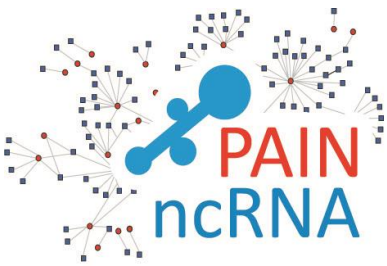


# FINAL PUBLISHABLE SUMMARY REPORT

**Non-coding RNAs in neurogenic and Neuropathic pain mechanisms and their application for risk assessment, patient stratification and personalised pain medicine**

Project duration: 01/10/2013 - 30/09/2017

Coordinator: Prof. Dr. Michaela Kress, Medical University Innsbruck

Website: http://ncrna-pain.eu/

The ncRNAPain project has received funding from the European Union's Seventh Framework Programme for research, technological development and demonstration under grant agreement n° 602133.

# EXECUTIVE SUMMARY

Chronic pain syndromes which develop after nerve damage, trauma, or surgery are characterized by persistent and severe pain. They induce anxiety and depression and greatly impair patients' quality of life. One out of five Europeans suffer from chronic pain, many of them for more than two years, some even longer. Chronic pain therefore constitutes not only a heavy burden for individual patients and their families, but also for national health systems in Europe since treatment costs take up 1.5 to 3 % of their gross domestic product (GDP) per year. Advancing scientific research in this field is thus a societal need and a crucial engagement for improved patient care.

ncRNAPain set out to further explore the biological mechanisms underlying chronic pain. Endowed with an overall funding budget of 6 million euros by the European Commission for four years, the project focused on non-coding RiboNucleic Acids (ncRNAs). The project aimed at decoding these biological molecules' role, which perform multiple vital actions in our genetic make-up and in the generation of chronic pain syndromes.

More specifically, researchers of the ncRNAPain project investigated ncRNAs in pain syndromes for which treatments are either inexistent or inadequate, such as neuropathic and neurogenic pain.

ncRNAPain sought to identify specific patterns of circulating ncRNA for improving diagnostic and prognostic precision, as well as their contribution to inter-individual variations in the response to painful stimuli and analgesic drugs. ncNRAs are suggested to be critical regulators of nociception and neuro-immune interactions, thereby making them ideal druggable targets for chronic neurogenic and neuropathic pain. ncRNAPain provides a novel understanding of the concerted function of ncRNAs in the control of the nociceptive system and reveals insights into signals used in the pain pathway, with special focus on neurogenic and neuropathic pain. Manipulating ncRNAs offers the possibility to control multiple targets including immune contributions, sensory processing and cognitive pathways. Based on recent developments it can be expected that ncRNAs and ncRNA derivatives will have fewer sequence-specific "off-target" effects than current drugs. Therefore, ncRNAs are expected to have superior advantages by targeting multiple pain-associated genes and ncRNA-based drugs may be the most appropriate therapy for the prevention or treatment of neuropathic pain.

# EXECUTIVE SUMMARY

ncRNAPain has brought together a multidisciplinary consortium of clinical partners, epidemiologists, neuroscientists, bioinformatics, and ncRNA experts to investigate ncRNAs specifically in neurogenic and neuropathic pain. ncRNAPain has identified and validated pain predisposing ncRNA patterns and polymorphisms as potentially useful biomarkers for pain. Innovative tools are developed to enable better patient stratification, for mechanism-based treatment selection and targeted prevention strategies for high risk individuals.

ncRNAPain investigated the role of ncRNA regulation of circuitries and processes modulating nociception and endogenous analgesia. Furthermore, the importance of ncRNAs in the cognitive, emotional and behavioral components of pain and their potential as druggable molecular targets for pain prevention and pain relief was assessed.

# PROJECT CONTEXT & MAIN OBJECTIVES

Chronic pain syndromes which develop after nerve damage, trauma or surgery are characterized by persistent and severe pain; they induce anxiety and depression and greatly impair patients' quality of life. One out of five Europeans suffer from chronic pain with most reporting that they endure it for more than two years, some even longer. Due to direct and follow-up costs they constitute a heavy burden for the health system. Ample evidence suggests that neuro-immune alterations in the peripheral and central nervous system play a major role in the pathophysiology of neurogenic and neuropathic pain.

Non-coding RNAs (ncRNAs), including the most extensively studied microRNAs (miRs) and Piwi-binding piRNAs, are intimately associated with normal cellular as well as pathological processes. Various diseases, including neuropathic pain disorders, reveal unique miR expression profiles which can be exploited as diagnostic and prognostic markers. Recent reports on miR modulation of both neuronal and immune processes further predict therapeutic potential for manipulating disease-modified ncRNAs in diseases affecting both the immune system and brain function, such as neuropathic pain disorders, Alzheimer's disease, Parkinson's disease, multiple sclerosis and anxiety-related disorders.

ncRNAs that function within both the nervous and the immune systems possibly act as 'negotiators' between these two interacting compartments (Fig. 1).

These 'neurimmiRs' primarily target transcription factor genes or other regulatory genes, which enables simultaneous modulation of both immune and neuronal processes including cognition through direct or indirect alterations of neuron–glia or brain-to-body signalling. Thus, a given miR controls multiple cellular pathways and miRs can act as "master switches" of the transcriptome or proteome, regulating multiple gene products and orchestrating multiple pathways including genes that encode cellular enzymes, trophic factors, receptor proteins and ion channels many of which are individually pursued as drug targets.

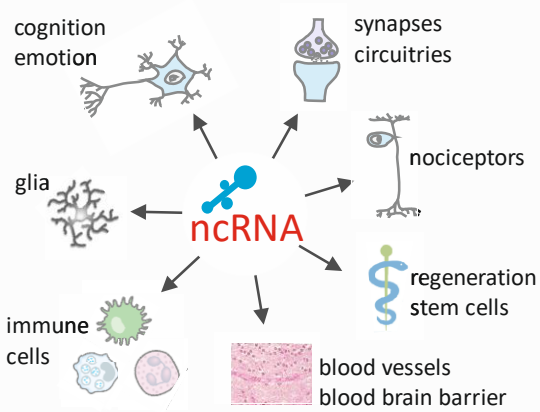


**Fig. 1** Targets of ncRNA regulation networks

# PROJECT CONTEXT & MAIN OBJECTIVES

Pain conditions have been suggested to deregulate the expression of miRs in pain pathways from primary afferent nociceptors to brain areas associated with emotional components of pain perception. miRs are frequently misregulated and expressed at aberrant levels in diseased tissue, and first evidence suggests that this applies to neurogenic pain in complex regional pain syndrome (CRPS). Altered ncRNA expression is frequently a consequence of genetic mutations, which may also cause loss or gain of function. This may account for inter-individual variation of pain sensitivity. However, the functional consequences of polymorphisms in ncRNA genes and/or their binding sites, the downstream targets of ncRNAs and the mechanisms by which ncRNAs regulate circuitries and processes modulating nociception and endogenous analgesia are as yet unresolved.

Therapeutic miR regulation has been thoroughly studied and widely established in cancer research but its impact and the therapeutic prospects of ncRNAs in the pain field are largely unexplored. Manipulation of ncRNAs offers the possibility to control multiple targets including neuro-immune interactions, nociceptive processing and cognitive pathways. Based on recent developments it is expected that ncRNAs and ncRNA derivatives will have few, if any, sequence-specific “off-target” effects. Thus, ncRNA based diagnostics and therapeutics may have superior advantages by targeting multiple pain-associated genes and ncRNA-based drugs may be the most appropriate therapy for the prevention or treatment of neuropathic pain.

The ncRNAPain consortium focused on the importance of miRs and novel ncRNAs in pain syndromes for which treatments are either inexistent or inadequate, such as neuropathic and neurogenic pain and headache. ncRNAPain assessed the role of miRs and novel ncRNA and the mechanisms by which they regulate nociception and pain as well as their contribution to inter-individual variations in the response to painful stimuli and analgesic drugs. Research results obtained by the ncRNAPain consortium could be translated into risk assessment, patient stratification and new drug targets for pain medicine.

The implementation of the multifaceted ncRNAPain work plan is a synergistic task that requires a consortium combining a variety of complementary expertise including knowledge of RNomics, expression profiling generation of ncRNA mimics and inhibitors, ncRNA regulated target genes and processes, together with expertise in glia biology, neuro-immune signalling, nociceptor morphology and physiology, pain pharmacology, electrophysiology, spinal cord circuits, and neurobiology of cognition and emotion.

# PROJECT CONTEXT & MAIN OBJECTIVES

Special expertise is required for genetic epidemiology and bioinformatic analysis of networks and network hubs, as well as thorough knowledge of all aspects of clinical pain research and rodent pain models including ethical aspects and legal requirements.

The ncRNAPain consortium was designed to meet these challenges. It combines the necessary multidisciplinarity and complementarity required, and makes use of already established longstanding interactions between the highly experienced partners involved: with its unique composition, ncRNAPain brings together experts in neurosciences, bioinformatics, as well as clinical and preclinical experts in neurogenic and neuropathic pain, including headaches. Thus, the project altogether contains 11 partner institutions and involves 15 research groups, each of which is essential for the project´s success. Hence, all partners are chosen to collectively constitute a consortium capable of achieving the ncRNAPain objectives.

Each of the partners has a long track record in a large number of projects at national, international and European level with a special focus on research in the field of pain research, non-coding RNAs or the conduction and analysis of large cohort studies

ncRNAPain pursued a translational research approach: Specifically novel ncRNA based pain therapy was validated in preclinical models during the project. To meet the urgent need for improved diagnostic standards and tools, ncRNPain explored the possibility to develop diagnostic kits and their potential usefulness for patient stratification and individual risk assessment.

# PROJECT CONTEXT & MAIN OBJECTIVES

**The overall objective of ncRNAPain was to better understand the generation of pain, its propagation, and how to ease pain and thus, help patients suffering from chronic pain.**

In detail, the project sought to achieve the following scientific objectives:

- Identify pain predisposing **ncRNA patterns as biomarkers** for pain and inter-individual variations in the response to painful stimuli and analgesic drugs
- Identify and validate **ncRNAs as druggable molecular targets** for pain prevention and pain relief
- Understand **ncRNA regulation of circuitries and processes** modulating nociception and endogenous analgesia
- Understand the role of **ncRNAs in the cognitive, emotional and behavioural components of pain**
- Identify **pain predisposing genetic polymorphisms** in ncRNAs and/or their binding sites
- Develop **ncRNA-based diagnostic tools** to enable better patient stratification, mechanism-based treatment and targeted prevention strategies for high-risk individuals

# SCIENTIFIC & TECHNOLOGICAL RESULTS

The ncRNAPain project is structured in 2 organizational and 6 thematic work packages (WP), and within each WP up to 11 partners work together to achieve the main tasks and objectives of this WP.

### Work package 1

This WP provided the organizational framework and all necessary support mechanisms to enable a smooth project workflow in ncRNAPain and to ensure that all contractual commitments can be met in time.

Efficient management and support structures were implemented and a web-based project management platform "**ProjectAngel**" was installed which is also used as intranet for data storage (PAINBASE) and file sharing between the partners. The progress of each scientific WP was presented by the WP leaders during the regular project meetings and also *ad hoc* by personal communication if the need arose, and assessed by the Steering Committee. Deliverables were uploaded to ProjectAngel by the responsible partner and quality controlled by the WP leader and the coordinator before submission to the EC. The consortium constantly monitored the benefit/burden balance of each WP, as well as the impact of the research concerned, not only in terms of scientific innovation, but also in terms of human dignity, and social and cultural impact. Initial activities focused on the preparation of documents required for ethical clearance. Detailed information were given on the study protocol, biometric plan, written consent, data and privacy protection measures (anonymization; pseudonymization), use of human material and the right to withdraw at all times without disadvantages.

Copies of ethical approval documents and permissions were provided before the start of any scientific work. To assure adherence to ethical standards, preclinical and clinical ethics supervisor panel performed audits at all partners concerned on a regular basis.

For animal experiments, coherence to the "3R"-principles "reduction, replacement, refinement" was ensured by the ethical supervisor panel who assisted partners with applications and formal requirements; information was given on the species and number of animals to be used, the nature of the experiments, the procedures to be carried out, and effort to minimize anticipated discomfort. Furthermore, details were provided on the implementation of procedures to ensure the welfare of animals during their lives (husbandry, minimizing harms, criteria for humane endpoints, inspection protocols).

# SCIENTIFIC & TECHNOLOGICAL RESULTS

## Work package 2

This WP aimed to identify altered expression patterns of ncRNAs/miRs in painful compared to non-painful diabetic neuropathies, in neurogenic pain (CRPS) after arm fracture or surgery in comparison to patients after arm fracture or surgery without CRPS (healthy healers), and in patients with nerve injuries with and without pain. In this unbiased screening study, clinical partners collected biomaterial from clinically well-characterized patient groups and matched controls according to pre-developed SOPs for patient assessment and sample processing. miRNAs and ncRNAs were isolated from plasma and leukocytes. Subsequently, differential expression of miRs/ncRNAs were analysed by expression profiling (RNA-Seq and micro-array analysis). Specific ncRNA expression patterns were expected to correlate to respective pain phenotypes, and such pain phenotype-specific ncRNA expression patterns were identified. Based on these data, ncRNAPain developed an ncRNA and miR panel of candidates for qRT-PCR analysis, which was analysed in detail with respect to its suitability for a diagnostic tool covering pain-predisposing ncRNAs/miRs. Subsequently, the candidate miRs were validated using independent cohorts of patients from clinical partners and identified miR/ncRNA candidates were further studied in preclinical models following a reverse-translational/translational paradigm. ncRNPAin analyses next-generation sequencing data, biomolecular networks, pathways, and clinical data to verify the biomarkers and develops decision making strategies with the future aim to provide a tool for an individualized treatment selection.

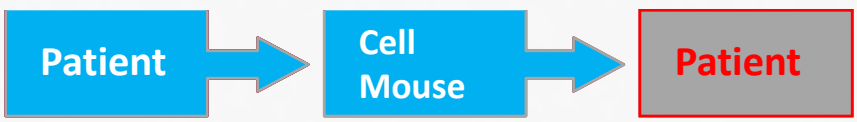


Fig. 2: Reverse translational/translational concept of ncRNAPain: scientific research facilitates the reverse translation of findings from patients to cellular and animal models and the translation of findings from these models to patients

# SCIENTIFIC & TECHNOLOGICAL RESULTS

**SOPs for clinical data collection and sample handling**

Standardised patient assessment and sample processing are essential for data homogeneity in the translational research of ncRNAPain. Therefore, SOPs were developed and implemented at all patient recruiting centres. For assessment of pain phenotypes, SOPs included complete history, diagnosis-related clinical questionnaires and scales (for quantification of pain, emotions, coping and executive cognitive function), targeted clinical (neurological status and technical investigations (plasma quantification of inflammation biomarkers), nerve conduction studies, autonomic screen), and quantitative sensory testing (QST). Standard sample collection and handling protocols were agreed on and used by all partners. Training was organised and provided for patient recruitment according to the SOPs. This training and quality assessment was repeated once yearly.

**Screening and differential expression analysis**

Clinical partners recruit diabetic neuropathy (with/without pain) patients, post fracture patients (CRPS/non-CRPS pain) and respective healthy volunteers according to the SOPs. The same numbers of participants as independent cohorts for the validating study were recruited in a similar manner. From patients and controls blood plasma and leukocytes were collected. Blood collection was performed carefully to avoid hemolysis which would disturb the individual miR profile. Blood was separated into plasma and leucocytes within half an hour, snap frozen and immediately stored at -80°C. We then isolated miRs and novel small ncRNAs from these samples by size fractionation and analyse differential expression of miRs by deep-sequencing (RNASeq) and by employing the miR qPCR panels. In addition, ncRNAs and/or miRs may be present in blood plasma as stable ribonucleo-protein complexes (RNPs). To that end, ncRNAPain investigated the presence of such uncharacterized RNP species in plasma samples. RNPs were enriched by size fractionation methods and we investigated differential expression of these RNA species by RNA-Seq. In a second approach, we isolated ncRNAs from the separated peripheral blood monocytic cells (PBMCs); subsequently, these samples were subjected to transcriptional profiling by employing RNA-Seq. ncRNA expression data were then computationally analysed using a data integration strategy. Specifically, integration of the generated ncRNA expression data with clinical data as well as publicly available information, and subsequent analyses of the integrated data in a network and pathway context enabled us to pinpoint the most promising candidates and prioritize ncRNAs for the development of a custom qPCR panel containing 96 miRs and longer ncRNAs.

# SCIENTIFIC & TECHNOLOGICAL RESULTS

**Development of a custom-made ncRNA qPCR panel**

Based on the identification of differentially expressed ncRNAs in plasma and leukocytes of pain patients, ncRNAPain generated a custom qPCR panel for identification of pain predisposing ncRNAs/miRs. To this end, the panel was used to quantitatively assess the prioritized differentially expressed ncRNAs/miRs from the above screens. Depending on the number of the identified ncRNAs it will be preferable to develop a small scale qPCR panel. Subsequently, the custom-made panel will be applied to screening of further pain patient cohorts like other painful neuropathies and to patients with migraine. Cooperation with a FP7 headache consortium has been implemented to this end. Further analysis of the role of the identified ncRNAs will be performed in preclinical studies.

# SCIENTIFIC & TECHNOLOGICAL RESULTS

## Work package 3

This WP aimed to identify those ncRNAs which can serve as clinically relevant targets based on the criteria of striking expression patterns, in vivo preclinical impact on neuropathic, and neurogenic pain and gene targets. Starting with profiling analyses in preclinical models, a selection of the most promising ncRNAs/miRs has been derived by performing expression analyses. Specific tools for manipulating the expression of key ncRNAs/miRs have been developed and employed to assess efficacy in preventing or reversing signatures of neuropathic or neurogenic pain in preclinical models. Transgenic mouse models for selected candidates have been generated and bioinformatics and molecular approaches been used to identify pain-related gene targets of regulated ncRNAs/miRs as a basis for understanding pathophysiological mechanisms and therapeutic relevance.

### Differential expression profiling in preclinical models

Via mouse micro array analysis which were validated by qPCR analyses, we investigated differential expression of ncRNAs, including miRs, in the following rodent model systems: dorsal root ganglia (DRG), spinal cord dorsal horn and brain tissue (prefrontal cortex, retrosplenial and entorhinal cortex, anterior cingulate cortex, insula and domains of the amygdala). Expression was assessed in preclinical models of neurogenic pain (tibia fracture model - TFM) or neuropathic pain (STZ diabetic neuropathic pain model (DPN) and spared nerve injury model (SNI)), and compared to corresponding sham treated controls. Conventional microarrays or qPCR panels were used for miR profiling. For all three models, specific patterns of deregulated miRs were identified, whereby the largest number miRs were obtained in the DPN model in DRG, spinal cord and brain. In contrast, only two miRs reached significance after all statistical corrections in DRG of the SNI model, whereas only minor differential expression was seen for several miRs in the spinal cord or brain following SNI. The weakest effects were observed in the TFM models.

# SCIENTIFIC & TECHNOLOGICAL RESULTS

**Assessment of expression patterns and regulation in preclinical models**

Markedly regulated ncRNAs/miRs were characterized with respect to their expression patterns in the aforementioned preclinical models via qPCR and in situ hybridisation on samples of peripheral nerves, DRG, spinal cord and brain tissues. miR/ncRNAs which consistently demonstrate significant regulation across assessment methods and models are considered as 'high priority' targets and there functional importance and target genes was assessed in more detail.

**Identification of ncRNA target genes**

Because human and rodent ncRNA sequences are structural but not necessarily sequence homologues, and structure prediction algorithms are still imprecise, we proposed an ncRNA target gene identification approach to identify mechanistically relevant ncRNA pathways. Using algorithms and a combination of online tools for predicting mRNA targets for ncRNAs/miRs, pain-relevant genes were identified in silico. A consensus approach using several algorithms for predicting mRNA targets is used to identify relevant genes which are targeted by ncRNAs/miRs of interest in preclinical models and patients. High-confidence candidates are validated by qRT-PCR analyses. Since the qPCR validation rate of predicted target genes in DRG samples was around 20% only and therefore considered rather inefficient to identify relevant pathways, in a second approach RNA Seq for miRs and long mRNAs was combined in the same samples and this approach significantly improved validation rates to approximately 75 %. For several miRs, luciferase assays were performed to validate miR regulation of selected target genes.

**Antisense oligonucleotides (ASO) and miR/ncRNA mimics**

To study the functional relevance of ncRNAs, including miRs, that are up-regulated in pain models, we utilize inhibitors/antisense oligonucleotides (ASO), which knockdown expression of miRs/ncRNAs, respectively. Similarly, we employ mimics which simulate and 'rescue' expression of targeted ncRNAs/miRs. Lentiviral and AAV based miR mimics or ASO are commercially available for a large number of miRs and respective tools for novel ncRNAs developed by ncRNAPain partners. Efficacy as well as specificity of these tools are tested on ncRNA expression levels in culture systems, e.g. neuroblastoma cell lines and primary neuron cultures, in vitro.

# SCIENTIFIC & TECHNOLOGICAL RESULTS

**Preclinical analysis of miRs/ncRNA inhibitors and mimics in vivo**

Efficacious delivery in vivo has been established in combination with a highly efficient transfection reagent or direct intrathecal or perineural injections in mice. Inhibitors (e.g. antisense oligonucleotides (ASO)) or mimics of the selected ncRNAs/miRs were tested in vivo for their impact on the development and maintenance of neurogenic pain (tibia fracture model) or neuropathic pain (DPN, SNI models). Sensory hypersensitivity to mechanical and temperature stimuli, signs of inflammation, temperature, edema, motor activity as well as ongoing pain behaviour has been assessed following ASO application. Selection criteria for miR candidates depended on numbers of deregulated candidates and involve homology to humans, stringency, overlap between tissues and pain models and common predicted targets. For one miR candidate, ASO injections induced a robust reduction in mechanical hypersensitivity in the SNI model. This candidate was further investigated to gain novel mechanistic insight.

**Generation of transgenic mouse models**

Null mutant mice, over-expressors or conditional alleles have been generated to explore the importance of selected miRs/ncRNAs in vivo. Targeting constructs have been generated by exploiting the CRSPR technology and ES cell targeting and selection of positive clones were performed. Blastocyst injection of positive clones, breeding of chimeras to establish germ-line transmission are undertaken and overseen identified specific miRs from expression profiling screens for which transgenic animals were generated first and are available. Altogether, 16 novel transgenic lines are generated and used.

# SCIENTIFIC & TECHNOLOGICAL RESULTS

## Work package 4

This WP4 examined the role of ncRNAs in the development and maintenance of morphological and functional changes in peripheral nociceptors and spinal nociceptive pathways resulting in sensitization and reduced endogenous inhibition. In addition to neurons, immune cells such as macrophages, microglia and lymphocytes as well as components of the blood-nerve- and blood-spinal-cord barriers are investigated since they participate in neuronal sensitization and chronic pain mechanisms. Combined *ex vivo*, *in vitro* and *in vivo* approaches are employed and the expression of key ncRNAs is manipulated using inhibitors/ASOs or mimics developed by ncRNAPain. Already known miRs and novel ncRNAs evolving from ncRNAPain were assessed in preclinical models of neurogenic and neuropathic pain (SNI, DPN, BF) using inhibitors and mimics and assessing expression of target genes, immune cell invasion and biophysical properties in peripheral nerve, DRG, and spinal cord dorsal horn. Inhibitors and mimics together with transfection reagents or viral constructs have been incubated with DRG neurons, dorsal horn neurons, primary microglia or astrocytes in culture. For in vivo assays, inhibitors for knockdown of miRs and ncRNA as well as ncRNA mimics were delivered intraneurally and intrathecally to mice with neuropathic or neurogenic pain in the DPN, SNI and TFM models. At defined time intervals after in vivo transfer, gene expression, electrophysiological and histological studies are performed in order to assess possible effects on sensory neurons, immune cells and microglia, astrocytes and dorsal horn neuron activities. Mice with a global or tissue specific deletion of specific ncRNAs are investigated likewise

# SCIENTIFIC & TECHNOLOGICAL RESULTS

**Primary nociceptive afferents**

In the preclinical models that were used in this programme of research we have evaluated ncRNA role on pain sensing primary afferents, the nociceptors, as well as peripheral nerves in vivo and in vitro. In order to assess miR expression specifically in sensory neurons, in situ hybridsation with specific primers and purified neuron preparations were used. Several miRs were found to be significantly upregulated in DRG neurons rather than non-neuronal cells. In line with these findings, intrathecal injection of ASO in the SNI model resulted in a resolution of mechanical hypersensitivity suggesting a corresponding effect on nociceptor function. As a cellular model of nociception, DRG neuron cultures are transfected with ncRNA compounds to test for alteration of neuron excitability, growth and survival, neurotransmitter release, calcium fluxes and ionic currents involved in hypersensitivity of nociceptors.

As transgenic mice became available, the functional properties of peripheral nociceptive neurons, changes in ligand-gated and voltage-dependent ion channel and metabotropic receptor expression and biophysical properties of peripheral neurons are assessed in the three preclinical models (DPN, SNI and BF models) using behaviour phenotyping in vivo followed by electrophysiology (single fibre recordings from skin-nerve preparation, whole cell voltage-clamp recordings of isolated DRG neurons, microfluorimetric calcium measurements).

**Microglia and immune cells**

miRs/ncRNAs candidates that are differentially expressed in cells associated with the immune system or have a known role in the regulation of innate immunity are investigated. Possible effects of ncRNA ASOs or mimics on immune cells are evaluated in freshly isolated macrophages as well as microglia cultures culture by measuring survival, morphological changes and the release of cyto(chemo)kines induced under conditions which mimic nociceptive facilitation. Immune cell invasion in skin, bone, peripheral nerve, dorsal root ganglia and spinal cord is investigated in ncRNA transgenic mice in SNI, DPN and bone fracture pain models.

# SCIENTIFIC & TECHNOLOGICAL RESULTS

**Astrocytes and blood-neuron barrier**

In peripheral nerves of neuropathic/neurogenic ncRNA transgenic or ncRNA mimic/antagonist treated wt mice, immunohistochemical (IHC) staining were performed to assess general nerve morphology, tight junction protein expression as surrogate marker for the blood-nerve-barrier and macrophage invasion patterns. IHC analysis of perfused-fixed spinal cords provide correlative information on expression of markers of pain-related activity including p-ERK and immediate early gene products in neurons, microglial markers Iba-1 and p-p38 MAPK, astrocytic marker GFAP and p-JNK and T cell markers CD3/4 and cytokines as well as by tight junction protein expression pattern of the blood-nerve-barrier.

**Spinal synaptic transmission**

In order to test for effects on spinal circuits, primary cultures of dorsal horn neurons are transfected with ncRNA inhibitors/mimics. Synaptic transmission is investigated by patch-clamp and calcium imaging and ncRNA regulated ionic conductances are characterized. Neuron activity has been recorded electrophysiologically in spinal cord slices. Basic parameters for neuron excitability and synaptic plasticity have been obtained with standard protocols (number of action potentials, activation thresholds, discharge patterns, miniature synaptic potentials, paired-pulse facilitation/depression). Finally, spinal cord in vivo-electrophysiology recording includes the assessment of nociceptive-specific (NS) and wide-dynamic range (WDR) neuron responses to mechanical and electrical stimulation of the hind paw. Windup of WDR neurons is assessed as an index of short-lasting central sensitization. Threshold responses, size of receptor fields and spontaneous activity will be evaluated in NS and WDR neurons under control conditions and following spinal application of mimics or ASOs/inhibitors or transgenic mice with a specific focus on the regulation of voltage-gated calcium channels and regulatory kinases and their interaction with miRs.

# SCIENTIFIC & TECHNOLOGICAL RESULTS

## Work package 5

WP5 aimed at establishing the existence of and competition between ncRNA regulators of emotional and cognitive components of pain behaviour, discovering the ways in which ncRNAs are involved in the context-dependent control of pain circuitries in the brain and in the consequent emotional and cognitive phenotypes. Converging evidence will be collected from humans and rodent models, specifically regarding changes in brain function and morphology. Based on current literature, ncRNAPain assesses changes in specific brain regions. Tools for experimental and therapeutic interference with regulatory processes will be used to identify ncRNA networks related to pain-associated cognitive and emotional changes. Biomarkers for these changes were then identified by comparing rodent expression profiling with data mining and state-of-the-art biostatistics analyses of patients' profiles and psychological stress-coping strategies based on predefined SOP-based questionnaires and clinical testing. Findings were validated by experimental interference with ncRNA functions in specific brain regions using ncRNA inhibitors or mimics and engineered mice. WP5 planned to experimentally identify those ncRNAs and their target genes that initiate and sustain pain associated emotional and cognitive deficits and find new ways to manipulate pain-related changes for re-achieving homeostasis.

### Cognitive and emotional status

In order to determine those ncRNA functions that contribute to quantitative trait features of pain behaviour, and to assess functional involvement of particular ncRNAs in such quantitative traits, a comparison of the data from explorative human screens with experimental interference studies in engineered cells and animals was required. Correspondingly, this demanded the development of a dedicated database for the storage and retrieval of the clinical, phenotypical, and biomolecular data. Additionally, it was necessary to perform in-depth bioinformatics analyses including unsupervised (hierarchical clustering, self-organized maps) and supervised methods (support vector machines, decision trees) to link ncRNA profiles with particular functions and with their predicted gene targets in a non-biased manner. To refine the working hypothesis and re-enforce the strength of the findings, the same readouts were employed in different experimental systems. This enabled us to identify the existence of specific pain-controlling ncRNAs in mice, parallel to continued efforts of screening already available and new collections of human body fluids and DNA samples for ncRNA-silencing mutations in target genes.

# SCIENTIFIC & TECHNOLOGICAL RESULTS

**Brain areas involved in neuropathic or neurogenic pain**

ncRNAPain investigates mice with neurogenic or neuropathic pain (DPN, SNI, TFM) and characterizes pain, anxiety and memory behaviour patterns by assessing possible deficits in elevated plus maze, startle responses, new object recognition and Morris Water Maze/Barnes Maze tests. Tools are developed and validated to apply mimics or inhibitors/ASOs for deregulated miRs and for ncRNA modulators exerting gain- or loss-of function of the candidate ncRNAs, and viral shRNA suppressors of their suspected target transcripts into specific brain areas (prefrontal cortex, the retrosplenial and entorhinal cortex, the anterior cingulate cortex, the insula and domains of the amygdala). Naïve and sham treated vs. mice with neuropathic or neurogenic pain are analyzed for functional alterations and over-expression of the stress-associated small immediate protein c-fos and the IL-I responding transcriptional activator Egr-1 in brain neurons. ncRNAPain combines behavioural tests of emotional and cognitive functioning under elicited pain in native rodent strains with molecular and cellular readouts.

**Cognitive components of pain**

Differential target gene expression analysis and electrophysiology are combined to assess functional cognition-related changes in specific brain areas. Standard ncRNA/miR in situ hybridization and immunohistochemistry of their validated target gene products in cortical brain sections are used in brain sections from preclinical models. To allow loss- and gain-of-function experiments *in vitro* and *in vivo*, engineered cell and mouse models are used for studying such ncRNAs. This involves conditional expression of candidate ncRNAs in brain tissues, and synthetic and chemically protected oligonucleotides will be used to neutralize the effects of the studied ncRNAs to explore the corresponding brain-to-body and body-to-brain messages.

# SCIENTIFIC & TECHNOLOGICAL RESULTS

Classical patch-clamp recordings from wt and transgenic mice with neuropathic or neurogenic pain are performed to assess ncRNA modulation of general neuron excitability, synaptic transmission, synaptic plasticity and inhibitory synaptic modulation by excitatory and inhibitory neuron transmitters in slice preparations obtained from e.g. prefrontal cortex or hippocampus. Cell cultures will be used to assess fast effects on neuron morphology. Animals transgenic for relevant ncRNA candidates may have altered cognitive processing and morphological changes and a deficit in fear conditioning has been discovered for one miR candidate.

**Emotional components of pain**

To assess anxiety- or depression-like behaviour in preclinical models elevated plus maze, open field test, sucrose preference and force swim test were performed. Special attention has been devoted to neurotransmitter systems (for example the cholinergic system) that are known to contribute to anxiety and inflammation and to be subject to ncRNA regulation, using ncRNA-derived tools. Electrophysiological recordings have been performed from neurons in slice preparations of relevant brain regions of wt and transgenic mice with or without neuropathic or neurogenic pain in order to assess deficits that require specific ncRNAs.

# SCIENTIFIC & TECHNOLOGICAL RESULTS

## Work package 6

WP6 assessed the occurrence and importance of polymorphisms in selected ncRNAs, their 3' UTR binding region, their target gene promoter region or their respective target gene for neuropathic or neurogenic pain. Studies have been undertaken looking for the association of human pain disorders with SNPs, haplotypes or specific mutations mostly in candidate genes such as ion channels. However, associations were either weak or specific mutations did not explain pain in large populations. ncRNAs and their binding sites regulate whole neuronal, immunological and endocrine circuitries. Thus ncRNA polymorphisms or their target RNAs are promising targets for identifying relevant pain mechanisms. Large chronic pain patient cohorts, defined by standard clinical diagnostic criteria, are not homogenous. From the SOPs used in the screening study in ncRNAPain with extensively characterized patients and ncRNAPain controls we defined core investigations, which will allowed assessment of large cohorts. In biological material from these subjects, polymorphism of miRs, novel ncRNAs and ncRNA target genes has been obtained for analyses of genotype – phenotype correlation using genome-wide association studies as well as targeted genotyping for SNPs in candidate genes which are not sufficiently covered by GWAS analysis. Based on our previous experience and other published studies we have extracted a large number of candidate SNPs. ncRNAPain applies the data integration strategy to prioritize the candidate SNPs for further functional analyses.

### Recruitment of patient cohorts

ncRNAPain SOPs were defined to characterize pain phenotypes in patients and controls. From these extensive SOPs, core parameters were selected which are sufficient to describe the phenotype and allow the screening of large cohorts. Inclusion criteria were opened to other painful and painless neuropathies like those with inflammatory or idiopathic aetiology. Since the recruitment of patient cohorts under clinical conditions requires a strongly standardized procedure we apply electronic case report forms implemented in "Askimed" (http://www.askimed.com/). Askimed allows a standardized data collection with extensive checking of plausibility as part of the quality control management. Staff has been trained, certified and re-trained each year. From all patients and controls, blood has been obtained for the collection of DNA, RNA, plasma and serum for further analyses. Together with academic cooperation partners (e.g. the DFNS and TREND consortium) we have DNA available from >1500 CRPS patients and >1500 patients with diabetic polyneuropathy. In addition we seek to attract other cooperation partners who are working in the same field or who have access to this type of patients.

# SCIENTIFIC & TECHNOLOGICAL RESULTS

**Selection of specific miRs/ncRNAs and deep sequencing**

In a first step we use complementary approaches to identify specific miRs/ncRNAs and their polymorphisms: these approaches are based on available data in public databases, bioinformatic tools for the prediction of miRs/ncRNAs and most importantly, the results from ncRNAPain. The selected polymorphisms not only include the miRs/ncRNAs but also polymorphisms in their promoters as well as in targeted genes and in genes which will be newly targeted if a polymorphism in a particular miRs/ncRNAs occurs. In case of a very high number of SNPs we prioritize the selection of the genes based on the known pathophysiology and affected pain pathways including ncRNAs for cytokine and inflammation regulation, regulation of proteases, resolvins and tissue repair, regulation of mast cells, blood vessels and endothelial function, opioid-receptor availability, regulation of glycolysis and ncRNAs involved in brain responses to chronic pain.

**Genotyping and association studies** were performed by two different but complementing approaches: 1. A SNP microarray including about 2.5 million common and rare variants has been applied in as many of the recruited patients as possible (>1500). A genome-wide association (GWA) analysis was performed comparing patients with about 6000 matched controls from the KORA Study for whom genotype data were already available. The GWA approach was selected for two main reasons: a) many SNPs in miRs/ncRNAs targeted genes can be analysed without expensive customized genotyping and b) GWA analyses on very well characterized diabetic and migraine cohorts but not on CRPS are available to date and adding CRPS cohorts in a collaborative effort will considerably increase the probability to identify genes associated with respective pain disorders. Therefore collaborations with migraine and headache groups needed to be established specifically with other consortia to increase the power of GWA in the pain field. 2. SNPs selected in ncRNAPain and which are not available on the SNP microarray or the hereby imputed SNPs will be genotyped in a targeted approach by multiplex genotyping using iPLEX technology on a Sequenom Massay Array platform. SNPs which fail with these methods are genotyped by KBioscience KASP assays or Taqman assays. The same technologies will be used in case of fine-mapping of genes which show a strong association signal. The analysis will be further extended to intermediate phenotypes in the patient group characterized by sensory profiles, pain-related cognitive function and clinical signs.

# SCIENTIFIC & TECHNOLOGICAL RESULTS

**Assessment of functional consequences of polymorphisms in cellular and preclinical models**

To understand the functional consequences of the identified ncRNA associated polymorphisms, mutated ncRNAs will be generated and cloned into viral vectors using standard protocols. Overexpression of mutated ncRNAs or target genes will be performed in neuroblastoma cell lines and primary neuron cultures (DRG, spinal neurons, hippocampal neurons, others). Survival assays, outgrowth analyses, neuropeptide release and biophysical parameters will be assessed in order to obtain first insight into potential functional deficits in the pain pathway that are causally associated with deficient ncRNA regulation due to loss or gain of function mutations of ncRNAs, promotor regions or ncRNA regulated genes. In vivo overexpression of mutant ncRNAs following local application of mutant ncRNA viral vectors will be performed in untreated wt mice and mice with neuropathic or neurogenic pathology for behavioural phenotyping to assess general changes of pain processing and any potentially increased susceptibility to develop neuropathic or neurogenic pain. Tissue samples from rodent models will be assessed for morphological changes and signs of neuro-immune deficits.

# SCIENTIFIC & TECHNOLOGICAL RESULTS

## Work package 7

The WP aims at translational results of ncRNAPain to develop tools for routine clinical use that enable physicians to make the correct mechanism-based pain diagnosis in an individual patient. Based on the knowledge provided by ncRNAPain, ncRNA patterns, SNP information, inflammation markers and clinical signatures are collected and databases for ncRNA patterns, ncRNA SNP patterns, psychological inventories, sensory tests and QST profiles together with functional clinical and laboratory data were generated. Bioinformatics/systems biology analyses were applied to reconstruct ncRNA-mRNA networks and identify network hubs which will be the basis for selection of ncRNA candidates that improve patient stratification and risk assessment for clinical purposes and for novel ncRNA based therapies. Easy to use small scale ncRNA based assay panels for clinical use will be developed. ncRNA based treatment strategies aiming at inactivating pain inducing ncRNAs or by substitution of ncRNAs for pain relief will be developed.

### Diagnostic ncRNA panel and improved clinical SOPs

In order to allow improved diagnosis and assignment of the most appropriate therapies, based on data derived from ncRNAPAin the custom-made qPCR panel/hybridization assay panel has been developed and a miR signature which is highly selective and specific for CRPS has been discovered. Based on these ncRNAPain outcomes, the ncRNA assay will be optimised for ncRNAs found to correlate best with the diagnosis of neurogenic and neuropathic pain.

A data integration approach will be used for the analysis of the biomolecular and clinical data and for the identification of predictive parameters for improved diagnosis and patient stratification as published. We characterized sensory phenotypes of patients with painful and painless diabetic neuropathy and assessed demographic, clinical, metabolic, and electrophysiological parameters related to the presence of neuropathic pain in a large cohort of well-defined DSPN subjects. This observational cross-sectional multi-center cohort study comprised detailed history taking, laboratory tests, neurological examination, quantitative sensory testing, nerve conduction studies, and neuropathy severity scores.

# SCIENTIFIC & TECHNOLOGICAL RESULTS

All parameters were analysed with regard to the presence and severity of neuropathic pain. Neuropathic pain was positively correlated with the severity of neuropathy and thermal hyposensitivity. A minority of patients with painful DSPN had a sensory profile, indicating thermal hypersensitivity that was associated with less severe neuropathy. Neuropathic pain was further linked to female sex and higher cognitive appraisal of pain as assessed by the pain catastrophizing scale, while parameters related to diabetes showed no influence on neuropathic pain with the exception of laboratory signs of nephropathy. These findings confirmed the value of comprehensive DSPN phenotyping and underline the importance of defining different sensory phenotypes for stratification of patients for analgesic treatment and drug trials.

These clinical and ncRNA related parameters will useful to adapt SOPs for clinical cohort recruitment. Thereafter, detailed analysis of large additional cohorts will be performed for initial validation. Patient subgroups with specific ncRNA and biographical fingerprints will be identified.

**ncRNAs for pain prevention and treatment**

Outcome from ncRNAPain will provide a sufficiently reliable basis and mechanism-based justification to transfer the gain knowledge into ncRNA based analgesic therapeutic applications for the benefit of patients and to select ncRNA candidates as potentially useful drug targets. The selection process used a combination of bioinformatics analysis, expression profiling in patients and preclinical models and ncRNA target genes and a careful literature search for regulated pathways and risk assessment to select the appropriate ncRNAs emerging from ncRNAPain as the most likely promising ones to yield clinical benefit. Bioinformatics screening algorithms will be used to search for sequence-dependent off-target effects (OTE). Certain OTEs will become evident from outcome of preclinical work packages and will both be used as exclusion criteria. Further challenges for the transition to the therapeutic arena - mainly regarding RNA stability and systemic delivery - will be addressed by usage of locked nucleic acids (LNA) or short seed-directed anti-ncRNAs, by chemical modifications (e.g) or the use of packaging carriers.

# SCIENTIFIC & TECHNOLOGICAL RESULTS

## Work package 8

The main objective of WP8 was to ensure coherent external communication of ncRNAPain activities, progress and achievements as well as the optimal management and exploitation of research outcomes and resulting Intellectual Property (IP).

### External project communication and networking

The ncRNAPain consortium was particularly devoted to ensure that the project's ideas are known and understood by a diversity of actors, inside and outside the "Clinical and experimental pain research" and "mi/ncRNA" research community. To ensure sustainability of the projec's impact as described above, dissemination activities targeted (alongside international scientific players) foundations, organizations, health care authorities, etc. committed to advancing awareness about the progress of ncRNAPain research at diverse levels.

Dissemination tasks were also focused towards European and International organizations dealing with clinical and experimental pain research and neuroscience, diabetes, and cytokines and inflammation, and headache and migraine such as the International Association for the Study of Pain (IASP), the European Federation of IASP Chapters (EFIC), the Federation of European Neuroscience Societies (FENS) and the Society for Neuroscience and the International Headache Society.

Scientific dissemination activities during the project's lifetime particularly focused on presenting objectives and research carried out in the frame of the ncRNAPain project at diverse events, including the organization of workshops, and in scientific publications, preferably as open access publications.

During the course of the project, publication output of the ncRNAPain consortium gained momentum. 40 articles were published up to date; further publications are currently in preparation and will be submitted shortly.

# SCIENTIFIC & TECHNOLOGICAL RESULTS

Spreading of ncRNAPain results and objectives by further scientific dissemination activities (e.g. poster presentations, talks given at scientific conferences, workshops etc.) were put in the focus. As outlined above, liaising with European and International organizations dealing with clinical and experimental pain research and neuroscience is of particular importance. Thus, beneficiaries were involved in organization of and active participation in presentations, refresher courses and workshops. The most important event in this respect was the IASP 16th World congress on Pain in Yokohama, Japan, from 26 to 30 September 2016. Claudia Sommer, PI of the UKW-Neuro group, headed the Scientific Program Committee. This major event on Pain Research offered an excellent platform for networking with other researchers and initiatives and to disseminate the ncRNAPain project research. Several beneficiaries of the ncRNAPain project actively contributed to the program by giving presentations, offering refresher courses and workshops as well as presenting posters.

Furthermore, Coordinator Michaela Kress gave a plenary lecture at the 2015 EFIC Conference in Vienna, Frank Birklein from partner UMC-Mainz was invited to give the prestigious Sunderland Lecture at the Annual Scientific Meeting of the Australian Pain Society 2016. Moreover, research activities and first results were presented by the Consortium members at several conferences of national and international Pain Associations, such as the European Pain Federation or the British Pain Society.

Further to scientific dissemination, major efforts were put into communication about and raising awareness for the project and its objectives. To this end the project website (http://ncrna-pain.eu) was set up as a major tool of information directed towards a broad audience, including the general public and patients. Targeted communication measures, e.g. leaflets, as well as regular press releases and news updates ensured that the project's innovative impact on Europe's health sector and economy in general as well as on patients' quality of life in Europe and around the world were continuously addressed.

# POTENTIAL IMPACT

Chronic pain is not only a burden to individual patients and their families but a major societal problem which costs European societies around 1.5 to 3 % of GDP (gross domestic product). 21 % of Europeans with chronic pain are unable to work as a result of their pain, and as few as 10 % return to work according to estimates in a survey study. 27 - 50 % of chronic patients worry about negative effects of their pain on relationships, their jobs, family and friends.

ncRNAPain as an innovative and ambitious project tackled ncRNAs in pain syndromes for which treatments are insufficient. ncRNAPain provided a major advancement in both the knowledge of how pain is generated, propagated and quenched, and in evidence-based diagnosis and treatment of pain syndromes. The consortium committed to translate pre-clinical and clinical results into solutions for the benefit of the patients, in order to allow for improved identification of high risk individuals, better prevention, and mechanism-based treatment strategies which are considered to be a significant benefit for their quality of life.

Specifically, ncRNAPain has an impact on the following areas:

### Scientific impact

The ncRNAPain project builds a strong scientific platform and improves innovation and competitiveness of European research. ncRNAPain set out to harmonize future initiatives of miR/ncRNA research in the pain field. All consortium members have excellent publication records during the project, thus promising excellent scientific impact. By combining the expertise of the individual researchers through mutual support and data exchange, future research activities are channelled into a joint collaborative effort which minimizes competition between partners, and accelerates scientific progress and innovation through collaboration. ncRNAPain partners generate new concepts and ideas which are vividly discussed within the consortium and will influence translational pain research in general.

ncRNAPain has stratified and accelerated research activities and generated corporate identity by continuous communication, data mining and sharing and regular exchange of ideas in order to expand European leadership in the field with a translational/reverse translational approach in the pain field. In order to assure comparability of findings between labs, standardisation routines have been agreed on for all preclinical models as well as standard operating procedures for patient recruitment, clinical assessment, psychological profiling, inflammation markers etc.

# POTENTIAL IMPACT

For the ncRNAPain project standardisation is a key enabler for interoperability, specifically for comparability of scientific data and for homogeneity of patient cohorts, which is in particular important for rare diseases like CRPS to ensure quick recruitment, scientific quality, open markets and thereby building stakeholder confidence. Standardisation already at early stages of the project has guaranteed high quality and uniformity of patient sample and data handling as well as comparability and reliability of preclinical results. It will foster future transition of research activities into Horizon 2020 as well as access to the market of the innovative solutions of ncRNAPain and thus help ensure the practical application of research results.

The ncRNAPain project provides innovative research beyond the state of the art and identifies and validates ncRNAs as novel druggable molecular targets. ncRNAPain identifies and develops ncRNA expression patterns or polymorphisms as biomarkers or predictors for pain. The project identifies pain predisposing genetic polymorphisms in ncRNAs, promoter regions and target genes, and provides novel understanding of how ncRNA expression patterns and polymorphisms in ncRNAs and/or their binding sites predispose for pain and cause inter-individual variations in the response to painful stimuli and analgesic drugs. Based on bioinformatic analyses ncRNA-based diagnostic tools for clinical application are available and also new druggable ncRNAs were identified as molecular targets. Through innovative research ncRNAPain will gain new knowledge of how ncRNAs regulate processes modulating nociception and endogenous analgesia and determine the importance of ncRNAs in circuitries and cognitive, emotional and behavioural components of pain. Insights into the concerted function of ncRNAs in the control of macromolecular complexes in neurons, glia and immune cells and signals used for neuro-immune communication in the pain pathway are obtained. ncRNAPain provides translational results, specifically for potential novel ncRNA based pain therapy which were validated in preclinical models during the project and for which patent applications will be arranged. To meet the urgent need for improved diagnostic standards and tools, diagnostic kits for patient stratification are developed.

The ncRNAPain project uncovers the biological role of ncRNAs in pain syndromes. Innovative novel concepts, ncRNA targets and pathways will become available which will be shared with other scientific disciplines. Novel ncRNAs discovered by the ncRNAPain consortium will be published and the searchable database PAINBASE will then be accessible as a potential new tool for the development of diagnostic parameters in other scientific disciplines. Innovative ideas and scientific excellence will ensure transition of ncRNAPain research activities into Horizon 2020.

# POTENTIAL IMPACT

## Health care providers and patients

Future improvement of the diagnostic and therapeutic strategies for chronic pain patients is a major socioeconomic and societal challenge (1) and expected to have a major positive impact on health care systems. Special emphasis has therefore been put on translational research and the development and validation of new diagnostic tools and therapies. Moreover, a balanced recruitment of clinical scientists, epidemiologists and basic researchers has encouraged translational research and expedites the successful development of clinically relevant results. The ncRNAPain consortium is strongly committed to a reverse translational/translation approach aiming at clinically applicable solutions and sustainability. This will definitely ease the conduction of adequate clinical studies in rare diseases.

Clinical training has been provided to the staff of clinical partners within the consortium which ensured improved clinical standards and standardised diagnostic of pain disorders with immediate benefit for hospital staff and recruited patients. Continuous training, and as evidence becomes gradually available, improved patient stratification and risk assessment with the diagnosis tool-box will provide accelerated diagnosis and improved mechanism-based treatment selection to patients of clinical partners. Clinical training will be provided for external institutions which will have translational impact on the diagnostic possibilities and patient stratification routines and individual risk assessment.

A diagnosis tool-box will offer significant improvements for assigning an individually tailored pain treatment to each pain patient or for high risk patients, to take evidence based preventive measures. Improved clinical guidelines and standard operating procedures for diagnosing neurogenic and neuropathic pain will facilitate patient stratification and mechanism based treatment selection. This will yield improved European and global guidelines for diagnosis and patient stratification aiming towards a personalised pain medicine.

Since treatments for chronic pain syndromes to date are insufficient, ncRNAPain will offer a major advancement in both the understanding and the diagnosis and treatment of pain syndromes. The consortium is committed to translate pre-clinical and clinical results into solutions for the benefit of the patients, by future developing novel cures for pain disorders which will be a significant benefit for their quality of life.

# POTENTIAL IMPACT

## Impact for the consortium

An outstanding team of experts generates impact by itself through their innovative research beyond the state of the art; and by combining the expertise of individual partners into mutual support and data exchange, research activities are channelled into common collaborative efforts rather than competition between partners. ncRNAPain synergizes convergent technology transfer by connecting well-established pain clinicians, neuroscientists, microglia experts and internationally renowned experts in bioinformatics, genetics, epidemiology and the ncRNA field together with the European SME leader in miR-research and technology. The consortium's interdisciplinary structure by itself ensures strong impact for the consortium partners and their scientific profiles and leadership.

The multi-disciplinary nature of this project has encouraged innovation and increased the competitiveness and impact of the research. ncRNAPain has strengthened the R&D capacities of all partners involved and thus will ensure transition of the scientific programme in a follow up initiative. This will contribute to one of the flagship initiatives of Europe 2020, i.e. the "**Innovation Union**", by promoting knowledge and innovation partnerships through links between education, business, research and innovation. International collaboration in a network of complementary scientific expertise has accelerated innovation and scientific progress of all partners and the consortium as a whole. ncRNAPain has established collaboration of preclinical and clinical scientists including experts in bio/neuroinformatics and provided all partners with updated information on technological and scientific state of the art within the consortium. ncRNAPain has initiated immediate pan-European partnership for technology transfer between scientific disciplines and preclinical and clinical scientific research. Established models, available tools and complementary expertise within the consortium guaranteed an immediate start to the research work. In order to achieve uniform high quality standards throughout the entire consortium, audits and mutual training of staff and partners with respective expertise were part of the work plan.

Project relevant data are shared e.g. by implementing a searchable object-oriented data base for mouse and human data which is filled with data that are already available from partners and continuously expanded by novel data from literature mining, and results of ncRNAPain.

# POTENTIAL IMPACT

The database is available in the protected area of the ncRNAPain website and accessible for all partners. Data sharing is one of the columns for efficient collaboration of all partners and ensure innovative research beyond the state of the art.

The ncRNAPain project has attracted and employed the best qualified scientific staff to strengthen the individual partners and the consortium as a whole. The ncRNAPain consortium expands scientific leadership beyond the state of the art in order to target pain syndromes whose treatments are inexistent or inadequate and provides knowledge on ncRNA related mechanisms of different pain syndromes as well as the inter-individual variation in the response to painful stimuli and analgesic drugs. The consortium represented a critical mass to attract more scientific interest and with it funding including the possibility for support from the pharmaceutical industry. ncRNAPain will develop measurable standards for quality assessment which makes it an innovation nucleus for transition into Horizon2020 and the basis of a follow up large scale program in the pain area.

Partners involved in the project expanded their research into innovative ncRNA science and gain novel insight in biological processes relevant to the health area. Regular future meetings of participants will intensify interaction between clinical researchers, basic scientists and in particular SME partners which tighten their network and facilitate generation and exchange of innovative ideas. Continuous interaction between partners guarantees efficient data acquisition, fosters joint publications in top journals and already ensured support and training of outstanding junior scientists. Through ncRNAPain, all partners benefit from new scientific insights which promote and support the global visibility and leadership of the consortium and its partners.

In the preparation process for this proposal many discussions between clinical partner, geneticists, bioinformatics experts and epidemiologists clearly pointed out the need to gain access to large patient cohorts in order to increase the power of association and intervention studies. It is emerging that individual consortia will not be able to phenotype enough cases but reasonable numbers will only be achieved by joint efforts of all pain research consortia in Europe or all over the world. ncRNAPain offers leadership and will set out to coordinate genetic studies and form a structured network of European pain research consortia with regular meetings and data exchange.

# POTENTIAL IMPACT

## Economic impact

Against the increased global competition, the ncRNAPain project has defined a strategy to support growth and job creation for innovation in Europe. The objectives outlined in this strategy clearly support the importance of improving Europe´s performance in research and innovation by the creation of new products or services, to generate growth and jobs and help address social challenges. ncRNAPain sets out to bundle all miR/ncRNA initiatives in pain research together under a common theme playing a crucial role in reaching the goals of growth, competitiveness and employment. The ncRNAPain consortium has been assembled to maximize the likelihood of a successful outcome with regard to innovation and exploitability.

ncRNAPain has attracted the global market leader SME in miR analysis who has an inherent commercial interest in developing diagnostic panels for clinical purposes currently in the oncology area. This can be extended to other subspecialties of the health arena, e.g. pain therapies. Competitors for ncRNA analyses and inhibitors or mimics are emerging on the global market and ncRNAPain has provided support for Europe's market leader to expand their leadership and innovative potential in ncRNA molecular biology by developing innovative products for scientific research.

ncRNAPain in a mutual exchange of ideas and technology expects to support European SME in ncRNA drug discovery and expand SME profile into clinically applicable products. This applies to the support of European SME in ncRNA drug discovery. The clinical as well as ncRNA-based components of the diagnostic tool-box represent a greatly improved and effective diagnostic approach for clinical use. Innovative product development with major advantages over conventional clinical SOPs contributes to the increase of European competitiveness. It is expected that ncRNAPain will attract global interest and thus not only improve the health of European citizens but also boost the innovative capacities of European Research and health-related industries. Support from ncRNAPain for the European SME market leader in ncRNA research tools will impact upon intermediate commercialization opportunities of assays and kits which will allow SME to successfully compete on the global market.

# POTENTIAL IMPACT

The clinical validation of novel ncRNA based drugs for pain therapy together with exploitation of diagnostic kits for global clinical use impacts on the future position and value of the European SME on the global market where pain killers according to the Visiongain report (http://www.visiongain.com/Report/867/World-Pain-Relieving-Drug-Market-2012-2022) will reach a value of 61.6 billion $ in 2012. The study forecasts that the overall market for pain relieving drugs will grow steadily in the next ten years. The world pain-treating drug market is a high revenue industry with a high public and healthcare profile which, however, needs more therapies tailored to individual needs.

The expected growth of the SME partner together with high profile education and training in an innovative research area will create new jobs and attract excellent young scientists who are employed by ncRNAPain related funds and industry or other sources and will further contribute to commercial progress and allow for success in the **"Europe 2020 strategy**" with its priorities to develop smart, sustainable and inclusive growth (http://ec.europa.eu/eu2020).

# POTENTIAL IMPACT

## Impact for Europe

ncRNAPain's committed to improving the health of European citizens and increasing the competitiveness and boosting the innovative capacity of European health-related industries and businesses while addressing a global health issue. Emphasis was put on translational research (translation of basic discoveries in clinical applications including scientific validation of experimental results) the development of methods for health promotion and prevention including promotion of child health, healthy ageing, diagnostic tools medical technologies, new therapies, as well as sustainable and efficient healthcare systems. Chronic pain syndromes occur at all ages and affect all societies in Europe and all over the world. The lack of knowledge and evidence-based standardized clinical routines for the diagnosis of pain, and the inexistent or insufficiency of current pain therapies are a global issue.

ncRNAPain is a consortium of international experts in Europe who have increased the European research profile. ncRNAPain improved the progress of European RTD in this area and the competitiveness and international visibility of European scientists.

In addition to continuous efforts to increase European pain research and improve the progress of European RTD, dissemination of results to the scientific and lay public leads to a timely awareness by society and patients of the novel and effective diagnostic and therapeutic applications, and ultimately promotes RTD culture and public understanding of science.

ncRNAPain tackles the improvement of health and wellbeing of European citizens, the increase incompetitiveness of European health related scientific research bodies, industries and services, as well as addressing the socio-economic dimension of health care and global health issues. Furthermore, novel ncRNA based drugs for curing chronic pain as an expected outcome of ncRNAPain will help to alleviate the burden of public health by curing chronic pain.

# POTENTIAL IMPACT

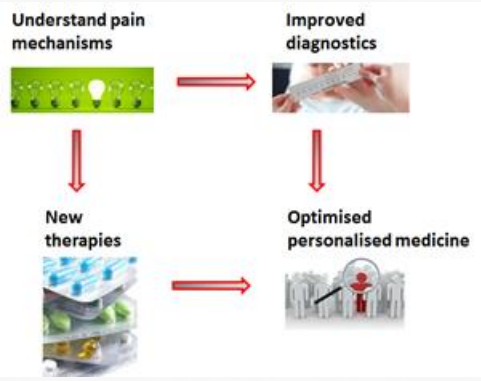


**Fig 3.** ncRNAPain impact

Research and innovation are key drivers to deepen knowledge of how pain is generated, propagated and quenched, to work towards the identification of ncRNAs as more effective diagnostic and/or treatment approaches, and translate pre-clinical and clinical results into solutions for the benefit of the patients. ncRNAPain results, impact and directions for future development ensure that the research that was started during the FP7 funded project will continue and contribute towards the scientific, medical and societal goals beyond its funding period. ncRNAPain provides essential information on topics ranging from diagnostic improvements and novel mechanistic insight to the development of new mechanism-based treatment options. Importantly, it offers concrete novel knowledge that is relevant for both the general public and policy makers.

Highly ambitious and visionary research requires substantial resources and bears the risk of not achieving its original goals. However, as our results give clear evidence for, research is indeed an effective investment to address and advance unmet medical needs in modern highly knowledge-driven societies within the European Union.

Taken together, ncRNAPain offered an important innovation in the health sector through the definition of new standards, development of new technologies and opening the road for the development of new drugs/therapies for pain syndromes whose treatments are inexistent or inadequate for the improvement of quality of life in Europe and around the world.