of inflammation. More likely however, at present we cannot exclude that hormonal and metabolic factors, or more importantly, medication influenced the expression of miR-155 in our patients, as was shown in breast cancer patients after chemotherapy (Sun et al., 2012) and patients with multiple sclerosis after Natalizumab treatment (Mameli et al., 2016). The influence of drug treatment is thus an important aspect that needs to be controlled for in future studies. Nonetheless, both miRNAs point towards a pathology in skin of patients with peripheral neuropathies and further suggest their clinical use in the differential diagnosis of painful neuropathies. In fact, skin biopsies where recently designated as “the most promising biomarker”, in case for hereditary types of neuropathies (Fridman et al., 2015), which together with specific miRNA signatures might find clinical practice.

Several questions remain unanswered with regard to the present findings. While not having measured miRNA expression in healthy controls thus rendering direct comparisons preliminary, remains the exact cellular location of miRNAs in skin and sural nerve biopsies unknown. Nevertheless, future morphologic (e.g. in-situ hybridization) analyses could reveal novel insights about the pathophysiologic role of miRNA in skin and/or sural nerve of patients, also with regard to downstream targets and/or immune cell infiltration. Another important question that remains, is how are the observed miRNA expression profiles found in peripheral tissues involved in the pathophysiology of pain? Results presented in Chapter 2 and continued in Chapter 3 point towards aberrant expression of miRNAs in WBC, sural nerve and skin of patients with chronic pain. However, aberrant expression alone cannot explain the induction or maintenance of chronic pain itself. But it is likely that after peripheral or central insults, immune cell changes in miRNA expression influence neuronal functioning, whether directly by suppressing or enhancing genes within neurons, glial cells or vice versa (as depicted in Figure 7, Chapter 3). In addition, also the inter-cellular

exchange of miRNAs via exosomes (Stoorvogel, 2012) and the recently described direct interaction of miRNAs with extracellular nociceptors (Park et al., 2014) are likely to be involved in the pathophysiology of chronic pain.

The present results in this chapter can thus not answer if deregulated miR-21, miR-146a and miR-155 expression is the cause or consequence of peripheral neuropathies, as profiles are highly selective depending on the cause. It does however strongly underpin the fact that neuropathies are not solely restricted to peripheral nerves and involve both skin and systemic expression, presumably via a reciprocal interaction. Despite being in its infancy, it also highlights the clinical use of miRNA expression signatures derived from simple blood and/or skin biopsies as biomarker for pain in peripheral neuropathies. miRNA based biomarkers could in the future overcome the poor individual treatment success of patients suffering from chronic pain, by miRNA-signature tailored treatment regimens.

Chapter 3: Increased miR-132-3p expression is associated with chronic neuropathic pain.

In this study we show that peripheral neuropathies, especially those of painful inflammatory origin, are associated with an increase of miR-132-3p in human white blood cells (WBC) and sural nerve biopsies. We furthermore translated patient findings into an animal model of chronic neuropathic pain, namely the spared nerve injury model (SNI). We found that miR-132-3p has a biphasic response after SNI, an early decrease followed by a much later increase, interestingly at a time when pain behavior was well established. These findings let us to pharmacologically modulate the expression of miR-132-3p *in vivo* via intrathecal catheters (i.t.). Strikingly, we were able to reverse pain behavior and aversion with i.t. injection of a miR-132-3p antagonist and furthermore induce pain with a miR-132-3p mimetic in naïve animals.

The reasons for choosing miR-132 in this study were diverse. miR-132 is widely expressed throughout the brain, regulated by neuronal activity and involved in multiple neuronal processes including numerous neurological

pathologies (Wanet et al., 2012). In greater detail, miR-132 regulates synapse structure and function (Bredy et al., 2011; Miller et al., 2012; Schratt, 2009; Soreq and Wolf, 2011), is necessary for neuronal plasticity (Elramah et al., 2014) and pathological pain. Indeed, deregulated expression of miR-132 was observed in neuropathic pain conditions in the hippocampus (Arai et al., 2013; Hori et al., 2013), the spinal cord (Zhang et al., 2015), and in bone cancer pain (Hou et al., 2016). Furthermore, miR-132 is known to not only be involved in neuronal processes, but exerting its actions in inflammatory cascades. Baltimore and colleagues first described that miR-132 was up-regulated in a human monocytic cell line after stimulation with pro-inflammatory agents (Taganov et al., 2006). Which was soon confirmed by many other independent findings in experimental conditions, as reviewed elsewhere (Wanet et al., 2012). This led Soreq and colleagues to establish the paradigm of “NeurimmiRs”, i.e. certain miRNAs that control transcripts involved in the neuroimmune interface (Soreq and Wolf, 2011). Interestingly, miR-132 was the first miRNA to be demonstrated to function within the neuroimmune interface, by controlling the bidirectional communication between specific neuronal circuits and inflammatory processes. In mice, miR-132 was shown to regulate cholinergic anti-inflammatory signals by targeting acetylcholinesterase and thus contributing in a brain-to-body resolution of inflammation (Shaked et al., 2009). The same group later on demonstrated the involvement of miR-132 in human inflammatory bowel disease (Maharshak et al., 2013), while other groups found miR-132 to be increased in WBC, serum and plasma of patients with arthritis (Beyer et al., 2015; Chatzikyriakidou et al., 2012; Murata et al., 2010; Pauley et al., 2008), in CRPS patients (Orlova et al., 2011), in WBC in multiple sclerosis (Mameli et al., 2016; Miyazaki et al., 2014), in serum in spinal muscular atrophy (Catapano et al., 2016), in periodontal ligament cells of Friedrich’s ataxia patients (Quesada et al., 2015) and numerous cancer subtypes (Wanet et al., 2012). miRNAs are known to control multiple cellular pathways and act as master-switches of entire signaling networks, in fact also miR-132 has many validated targets (Wanet et al., 2012). Comprehensively deploying further target prediction analysis of miR-132 revealed numerous

targets involved in the neuroimmune interface [miRBASE.org: http://mirbase.org/cgi-bin/mirna_entry.pl?acc=MI0000449, (Kozomara and Griffiths-Jones, 2011)]. In addition, in context of the current thesis, miR-132 is predicted to target several ion channels, potentiate glutamate, NMDA, or K^+-mediated depolarization, thus affecting cell excitability and regulating neurotransmission. One of these predicted targets is the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR-) GluA1. AMPA receptors are known to be involved in inflammatory (Choi et al., 2012; Leinders et al., 2014) and neuropathic pain (Xu and Yaksh, 2011) and down-regulation of GluA1 is expected to reduce pain behavior (Hartmann et al., 2004). Interestingly, in the current study we found that SNI up-regulated GluA1 and GluA2 levels, but also increased miR-132 rendering these effects as contradictory at first glance. However, it is worth mentioning that SNI induces multiple effects beyond the proposed miR-132 mechanisms, including induction of other miRNAs, pro-nociceptive factors and morphological changes, some of which may induce the increased GluA1 expression. Thus, the final outcome of GluA1 expression changes is dependent on as of yet unknown more complex mechanisms, which need to be addressed in further experiments. Another unique aspect of the current study is the association of miR-132 with avoidance behavior, especially avoidance of evoked painful stimuli. The place escape avoidance paradigm (PEAP) measures and dissociates the affective/motivational component of pain, and was based on the assumption that if an organism escapes and/or avoids a noxious stimulus, then this stimulus can be determined as aversive to the organism (LaBuda and Fuchs, 2000). Based on the testing paradigm with a suprathreshold von Frey filament, the animal has a choice to move to an aversive area in order to avoid noxious stimulation. If however the animal does not perceive suprathreshold stimulation as being painful, after for example interfering pain processing with various analgesic compounds, it will not move to the aversive area, which has been used throughout many other studies (Fuchs and McNabb, 2012). In our study we were the first to show that pharmacological

inhibition of miR-132-3p reduced the aversive nature of pain in the PEAP, thus miR-132 possibly also interferes with higher brain processing of painful stimuli.
Taken together, these data provide strong evidence that increased miR-132-3p expression is not only associated with human neuropathic pain, but can also be translated into and modified in animal models of NP. Hence, miR-132 presents an attractive opportunity for future biomarker development and targeted gene-regulatory therapies in pain conditions and possibly also other clinical conditions. However, significant challenges associated with methodology and downstream target expression warrants additional studies before clinical utilization in patients suffering from pain can be achieved. For example, the translation of pre-clinical findings into clinical value is hampered by the unstable nature of RNA molecules. A possible way to circumvent these issues can be achieved by modifying RNA molecules in a way that improves their circulatory and targeting properties. Modifications can be performed in several ways, often by the attachment of specific chemical groups (Juliano et al., 2008). In our study we choose to use locked nuclei acid (LNA), a modification with methylene bridges that constrains the ribose ring and forms duplexes with a target RNA sequence. This renders the construct resistant to intracellular degradation. Of note, Roche Ltd. (Miravirsen) currently deploys the LNA technology successfully as a therapeutic compound for the treatment of chronic hepatitis C in phase II of clinical development, in combination with other antiviral drugs (NCT02452814) further underscoring the relevance of the present findings that miRNA-based therapy with LNA molecules present relevant future analgesic entities. In our study we used i-Fect^{TM}, a transfection agent to improve the cellular uptake of the LNA inhibitors. This may not very easily translate into clinical relevance, as transfection agents and LNAs itself may have severe off-target effects by passive accumulation in other than the diseased tissue (Mook et al., 2010). Possible ways to cope with passive accumulation in unwanted tissue sites are either the specific modification of LNAs to ensure the precise delivery to certain tissues, or as used in our study a local delivery, i.e. the i.t. injection of LNA drug carriers. With respect to the invasive nature of i.t. administrations, more viable delivery routes for example

orally or intravenous injections might be more clinically relevant. However, when using systemic routes over the more invasive i.t. route one has to keep in mind that new challenges are encountered in form of the blood-brain and blood-spinal cord barriers. Interestingly, Lee and colleagues recently proposed a mechanism in which the drug carriers are directly targeted towards myelinated or unmyelinated peripheral nerves themselves (Lee et al., 2013). Another possible approach would be to target them towards the distorted microenvironment that is characteristic of the given disease process (Torchilin, 2014). There is thus a great potential for the use of miRNAs as analgesics for the various different pain syndromes, when the delivery and targeting approach has been optimized for the given syndrome. Our study thus represents a milestone for the use of miRNAs as biomarkers and as targeted gene-regulatory molecule for the therapy of various pain conditions in the future. Future translational studies will further unveil the exact role of miRNAs in chronic pain and might lead to the first human clinical trials.

Chapter 4: Increased cutaneous miR-let-7d expression correlates with small nerve fiber pathology in patients with fibromyalgia syndrome.

In this chapter we show that FMS is associated with aberrant expression of miR-let-7d. We first analyzed the expression of all annotated human miRNAs in WBC of a subset of FMS patients and found 51 to be differentially expressed, when compared to healthy controls. Individually analyzing selected candidates we found that miR-103, miR-146a and miR-let-7d were aberrantly expressed when measured throughout the entire cohort. Emphasis was then put on miR-let7d as an innovative target based on its correlation with the mean pain intensity over the last 4 weeks. Interestingly, we also show that miR-let-7d not only correlated with reduced small nerve fiber density but also provided a mechanism for impaired cutaneous small nerve fiber homeostasis via the insulin-like growth factor-1 (IGF-1R) pathway.

FMS is associated with decreased IENFD (Caro and Winter, 2014; de Tommaso et al., 2014; Doppler et al., 2015; Giannoccaro et al., 2014; Kosmidis et al., 2014; Oaklander et al., 2013; Üçeyler et al., 2013), corneal small fiber loss (Ramirez et al., 2015), but paradoxically with small fiber hyperexcitability (Serra et al., 2014). At first glance this may sound conflicting, since hyperexcitability in nociceptive fibers is known to provoke pain and a reduction or loss of these fibers would not fit into this paradigm. This so-called paradox of FMS has been the subject of many discussions between FMS experts throughout the field. Whereas some suggest a common causal phenomenon, that both increases the excitability and destroys them, do others rather point towards an associative character of reduced small nerve fibers and highlight the many CNS changes in FMS. For example, the findings of Doppler et al (2015) are in favor of a common factor, as they present evidence for thinned fibers that might actually represent a progressed stage of degeneration and thus might lead to abnormal hyperexcitability of neighboring axons. The aforementioned common phenomenon might be related to specific genetic mutations, metabolic disorders, inflammation, or external factors such as drug intake. However, whether the reduction of skin innervation and thinning of nociceptive fibers in the skin of FMS patients represents an epiphenomenon or is closely related to the underlying pathophysiology of FMS remains to be clarified and cannot be answered by the results of this thesis. Furthermore, it needs to be determined whether only dermal small nerve fibers are involved or also those that innervate muscle and fascia. Indeed, fibromyalgia patients rather report deep musculoskeletal pain than superficial pain, compared to patients with other chronic pain conditions. Critics of the small nerve fiber pathology as underlying cause of pain in FMS, rely on the latter fact that pain is felt throughout the entire body. Moreover, they rightfully ask, how are these findings responsible for the many comorbid symptoms (fatigue, sleep, memory and mood problems) in FMS that are often more detrimental for patients than the pain? As mentioned earlier, they refer to the “overwhelming” evidence that the CNS is playing the major pathogenic role (Clauw, 2014, 2015). Indeed, there is evidence for a strong relationship between

neurotransmitter changes being causative for the abnormalities of pain sensations, comorbid symptoms, and improvement of clinical features after administration of centrally acting analgesics (Harris et al., 2013). Moreover, another hypothesis is that these abnormalities might be due to neuroplastic changes leading to structural and functional reorganization, as of note in most chronic pain conditions. In particular, these neuroplastic changes in the brain of FMS patients were shown to be secondary, rather than primary to their accompanying affective disorder (Hsu et al., 2009), meaning that the changes may contribute to the pathophysiology of FMS but cannot explain the cause of symptoms. Perhaps the most striking argument is that a reduction of small nerve fibers was found in many other chronic pain conditions and only concerns a subset of FMS patients. It furthermore rather poorly correlates with the presence or severity of pain, and can thus merely explain a deficit in temperature or pain sensation (Lefaucheur, 2016). It is worth mentioning, that support for the correlation between IENFD and the presence or absence of pain was presented by findings in other conditions, for example patients with postherpetic neuralgia (Oaklander, 2001) and acute motor Guillain-Barré syndrome (Ruts et al., 2012). However, yet no other study reproduced these findings in FMS patients. Taken together there is evidence for CNS related abnormalities and peripheral alterations, possibly also an interaction of the latter two, thereby contributing to the multimodality of symptoms in FMS. There is no doubt that at least a subgroup of FMS patients have an underlying small nerve fiber pathology. Furthermore, functional tests, morphological and neurophysiological data undoubtedly point towards the fact that there is something wrong with nociceptor function and morphology. Even if it is presently not possible to explain the association with all FMS symptoms, these findings were encouraging to further research the pathophysiology. We thus asked ourselves if miRNAs are involved in the pathomechanism, and how they relate to the small fiber impairments in FMS.

Aberrant miRNA expression in CSF (Bjersing et al., 2013) and serum (Bjersing et al., 2015; Cerda-Olmedo et al., 2015) of FMS patients has been shown before, especially the relation of serum miRNAs to symptom severity

seemed intriguing. However, striking differences in methodology, standardized patient recruitment and assessment warranted additional studies. By clinically well characterizing FMS patients and comparing results to age- and gender-matched healthy controls we were able identify miR-let-7d as potential culprit of impaired cutaneous small nerve fiber homeostasis. The let-7 family were the first miRNAs identified to play important roles in cell proliferation, differentiation, and brain development and have furthermore shown to be prognostic markers for several malignancies (Roush and Slack, 2008), among them experimental neuropathic pain (Brandenburger et al., 2012). Interestingly, several components of the IGF1/IGF-1R signaling pathway are validated to be under tight control of let-7 miRNAs (Jung and Suh, 2014). Let-7d was shown to target IGF-binding proteins (IGFBP), the IGF-1R, and IGF2 [For more detailed information please review: DIANA-TarBasev7.0: http://diana.imis.athena-innovation.gr/DianaTools/index.php?r=tarbase/index&mirnas=hsa-let-7d-5p,(Paraskevopoulou et al., 2013)]. The IGF1/IGF-1R signaling pathway is involved multiple processes, among them muscle regeneration (Hayashi et al., 2004), neurite outgrowth (Ozdinler and Macklis, 2006), peripheral nerve regeneration (Kanje et al., 1989), and inflammation (Smith, 2010). Upon binding of IGF-1 to IGF-1R a receptor tyrosine kinase receptor, multiple downstream signaling cascades are initiated, leading to the activation of the phosphatidylinositol-3 kinase (PI3K), rapamycin (mTOR) and multiple other pathways, depending on cell-type and status (Laviola et al., 2007). The IGF1-pathway is under close control of IGFBPs. Intriguingly, IGFBP5 was shown to be elevated in sural nerve samples of patients with diabetic neuropathy and leading to progressive neurodegeneration in transgenic mice (Simon et al., 2015). The same group also reported decreased IGF-1R activation; do however also report that the mechanisms are not clear. By controlling multiple components of the pathway, let-7d might thus be the missing link. Indeed, in the current study we found an increased let-7d expression, and a reciprocal decrease in IGF-1R mRNA, selectively in patients that show a reduction in the quantity of small nerve fibers. To date this is the first study measuring let-7d in the skin of patients in

relation to IGF-1R. However, while the current study opens promising new insights of the small nerve fiber pathology in FMS, several questions remain unanswered and need to be addressed in future projects. In particular in relation to the different cells in the epidermal layer, and how they influence the regeneration of intraepidermal nerve fibers. Furthermore, as discussed above, quantitation of small nerve fibers is at present an interesting observation for a subset of FMS patients but too early to be used as a diagnostic marker for FMS. The same holds true for miRNAs, regardless of their clear pathophysiologic role in subsets of FMS patients. Nonetheless, an objective biomarker would dramatically improve diagnostics and would open new treatment possibilities for FMS patients. Hence, the current study might have important implications in improving diagnostic criteria of FMS and presents a milestone in the miRNA-related pathophysiology of small nerve fibers in FMS.

Back to the clinical problem

The results presented in this thesis point towards the significance of miRNAs in chronic pain. In experimental neuropathic pain miR-132-3p was shown to reverse pain behavior and aberrant miRNA expression profiles of miR-132-3p, miR-146a, miR-155 and miR-21 were shown to have potential diagnostic validity in characterizing peripheral neuropathies, especially differentiating painful from painless ones. In fibromyalgia syndrome, miR-let-7d was associated with pain and shown to be involved in the small nerve fiber pathology. These findings highlight the importance of miRNAs in diagnosis of - and the pathophysiology of chronic pain.

Current treatment paradigms of chronic neuropathic pain display relatively poor responses and often severe side-effects. This is in part due to the limited amount of standardized randomized controlled trials and the difference in national and international recommendation guidelines. But also the multifaceted nature of pain impairs treatment efficacy. This is why opioids or a combination of drugs are often the most effective choice for treating moderate to severe chronic pain. Indeed, opioids act at all levels of the neural circuitry of pain and also

improve the emotional state of patients. But unfortunately do patients rapidly develop tolerance and other side effects due to chronic opioid use. Hence, more efficacious and specific medications are needed for all chronic pain syndromes. Both the novel and specific mode of action and the ability to impact entire signaling networks as master switches, triggered enthusiasm for miRNAs as novel therapeutic analgesics. Possible treatment options may directly interfere with miRNA regulation via miRNA antagonists or mimetics. This can furthermore be targeted towards a single miRNA or multiple miRNAs simultaneously. In addition, as rapid changes in miRNA expression after nerve injury may imply early treatment, therapies modulating miRNA expression in early phases of neuropathic pain may prevent the transition from acute to chronic pain. However, several difficulties with regard to delivery approach, cellular uptake, specificity and toxicity remain for neuronal miRNA therapeutics and thus leaving the potential therapeutic impact of miRNAs largely unexplored. Also, understanding the differential regulation of target genes throughout the progression of chronic pain as well as understanding the specific roles of pain related miRNAs in the various cell and tissue types will be one of the great challenges in the future. It is thus not expected that miRNA- based drugs will reach a clinical phase in the near future, however, insights about these chemicals gathered by translational studies as presented in this thesis will drastically augment knowledge about possible ways to treat chronic pain with miRNA modulators. On the other hand, based on and in analogy to recent developments in for example oncology, specific miRNA profiles characteristic for a given peripheral neuropathy may be useful for diagnostic and prognostic assessments that may lead to a more targeted treatment. However, there is still a long way to go and a lot of similar experimental and clinical studies necessary until miRNA-modulating drugs will be developed for the treatment of pain in patients.

Conclusion

Altogether, these findings provide novel insights into the pathophysiological mechanism of miRNAs in chronic pain. Although a transition from bench to beside is preliminary, the demonstrated results suggest that altered expression of miRNAs are associated with the degree of pain experienced by patients. They furthermore suggest that specific miRNA expression profiles can be used in the clinical differential diagnosis of patients with neuropathies in the future. Also the implication of altered miRNA function in the pathophysiology of small nerve fiber related impairments in FMS was highlighted in this thesis, and point towards the possibility to use cutaneous miRNA expression profiles to diagnose FMS subgroups within the disease. Additional research is required to identify if these miRNA profiles are expressed individually in patients, and to advance understanding about the factors that generate these differences. Findings from future studies may ultimately result in adequate anti-pain medication based on miRNA profiles, which deals with the intractable nature of chronic pain.

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Appendix

Appendix A: List of Figures and Tables

Chapter 1

Chapter 2

Chapter 3

Chapter 4

Appendix B: Affidavit

Affidavit

I hereby confirm that my thesis entitled *microRNAs in chronic pain* is the result of my own work. I did not receive any help or support from commercial consultants. All sources and / or materials applied are listed and specified in the thesis.

Furthermore, I confirm that this thesis has not yet been submitted as part of another examination process neither in identical nor in similar form.

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Eidesstattliche Erklärung

Hiermit erkläre ich an Eides statt, die Dissertation *microRNAs bei chronischen Schmerzen* eigenhändig, d.h. insbesondere selbständig und ohne Hilfe eines kommerziellen Promotionsberaters, angefertigt und keine anderen als die von mir angegebenen Quellen und Hilfsmittel verwendet zu haben.

Ich erkläre außerdem, dass die Dissertation weder in der gleichen noch in ähnlicher Form bereits in einem anderen Prüfungsverfahren vorgelegen hat.

…… ……

Ort, Datum Unterschrift

Appendix C: List of Publications with full reference

2016

- **Leinders, M.**, Doppler, K., Klein, T., Deckart, M., Rittner, H., Sommer, C., Üçeyler, N., 2016a. Increased cutaneous miR-let-7d expression correlates with small nerve fiber pathology in patients with fibromyalgia syndrome. *Pain* 157, 2493-2503.
- **Leinders, M.**, Üçeyler, N., Pritchard, R.A., Sommer, C., Sorkin, L.S., 2016. Increased miR-132-3p expression is associated with chronic neuropathic pain. *Exp Neurol,* 283, 276-86.
- **Leinders, M.**, Thomann, A., Üçeyler, N., Sommer, C., 2016. Aberrant microRNA expression in patients with painful peripheral neuropathies. *Submitted Neurobiol Dis*.
- Pritchard, R.A., Falk, L., Larsson, M., **Leinders, M.**, Sorkin, L.S., 2016. Different phosphoinositide 3-kinase isoforms mediate carrageenan nociception and inflammation. *Pain* 157, 137-46.
- Wigerblad, G., Yin, H.Z., **Leinders, M.**, Pritchard R.A., Koehrn, F., Huganir, R.L., Weiss, J.H., Sorkin L.S., Svensson, C.I., 2016. Inflammation-induced GluA1 trafficking and membrane insertion of Ca2+ permeable AMPA receptors in dorsal horn neurons is dependent on spinal tumor necrosis factor, PI3-kinase and protein kinase A. *Exp Neurol, EXNR-16-644.*

2014

- **Leinders, M.**, Koehrn F.J., Bartok B., Boyle D.L., Shubayev V., Kalcheva I., Yu N.K., Park J., Kaang B.K., Hefferan M.P., Firestein G.S., Sorkin L.S., 2014. Differential distribution of PI3K isoforms in spinal cord and dorsal root ganglia: potential roles in acute inflammatory pain. *Pain* 155, 1150-60.

2013

- **Leinders, M.**, Knaepen, L., De Kock, M., Sommer, C., Hermans, E., Deumens, R., 2013. Up-regulation of spinal microglial Iba-1 expression persists after resolution of neuropathic pain hypersensitivity. *Neurosci Lett, 554, 146-50*.

Appendix D: Approval of a "Dissertation Based on Several Published Manuscripts"

Julius-Maximilians-
UNIVERSITÄT
WÜRZBURG

Graduate School
Life Sciences

Approval of a "Dissertation Based on Several Published Manuscripts"

for the doctoral researcher

Mathias Leinders

(Name)

who has accomplished a publication record significantly above average as documented in the attachment.

The **Section Speakers and the Thesis Committee** therefore approve a "Dissertation Based on Several Published Manuscripts".

The **Thesis Committee** additionally confirms that the doctoral researcher has fulfilled all requirements of the GSLS program "life science".

Thesis Committee

Supervisor	Name	Date	Signature
1	Prof. Dr. C. Sommer	02.08.2016	
2	Prof. Dr. U. Fischer		
3	PD. Dr. A. Schmitt-Böhrer		
4 (if applicable)			

Section Speakers

Speaker	Name	Date	Signature
1	Prof. Dr. E. Asan		
2	Prof. Dr. P. Pauli		
3 (if applicable)	Prof. Dr. Sendtner		

Julius-Maximilians-
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WÜRZBURG

Graduate School
Life Sciences

Approval of a "Dissertation Based on Several Published Manuscripts"

for the doctoral researcher

Mathias Leinders

(Name)

who has accomplished a publication record significantly above average as documented in the attachment.

The **Section Speakers and the Thesis Committee** therefore approve a "Dissertation Based on Several Published Manuscripts".

The **Thesis Committee** additionally confirms that the doctoral researcher has fulfilled all requirements of the GSLS program "life science".

Thesis Committee

Supervisor	Name	Date	Signature
1	Prof. Dr. C. Sommer		
2	Prof. Dr. U. Fischer	1.8.2016	
3	PD. Dr. A. Schmitt-Böhrer		
4 (if applicable)			

Section Speakers

Speaker	Name	Date	Signature
1	Prof. Dr. E. Asan		
2	Prof. Dr. P. Pauli		
3 (if applicable)	Prof. Dr. Sendtner		

Julius-Maximilians-
UNIVERSITÄT
WÜRZBURG

Graduate School
Life Sciences

Approval of a "Dissertation Based on Several Published Manuscripts"

for the doctoral researcher

Mathias Leinders

(Name)

who has accomplished a publication record significantly above average as documented in the attachment.

The **Section Speakers and the Thesis Committee** therefore approve a "Dissertation Based on Several Published Manuscripts".

The **Thesis Committee** additionally confirms that the doctoral researcher has fulfilled all requirements of the GSLS program "life science".

Thesis Committee

Supervisor	Name	Date	Signature
1	Prof. Dr. C. Sommer		
2	Prof. Dr. U. Fischer		
3	PD. Dr. A. Schmitt-Böhrer	2016/08/17	A. Schmitt-Böhrer
4 (if applicable)			

Section Speakers

Speaker	Name	Date	Signature
1	Prof. Dr. E. Asan		
2	Prof. Dr. P. Pauli		
3 (if applicable)	Prof. Dr. Sendtner		

Appendix E: Statement On Individual Author Contributions

Julius-Maximilians-
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Graduate School
Life Sciences

"Dissertation Based on Several Published Manuscripts"

Statement of individual author contributions and of legal second publication rights

(If required please use more than one sheet)

Publication (complete reference): Leinders M, Doppler K, Rittner K, Deckert M, Klein T, Sommer C, Üçeyler N: Increased cutaneous miR-let-7d expression correlates with small nerve fiber pathology in patients with fibromyalgia syndrome. Pain 2016 Jul 15, [Epub ahead of print].

Participated in	**Author Initials,** Responsibility decreasing from left to right				
Study Design Methods Development	Leinders M	Üçeyler N	Sommer C		
Data Collection	Leinders M				
Data Analysis and Interpretation	Leinders M	Üçeyler N	Sommer C		
Patient recruitment and assessment	Doppler K	Rittner K	Deckert M Klein T	Üceyler N Sommer S	Leinders M
Manuscript Writing Writing of Introduction Writing of Materials & Methods Writing of Discussion Writing of First Draft	Leinders M Leinders M Leinders M Leinders M Leinders M	Üçeyler N	Sommer C		

Explanations (if applicable): An extra row was added, "patient recruitment and assessment". These authors were involved in the recruitment and assessments of patients of the multiple pain centers. These persons however did not participate in any of the other listed points.

Publication (complete reference): Leinders M**,** Üçeyler N, Pritchard R, Sommer C, Sorkin LS: increased miR-132 expression is associated with chronic neuropathic pain. Exp Neurol. 2016 Jun 24; 283 (Pt A):276-286.

Participated in	**Author Initials,** Responsibility decreasing from left to right				
Study Design Methods Development	Leinders M	Sorkin LS	Sommer C	Üceyler N	
Data Collection	Leinders M				
Data Analysis and Interpretation	Leinders M	Sorkin LS			
"Animal maintenance, Lab technician"	Pritchard R				
Manuscript Writing Writing of Introduction	Leinders M Leinders M	Sorkin LS	Sommer C	Üceyler N	

Writing of Materials & Methods Writing of Discussion Writing of First Draft	Leinders M Leinders M Leinders M				

Explanations (if applicable): An extra row was added, “Animal maintenance, Lab technician”. Pritchard R assisted in taking care of my animals in the vivarium at UCSD, provided support as a lab technician in maintenance of machines & solutions, surgery utensils, and as a reference point for vivarium related tasks.

Publication (complete reference): Leinders M, Thomann A, Üçeyler N, Sommer C: microRNA expression profiles in patients with Polyneuropathy.

Participated in	**Author Initials,** Responsibility decreasing from left to right				
Study Design Methods Development	Leinders M	Sommer C			
Data Collection	Leinders M	Üçeyler N	Thomann A		
Data Analysis and Interpretation	Leinders M	Üçeyler N	Sommer C		
Patient recruitment and asessment	Üceyler N	Sommer C	Leinders M		
Manuscript Writing Writing of Introduction Writing of Materials & Methods Writing of Discussion Writing of First Draft	Leinders M Leinders M Leinders M Leinders M Leinders M	Üçeyler N	Sommer C		

Explanations (if applicable):

The doctoral researcher confirms that she/he has obtained permission from both the publishers and the co-authors for legal second publication.

The doctoral researcher and the primary supervisor confirm the correctness of the above mentioned assessment.

Leinders, Mathias	28.10.2016	Würzburg	
Doctoral Researcher’s Name	Date	Place	Signature

Sommer, Claudia, Prof. Dr.	28.10.2016	Würzburg	
Primary Supervisor’s Name	Date	Place	Signature

Appendix F: Curriculum Vitae

Curriculum Vitae

Education

2013	Ph.D. student, Department of Neurology, University hospital Würzburg, Würzburg, DE. Thesis defense date December 2016
2012	M.Sc. (clinical Neuroscience), Maastricht University and University of California San Diego, San Diego, USA
2010	B.Sc. (medicine and molecular biology), Maastricht University, Maastricht, NL
2006	University-entrance diploma (Abitur) and certified vocational training (Biological-technical assistant), Lessing-Gymnasium und Berufskolleg, Düsseldorf, DE

Research Experience

2015	Visiting Research fellow, Anesthesia Research Laboratories, UCSD, USA (8 months), BaCaTeC®
2014	Visiting Research fellow, Anesthesia Research Laboratories, UCSD, USA (6 months), BaCaTeC®
2012	Stipend at Department of Neurology, University hospital Würzburg, Würzburg, DE (6 months)
2012	Visiting Research fellow, Anesthesia Research Laboratories, UCSD, USA (12 months)
2011	Student Assistant (The role of microglia in the development and resolution of nerve injury-induced pain hypersensitivity), Maastricht University, Department of Anesthesiology, Maastricht University Medical Center+, Maastricht, NL (7 months)
2009	Student Assistant (Stress and depression as risk factors for Alzheimer's disease), Maastricht University, Department of Cellular and Molecular Neuroscience, Maastricht, NL (12 months)

Grant Support/ Earning Power

Active	BaCaTeC (Bavaria California Technology Center). Research collaboration grant. miRNA novel key players in chronic pain? Mathias Leinders, PI (7,500 EUR).

Current collaborative partners

Université catholique de Louvain, Institute of Neuroscience, Brussels, Belgium (Dr. Ronald Deumens, Prof. Dr. Emanuel Hermanns)

University of California San Diego, Department of Anesthesiology, San Diego, United States of America (Prof Dr. Linda Sorkin, Prof Dr TL Yaksh, Prof. Dr. Mark Wallace)

University of Würzburg, Department of Neurology, Würzburg, Germany (Prof. Dr. Claudia Sommer, PD Dr. Nurcan Üçeyler, Prof. Dr. Klaus Toyka)

The Hebrew University of Jerusalem, Department of Biological Chemistry, Jerusalem, Israel (Prof. Dr. Hermona Soreq)

ncRNA pain, European collaboration consortium (http://www.ncrna-pain.eu)

Journal Review

Neural Regeneration Research
PLOS ONE
BMC Anesthesiology
Pain

Current

2013-2016 Ph.D. at Department of Neurology, University hospital Würzburg, Germany (defense date April 2016)
- Introduction to clinical Neurology & Physiology of different diseases
- Study the role of microRNAs in Fibromyalgia Syndrome and peripheral neuropathies
- Introduction to clinical research settings & clinical trials
- Managed the interaction of physicians and basic scientists in form of translational research

Internships:

2005 Institute of animal experiments, University of Düsseldorf, Germany
- Introduction to the work with animal models and applications

2006 Institute of forensic medicine, University of Düsseldorf, Germany
- Gained practical experience in experimental analysis and autopsy
- Assisted in clinic toxicological analysis and forensic toxicological analysis (GC/LC/MS)

2009 Division of Neuroscience, University of Maastricht, Netherlands
- Introduction to the work of Prof. Daniel van den Hove/ Annerieke Sierksma and Prof. Jos Prickaerts (application of DNA-isolation, PCR, Western blot, ELISA, microtome function and antibody-staining)
- Got in touch with several researchers and obtained great insights into a life of being a researcher (2 months) Pain Management and Research Center, Department of Anesthesiology, Academic Hospital Maastricht

2011 Department of Anesthesiology, Maastricht University Medical Center
- Introduction to clinical pain research under Supervision of Dr. Ronald Deumens. (4 months)

2011-2012 Anesthesia Research Labs, University of California, San Diego.
- Molecular inflammatory processes leading to sensitization pain pathways in the spinal cord.
- Gained expert practical experience in different behavioral animal experiments and surgery
- Gained practical experience in pharmacological interventions
- Gained good practical knowledge in Subcellular fractionations, Western-blots, Immunohistochemistry and data analysis.

Invited Presentations

2016 "microRNAs in Fibromyalgia Syndrome" Summer School, Department of Psychology, University of Würzburg, Germany

2016 "microRNAs in peripheral Neuropathies" Pain Meeting, Université catholique de Louvain, Brussels, Belgium

2015 "The role of microRNAs in chronic Neuropathic pain" Poster presentation at SfN, Chicago, USA

2014 "microRNAs as potential biomarkers in Fibromyalgia Syndrome" Poster presentation at 15th World Congress on Pain, Buenos Aires, Argentina

2013 "PI3-K novel pain targets?" 11th Dutch Endo-Neuro-Psycho Meeting, Lunteren, Netherlands

2012 "Expression and functional relevance of PI3-K in carrageenan-induced pain behavior" Poster presentation at 14thWorld Congress on Pain, Milan, Italy

Publications/Abstracts

Leinders M, Üçeyler N, Pritchard R, Sommer C. Sorkin LS: increased miR-132 expression is associated with chronic neuropathic pain. *Submitted exp Neurol*

Publications/Abstracts

Leinders M, Üçeyler N, Pritchard R, Sommer C. Sorkin LS: increased miR-132 expression is associated with chronic neuropathic pain. *Submitted exp Neurol*

Leinders M, Thomann A, Üçeyler N, Sommer C: microRNA expression profiles in patients with Polyneuropathy. *Submitted Neurobiol Dis*

Leinders M, Doppler K, Rittner K, Deckert M, Sommer C, Üçeyler N: microRNAs as potential biomarkers in Fibromyalgia Syndrome. *Submitted Pain*

Leinders M & Andrade P, Visser-Vandewalle V, Daemen M, Steinbusch HW, Buurman WA, Hoogland G: Inflammation and chronic pain: correlation between inflammatory profiles and pain evaluation in humans. *Submitted EJP*.

Leinders M, Koehrn FJ, Bartok B, Boyle DL, Shubayev V, Kalcheva I, Kyung Y, Park J, Kaang Bong-Kiun, Hefferan M, Firestein GS, Sorkin LS: Differential Distribution of PI3K Isoforms in Spinal Cord and Dorsal Root Ganglia: Potential Roles in Acute Inflammatory Pain. *Pain 2014 Jun;155(6):1150-60*.

Leinders M, Knaepen L, De Kock M, Sommer C, Hermans E, Deumens R. Up-regulation of spinal microglial Iba-1 expression persists after resolution of neuropathic pain hypersensitivity. Neurosci Lett. 2013 Oct 25;554:146-50.

R. A. Pritchard, M. Larsson, L. Falk, **Leinders M.** and L.S. Sorkin. Different Isoforms of PI3Kinase mediate Carrageegan-Induced Nociception and Inflammation in the Periphery. *Pain. 2016 Jan;157(1):137-46*.

Leinders M, Doppler K, Rittner K, Deckert M, Sommer C, Üçeyler N (2014). 15th World Congress on Pain (IASP), Buenos Aires (ARG). MicroRNAs as potential biomarkers in Fibromyalgia Syndrome.

M. Leinders, F. Koehrn, B. Bartok, G. S. Firestein, L. Sorkin (2012). 14th World Congress on Pain (IASP), Milan (IT). Expression and functional relevance of spinal phosphoinositide 3-Kinases in carrageenan-induced pain behavior.

L. Sorkin, **M. Leinders**, F. Koehrn (2012). The Society for Neuroscience (SFN) 42nd Annual Meeting, New Orleans (US). Expression and functional relevance of spinal phosphoinositide 3-Kinases in carrageenan-induced pain behavior.