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On the prospect of clinical utilization of microRNAs as biomarkers or treatment of chronic pain - A comment on: *Increased miR-132-3p expression is associated with chronic neuropathic pain*

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In the recent issue of Experimental Neurology, a study by *Leinders et al*. investigated the role of microRNAs (miRNA) in chronic neuropathic pain (NP) (Leinders et al., 2016). Specifically, the potential biomarker utility and pro-nociceptive effects of miR-132-3p were assessed in patients with neuropathy (painful and non-painful) vs. healthy controls and in a spared nerve injury model in mice (SNI). Several notable results were derived from the miR-132-3p expression analysis conducted in patients vs. healthy controls, i.e. miR-132-3p was significantly up-regulated in the white blood cells (WBC) in chronic NP patients, perhaps signifying miRNA-mediated neuro-immune mechanisms in pain generation (Soreq and Wolf, 2011). Moreover, a significant increase of miRNA-132-3p was found in sural nerve biopsies of neuropathy patients with pain versus patients without pain (n=81) and perhaps more eloquent, a significant positive correlation between miR-132-3p expression and pain levels was observed. In the context of the latter finding, the 1.2 fold-change observed in miR-132-3p expression is relatively modest, and as stated by the authors it is unclear to which extent < 2-fold-changes of miRNA-expression have significant biological implications (Dalman et al., 2012). The data on miR-132-3p expression in a cohort of patients suffering from chronic NP constitutes a contribution to the highly sparse current knowledge regarding miRNAs as modulatory molecules and biomarkers of pain conditions (Andersen et al., 2014, Kynast et al., 2013) first explored by *Orlova et al*. (2011) in complex regional pain syndrome (CRPS). Interestingly, this first study in the field also found aberrant expression of circulatory miR-132 in the CRPS group, although it was not specified whether this was the "-3p" (sense) or "-5p" (antisense) miRNA strand (Orlova et al., 2011). The study by *Leinders et al*. applied a much-revered back-translational research approach in which a "bedside observation", i.e. miRNA-132-3p expression data obtained from 111 patients suffering from neuropathy and 30 healthy controls were further explored in a preclinical model of neuropathic pain and hyperalgesia. In the SNI model miR-132-3p expression levels were found increased in the dorsal root ganglia and spinal cord of rats after injury and subsequent pharmacological modulation of miR-132-3p expression with miRNA antagonists applied intrathecally, dose-dependently reversed mechanical allodynia and eliminated pain behavior in a place escape avoidance paradigm. Moreover, intrathecal administration of miR-132-3p mimetic in naïve rats dose-dependently generated pain behavior.

The need for novel and effective treatment options in neuropathic pain disorders

It is well-established that NP conditions, caused by a disease or a lesion affecting the somatosensory nervous system, have a pronounced detrimental effect on quality-of-life and represents a substantial individual and societal economic burden (Attal et al., 2011, IASP, 2011, Langley et al., 2012). A large survey conducted in the French general population estimated the total point prevalence of chronic pain with neuropathic characteristics to be 6.9% [95% CI: 6.6–7.2] and found that ≈ 74% of these respondents experienced pain classified as "moderate-to-severe" (Bouhassira et al., 2008). Despite a large variety of etiologies of NP, it is now regarded as a distinct clinical entity wherein a common feature is the generally accepted notion that traditional analgesics are ineffective in providing pain amelioration (Baron et al., 2010, Finnerup et al., 2015). Several epidemiological studies have shown that a large fraction of patients with neuropathic pain currently receive suboptimal treatment (Dworkin et al., 2012, Martinez et al., 2014). Two of the primary reasons behind this inadequate treatment appear to be diagnostic accuracy and ineffective analgesic drugs for NP (Martinez et

al., 2014). For instance, a recent meta-analysis on pharmacotherapy for neuropathic pain found relatively high combined *numbers needed to treat* (NNT) for common first line drugs such as pregabalin (NNT = 7.7) and gabapentin (NNT = 7.2) (Finnerup et al., 2015). Hence, there is definite room for treatment optimization and forward-thinking research initiatives to improve patient outcomes.

MicroRNAs and their role in nociceptive processing and clinical pain conditions

MiRNAs are a class of gene regulating molecules exhibiting their functions post-transcriptionally by inhibiting the expression of target mRNAs. First discovered in humans in 2000 (Pasquinelli et al., 2000), they have since been shown to be essential to almost all known biological processes as well as numerous pathophysiological conditions (Kinet et al., 2013, Winter and Diederichs, 2011), including chronic pain conditions (Andersen et al., 2014). Approximately 1.500 miRNAs are encoded within the human genome and since *in silico* predictions and genome-wide identification of miRNA targets shows that each miRNA can inhibit the expression of hundreds of different mRNAs, it is estimated that at least 50% of the human protein coding genes are under miRNA regulation (Jonas and Izaurralde, 2015). Notably, miRNAs can exert their regulatory function of gene expression in several different ways, i.e. by mRNA cleavage, translational repression or mRNA deadenylation within the cells where they were initially transcribed (Jonas and Izaurralde, 2015, Winter et al., 2009). However, more recently it has been asserted that miRNAs are also exchanged between cells as a means of inter-cellular communication e.g. via exosomes (Stoorvogel, 2012), and that at least some miRNAs present in the extracellular space may directly interact with surface receptors (Fabbri et al., 2012). For instance in the context of pain, it was recently shown that the miRNA Let-7b acts as an agonist on toll-like receptor 7 and transient receptor potential cation channel, A1 (TRPA1) and hence constitute a novel pain mediator (Park et al., 2014).

With the rapid increase in published papers exploring miRNA as key players in tuning nociceptive processes and as biomarkers in pain conditions, a couple of key questions arise: 1) does miRNA expression assessed in WBCs, cerebrospinal fluid (CSF) or serum constitute a viable biomarker strategy for the purpose of diagnosis, prognosis, prediction, patient stratification etc.? 2) Could miRNA-based analgesic therapy of nociceptive and neuropathic pain be an avenue for future drug development and which prerequisites (especially drug-delivery related challenges) must be met to attain progress in this regard? In the sections below conceptual outlines of approaches to applying miRNA for biomarker and treatment purposes in the future are provided.

The potential use of microRNA as biomarkers in pain conditions

While the idea of using miRNAs quantified from various bio fluids and biopsies as biomarkers for diagnosis, prognosis and segmentation, are highly progressed in fields such as oncology (Calin and Croce, 2006, Henriksen et al., 2014) and cardiology (Kinet et al., 2013, Weber et al., 2010), the concept have only quite recently been applied in pain conditions. Advantages associated with using miRNAs for biomarkers purposes are their relative stability (when linked to proteins or contained in extracellular vesicles), their apparent sensitivity and their presence in virtually all biofluids (Jung et al., 2010, Kemppainen et al., n.d., Tomaselli et

al., 2012). The first study investigating the association between circulating miRNA signatures and pain in a clinical cohort was conducted by *Orlova et al*. (2011). Here, whole blood samples were obtained from patients with CRPS and miRNA expression was quantified alongside cytokines and numerous clinical parameters, upon which the data were compared to healthy matched controls. Subsequent correlational analyses revealed that several of the aberrantly expressed miRNAs were significantly correlated with pain levels, miR-150 expression was correlated with the frequency of migraine attacks within the cohort and multiple miRNAs was found to correlate with the levels of circulating cytokines. Similarly, a recent study investigated miRNA expression profiles in CSF of patients suffering from fibromyalgia and found 10 miRNAs to be differentially expressed between affected patients vs. healthy controls, amongst which decreased levels of miR-145-5p were associated with increased pain intensity and fatigue (Bjersing et al., 2013). More recently, aberrant circulating miRNA profiles have also been shown in conditions such as migraine, osteoarthritis and rheumatoid arthritis (Andersen et al., 2016, Beyer et al., 2015, Furer et al., 2010, Pauley et al., 2008, Tafuri et al., 2015). Despite the mentioned advantages and promising findings on miRNAs as biofluid biomarkers in pain conditions numerous obstacles adhere, particularly related to standardization of quantification procedures, normalization and appropriate housekeeping miRNAs, as well as the lack of comprehensive normative data grouped by age, gender, obesity, hormonal, and metabolic factors (Allegra et al., 2012, Blondal et al., 2013, Tomaselli et al., 2012).

Considerations on the design and delivery of RNA-interfering medicines in neuropathic pain disorders

As for therapeutic utilization, the ability of microRNAs to post-transcriptionally modulate the expressional profile of effector molecules (proteins), and their dysregulated expression levels in most known diseases, implies an obvious potential either as therapeutic targets or as a drug themselves (Hammond, 2015). However, the translation of *in vitro* findings into *in vivo* or clinical value is severely hampered by the unstable nature of the RNA molecule (Miele et al., 2012). This is largely mediated through RNA degradation by nucleases present in many compartments of the human organism, especially in the systemic circulation, which lowers the circulatory properties of RNA molecules such as miRNAs. In addition, ‘naked’ RNA molecules are only taken up by cells to a relatively low degree, further decreasing the usability in a clinical setting (Miele et al., 2012). Such issues can be circumvented by employing different measures, namely by modifying the RNA molecule itself or by packaging the RNA molecule into drug carriers that can improve their circulatory properties and targeting towards specific areas of disease.

Modifications of the RNA molecule can be performed in several ways, often by the attachment of specific chemical groups (Juliano et al., 2008). A highly used chemical modification that stabilizes the RNA structure is the creation of a phosphorothioate in the oligonucleotide backbone, but other and more stable constructs are receiving increasing attention including the 2’OH modification, peptide nucleic acid (PNA), hexitol nucleic acid (HNA) and (as used by *Leinders et al*.) locked nucleic acid (LNA) (Juliano et al., 2008). LNAs are characterized by a methylene bridge that constrains the ribose ring and creates a favourable conformation for binding a target RNA sequence. The LNA molecule and the resulting duplex with a target RNA sequence is therefore resistant to endo- and exonuclease activity (Petri et al., 2009, Vester and Wengel,

2004). LNAs has been used as a modulator of several types of RNA molecules in different settings, especially targeting specific miRNAs to reduce or abolish their inhibition on target mRNA sequences (Zhang et al., 2012). E.g. in a cancer context, targeting of an oncogenic miRNA would release tumor suppressor mRNA sequences to be translated into functional proteins (Henriksen et al., 2014, Møller et al., 2013). Most prominently, the LNA technology has been used as a therapeutic compound for the treatment of chronic hepatitis C virus lead by the Danish biotech company Santaris Pharma (now Roche Ltd). By targeting miR-122, which is a conserved, liver-specific miRNA, with LNAs, they were able to reduce both the viral load and the resulting liver-related disease symptoms in rodents and primates (Elmén et al., 2008a, 2008b, Lanford et al., 2010). Interestingly, this LNA-mediated knockdown was reversible with levels of miR-122 increasing to more than 50 % of its original expression level one week after treatment (Elmén et al., 2008b). We note that indications of the expressional profile changing over time past administration of the drug was also observed by *Leinders et al*., and this raises the question as to whether a stable knockdown can be achieved and which number of drug administrations this would necessitate. The LNA drug (Miravirsen) is currently in phase II of clinical development for the treatment of hepatitis C in combination with other antiviral drugs (NCT02452814). It is therefore likely that the LNA technology will constitute a relevant drug entity in the future, hereby underscoring the relevance of the findings by *Leinders et al*. and other previous studies indicating that miRNA-based therapy could be relevant for analgesic purposes.

The cellular uptake of the LNAs may need to be improved in order to reach clinical benefit from the miRNA knockdown. In addition, even though the LNAs have a characteristic high stability and good circulatory properties *in vivo*, passive accumulation of these molecules in most tissues of the body would likely lead to severe off-target effects, especially if the targeted miRNA has an endogenous role in other tissues than the diseased one (Mook et al., 2010). In order to increase the cellular uptake of the LNAs, *Leinders et al*. used both a cholesterol modification of the LNA molecule and a subsequent packaging into a lipid nanoparticle by way of a commercial transfection reagent (i-Fect^{TM}). While not completely irrelevant, the use of commercial transfection reagents for drug delivery may not easily translate into clinical relevance, since these reagents are optimized for another purpose (e.g. transfection of cells *in vitro*). The resulting lipid nanoparticle will therefore have a number of 'design flaws' with respect to administration in a clinical setting, such as reduced circulatory properties, unspecific uptake in surrounding cells, toxicity and short storage life (Allen and Cullis, 2013). Still, the main component of these transfection reagents, i.e. cationic lipids, are not without clinical relevance (Zimmermann et al., 2006). Cationic lipids are optimal for mediating a high uptake of a nanoparticle-nucleic acid construct, since they readily interact with the negatively charged cell membrane, however, they can also mediate detrimental side effects due to their chemical properties (Allen and Cullis, 2013, Filion and Phillips, 1997). It is therefore important to optimize the lipid composition of the drug carrier in order to reduce side effects and still mediate a high level of cell uptake and subsequent miRNA inhibition (Heyes et al., 2005, Semple et al., 2010).

Direct tissue targeting of RNA-interfering medicines for future therapy of neuropathic pain disorders

With an optimal design of the nanoparticle by tuning the particle surface charge, it is possible to mediate efficient RNA interference both in primates and in humans (Kranz et al., 2016, Zimmermann et al., 2006). Nevertheless, this will not necessarily secure specific delivery of the LNAs, because tuning of the nanoparticle surface charge can only ensure precise delivery to certain tissues in the human body. An important aspect for the efficacy of treatment in *Leinders et al*. is delivery of the LNAs to areas of the CNS via intrathecal injection. This administration route is inarguably relevant in order to achieve local delivery of the drug, but it may also be too invasive as compared to a drug formulation administrated orally or via intravenous injection (although this route is feasible in certain clinical scenarios (Bruel and Burton, 2016)). However, by using these more viable traditional administration routes, new challenges are encountered in the form of the blood-brain and blood-spinal cord barriers (BBB and BSCB, respectively), which severely reduce the transport of drugs and drug carriers from the periphery to the CNS (Rossi et al., 2013). One way to overcome this challenge could be to target the drug carriers towards the myelinated, peripheral nerves themselves (Lee et al., 2013), and deliver LNAs to reduce the expression of ion channels like transient receptor potential cation channel V1, TRPA1 and Nav1.7. For central delivery of the LNAs, which requires passage of the BSCB, a dual-targeting approach could be employed, first traversing the barrier membrane via transferrin receptor-mediated transcytosis followed by direct delivery to, or modulation of, specific neurons e.g. by targeting the N-methyl-D-aspartate receptor (Johnsen and Moos, 2016, Rungta et al., 2013). Another approach to use for improving the drug delivery in pain-related disorders and decrease side effects could be to take advantage of the distorted microenvironment characteristic of the particular disease process (Torchilin, 2014). Hence, nanoparticles could be designed to only release its contents when stimulated with specific enzymes or changes in the redox state (Bruun et al., 2015, Gjetting et al., 2014, Poon et al., 2011, Torchilin, 2014). For example, this would mediate drug release at sites with high expression of matrix metalloproteinases or hypoxia, as seen in neuropathic pain, and thus decrease the amount of off-target effects (Cameron et al., 2001, Ji et al., 2009, Lakhan et al., 2013, Lim et al., 2015, Thomson et al., 2011). We believe that with optimization of the delivery approach, the concept brought forward by *Leinders et al*. and other studies exploring the role of miRNAs in pain processing could hold a great potential in the future treatment of neuropathic pain.

In summary, as shown by *Leinders et al*. miRNAs are aberrantly expressed in chronic NP patients and are capable of greatly modulating nociceptive signaling in animal models of NP. Hence, these small noncoding RNAs molecules are an attractive opportunity for future biomarker development and targeted gene-regulatory therapy in pain conditions as well as in numerous other clinical conditions. However, missing pieces of the puzzle and significant challenges associated with methodology and drug delivery warrants additional studies before clinical utilization in patients suffering from pain can be achieved.

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