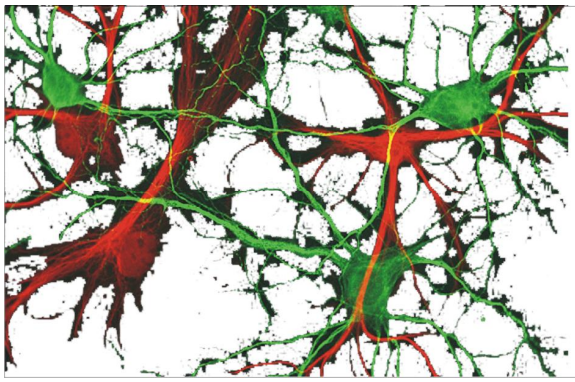

GLORIA

(glial-opioid receptor interface in analgesia)

**Understanding chronic pain and new druggable targets:**

**Focus on glial-opiod receptor interface**

# GLORIA

# FINAL REPORT

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**1 Executive summary ..... 3**
**2 Summary description of project context and objectives ..... 4**
**3 Description of the main S&T results/foregrounds ..... 6**
3.1 WP1 Mapping of pheno- and genotypes in chronic pain ..... 6
3.1.1 Main objectives ..... 6
3.1.2 Main results ..... 6
3.1.3 Conclusions ..... 10
3.2 WP2 Mapping of phenotypes in chronic pain using advanced proteomics ..... 11
3.2.1 Main objectives ..... 11
3.2.2 Main results ..... 11
3.2.3 Conclusions ..... 13
3.3 WP3 In vivo studies of glial cell activation and pain regulation by imaging ..... 13
3.3.1 Main objectives ..... 13
3.3.2 Main results ..... 13
3.3.3 Conclusions ..... 16
3.4 WP4 Glial activation in animal models of chronic pain ..... 17
3.4.1 Main objectives ..... 17
3.4.2 Main results ..... 17
3.4.3 Conclusions ..... 20
3.5 WP5 Role of opioid receptors on glial cells in chronic pain: conditional knockouts ..... 21
3.5.1 Main objectives ..... 21
3.5.2 Main results ..... 21
3.5.3 Conclusions ..... 22
3.6 WP6 Novel compounds for managing chronic pain ..... 22
3.6.1 Main objectives ..... 22
3.6.2 Main results ..... 23
3.6.3 Conclusions ..... 24
**4 Potential impact, main dissemination activities and exploitation of results ..... 25**
4.1 Impact ..... 25
4.2 Main dissemination activities ..... 27
4.3 Exploitation of results ..... 32
**5 Website and contact details ..... 34**

## 1 Executive summary

Chronic pain affects 20% of the adult population in the EU, decreases the quality of life of the patient and causes significant costs to the society. Currently available analgesics are either not effective enough or cause serious adverse effects. The aim of GLORIA (Glial-Opiod Receptor Interface in Analgesia) was to study neuroinflammation and the role of glial activation in chronic pain conditions fibromyalgia (FM); osteoarthritis (OA); rheumatoid arthritis (RA); neuropathic pain (NP), and opioid tolerance and hyperalgesia (OIH). Studies included both chronic pain patient cohorts and respective experimental disease models. The goal was also to design and synthesize novel analgesic molecules that either target glial activation via Toll-like receptor (TLR), or are opioid analogues or glial cell line-derived neurotrophic factor (GDNF) family ligand (GFL) mimetics.

We discovered clinical pain phenotypes using epidemiological and data science methods: remaining pain and widespread pain in early RA, and persistent pain after major surgery. The information will help to recognise patients at risk for developing chronic pain and to initiate early interventions to prevent pain becoming chronic. We identified several potential, neuroinflammation-related, protein biomarkers associated with chronic pain, and genetic and epigenetic markers associated with persistent pain after major surgery. Using positron emission tomography (PET) we were able to provide first clinical evidence on the role of glia activation in the pathophysiology of FM. We also showed that neuroinflammation is present in all assessed chronic pain conditions, but with different profiles. Thus, the clinical evidence from GLORIA suggests the immune system as a target for potential future drug development for different chronic pain conditions.

We showed that glial cells are activated also in experimental neuropathic pain models, and that, like in humans, there are differences between sexes in the development of pain-like behaviour. We showed for the first time that opioid receptors in glial cells participate in the development of NP, and tolerance to opioid analgesia. In the preclinical models of opioid tolerance and OIH, glial cell activation pattern had similarities with the NP models, but without sex differences. However, we found differences between the sexes related to microglial receptors in certain NP and RA models.

We found new compounds showing activity on TLR4 and discovered new 7β-hydroxy-8-ketone opioid derivatives. We also found several new compounds that act as GFL mimetics, which attenuated pain-like behaviour, protected sensory neurons and reduced microglial reactivity in experimental neuropathic pain models, making GFL mimetics promising new molecules for further development as disease-modifying drugs for the treatment of NP.

In conclusion, GLORIA provides extensive evidence for the role of glia activation and neuroinflammation in chronic pain. We have made several novel and important findings on the pathophysiology of chronic pain and found novel diagnostic tools for chronic pain patients. We have also discovered novel molecules with therapeutic potential. The outcomes of GLORIA have the potential to translate its findings into more effective and targeted diagnosis and treatment of chronic pain. The outcomes of GLORIA will thus benefit both patients and clinicians, research, pharmaceutical industry and the whole society.

## 2 Summary description of project context and objectives

Chronic pain is prevalent; it decreases the quality of life of the patient and causes significant costs to the society. It has been estimated that one European in five and one in three of the over 70-year olds has chronic daily pain that requires treatment. The costs of chronic pain can be calculated in decreased quality of life and economic losses (private and societal) due to lost working days and medical expenses. Chronic pain patients often experience muscle and joint wasting (due to immobilisation), depression, anxiety, disturbed sleep, cognitive impairment, loss of appetite, isolation, and possibly even suicidal thoughts. Their reduced abilities are usually the cause of poor job performance, dependence on medication as well as on family or other caregivers, and excessive use of professional healthcare systems. This amounts to a significant financial burden to the family, friends, employees, and health care systems. The burden of suffering that pain inflicts on individuals and the costs it inflicts on society clearly illustrate the urgent need for European governments and institutions to include the societal impact of pain on their policy agendas. Whereas acute pain is an important symptom, a signal that requires attention, and is the most common reason for people to seek help from medical care, the protective nature of pain loses its relevance the more chronic it becomes. The categorisation of chronic pain to nociceptive, inflammatory, neuropathic and dysfunctional (e.g. fibromyalgia) is crude and, according to the current understanding of pain, many pathophysiological mechanisms overlap. Cognitive or mood factors are also involved in all types of pain.

Currently available analgesics are either not effective enough or patients cannot take them due to adverse effects. Analgesic efficacy could be improved by targeting pain mechanisms that are specific for different conditions: fibromyalgia (FM), osteoarthritis (OA), rheumatoid arthritis (RA), and neuropathic pain (NP), and by improving the safety of the current analgesics, such as opioids. So far, most analgesics have been targeted to symptom relief. Only inflammatory pain has benefited from disease-modifying drugs. Understanding the pathophysiological mechanism of the underlying disease that causes pain may provide new insight to more targeted, personalised pain therapy that also has a better safety profile. Neuroinflammation is an emerging field of research in pain. Inflammatory pain can cause antibody-mediated nerve damage and neuropathic pain involves, regardless of its origin, the interaction of neuronal, glial and immune cells.

GLORIA focused on the role of glial activation by tissue and nerve injury, inflammation, and high doses of opioids in chronic pain patients and in respective experimental disease models. Glial cells (microglia and astrocytes) are highly reactive immunocompetent cells of the central nervous system that can be activated by inflammation, infection, nerve/tissue damage, and stress. They have emerged as important targets in understanding the mechanisms of both neuropathic and inflammatory pain as well as the role of neuroinflammation in the development of central pain sensitisation. Opioid-induced hyperalgesia and tolerance have been associated with glial cell activation raising the intriguing possibility that activation of the opioidergic system, in response to chronic pain, could induce glial activation via both exogenous and endogenous opioid receptor ligands.

Individual differences in glial activation and pain perception were used to design different research lines to develop personalised analgesics. We used well-validated *in vivo* models relevant for RA, OA, FM, and NP. Glial activation in these models was studied using novel methodologies to better understand the pathophysiology of these conditions. GLORIA also focused on the design and synthesis of three lines of new analgesic molecules, those that target the activation of glia or are GDNF family ligand (GFL) mimetics, and improved opioid analogues.

**Main objectives of GLORIA were to:**

1. Phenotype and genotype four different chronic pain conditions in humans (NP, FM, OA, RA) and to use/develop respective animal models for the development of new targeted analgesics.
2. Determine the role of glial cell activation in these four chronic pain conditions and in the respective disease-specific animal models of chronic pain and opioid tolerance and hyperalgesia (OIH).
3. Define the role of the opioid receptor in glial activation by using conditional knockout mice and different *in vitro* and *in vivo* methods in animal models of NP, FM, OA, and RA.
4. Design and synthesise new compounds for the management of chronic pain and to test them in disease-specific animal models.

**Main lines of research in GLORIA aiming to reach the objectives:**

WP1: Mapping of pheno- and genotypes in chronic pain
The main goal was to identify biomarkers for chronic pain and those related to the glia-TLR4-opioid systems. We also performed systematic pathway analyses to tag new potential genes via novel systems biology bioinformatics methods.

WP2: Mapping of phenotypes in chronic pain using advanced proteomics
We used advanced proteomics to identify biomarkers for pain patterns and for different phenotypes in chronic pain. We established a proteomic platform, performed epidemiological characterization of pain patterns and geno-phenotypes in large patient cohorts and identified biomarkers for pain phenotypes in RA, OA, FM and NP.

WP3: In vivo studies of glial cell activation and pain regulation by imaging
We studied the role of neuroinflammation in chronic pain. We assessed endogenous pain modulation and resting state networks with functional magnetic resonance imaging (fMRI) in chronic pain patients; established patterns of neuroinflammation by analysing cerebrospinal fluid and developed methods to assess glia cell activation using positron emission tomography (PET) in humans, and developed cellular models for quantitative characterization of opioid receptor-mediated signalling dynamics by functional fluorescence microscopy imaging (fFMI).

WP4: Glial activation in animal models of chronic pain
The aim of this work was to examine the role of glia cells and opioid receptors expressed in them in *in vivo* models representing inflammatory, nociceptive, neuropathic, and fibromyalgia-type pain.

WP5: Role of opioid receptors on glial cells in chronic pain: conditional knockouts
We established genetic *in vivo* models to study the role of mu and delta opioid receptors in glial cells in NP. We aimed to identify key opioid receptor populations expressed by glial cells in the development and persistence of chronic pain and opioid tolerance/hyperalgesia.

WP6: Novel compounds for managing chronic pain
We searched for novel, more potent compounds to target pain-related receptors using computational methods, and optimised existing drugs for improved safety by combining computational methods with synthesis of derivative molecules and *in vitro/vivo* testing of the compounds. The focus was on two lines of molecules: those that target opioid receptors/glia and GFL mimetics.

# 3 Description of the main S&T results/foregrounds

## 3.1 WP1 Mapping of pheno- and genotypes in chronic pain

### 3.1.1 Main objectives

The objective of WP1 was to identify potential biomarkers for persistent pain, with a special focus on pain-related genetic and epigenetic markers that are accessible in biological materials from healthy subjects or pain patients. The main hypothesis involved the glial-opioid interface, the evidence suggesting important roles for the opioid (*e.g. OPRM1*) and Toll-like receptor (e.g. *TLR4*) genes as primary candidates associating with persistent pain. In addition, computational systems biology analyses were used to tag further candidate genes in the clinical context of persistent pain due to various causes. Thereby, WP1 analysed patterns of genetic, epigenetic, clinical, psychological and demographic parameters, which may allow identifying subgroups of subjects that share pain phenotype features.

Several methods of machine-learning and artificial intelligence were applied in addition to contemporary next-generation gene sequencing and epigenotyping methods. This resulted in (i) the identification of pain-related subgroups of patients, (ii) the association of genetic markers allowing to identify patients belonging to these phenotype groups based on genetic markers and (iii) the association of epigenetic markers with the pain phenotype of persisting pain after major surgery.

### 3.1.2 Main results

***Phenotypes of pain including persistent pain***
Using rule-based analyses and/or machine-learned data projections, pain-related phenotypes emerged in healthy subjects who had participated in experimental pain studies as well as in patients who had undergone breast cancer surgery.

**Firstly**, the sensory phenomena, resembling those in neuropathic pain, induced by topical capsaicin, a TRPV1 agonist, application were observed in healthy subjects when quantitative sensory testing (QST) data were projected on an artificial neuronal network. The data were subsequently visualised

usig a cluster structure by means of the so-called U-matrix, which is an add-on to the self-organising map of the Kohonen type, depicting the distances between data points in the high-dimensional as a third dimension on the two-dimensional map. From this projection, subgroups comprising approximately 20% of the subjects were identified. They displayed 60% of the pain phenotype patterns described in neuropathic pain patients.

**Secondly**, healthy volunteers could be subgrouped with respect to their response to local ultraviolet-B (UV-B) irradiation or capsaicin application. While heat pain thresholds were the most relevantly affected QST parameter with both sensitisation procedures, UV-B additionally modulated the sensitivity to cold stimuli. Moreover, self-organising maps suggested that there were subgroups that responded to topical application of capsaicin with different sensitivities to pressure pain. The subgroups could subsequently be attributed to sex differences as women were more sensitive to pressure pain stimuli than men.

**Thirdly**, pain-related phenotype subgroups with respect to chronification were found in a cohort of 1,000 women who were followed-up for three years after breast cancer surgery for the evolvement of postsurgery pain. Based on predefined rules regarding the intensity of pain during repeated assessments over three years, two major subgroups of subjects were identified: the “persistent pain” and the “non- persistent pain” phenotypes. A diagnostic tool was created based on machine-learned methods. The tool included 21 rules involving psychological, demographic and clinical factors. Application of this rule-based classifier (biomarker) enabled a highly accurate identification of patients who are not at risk to develop persistent pain. This can be used to identify patients in whom complex time-consuming preventive therapies are unecessary, allowing them to more quickly return to normal life after breast cancer surgery. Further analysis indicated that a short questionnaire of less than 10 psychological items provided almost similar diagnostic accuracy for identifying patients at risk, or not at risk, for persistent pain. This short questionnaire was developed by applying supervised machine-learning feature selection methods to data acquired from standard questionnaires comprising more than 50 items.

**Fourthly**, further analyses of the time courses of pain during the three years after breast cancer surgery in the above-mentioned patient cohort indicated that pain can take three major types of temporal developments. Three subgroups of patients were identified as best describing the temporal pattern of pain from 6 months after breast cancer surgery. While pain decreased in cluster #1 and remained stable in cluster #2, a subgroup of patients (cluster #3, 11%) had high pain levels that tended to increase over time. Early (one month after surgery) pain symptoms did not provide enough information to identify this patient subgroup; however, it provided sufficient information to identify patients who are unlikely to develop persistent pain.

***Genetic markers of persistent pain***

A genetic component contributing to the individual perception of pain and the risk for chronification has been a research subject for more than half a century. Accumulated evidence involves nowadays more than 500 genes in these processes. While together with other lines of experimental and clinical research this allows to pursue specific hypotheses, such as the implication of opioid and Toll-like

receptor genes in pain persistence pursed in the GLORIA project, our understanding of the genetic architecture of pain and its progression toward persistence is still incomplete.

Therefore, to approach the association of genetic patterns underlying the pain-related phenotypes identified within WP1 at a broader level than the hypothesis-related restriction to the main players at the glial-opioid interface, a systems-biology analysis of persistent pain preceded the selection of a set of candidate genes. Here, the increasing availability of "big data" enabled data mining and knowledge discovery by means of machine-learning, which combined the knowledge of 535 genes identified empirically as relevant to pain with the knowledge about the functions of thousands of human genes. Starting from an accepted description of chronic pain as displaying systemic features described by the terms "learning" and "neuronal plasticity," a functional genomics analysis was applied, which identified a subset of n = 34 pain genes to be annotated with both Gene Ontology (GO) terms. Published empirical evidence supporting their involvement in chronic pain was identified for almost all these genes, whereas such evidence was virtually absent in a randomly selected set of 34 other human genes.

This set was included in a next-generation sequencing (NGS) panel comprising a total of 77 genes, which was developed from thee different lines of evidence. Specifically, the **first** line comprised the above-mentioned n = 34 genes controlling biological processes of learning and of neuronal plasticity. The **second** line of evidence included n = 13 genes reported to carry variants that modulated the risk or the intensity of pain in at least two different clinical settings of persistent pain. They had emerged in a separate analysis of a set of 110 genes carrying variants reported to be associated with modulation of the clinical phenotype of persisting pain in eight different clinical settings. Unsupervised machine-learning aimed at functional clustering was applied and finally, a subset of n = 13 genes was found, comprising those most consistently involved in persisting pain. On this basis, two groups of biological processes, the immune system and nitric oxide signaling, emerged as major

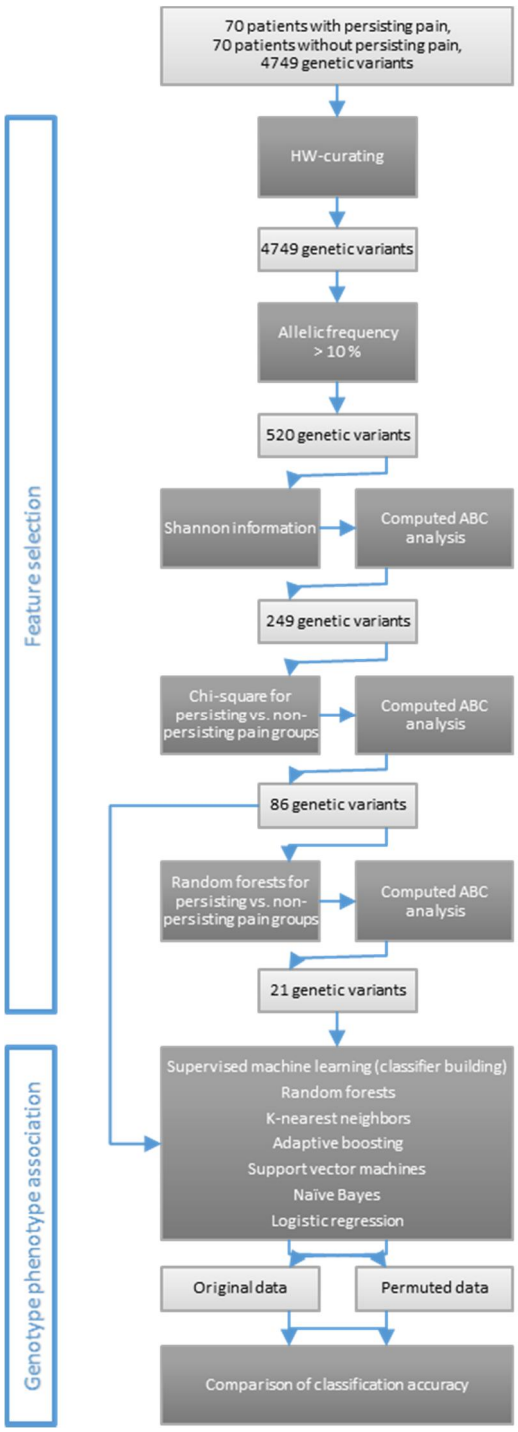


**Figure 1. Flowchart of the data analysis.** Feature selection started with the full NGS information. Subsequently, variants not in the Hardy-Weinberg equilibrium and those rarer than 10% allelic frequency were eliminated. The next steps involved analysis of information content using the Shannon information criterion and variants most unequally distributed among phenotype groups, based on the $\chi 2$ statistic, selected as the "A" subset of a computed ABC analysis, i.e. the "most profitable" item. Finally, the genetic variants were ranked for their importance in a random forests classifier, and only the most relevant items in ABC set "A" were maintained. This selection was used for genotype versus phenotype associations, once as original genotype per patient, and again with permuted genetic information.

players in sensitisation to persisting pain. This is biologically highly plausible and in agreement with other lines of pain research such as the GLORIA hypothesis of an immune system component in persistent pain. Finally, from the **third** line of evidence n = 30 genes were selected as those repeatedly shown during the last several years to play a role in the modulation of persistent pain, or that had only recently been reported as novel genetic modulators of persistent pain and were therefore included to keep the gene panel as up-to-date as possible.

To address the role of genetic variants in persistent pain, novel Ampliseq$^{TM}$-based NGS panels were developed and validated for (i) opioid and Toll-like receptors, (ii) TRP ion channels, and (iii) the n = 77 candidate pain modulating genes described above. This provided the advantage of unrestricted access to the complete coding and regulatory DNA sequence over genotyping selected functional genetic variants, which has been a research focus until recently. However, the resulting large amount of genetic information posed challenges to the bioinformatics analysis aimed at the association of the complex genotypes with pain-related phenotypes.

Therefore, a novel bioinformatical approach (Fig. 1) was developed to deal with the amounts of “big data” derived by means of NGS. The data analysis was performed in three main steps comprising (i) feature selection based on supervised machine-learning methods and item categorization techniques to identify most informative genetic variants, followed by (ii) unsupervised machine-learning based exploration of the resulting reduced genetic data for a group structure that reflected the pain phenotype group structure. Agreement between genotype and phenotype data structures would encourage an association between the two data sets. This was addressed subsequently by means of (iii) supervised machine-learning based genotype phenotype association, using several types of artificial intelligence algorithms.

The analytical approach was first applied on a data set comprising NGS based genotype information about *TRPA1* and *TRPV1* ion channels acquired in healthy volunteers who had undergone experimental sensitisation to heat stimuli following topical application of capsaicin cream. Specifically, *TRPA1*/*TRPV1* exomic sequences derived by NGS were assessed in n = 75 healthy volunteers. The genetic information comprised 278 loci. Gaussian mixture modeling indicated two phenotype groups with high or low capsaicin-induced hypersensitisation to heat. Unsupervised machine learning implemented as swarm-based clustering suggested differences in the genetic pattern between these phenotype groups. Several methods of supervised machine learning predicted the phenotype group association consistently better when based on the observed genotypes than when using a random permutation of the exomic sequences. Of note, *TRPA1* variants were more important for correct phenotype group association than *TRPV1* variants.

Following successful validation of the bioinformatical method described above, NGS was performed for the two extreme pain phenotype groups (pesitent pain or no persistet pain) identified in the cohort of patients treated for breast cancer. During these analyses, the genetic information was reduced from the original 4,748 variants found in the 77 genes to 21 relevant variants located in 13 different genes. The pattern of variants supported an association of NGS-derived genotypes with pain phenotypes. Supervised machine learning-based classifiers, trained with 2/3 of the data, identified the correct pain phenotype group in the remaining 1/3 of the patients with an accuracy of about 70%.

Finally, the systems-biology analyses employing machine-learned techniques for knowledge discovery in big data, such as the Gene Ontology knowledge base for the biological roles of genes or the DrugBank database listing the targets of currently available drugs, were used to approach possible novel analgesic drugs. With the contribution of WP1, a computational functional genomics-based approach at reducing sets of genes to the most relevant items was proposed. It is based on the importance of the gene within the polyhierarchy of biological processes characterizing the disease. The proposed method uses a gene importance score derived from the location of the gene-related biological processes in the DAG (directed acyclic graph). It attempts to recreate the roles of the original gene set with the least number of genes in descending order of importance. A subset of 29 best-scoring genes out of >500 were obtained as candidates for potential analgesic targets. Interestingly, most of them were targets of novel drugs already under clinical development.

***Epigenetic markers of persistent pain***

Changes in DNA methylation have been repeatedly shown to contribute to the development and treatment responsiveness of persistent pain. This has been observed at both single gene and global DNA methylation levels. In the present epigenetic analysis, the methylation status of *TLR4*, *OPRM1* and *LINE1* was assessed for possile association with the persistent postsurgical pain. DNA samples and pain data were available from the extreme phenotype subgroups of the patients treated for breast cancer identified as described above. DNA methylation levels were quantified using Pyrosequencing™ assays. Unsupervised machine-learning was employed for epigenotype-phenotype association. The present analysis could not provide support for a specific epigenetic modulation of persistent postoperative pain via methylation of two key genes of the glial-opioid interface.

### *3.1.3 Conclusions*

WP1 made extensive use of contemporary and novel methods of data science including machine-learning and artificial intelligence used for unsupervised data structure detection to identify pain-related phenotypes in healthy volunteers and patients at risk of or displaying persistent pain after major surgery. As a result, subgroups of subjects with respect to pain phenotypes emerged in all data sets. Importantly, these subgroups were always biologically plausible with respect to the parameters in which they differed from other subgroups in the respective cohorts. That is, in healthy volunteers, subgroups were based on the subjects' sex and on the sensitivity patterns to particular pain stimuli. In pain patients, the subgroups were distinct with respect to the time course of pain during three years after surgery, and they were accessible via algorithms including psychological, demographic and clinical factors.

WP1 also made extensive use of contemporary methods of next generation sequencing of relevant regions of pain-related genes and of DNA methylation analysis. Genetic and epigenetic information, centered on the glial-opioid interface but not restricted to it, provided (i) bases for the discovery of new candidate genes for the modulation of clinical persistent pain, (ii) the identification of novel drug targets based on computational functional genomics analysis of genes that have repeatedly been implicated in persistent pain, and (iii) for the association of genotypes acquired in the present cohorts

with pain-related phenotypes identified in the present cohorts of healthy subjects and pain patients. Using NGS-derived genetic information on the coding and regulatory sequences of 77 genes, machine-learned analysis indicated that the genotypes provide useful information for the allocation of the patients to either a “persistent pain” or “non-persistent pain” phenotype group in a three-year follow-up after breast cancer surgery. Genetic information, obtained by NGS, provided a similar diagnostic accuracy for persistent pain as non-genetic predictors. Moreover, a particular biological implication in breast cancer could be observed for most of the highest-ranking genes.

The results emphasize the utility of data science, including machine-learning and artificial intelligence, in pain research. The results further emphasise the utility of novel next-generation sequencing based methods in pain research. The results support a subgroup structure among all accessed pain phenotypes and encourage the establishment of genetic and epigenetic biomarkers, along with sociodemographic and psychological factors, to be used in the clinical setting of persistent pain.

## *3.2 WP2 Mapping of phenotypes in chronic pain using advanced proteomics*

### *3.2.1 Main objectives*

WP2 worked on the usage of advanced techniques in order to find biomarkers for pain patterns and different phenotypes in chronic pain. The specific objectives were to establish a proteomic platform, perform pilot studies on cerebrospinal fluid (CSF) samples and to perform epidemiological characterization of pain patterns and pain phenotypes in cohorts of patient groups with different kinds of pain. These include a cohort with fibromyalgia, widespread pain, a cohort of patients in the early phase of rheumatoid arthritis, an inflammatory joint disease characterized by recurrent joint pain, and finally, a cohort of patients with breast cancer surgery, of whom some developed neuropathic pain.

### *3.2.2 Main results*

***Epidemiological characterization of pain patterns in early RA***

We have made a thorough epidemiological work in order to define relevant and stable clinical phenotypes that could later be related to biomarkers and candidate proteins. First, we characterised the condition of remaining pain, i.e. pain that remains in spite of adequate anti-rheumatic treatment in RA. This work revealed that remaining significant pain after adequate treament is common in RA, and almost 20% of the patients with a good response to treatment displayed remaining significant pain. We have also shown that remaining pain after inflammation control one year after diagnosis strongly associates with widespread pain (WSP) three years after diagnosis in early RA. Next, we have validated a register-based phenotype for WSP. We used a pain mannequin, adapted from the Standardised Nordic questionnaire for analysis of musculoskeletal symptoms and assessed widespread pain in the early RA cohort three years after diagnosis. 729 patients were available; 60 (8%) had widespread pain three years after RA-diagnosis. The widespread pain-patients had statistically significantly worse health-related quality of life (SF-36) scores in all components at three

years and the pain-related clinical measures were significantly worse already at diagnosis of RA, while the inflammation-related measures did not differ. Moreover, WSP phenotype associates with markedly higher scores for depression and anxiety as measured with the HADS-instrument. This data thus further support that registry-based WSP phenotype seems to mirror a generalised pain condition and represents a pain phenotype in RA. In the analysis we excluded patients reporting pain problems before diagnosis of RA. Thus, the register-based WSP phenotype should not be biased by RA-patients having widespread pain already at baseline, but instead represents patients who have developed a pain condition during the early RA course. We have also found an association between the development of WSP and a genotype which will be further explored. We conclude that RA patients who had developed widespread pain three years after their diagnosis had a worse overall health status at that time and differed already at the time of diagnosis from the patients who did not develop widespread pain, both with respect to clinical presentation and genetic make-up. We have also started another approach by using the machine-learning method (together with JWGU, WP1) for comprehensive analysis and phenotyping of persistent pain in the EIRA cohort. These results are preliminary, but for the first time identify a group of patients who have persistent pain during the early years of rheumatoid arthritis.

***Proteins in CSF associated with pain; the "Pain proteome"***

In order to define a list with proteins of interest as biomarkers in pain conditions, we investigated the CSF proteome of FM in comparison with CSF from RA patients and CSF from controls (sampled due to investigation of non-inflammatory diseases). We also studied the inflammation-related protemics in the serum of the postsurgical NP patients. These results are still being analysed.

We investigated proteins found in human CSF with respect to known **"**pain**"** genes in a cohort of patients with dysfunctional pain (FM), inflammatory pain (RA) and non-pain controls utilising a mass spectrometry-based quantitative proteomics method combined with multivariate data analysis to explore quantitative differences between these cohorts of patients. Our findings support notable presence of pain-related proteins in CSF, yet with specific domains including inflammatory responses, neuropeptide signaling and hormonal activity. We have investigated molecular functions of significantly altered proteins and demonstrate the presence of 176 known pain-related proteins in CSF. In addition, we found ten proteins potentially associated with pain in FM and RA: neural cell adhesion molecule L1, complement C4-A, lysozyme C, receptor-type tyrosine-protein phosphatase zeta, apolipoprotein D, alpha-1-antichymotrypsin, granulins, calcium/calmodulindependent protein kinase type II subunit alpha, mast/stem cell growth factor receptor Kit, and prolow-density lipoprotein receptor-related protein 1. These proteins are novel in the context of FM but are known to be involved in pain mechanisms including inflammatory response and signal transduction.

***Identification of candidate proteins involved in development of neuropathic pain***

Proteins were studied as potential biomarkers for the development of neuropathic pain in women who were operated for breast cancer. Serum samples taken before surgery and at a follow-up 4-9 years later, were analysed from two groups of patients who had a surgical nerve injury that had or had not led to chronic neuropathic pain. A proximity extension analysis (PEA) with an inflammatory panel was used, based on the hypothesis of GLORIA that neuroinflammation plays an important role

also in neuropathic pain. Preliminary results suggest that a group of inflammatory biomarkers seems to be associated with chronic neuropathic pain.

### 3.2.3 Conclusions

We have defined and validated clinical pain phenotypes (remaining pain and widespread pain) in early RA with epidemiological methods. We have shown that remaining pain is common in early RA, and pain may persist despite good clinical response to anti-rheumatic treatment. We have identified potential biomarkers associating with development of widespread pain in RA that will be further validated. We have used machine-learning analysis to define additional clinical pain phenotype, persistent pain, in early RA. We have established a platform for mass spectrometry-based mapping of candidate peptides in clinical patient cohorts, and formed a target list for further linking of biomarker data with clinical pain data. Human inflammatory biomarker associations with persistent pain suggest the immune system as a target for potential future drugs for persistent pain.

Moreover, we have performed a mapping of the ”pain proteome” of CSF in FM and the identification of candidate biomarkers associated with FM. We found that “pain proteins” detected in CSF are typically related to synaptic transmission, inflammatory responses, neuropeptide signaling, and hormonal activity. In addition, we found ten proteins potentially associated with chronic pain in FM and RA: neural cell adhesion molecule L1, complement C4-A, lysozyme C, receptor-type tyrosine-protein phosphatase zeta, apolipoprotein D, alpha-1-antichymotrypsin, granulins, calcium/calmodulin-dependent protein kinase type II subunit alpha, mast/stem cell growth factor receptor Kit, and prolow-density lipoprotein receptor-related protein 1. These proteins might be of importance for understanding the mechanisms of dysfunctional/inflammatory chronic pain and also for use as potential biomarkers.

## 3.3 WP3 In vivo studies of glial cell activation and pain regulation by imaging

### 3.3.1 Main objectives

The main objectives of WP3 were to establish the methodology for the assessment of glial cell activation using positron emission tomography (PET) in humans, to establish patterns of neuroinflammation by analysing cerebrospinal fluid and to assess endogenous pain modulation using functional magnetic imaging (fMRI) in chronic pain patients, and to develop cellular models for quantitative characterisation of opioid receptor-mediated signalling dynamics by functional Fluorescence Microscopy Imaging (fFMI).

### 3.3.2 Main results

***Assessment of glia cell activation using positron emission tomography (PET) in humans***

The first objective was to establish the use of a new generation of PET ligands ([$^{11}$C]PBR28) for *in vivo* quantification of glial activation in human pain patients. As glial cell activation has been linked to the “sickness response” during infections/inflammation in humans, we chose to examine patients

suffering from fibromyalgia (FM) as they have many symptoms in common with "sickness response", such as fatigue, increased pain sensitivity and dysregulation of sleep and mood. In order to disentangle witch kind of glia cells were activated, we used two PET ligands: the [$^{11}$C]PBR28, a general high sensitivity marker for glial cell activation of microglia as well as astrocytes, and [$^{11}$C]-$_L$-deprenyl-$D_2$, which is regarded to be specific for astrocytes. Finally, glial cell activation was analysed in relation to clinical symptoms.

In FM patients, the distribution volume ($V_T$) of [$^{11}$C]PBR28 was elevated in several brain regions compared with healthy control subjects, including dorsolateral prefrontal cortex (dlPFC), dorsomedial PFC (dmPFC), primary somatosensory and motor cortices (S1/M1), precuneus, posterior cingulate cortex (PCC), supplementary motor area (SMA), supramarginal gyrus (SMG), and superior parietal lobule (SPL). Additionally, a region-of-interest (ROI) analysis of the anterior midcingulate cortex (aMCC) revealed elevated $V_T$ in the FM patients that approached statistical significance ($p$ = 0.071). There were no regions where control $V_T$ was significantly higher than FM $V_T$. Higher subjective ratings of fatigue in FM patients were associated with higher standardised uptake value ratio (SUVR) of [$^{11}$C]PBR28 in the anterior and posterior middle cingulate cortices.

Regarding the [$^{11}$C]-$_L$-deprenyl-$D_2$ analysis group, a marker specific for astrocytes, comparisons revealed no significant difference between FM patients and healthy control subjects across the ROIs extracted from the [$^{11}$C]PBR28 voxelwise analysis (p's > 0.53, uncorrected). The ROIs aMCC, dLPFC, dmPFC, frontoinsular cortex, S1/M1, PCC, precuneus, pMCC, SMA, and SPL were included.

***Patterns of neuroinflammation by analysing cerebrospinal fluid***

Patterns of neuroinflammation were established by assessing proinflammatory substances (cytokines/chemokines) in the cerebrospinal fluid (CSF) of chronic pain patients suffering from FM, RA and OA.

We found different profiles of cytokines in the CSF with higher interleukin (IL)-1β in patients with inflammatory, prostaglandin associated pain (RA), associated with decreased vagal activity and higher IL-8 in patients with nociplastic pain (pain due to altered nociception; FM). Furthermore, higher CSF concentrations of IL-8 and monocyte chemoattractant protein 1 (MCP1) were seen in patients with knee osteoarthritis (OA) compared with controls, indicating neuroinflammation. Symptom severity correlated with IL-6 and IL-8 levels in synovial fluid, but it was inversely associated with IL-6 and IL-8 levels in CSF, indicating that neuroinflammation in OA may be an adaptive, possibly neuroprotective mechanism promoting symptom reduction. The MCP1 concentrations correlated across all compartments i.e., synovial fluid, serum and CSF in female, but not male patients, indicating that MCP1 could be involved in neuro-immune, blood borne joint-to-central nervous system (CNS) signaling, however, with profound sex differences.

***Assessment of endogenous pain modulation using functional magnetic imaging (fMRI) in chronic pain patients***

FM patients were analysed to establish if the receptor used as a ligand in the PET studies assessing glial activation (PBR28), called translocator protein (TSPO), also is of physiological importance. We

analysed the relationships between genetically inherent TSPO high affinity binding (HAB) versus mixed/low affinity binding (MLAB) and FM symptoms as well as cerebral processing of nociceptive stimuli (functional magnetic resonance imaging, fMRI). All subjects were genotyped for the TSPO *rs6971* polymorphism, phenotyped regarding pain intensity (SF-36 bodily pain) and fibromyalgia severity (fibromyalgia impact questionnaire) and a subgroup was also assessed with fMRI.

Higher pain intensity ratings and higher FM impact was seen in the genetically inferred TSPO HAB compared with MLAB. No statistically significant group differences were seen regarding ratings of depression/anxiety or pressure pain sensitivity. The calibrated thumb pressure (P50) used to assess pain related cerebral activation did not differ between TSPO HAB (286 ± 172 kPa) and MLAB patients (233 ± 110 kPa) in the fMRI cohort. There were no brain regions where the HAB group had greater pain-related brain activations (BOLD) than MLAB. Conversely, the MLAB group had greater activity in several brain areas, including brain regions associated with pain processing such as dorsolateral prefrontal cortex (dlPFC), primary and secondary somatosensory cortex, insula and anterior cingulate cortex. Psychophysiological interaction (PPI) analysis of pain-evoked functional connectivity revealed significant positive connectivity between the right dlPFC and the right parietal cortex in TSPO HAB patients, compared to MLAB, i.e., within the fronto-parietal network. There were no regions where TSPO MLAB had higher dlPFC connectivity compared with HAB.

***Quantitative characterisation of opioid receptor-mediated signalling dynamics by functional Fluorescence Microscopy Imaging (fFMI)***

The overall goal for functional Fluorescence Microscopy Imaging (fFMI) was to quantitatively characterize at the single-cell and single-molecule level interactions between the mu-opioid (MOP) and the serotonin 5-$HT_{1A}$ receptors, and the effects of selected agonists on these interactions. To this aim, we developed genetically modified cell lines (human (HEK293) and rat (PC12)) stably transformed to express mu-opioid (MOP) and serotonin 5-$HT_{1A}$ receptors fused with spectrally distinct fluorescent proteins (eGFP-MOP (green) and Tomato-5-$HT_{1A}$ (red)). We used advanced fluorescence microscopy and spectroscopy-based methods with the ultimate, single-molecule sensitivity and a sub-microsecond temporal resolution, Fluorescence Correlation and Cross-Correlation Spectroscopy (FCS/FCCS), to quantitatively characterise in live cells MOP and 5-$HT_{1A}$ receptor surface density and the extent of binding to one another. We characterised how stimulation with selected opioids (e.g. morphine, fentanyl, codeine, oxycodone) and agonists at the 5-$HT_{1A}$ receptor (e.g. buspirone and its newly synthesised analogues) affects these interactions. Finally, $Ca^{2+}$ was monitored in single cells using the ratiometric dye Fura Red, to monitor changes in cellular signalling.

We successfully established the cellular models and performed the measurements. Our data show that MOP and 5-$HT_{1A}$ receptor heterodimers exist in unstimulated cells, and that about 20% of the receptors are bound to one another in cells expressing similar amounts of these receptors. For all opioids tested, long-time exposure to high levels of opioids (750 nM, 16 hour incubation as cell doubling time is approximately 34 hours for HEK293 cells and about 3-4 days for PC12 cells) significantly increased heterodimerisation, 40% – 60% of MOP–5-$HT_{1A}$ heterodimers. The extent to which different opioids facilitated the process of hereodimerisation was different for different opioids, with fentanyl causing the highest and oxycodone the lowest degree of receptor-receptor association.

Differences between the effects of morphine and oxycodone were not statistically significant, whereas difference between the effects caused by morphine or codeine, and morphine or fentanyl were statistically significant. Long-time combinatorial treatment with equimolar concentrations of morphine and buspirone or its analogues (16 hour treatment with 750 nM morphine and 750 nM buspirone, or its analogs), effectively abolished MOP–5-$HT_{1A}$ heterodimerisation. This was also true for the newly synthesised buspirone analogues. However, statistical significance between the effects caused by buspirone or any of the analogues was not observed.

### *3.3.3 Conclusions*

***Assessment of glia cell activation using positron emission tomography (PET) in humans***

Our results provide the first *in vivo* evidence supporting the role of glial activation in the pathophysiology of fibromyalgia and suggest that microglia, but not astrocytes, may be activated. Furthermore, the degree of glial activation was associated with fatigue, a major symptom of fibromyalgia. Overall, our data support glial modulation as a potential therapeutic strategy for fibromyalgia.

***Patterns of neuroinflammation by analysing cerebrospinal fluid***

Our results indicated that neuroinflammation was present in all assessed pain conditions (FM, RA, OA), but with different profiles, e.g. elevated (IL)-1β in inflammatory nociceptive pain (RA) and elevated IL-8 in pain conditions where dysfunctional endogenous pain modulation has previously been reported such as FM (nociplastic pain) and OA (nociceptive and nociplastic pain), suggesting that IL-1β is involved in inflammatory pain, and IL-8 in disturbances of autonomic nervous system and dysfunctional pain modulation. In addition, MCP1 levels were elevated in OA patients, and the data suggest that MCP1 has a special role in neuro-immune signalling in women. Overall, our findings provide new possibilities for the development of more targeted treatment strategies aiming to modulate neuroinflammation in chronic pain patients.

***Assessment of endogenous pain modulation using functional magnetic imaging (fMRI) in chronic pain patients***

To our knowledge, this is the first evidence of a genetic functional polymorphism affecting pain severity in FM patients. In addition to more severe FM symptoms in patients with genetically inferred TSPO HAB, functional connectivity analysis revealed that TSPO HAB was associated with higher pain related functional connectivity between structures within the frontoparietal network, a network which has been reported to be of major importance for vigilance as well as the anticipation and appraisal of pain.

***Quantitative characterisation of opioid receptor-mediated signalling dynamics by functional Fluorescence Microscopy Imaging (fFMI)***

Our study suggests that mu-opioid (MOP) and serotonin 5-$HT_{1A}$ receptors form heterodimers in unstimulated cell (*e.g.* not exposed to externally introduced receptor agonist). While the extent of heterodimerisation in unstimulated cells is limited (» 20%), long-term exposure to high concentrations of opioids differentially increases the degree of heterodimerisation. Combinatorial

treatment with the morphine and 5-$HT_{1A}$ receptor agonist buspirone or its newly synthesised analogues, prevents excessive heterdimerisation.

## 3.4 WP4 Glial activation in animal models of chronic pain

### 3.4.1 Main objectives

The main objectives for WP4 focused on the role of glia cells and glia-expressed opioid receptors in animal models representing the inflammatory, nociceptive, neuropathic, and dysfunctional pain patient populations in WP3. These models were the collagen antibody-induced arthritis (CAIA) model, the partial medial meniscectomy model (MMT), representing RA (inflammatory) and OA (nociceptive) pain, a fibromyalgia model and different neuropathic pain models. In addition, models of opioid-induced hypersensitivity and tolerance were established and used.

### 3.4.2 Main results

***Nerve injury-induced pain***

WP4 focused on neuropathic models that represent clinical neuropathic conditions such as traumatic nerve injury and diabetic polyneuropathy. In these models, we have studied the activation of glial cells and gender differences. We also evaluated the effects of potential new disease-modifying drugs targeting the receptors of neurotrophic factors belonging to the glial cell line-derived neurotrophic factor (GDNF) family ligands. Using immunohistochemistry, we found that animals with traumatic nerve injury show upregulation of glial markers in the spinal cord and dorsal root ganglia. In particular, the number of microglia/macrophages in the spinal cord and dorsal root ganglia is increased. These data were also confirmed by analysis of gene expression profile. In general, it seems that microglial activation is quite similar at the spinal level in different traumatic nerve injury models. However, our results with flow cytometry, that separates pro- and anti-inflammatory microglia, suggested that there might be differences in glial activation between different nerve injury models in the percentage of proinflammatory type microglial cells.

In studies where we used a model of diabetic neuropathy, immunohistochemistry showed some changes in neuronal and glial marker expression in the spinal cord and the dorsal root ganglia. However, this microglial change depended on the rat strain and the time point when the markers were assessed, which made it difficult to draw clear conclusions.

We also studied whether there are any differences in the pain behaviour and expression of neuronal and glial markers between the sexes in rats with traumatic nerve injury. Our studies indicated that female rats seem to develop mechanical hyperalgesia in response to traumatic nerve injury differently from male rats. We have performed several studies to elucidate the mechanisms behind the differences between sexes. For example, we have studied differences in the expression of neuronal markers in dorsal root ganglia and in the glial activation between the sexes. We have also performed proteomics and transcriptomics analyses in order to analyse possible differences which could explain the behavioural findings.

In order to further investigate sex-dependent differences in neuropathic pain-like behaviour we used mutant mice for the delta opioid receptor (DOR) in glial cells, and studied the DOR-Cx3cr1-Cre mutants for DOR in microglia and the DOR-GFAP-Cre mutants for DOR in astrocytes in the partial sciatic nerve ligation neuropathy model. In the mutants for DOR in microglia, neuropathy led to hypersensitivity to all thermal and mechanical modalities. Both sexes in control Cre- animals showed comparable hyperalgesia. There was no sex difference for heat and mechanical hypersensitivity in microglial DOR-deficient Cre+ mice while female Cre+ mice showed attenuated cold allodynia as compared with Cre+ males. In the mutants for DOR in astrocytes, the neuropathy produced hypersensitivity to both touch and cold. The two sexes of control Cre- animals showed comparable touch and cold hyperalgesia. There was also no sex difference in the neuropathic hypersensitivities between female and male astrocytic DOR-deficient Cre+ mice.

We tested the effect of GDNF family ligand (GFL) mimetics in acute nociceptive pain models and in models of experimental neuropathic pain. GFL mimetics are promising lead compounds for the disease-modifying treatment of neuropathic pain. Tested GFL mimetics alleviated both cold and mechanical allodynia in the traumatic nerve injury model of neuropathy, and in the model of diabetic neuropathy. One of these compounds also attenuated glial activation in the dorsal root ganglia. GFL mimetics did not influence mechanical or cold sensitivity in healthy animals. They were well tolerated and produced no toxic or adverse effects in rats.

Taken together, our results show that microglial cells are activated in neuropathic pain models, there seem to be some differences between sexes in the traumatic nerve injury model, and GFL mimetics are interesting new molecules for further development as disease-modifying drugs for the treatment of neuropathic pain.

***Opioid-induced hyperalgesia and tolerance***

Development of tolerance is a well-known pharmacological characteristic of opioids and a major clinical problem. We established opioid tolerance and hyperalgesia in several experiments and we studied the mechanisms of opioid-induced tolerance, especially the role of glial cells in its development in rats. In addition, we studied the differences between sexes as well as the effect of drugs, such as ketamine, on opioid tolerance.

In addition to the known neuronal mechanisms of opioid tolerance, the activation of glia has emerged as one potential mechanism. We studied the activation of microglia and astrocytes in morphine tolerance and opioid-induced hyperalgesia in rats using immunohistochemistry, flow cytometry and RNA sequencing in spinal cord and brain regions. Our results suggest that glial activation associated with opioid tolerance and opioid-induced hyperalgesia occurs mainly at the spinal cord level but not in the brain. Our transcriptome data suggest that the microglial activation pattern after chronic morphine treatment has similarities with that of neuropathic pain. In addition, we examined whether there are any differences in pain behaviour and glial markers between the sexes in rats with opioid tolerance and opioid hyperalgesia. In general, it seems that there are no major differences between sexes.

We have also studied the role of the main morphine metabolite, morphine-3-glucuronide in opioid tolerance. This metabolite may contribute to the development of opioid tolerance as well as allodynia after chronic morphine treatment.

In order to study the mechanisms of the development of opioid tolerance and hyperalgesia and how to attenuate them, ketamine (an NMDA antagonist) and its active metabolites were studied in morphine and oxycodone tolerance. Ketamine attenuated both morphine and oxycodone tolerance. However, ketamine and norketamine attenuated more effectively morphine tolerance than they attenuate oxycodone tolerance. Interestingly, ketamine also significantly increased morphine brain concentrations but not oxycodone brain concentrations, which may partly explain the difference.

Our results suggest that glial cells have a role in opioid tolerance but there seem to be no major differences between sexes. Ketamine and its metabolites potentiate the analgesic effects of opioids during chronic opioid treatment.

***Osteoarthritis-induced pain***

Despite carefully examining two different models of osteoarthritis based on partial menisectomy and anterior crusiate ligament tear and using different assays of pain-like behaviour, including measures of evoked and spontantouse activity, in both male and female mice, we were not able to establish a model system for osteoarthritis-induced pain. While both the meniscal and ligament tear models generated a pathology that resembles the joint pathology of human osteoarthritis, the mice did not display signs of enhanced sensitivity to mechanical or thermal stimuli or changes in burrowing activity or gait. This may reflect the poor correlation between joint pathology and pain in humans. Furthermore, we did not observe any change in spinal microglial or astrocyte reactivity in either model.

***Models of rheumatoid arthritis (RA) pain***

The collagen antibody-induced arthritis (CAIA) model was used for studies of arthritis-induced pain. We reproduced our earlier finding that sensitivity to mechanical stimulation was significantly increased, not only during ongoing joint inflammation but also for many weeks after the inflammation had resolved. We refer to the two phases as the inflammatory and the late (“post-inflammatory”) phases. The long-lasting change in pain-like behaviour is an important feature of the CAIA model as it reflects part of the problem for patients with rheumatoid arthritis – that pain persists despite medically controlled disease activity, the remaining pain.

Using the CAIA model and immunohistochemistry we observed that microglia displayed signs of increased reactivity in both male and female mice during the inflammatory and late phase while astrocytes only displayed signs of increased reactivity during the post-inflammatory phase. We found that spinal TLR4 was critical for the development of CAIA-induced hypersensitivity as blocking the action of the endogenous TLR4 ligand HMGB1 attenuated the pain-like behaviour both in the inflammatory and post-inflammatory phase. Injection of HGMB1 into CSF activated spinal glia and induced pain-like behaviour in a TLR4 dependent manner. Importantly, however, even though we did not detect any phenotypic difference in microglia isolated from the spinal cords of male and female mice subjected to CAIA, blocking the action of spinal microglia reversed the mechanical

hypersensitivity only in male mice. Furthermore, blockig the action of spinal microglia or deleting TLR4 in spinal microglia only prevented HMGB1-induced pain-like behaviour in male and not female mice. Interestingly, activation of spinal TLR4 by injection of HMGB1 evoked differential protein expression in male and female mice. Thus, taken together, even though HMGB1 is coupled to pain in both male and female mice, there appear to be a strong spinal microglial/TLR4 sex dimorphisms associated with arthritis-induced pain-like behaviour.

### *3.4.3 Conclusions*

In conclusion, in control animals that contain DOR in glial cells, there was no sex-dependent difference in traumatic neuropathic pain-like behaviour. In mice lacking DOR in microglia, neuropathic females showed an attenuated reaction to noxious cold as compared with males. This indicates that the absence of microglial DOR diminishes cold allodynia in females and hence that DOR expressed by microglia contributes to cold neuropathy-associated hypersensitivity in the females. Previously, the role of the endogenous DOR activity on neuropathic-like behaviour was investigated by using animals harbouring full DOR deletion or deletion in peripheral Nav1.8 nociceptive neurons. Both of these models showed an aggravated neuropathic pain behaviour, indicating a protective role of DOR and specifically of DOR in nociceptors. Here we show for the first time that DOR in female microglia contributes to cold pain in a neuropathic model.

In general, when using immunohistochemistry and assessment of changes in morphology as a readout for microglial reactivity, it seems that microglial activation is quite similar at the spinal level in different traumatic nerve injury models. However, using flow cytometry, that separates pro- and anti-inflammatory microglia, the picture is more complex and suggests that there might be differences in glial activation between different nerve injury models in the percentage of proinflammatory type microglial cells. Taken together, our results show that microglial cells are activated in neuropathic pain models, and that there are some differences between sexes in the development of pain-like behaviour after traumatic nerve injury. At least on later stages astrocytes rather than microglia may play a role in the sex dimorphism seen in response to noxious stimuli in traumatic nerve injury models.

Our results also suggest that glial activation associated with opioid tolerance and opioid-induced hyperalgesia occurs mainly at the spinal cord level but not in the brain, that there are similarities in glial activation in neuropathic pain models and opioid-induced hyperalgesia/tolerance, and that there are no major differences between the sexes.

Modelling osteoarthritis-induced pain in mice using surgical models is challenging. Even though the models used generated a pronounced knee joint osteoarthritis-like pathology we were not able to detect reproducible changes in behavior. The absence of detectable changes in evoked and spontaneous behavior dispite cartilage distruction indicate that this pathological feature is not critical for development of pain-like behavior.

Antibody-driven inflammatory arthritis has a long-term impact on spinal glial reactivity and mechanical hypersensitivity, both outlasting the joint inflammation by many weeks. The HMGB1-

TLR4 axis is an important contributor to spinal pain signal transmission, most likely via activation of spinal microglia. Suprisingly, while HMGB1 induces pain-like behaviour in both male and female mice, the TLR4-microglia aspect was only associated with nociception in male mice. Thus, the role of microglia and TLR4 role in pain is subject to sex dimorphism in arthritis-induced pain.

GFL mimetics attenuated nerve injury-induced pain-like behaviour, protected sensory neurons and reduced microglial reactivity in male rats. Therefore, they are promising new molecules for further development as disease-modifying drugs for the treatment of neuropathic pain. As there were no obvious sex specific differences seen in the neuronal or microglial cells in nerve injury-induced models of experimental neuropthy, we expect GFL mimetics to exert similar effects in female rats.

## *3.5 WP5 Role of opioid receptors on glial cells in chronic pain: conditional knockouts*

### *3.5.1 Main objectives*

The objectives of WP5 were to investigate the role of mu and delta opioid receptors on astrocytes or microglial cells in neuropathic pain by using conditional knockout mice and to evaluate selected compounds from WP6 for analgesia in opioid receptor knockout mice.

More specifically, the objectives were to create different conditional knockout mouse lines for opioid receptors in glial cells, characterise them for opioid receptor deletion in the microglia and astrocytes, and to evaluate the mutant animals for neuropathic pain-like behaviours, persistence of chronic pain and opioid tolerance/hyperalgesia. The objectives for the evaluation of selected compounds from WP6 were to first determine their agonistic or antagonistic properties in ligand binding and receptor activation assays in transfected cells *in vitro* and then to evaluate compounds identified as opioid agonists for analgesia in the mutant models for opioid receptors.

### *3.5.2 Main results*

We successfully produced and analysed genetic murine models for selective deletion of mu or delta opioid receptors in astrocytes and microglia. When delta receptors (DOR) were absent from astrocytes, the behavioural pain response to cold exposure was attenuated in both male and female mice. The response to cold pain was also attenuated in females devoid of delta receptor in microglia. Thus, we show for the first time that DOR in astrocytes and more moderately in microglia contributes to cold pain under a neuropathic condition.

Besides neurons, glial neuroinflammatory cells are known to be involved in chronic pain development and maintenance. Taken together, our results show that DOR activity in neurons lowers neuropathic pain behaviour while DOR activity in astrocytes and microglia augments this type of pain. When DOR was absent in astrocytes, the administration of a DOR activator or agonist still elicited normal analgesia, indicating that this receptor population does not influence analgesia. A chronic treatment by agonist led to a decline in analgesia over time, also called tolerance. This tolerance was absent

in the mutants for astrocytic delta receptors while it developed normally in controls containing the receptor.

When mu receptors (MOR) were absent from microglia, analgesia occured normally indicating that this receptor population on microglia does not influence analgesia. Chronic treatment with an opioid agonist led to analgesic tolerance. However, no hyperalgesia due to the treatment occurred, indicating that tolerance can be dissociated from opioid-induced hyperalgesia. Tolerance to analgesia was attenuated in the mutants lacking MOR in microglia, suggesting a role for this receptor population in the development of tolerance. We also showed that microglia express MOR by analysing transcriptomic data and a novel fluorescent preclinical model.

### *3.5.3 Conclusions*

The absence of DOR from astrocytes, and to a lesser extent from microglia, attenuated the behavioural response to cold stimuli in neuropathy. This implies that specific DOR populations in glial cells favour neuropathic pain as opposed to the receptors in peripheral nociceptive neurons that protect from pain. Glial receptors also control the development of analgesic tolerance trigerred by a chronic opioid agonist treatment.

The absence of mu receptors in microglia also tempers analgesic tolerance, suggesting that this MOR population contributes to the development of tolerance to mu opioid-targeted analgesia. Similarly to the results on DOR mutants, these findings indicate that the microglial MOR population promotes analgesic tolerance as compared with the receptors in peripheral nociceptive neurons that do not intervene in tolerance development.

Therefore, we show for the first time the participation of opioid receptors expressed by glial cells in the development of neuropathy-induced pain behavior and tolerance to analgesia. Our results confirm previous finding on the importance of glial cell activation in general in chronic pain and tolerance. Furthermore, they specify that the glial opioid receptors are important contributors of pain aggravation by glial cells as well as of analgesic tolerance.

## *3.6 WP6 Novel compounds for managing chronic pain*

### *3.6.1 Main objectives*

WP6 aimed to discover and optimise compounds to be used as (1) therapeutic molecules, for the management of chronic pain; (2) pharmacological probes, to understand the specific roles and interplay of the Toll-like receptor 4 (TRL4) and opioid systems in glial activation. WP6 used various computational and medicinal chemistry approaches towards compound design and synthesis in a tripartite feedback loop with pharmacological characterisation (design ↔ synthesis ↔ biological activity). Cell-based reporter assays, binding assays, and receptor phosphorylation as well as other *in vitro* primary functional testing assays were used. The most promising compounds were assessed in various experimental models.

The specific objectives were to discover novel active compounds: TLR4 inhibitors, mu, kappa, delta, opioid receptor (OR) agonists, and GFL mimetics; and to optimize existing and novel compounds for their binding and selectivity towards TLR4, ORs, and across the mu, kappa, delta ORs by means of iterative cycles of computational analyses, synthesis of congeneric series of analogues, and *in vitro* testing. The aim was also to evaluate early ADME properties and pharmacokinetic behaviour of the most promising compounds; and to study their cytotoxicity on human cell lines, as well as to test available GFL mimetics for their ability to activate RET, support neuronal survival and neurite formation *in vitro*, and the ability to alleviate pain, protect sensory neurons and influence microglia in animal models of experimental neuropathy.

### *3.6.2 Main results*

***Targeting TLR4***

Toll-like receptor (TLR4) belongs to the pattern recognition receptor family, a key component of the innate immune system. TLRs detect invading pathogens and initiate an immediate immune response to them, followed by a long-lasting adaptive immune response. It was an important target for our research since TLR4 has been associated with the development and maintenance of neuroinflammation and neuropathic pain. To find drug-like compounds acting on TLR4 we had to perform a complex multistep process ranging from virtual screening of thousands of compounds to further validation of chemical candidates by experimental testing. Several series of compound derivatives were obtained from commercial source during the project. We selected 13 compounds for further investigation and testing. From these, three candidates have shown activity by *in vitro* measurements, as well as binding to the membrane fraction of the TLR4 expressing cell line.

***Targeting opioid receptors***

It is well known that the opioid receptors are good targets for the development of therapeutics against pain. We concentrated on the three opioid receptors called mu, kappa and delta. The gold standard morphine molecule was used as a comparative scale for our novel compounds. We tried to develop not only morphine analogues but also compounds that are structurally different from morphine, and would target opioid receptors, since morphine has significant side effects such as addiction, constipation etc. The process of discovery of novel opioid compounds included the development of new synthetic methods to access the previously unexplored 7,8-position of the opioid core, and experimental evaluation of their biological activity towards the mu, delta and kappa opioid receptors. Comprehensive computational modeling of the opioid derivatives, molecular docking using the crystal structures of the opioid receptors, and computational studies on the structure-activity relationships and binding selectivity of the new opioid derivatives were employed to explain the observed biological activities. The work led to the discovery of new 7β-hydroxy-8-ketone opioid derivatives, which have shown a significant biological effect by acting as antagonists on mu and delta opioid receptors.

***Development of GFL mimetics***

GDNF family ligands (GLFs) are rare protein molecules with capacity to support and restore damaged neurons. Due to this unique ability, neurotrophic factors have recently attracted much attention as potential therapeutics for treatment of nervous system diseases including neuropathic

pain. However, GFL proteins themselves are poor drugs, because they are unable to penetrate tissue barriers, poorly spread in tissues, may produce side-effects as they can bind to different receptors, are expensive and difficult to produce and deliver to patients. Therefore, we concentrated on a methodology that uses small molecules mimicking neurotrophic factors that have improved pharmacological characteristics. We investigated two GLF receptor complexes, namely GFRalpha1/RET and GFRalpha/RET. We also searched for compounds selectively targeting GFRalpha1 and GFRalpha proteins. It was a comprehensive research using state-of-the-art computational methods as virtual screening, quantitative structure-activity relationships, structure-based drug design, molecular docking and molecular dynamics approaches. Moreover, all these approaches were coupled on various stages of the development with experimental *in vivo*/*in vitro* tests for the confirmation of the potential drug candidates. Thus, several dozens of potential compounds were discovered. The best candidates were further refined by experimental evaluations for their biological activity towards GFRalpha1/3 and/or GFRalpha1/3/RET. The final results showed that two compounds have good activity towards GFRalpha1/3/RET complex. Additional ADMET (absorption, distribution, metabolism, excretion, toxicity) experiments elucidated that one of these candidates can be used as a potential lead compound for future drug development. Some of the synthesised compounds were tested in animal models of experimental neuropathy. Apart from the ability to alleviate hypersensitivity to mechanical and/or thermal stimuli, these compounds were able to attenuate glial activation in the tissues of experimental animals. In addition, we discovered a novel compound targeting GFRalpha1 selectively. This compound is very promising due to its selectivity, however, it requires further optimisation to be tested *in vivo.*

### *3.6.3 Conclusions*

Drug development is a complex process involving a lot of research effort and money. However, within the framework of GLORIA project we have managed to achieve our milestones thanks to the collaboration between the partners of the project. The main goal of WP6 was to discover new potential drug candidates active on several protein targets related to pain. To achieve this goal, a huge amount of research was carried out based on state-of-the-art computational methods and comprehensive experimental tests.

To conclude the main findigs of WP6, the work towards TLR4 targeting compounds led to the discovery of three compounds showing activity on TLR4 by *in vitro* measurements. The research for OR targeting compounds led to the discovery of new 7β-hydroxy-8-ketone opioid derivatives, and four compounds showed significant biological effect in *in vitro* by acting as antagonists on mu and delta opioid receptors. For the GFL mimetics, three different groups of compounds were developed, and in total, six compounds showed *in vitro* activity toward GFRalpha1 and GFRalpha1/RET. For the GFRalpha3/RET targeting compounds, one compound showed good results in *in vitro* tests.

# 4 Potential impact, main dissemination activities and exploitation of results

## 4.1 Impact

Inflammatory pain, neuropathic pain and nociceptive pain are common conditions in the population. Presently, there is both lack of sufficient information on the mechanisms for these pain conditions, and there is also an unmet need to map and characterise non-inflammatory pain and widespread pain in inflammatory arthritis and RA. We have used different epidemiological approaches to identify patients at risk for development of chronic pain in relation to RA, and these data are expected to contribute to new strategies concerning early identification of patients using registers with the same parameters. An early identification of unmet needs in RA is already implemented at the Rheumatology department of Karolinska University Hospital in Stockholm, Sweden. This new strategy will hopefully result in earlier interventions in patients at risk for the development of chronic pain in addition to their rheumatic disease; for example thorough investigation of sleep patterns, concomitant fatigue and depressive symptoms. These comorbidities could be managed in parallell to immunosuppression of the joint disease, thereby potentially optimising the patient´s health and decreasing the risk of continous pain and development of widespread pain.

Through the GLORIA project, we have also achieved important information on candidate proteins and biomarkers that associate with pain conditions, such as fibromyalgia, RA and neuropathic pain after breast cancer surgery. The fact that we have studied potential biomarkers in the target organ, central nervous system (CNS), makes further impact on the utility of the data. This information will be further validated and valuable for several reasons: First, biomarkers associating with fibromyalgia will provide more insight into the pathogenesis of the disease, much of which has not yet been elicited. Understanding the pathophysiological mechanisms will lead to further development of treatment strategies. It will also be possible to compare different pain conditions concerning biomarkers, and we have preliminary data on similar groups of proteins being important both in fibromyalgia and widespread pain in RA. Second, the identification of biomarkers for pain development in inflammatory arthritis and dysfunctional pain is potentially of high clinical interest, and may be used both for identifying patients at risk of developing a chronic pain condition, but also for monitoring the disease and for treatment follow-up in patients with complex chronic pain conditions.

Identification of biomarkers for the development of neuropathic pain after breast cancer surgery is also a valuable tool for improved prediction of which patients will develop a persistent pain condition, and could thereby be subject to early intervention of contributing factors. On the other hand, it would also be possible to identify those patients in whom such a development is unlikely and who therefore can be spared unnecessary treatments and guided toward early return to their usual activities after major surgery.

Likewise, in OA further studies of several biomarkers, including MCP-1, may pave the way for interventional targeted studies that may succeed in decreasing OA pain in a more specific way.

Increased understanding of the regulation of inflammation in OA (shown to be distinct from RA) would facilitate development of efficient new biological drugs (i.e., drugs affecting proinflammatory cytokines) in OA, in analogy with the success seen in RA.

Finally, the concept of inflammation-associated pain and inflammation-induced pain sensitisation is a promising area with several implications. Thus, the pharma industry has a huge interest to develop further biological treatments not only as immunosuppressants, but also as targeted therapies that may intervene with the processes of pain sensitisation. Here, the GLORIA project has provided new information. The mechanisms studied in our project focusing on TSPO-related effects on symptoms, pain sensitivity as well as the cerebral correlates for pain anticipation and processing in health, inflammatory (RA) and centralised pain (FM) are of vital importance for future drug development. Furthermore, PET could be developed as a tool for grading of glial cell activation in pain patients and used for treatment follow-ups in drug development and studies on the function of existing drugs. Also, the increased understanding of the role of glial cell activation in chronic pain and in long-term opioid treatment in humans is important for the attempts to prevent opioid tolerance and hyperalgesia.

The role of glial cell activation in neuroinflammation was rendered further support by animal studies, where activation of glia with concomitant neuroinflammation was reported in both nociceptive as well as neuropathic pain in association with increased pain sensitivity. In addition, prolonged administration of opioids activated glia cells which were implicated in the development of opioid tolerance and opioid induced hyperalgesia. Taken together, glia cell activation seems to have an important role in the development of chronic pain. However, the glia-related data from the different experimental disease models also indicate that pain pathology may not always alter glial reactivity in the CNS. Also, the impact of glial cells and pharmacological tools targeting glia may vary between sexes. However, a clear sex dimorphism was only detected in certain disease models. Thus, the role of glial cell activation in neuroinflammation and pain may not be generalisable between different animal models and sexes and it is plausible that this also applies to human pain conditions.

Cellular and molecular mechanisms are crucial for understanding chronic pain. Cell surface receptors are the primary sites of action of pain causing/relieving substances. Cell surface receptors transmit pain by initiating a series of chemical interactions that take place in a well-defined order and involve several neuromodulatory and neurotransmitter systems, most notably the opioid system and the monoamine systems, which include dopamine, noradrenaline, adrenaline and serotonin receptors. Our study suggests that interactions between the mu-opioid (MOP) and the serotonin 5-$HT_{1A}$ receptors are potential druggable targets and that combinatorial treatment with opioids, MOP agonists extensively used in the management of severe chronic pain, and serotonin 5-$HT_{1A}$ receptor agonists, such as buspirone or its newly synthesised analogues, may be advantageous for long-term treatment of chronic pain. Also, our results on the specific regulation of cold allodynia in female animals devoid of microglial delta opioid receptor may have a potential impact on the treatment of neuropathic pain. We also showed a specific attenuation of pain and analgesic tolerance in preclinical models where opioid receptors are absent from glial cells.

New compounds devoid of activity at the glial opioid receptors may be identified to develop molecules causing less analgesic tolerance than the currently used opioid analgesics. In addition, the developed GFL mimetics can significantly improve the current management of neuropathic pain offering the option to restore lesioned neurons and reduce glial activation at the lesion site. Thus, they would be among the first disease modifying drugs targeted for neuropathic pain.

The entire process of drug development is challenging, time consuming, expensive, and requires consideration of many aspects. To meet these challenges, several multidisciplinary approaches are required for the process of drug development; collectively these approaches would form the basis of rational drug design. During the project, we developed methods that can be used as standards for research in this area, as more efficient and potent drugs are needed for the treatment of chronic pain which is an increasing problem in the modern western world. In the GLORIA project we have discovered several new promising compounds that could serve as lead compounds for the development of novel, more efficient drugs against chronic pain. At the moment, we are planning further development of these compounds. We foresee to file a patent application to protect our invention in the future, however, to avoid patent cliff problem we are looking for partners to proceed with our research. We have been in contact with leading pharmaceutical companies in the field of analgesics development. However, the compounds are still at a too early preclinical development stage to be immediately in-licensed. As our approach was considered interesting and valid, and the results were regarded as promising, we also consider further development of our compounds, presumably until late optimised leads or investigational new drug (IND) status application stage within our consortium. For this purpose we will need to attract additional funding from public sources, industrial and venture capital funds.

Apart from the scientific output in the form of published results and the many remaining articles to be written and published, an important feature of GLORIA has been the establishment of a joint translational research platform across several European countries.

## *4.2 Main dissemination activities*

GLORIA consortium has been very active in disseminating results to the scientific community and health care professionals as well as to the general public. In total 66 peer-reviewed articles have been published: 57 scientific articles and 9 review articles. In addition, 128 oral presentations and 64 poster presentations have been held. GLORIA has organized courses intended for M.Sc. and PhD students and open symposia. GLORIA has actively communicated also with elementary, middle, and high school students to spread awareness of the research done in GLORIA, as well as organised meetings with patient organisations. The key dissemination activities are listed below.

Publications

- **Differential Spinal and Supraspinal Activation of Glia in a Rat Model of Morphine Tolerance.** Jokinen V, Sidorova Y, Viisanen H, Suleymanova I, Tiilikainen H, Li Z, Lilius TO, Mätlik K, Anttila JE, Airavaara M, Tian L, Rauhala PV, Kalso EA. Neuroscience 375: 10-24, 2018

- **Brain glial activation in fibromyalgia – a multi-site positron emission tomography investigation.** Albrecht DS, Forsberg A, Sandstrom A, Bergan C, Kadetoff D, Protsenko E, Lampa J, Lee YC, Olgart Höglund C, Catana C, Cervenka S, Akeju O, Lekander M, Cohen G, Halldin C, Taylor N, Kim M, Hooker JM, Edwards RR, Napadow V, Kosek E, Loggia ML. Brain Behav Immun 75: 72-83, 2019
- **The translocator protein gene is associated with symptom severity and cerebral pain processing in fibromyalgia.** Kosek E, Martinsen S, Gerdle B, Mannerkorpi K, Löfgren M, Bileviciute-Ljungar I, Fransson P, Schalling M, Ingvar M, Ernberg M, Jensen KB. Brain Behav Immun 58: 218-227, 2016
- **Gene-to-gene interactions regulate endogenous pain modulation in fibromyalgia patients and healthy controls – antagonistic effects between opioid and serotonin related genes.** Tour J, Löfgren M, Mannerkorpi K, Gerdle B, Larsson A, Palstam A, Bileviciute-Ljungar I, Bjersing J, Ingvar M, Ernberg M, Schalling M, Kosek E. Pain 158(7): 1194-1203, 2017
- **The human CSF pain proteome.** Khoonsari P.E, Ossipova E, Lengqvist J, Svensson CI, Kosek E, Jakobsson PJ, Kultima K, Lampa J. J Proteomics 190: 67-76, 2019
- **Exploring the transcriptome of resident spinal microglia after collagen antibody-induced arthritis.** Fernandez-Zafra T, Agalave AM, Sandor K, Gao T, Su J, Jurczak J, Estelius J, Lampa J, Wiesenfeld-Hallin Z, Xu XJ, Denk F, Svensson CI. Pain 2018
- **A novel small molecule GDNF receptor RET agonist, BT13, promotes neurite growth from sensory neurons in vitro and attenuates experimental neuropathy in the rat.** Sidorova YA, Bespalow MM, Wong AW, Kambur O, Jokinen V, Lilius TO, Suleymanova I, Karelson G, Rauhala PV, Karelson M, Osborne, PB, Keast JR, Kalso EA, Saarma M. Front Pharmacol 8: 365, 2017
- **Spinal versus brain microglial and macrophage activation traits determine the different neuroinflammatory responses and analgesic effcets of minocycline in chroninc neuropathjic pain.** Li Z, Wei H, Piirainen S, Chen Z, Kalso E, Pertovaara A, Tian Li. Brain Behav Immun 58: 107-117, 2016
- **Ketamine coadministration attenuates morphine tolerance and leads to increased brain concentrations of both drugs in the rat.** Lilius TO, Jokinen V, Neuvonen MS, Niemi M, Kalso EA, Rauhala PV. Br J Pharmacol 172 (11): 2799-813, 2015
- **Morphine-induced hyperalgesia involves mu opioid receptors and the metabolite morphine-3-glucuronide.** Roeckel LA, Utard V, Reiss D, Mouheiche J, Maurin H, Robé A, Audouard E, Wood JN, Goumon Y, Simonin F, Gaveriaux-Ruff C. Sci Rep 7(1): 10406, 2017
- **Synthesis of 7β-hydroxy-8-ketone opioid derivatives with antagonist activity at mu- and delta-opioid receptors.** Ahonen TJ, Rinne M, Grutschreiber P, Mätlik K, Airavaara M, Schaarschmidt D, Lang H, Reiss D, Xhaard H, Gavériaux-Ruff C, Yli-Kauhaluoma J, Moreira VM. Eur J Med Chemistry 151: 495-507, 2018

- **Pattern of neuropathic pain induced by topical capsaicin application in healthy subjects.** Lötsch J, Dimova V, Hermens H, Zimmermann M, Geisslinger G, Oertel BG, et al. Pain 156(3): 405-14, 2015
- **Quantitative sensory testing response patterns to capsaicin- and ultraviolet-B-induced local skin hypersensitization in healthy subjects: a machine-learned analysis.** Lötsch J, Geisslinger G, Heinemann S, Lerch F, Oertel BG, Ultsch A. Pain 159(1): 11-24, 2018
- **A data science approach to candidate gene selection of pain regarded as a process of learning and neural plasticity.** Ultsch A, Kringel D, Kalso E, Mogil JS, Lötsch J. Pain 157(12): 2747-57, 2016
- **Machine-learning-derived classifier predicts absence of persistent pain after breast cancer surgery with high accuracy.** Lötsch J, Sipilä R, Tasmuth T, Kringel D, Estlander AM, Meretoja T, Kalso E, Ultsch A. Breast Cancer Res Treat 2018
- **A machine-learned analysis of human gene polymorphisms modulating persisting pain points to major roles of neuroimmune processes.** Kringel D, Lippmann C, Parnham MJ, Kalso E, Ultsch A, Lotsch J. Eur J Pain 22(10): 1735-56, 2018
- **Next-generation sequencing of human opioid receptor genes based on a custom AmpliSeq library and ion torrent personal genome machine.** Kringel D, Lötsch J. Clin Chim Acta 463: 32-38, 2016
- **Next-generation sequencing of the human TRPV1 gene and the regulating co-players LTB4R and LTB4R2 based on a custom AmpliSeq panel.** Kringel D, Sisignano M, Zinn S, Lötsch J. PLoS One 12(6): e0180116, 2017
- **Development of an AmpliSeq™ panel for next-generation sequencing of a set of genetic predictors of persisting pain.** Kringel D, Kaunisto MA, Lippmann C, Kalso E, Lötsch J. Front Pharmacol 9: 1008, 2018
- **Machine-learned selection of psychological questionnaire items relevant to the development of persistent pain after breast cancer surgery.** Lötsch J, Sipilä R, Dimova V, Kalso E. Br J Anaesth 121(5): 1123-1132, 2018
- **Remaining pain is common in early rheumatoid arthritis patients treated with methotrexate.** Altawil R, Saevarsdottir S, Wedrén S, Alfredsson L, L Klareskog L, Lampa J. Arthritis Care Research 61(8): 1061-8, 2016
- **In vivo evidence of a functional association between immune cells in blood and brain in healthy human subjects.** Kanegawa N, Collste K, Forsberg A, Schain M, Arakawa R, Jucaite A, Lekander M, Höglund CO, Kosek E, Lampa J, Halldin C, Farde L, Varrone A, Cervenka S. Brain Behav Immun 54:149-57, 2016
- **Evidence of fatigue, disordered sleep and peripheral inflammation, but not increased brain TSPO expression, in seasonal allergy: A [11C]PBR28 PET study.** Tamm S, Cervenka S, Forsberg A, Estelius J, Grunewald J, Gyllfors P, Karshikoff B, Kosek E, Lampa

J, Lensmar C, Strand V, Åkerstedt T, Halldin C, Ingvar M, Olgart Höglund C, Lekander M. Brain Behav Immun 68: 146-157, 2018

- **Evidence of different mediators of central inflammation in dysfunctional and inflammatory pain - Interleukin-8 in fibromyalgia and interleukin-1 β in rheumatoid arthritis.** Kosek E, Altawil R, Kadetoff D, Finn A, Westman M, Le Maître E, Andersson M, Jensen-Urstad M, Lampa Jon. J Neuroimmunology 280: 49-55, 2015
- **Functional association between brain microglia and blood immune cells in healthy human subjects.** Kanegawa N, Collste K, Forsberg A, Schain M, Arakawa R, Jucaite A, Lekander M, Olgart Höglund C, Kosek E, Halldin C, Farde L, Varrone A, Cervenka S. Brain Behav Immun 54: 149-57, 2016
- **Intrinsic brain connectivity in chronic pain: A resting-state fMRI study in patients with rheumatoid arthritis.** Flodin P, Martinsen S, Altawil R, Waldheim E, Lampa J, Kosek E, Fransson P. Front Hum Neurosci 10: 107, 2016
- **Differences in neuroimmune signalling between male and female patients suffering from knee osteoarthritis.** Kosek E, Finn A, Ultenius C, Gedin P, Hugo A, Gladh I, Andersson M, Svensson C, Ahmed A. J Neuroimmunol 321: 49-60, 2018
- **Dynamic lateral organization of opioid receptors (kappa, $mu_{wt}$ and $mu_{N40D}$) in the plasma membrane at the nanoscale level.** Rogacki MK, Golfetto O, Tobin SJ, Li T, Biswas S, Jorand R, Zhang H, Radoi V, Ming Y, Svenningsson P, Ganjali D, Wakefield DL, Sideris A, Small AR, Terenius L, Jovanović-Talisman T, Vukojević V. Traffic 19(9): 690-709, 2018
- **Characterization of neuroinflammation and periphery-to-CNS inflammatory cross-talk in patients with disc herniation and degenerative disc disease.** Palada V, Ahmed AS, Finn A, Berg S, Svensson C, Kosek E. Brain Behav Immun 75: 60-71, 2019
- **Microglia Express Mu Opioid Receptor: Insights from Transcriptomics and Fluorescent Reporter Mice.** Maduna T, Audouard E, Dembélé D, Mouzaoui N, Reiss D, Massotte D, Gaveriaux-Ruff C. Front Psychiatry 2018 (in press)
- **Ethanol and Naltrexone Have Distinct Effects on the Lateral Nano-organization of Mu and Kappa Opioid Receptors in the Plasma Membrane.** Tobin SJ, Wakefield DL, Terenius L, Vukojević V, Jovanović-Talisman T. ACS Chem Neurosci 2018 (in press)
- **Discovery of 12-thiazole abietanes as selective inhibitors of the human metabolic serine hydrolase hABHD16A.** Ahonen TJ, Savinainen JR, Yli-Kauhaluoma J, Kalso E, Laitinen JT, Moreira VM. ACS Med Chem Lett 2018 (in press)

Upcoming publications

- **Machine-learned analysis of global and glial/opioid intersection related DNA methylation in patients with persisting pain after breast cancer surgery.** Kringel D, Kaunisto MA, Kalso E, Lötsch J. (submitted)

- **A data science approach to the prediction of pain phenotypes in rheumatoid arthritis from early acquired parameters in a registry study.** Lötsch J, Klareskog L, Alfredsson A, Silberberg G, Lampa J. (submitted)

Oral presentations at conferences

- **9th Congress of the European Pain Federation (EFIC), September 2015, Vienna, Austria**
  - E Kalso: Translating systematic review evidence into clinical practice
- **IASP 16th World Congress on Pain, September 2016, Yokohama, Japan**
  - J Lötsch: Pharmacogenetic Implications in Pain Treatment
  - C Svensson: The role of spinal HMGB1, TLR4 and microglia activation in arthritis-induced pain in male and female mice
  - E Kalso: Biological mechanisms to explain lack of analgesic benefit
  - E Kalso: Clinical Pharmacology of Opioids
- **Belgian Conference of Rheumatology, September 2016, Brussels, Belgium**
  - J Lampa: Silence and Science behind chronic pain in RA and SpA
- **Pain Mechanisms and Therapeutics Conference, June 2016, Taormina, Italy**
  - Y Sidorova: Small molecule mimetics of glial cell line-derived neurotrophic factor family ligands for the treatment of neuropathic pain
- **European Pain School, June 2017, Siena, Italy**
  - C Svensson: Novel mechanisms of pain mediated by autoantibodies in conditions like rheumatoid arthritis?
  - C Svensson: Role of HMGB1 and TLR4 in spinal nociceptive signal transmission in models of arthritis-induced pain
- **10th Congress of the European Pain Federation (EFIC), September 2017, Copenhagen, Denmark**
  - E Kosek: Central pain processing in fibromyalgia
- **IASP 17th World Congress on Pain, September 2018, Boston, USA**
  - C Svensson: Bedside to bench: Elucidating mechanisms of articular and non-articular arthritis pain
  - E Kosek: Nociplastic pain, neuroinflammation and how the term should be used
  - E Kalso: Cancer Pain: Current strategies for safe and effective relief
  - E Kalso: New approaches to identifying individuals at risk of chronic postsurgical pain

Presentations at business meetings

- **9th Annual ELSCEO Forum & Exhibition 2015, Zurich, Switzerland**
- **BioFit 2015, Strasbourg, France**
- **15th Annual Biotech in Europe Forum 2015, Basel, Switzerland**

Theses

- **New insights into enhancing morphine analgesia: from glia to pharmacokinetics.** T Lilius. November 2014
- **Intrinsic Brain Activity in Health and Disease.** P Flodin. September 2015
- **Pain, Mechanisms of fatigue and autonomic function in rheumatoid arthritis.** R Altawil. December 2016
- **Inflammatory Polarization of Immune Cells in Neurological and Neuropsychiatric Disorders.** Z Li. November 2016
- **Opioid analgesia: modulation by drug interactions and glial activation.** V Jokinen. December 2017
- **Neuroimmune mechanisms in chronic inflammation – Translational studies of the inflammatory reflex.** J Estelius. June 2018

Other

- **Opioids and Pain.** Book chapter by C Stein and C Gaveriaux-Ruff. Oxford Handbook of the Neurobiology of Pain, July 2018, edited by JN Wood
- **Meeting with the Swedish patient association "Rheumatikerförbundet".** October 2014, Stockholm, Sweden

Software

Within WP1, several bioinfomatic methods had to be newly developed. Implementations in R have been developed with the contribution of WP1 and made freely available:

- **R library "Umatrix"**: Lötsch, J., Lerch, F., Djaldetti, R., Tegeder, I., Ultsch, A.: Idendtification of disease-distinct complex biomarker patterns by means of unsupervised machine-learning using an interactive R toolbox (Umatrix), BMC Big Data Analytics, pp. 1-17, 2018. R library avalaibe at https://cran.r-project.org/package=Umatrix
- **R library "dbtORA"**: Lippmann C, Kringel D, Ultsch A, Lötsch J. Computational functional genomics-based approaches in analgesic drug discovery and repurposing. Pharmacogenomics 19(9): 783-797, 2018. R library avalaibe at https://github.com/IME-TMP-FFM/dbtORA

## *4.3 Exploitation of results*

The results of GLORIA have been important both in a clinical context and for further research in the area of chronic pain. An important exploitation of the results in the RA cohorts is that chronic pain and remaining pain is common in early RA, and comprises an unmet need for the disease. The results have led to initiation of a programme for early identification of unmet needs in RA, with the purpose that this new strategy will first result in a thorough characterisation of early RA concerning sleep patterns, different aspects of fatigue and depression. In patients at risk for the development of

chronic pain (with high disability at diagnosis, high pain, and low systemic inflammation at onset) we will then offer a detailed pain analysis and adequate non-pharmacological interventions on a single-patient level. These may include information and occasionally optimisation of exercise by physiotherapists, psychologist contact and decision if the patient should go through a cognitive-behavioural therapy program.

To continue the work done in GLORIA, we will perform more indepth exploratory analysis of the associations between several pro-inflammatory substances in CSF and symptom severity in order to identify biomarkers for pain that could also become future treatment targets. The identified biomarkers will be further validated and their potential causative action will be investigated in experimental pain models. We will use the models from GLORIA WP4 that are specifically designed for studying mechanisms involved in pain sensitisation in relation to joint inflammation. Identification of biomarkers for neuropathic pain development after breast cancer surgery will be further exploited, and candidate biomarkers and biomarker patterns will be studied concerning the causality of pain sensitisation and effects on glia activity and opioid receptor interaction/activation in the experimental pain models. It is also a valuable tool for improvement of the prediction of which patients will develop a pain condition, thereby subject to early intervention of contributing factors. Likewise, in OA further studies of several biomarkers, including MCP-1, may pave the way for interventional targeted studies with the goal of decreasing OA pain in a more specific way. In addition, genetic research in pain medicine has been directed to the recognition of genes in which variants influence pain behaviour, post-operative drug requirements, and the temporal developments of pain toward persistence. The data science and machine-learning methods developed within GLORIA can be used for (i) the refinement of genetic and epigenetic biomarkers for persistent pain and (ii) the inclusion as novel functional genomics approaches in drug discovery and drug repurposing. Respective publications are mostly available as open access.

Also, intensive development of a new generation of PET ligands is ongoing with the aim of distinguishing different profiles of activated glia cells, e.g. protective or pro-inflammatory. Once in place, the effects of various treatments on glia cell activation and neuroinflammation can be studied and better understood, which is a prerequisite for the development of targeted treatment strategies as well as development of totally new treatment strategies. In addition, fFMI has proven to be resourceful for probing at the single-cell and the single-molecule levels basic molecular mechanisms underlaying chronic pain. fFMI has enabled us to quantitatively characterize in live cells receptor-receptor interactions, thus probing fundamental mechanisms through which chronic pain is brought about, and to examine the effects of available and prospective medications at the primary site of their action, the cell surface receptors in the plasma membrane. While the results obtained are of basic character, the methodological advancements, cellular models, experimental procedures and protocols are universally applicable and will be used in future studies.

The experimental mouse models for opioid receptors developed within GLORIA can be exploited in the search of analgesics with low tolerance potential. The future work based on GLORIA will be devoted to the characterization of opioid receptors in inflammatory cells in the central nervous system and other tissues as well as to the role of glial opioid receptors in other models of chronic pain. In general, the results from experimental pain models could be exploited to evaluate the

properties of novel candidate analgesics based on the delta opioid receptor and GFL mimetics. The published (and to be published) results within GLORIA project can serve as a basis for comprehensive research for pain therapeutics. In particular, we plan to test optimized GFL mimetics in nerve injury-based models of experimental neuropathy. The goal is to develop compounds with improved potency and efficacy in *in vitro* and *in vivo* tests, in combination with better pharmacokinetic properties. As so far we saw no sex-specific differences in microglial activation or neuronal markers, we will run the initial screening of the compounds only in males. Final GFL mimetics will be tested in animals of both sexes.

Based on the work supported by GLORIA we are changing gear with regards to the establishement of animal models of OA. We have already secured funding to examine if we can reproduce work showing that an OA model induced by repeated bone/joint loading at a force that causes microdamages leads to persistent changes in pain-like behaviour. If successful, we will examine the role of glia and TLR4 in this model, and potential sex dimorphism.

We will continue to investigate factors in the CNS and the periphery that maintain antibody-induced hypersensitivity beyond resolution of inflammation, which a particular focus on pharmacology and sex. Such studies are important as we in one way have to re-evaluate earlier reported data that was generated using only one sex of mice. Gaining a better understanding for when pain physiology and pain-relieving mechanisms are different between sexes in animal models of chronic pain may help in improving the translational aspect of pain research.

In conclusion, GLORIA has contributed with important clinical and basic knowledge, and these results will also be further exploited in new projects, and contribute to completing novel grant applications, including EU-funded projects.

## *5 Website and contact details*

The GLORIA website can be found at http://gloria.helsinki.fi/. In addition to the main language English, the website has been translated into Chinese, German, Estonian, Spanish, French, Portuguese, Finnish and Swedish (Fig. 2).


**Figure 2. Screenshot from GLORIA website.**

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**Chemedest OÜ**


GLORIA
(glial-opioid receptor interface in analgesia)