

Epigenetic and Proteomic Signatures for Chronic Pain Patients after Total Knee Replacement

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**EPIGENETIC AND PROTEOMIC
SIGNATURES FOR CHRONIC PAIN
PATIENTS AFTER TOTAL KNEE
REPLACEMENT**

**BY
ROCCO GIORDANO**

DISSERTATION SUBMITTED 2021



AALBORG UNIVERSITY
DENMARK

EPIGENETIC AND PROTEOMIC SIGNATURES FOR CHRONIC PAIN PATIENTS AFTER TOTAL KNEE REPLACEMENT

by

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AALBORG UNIVERSITY
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Dissertation submitted

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CV

Rocco received a B.Sc. degree in health biotechnologies and a M.Sc. in medical biotechnology from the Catholic University of Sacred Heart, Rome, Italy. Subsequently, he worked as research assistant in several laboratories at the Catholic University of Sacred Heart, Rome, Italy, acquiring expertise in new high throughput molecular techniques to evaluate the expression of non-coding RNA and proteins. In 2016, he worked at IRCCS Fondazione Don Carlo Gnocchi, Rome, Italy increasing his interest in pain research and genomic biomarkers. In September 2016, he enrolled as Ph.d. fellow at Center for Neuroplasticity and Pain under the supervision of Prof. Lars Arendt-Nielsen at Aalborg University, Denmark. He is interested in epigenetics, transcriptomics, proteomics and molecular biology behind the pain process. He focused his research on evaluating potential circulating genomic biomarkers for chronic pain after surgery in patients affected by knee osteoarthritis. He has been involved in several dissemination activities through conferences, abstract and poster submission for international congresses.

ENGLISH SUMMARY

Osteoarthritis (OA) is the most frequent painful musculoskeletal diagnosis in the older population and the most prominent cause of disability. Total knee replacement (TKR) is expected to provide pain relief for patients with severe OA, however, around 20% of knee OA (KOA) patients will experience chronic post-operative pain after TKR surgery. Several studies have found high preoperative pain intensities and sensitization of central pain pathways as predictors for chronic post-operative pain following TKR. Recently a study found preoperative proinflammatory cytokines to be associated with chronic post-operative pain following TKR. Preclinical data shows that proinflammatory cytokines sensitize the peripheral nerve endings, which may eventually lead to central sensitization, indicating that preoperative increase levels of inflammatory markers could act as prognostic biomarkers for chronic post-operative pain following TKR. It is now apparent that epigenetic modifications, like the actions of noncoding RNAs (e.g. microRNAs, lncRNAs, siRNAs, circRNAs), may confer susceptibility to OA, which could open up new avenues for alternative therapeutic approaches. Several studies have illustrated the possible diagnostic potential of circulating non-coding RNAs in OA, indicating that this family of molecules may act as potential predictors for the development and progression of knee and hip osteoarthritis.

Real-time poly-chain reaction (RT-qPCR), quantitative and qualitative techniques allowed the evaluation of the circulating ncRNAs in serum samples, through the isolation and retro-transcription of total RNAs. This methodology reduces non-specific results and reduces the difficult handling of the big amount of data obtained through other molecular biology techniques. Furthermore, a new proteomic approach, i.e. the proximity extension assay (PEA), has been used which gives a specific and standardized overview of inflammatory markers involved in the pathology.

This PhD-project includes three original studies that focus on the transcriptional, post-transcriptional and translation processes in the serum of patients with osteoarthritis, investigating factors that modulate the perception of pain.

The first study aimed to investigate the preoperative association of long non-coding RNAs (lncRNAs) and chronic post-operative pain, showing that patients with pain 1-year after surgery exhibited down-expression of three lncRNAs preoperatively when compared with patients with post-surgical full recovery (Study I).

The second study evaluated preoperative microRNA's (miRNA's) profile as potential predictive biomarkers for post-operative pain. Twenty-one miRNAs were analyzed,

and three miRNAs showed a pre-operative differential level of expression in patients with low pain relief after surgery compared to patients with high pain relief. The miRNAs dysregulated showed a predictive value for the pain relief after surgery pointing to possible use for these miRNAs as potential biomarkers for post-operative pain (Study II).

The third study aimed to evaluate the preoperative serum expression of a panel of 92 inflammatory cytokines and highlight correlation with pain intensity in patients with KOA. Patients with KOA showed ten cytokines with significantly lowered expression in the serum and five cytokines with higher expression levels when compared to healthy participants. Specifically, FGF-21 and 4E-BP1 were associated with pain in KOA, and TWEAK, FGF-21, CSF-1, IL6 were identified as independent predictors for pain intensity (Study III).

In conclusion, the current Ph.d. thesis provides the first evidence of the interaction between post-transcriptional modifications and post-operative pain, which is an important step forward and offers advantages for future exploration of the involvement of epigenetic modifications in the pain field.

DANSK RESUME

Slidgigt er den hyppigste muskuloskeletale lidelse hos ældre og er desuden den største årsag til smerter og funktionsnedsættelse i denne populationsgruppe. Hos patienter med svær slidgigt vil en operation med total udskiftning af knæet (TRK) forvente at lindre smerten, dog vil ca. 20% opleve at få kronisk post-operativ smerte efter TKR-operationen. Flere nye studier har vist, at der er en mulig sammenhæng mellem en høj præ-operativ smerteintensitet, tilstedeværelsen af inflammatoriske stoffer og udviklingen af kronisk post-operativ smerte efter TKR. For nyligt viste et studie, at præ-operative proinflammatoriske stoffer var forbundet med udviklingen af kronisk post-operativ smerte. Endvidere, prækliniske data viser, at proinflammatoriske stoffer sensibiliserer de perifere nerveender, hvilket til sidst kan føre til central sensibilisering af nervesystemet og deraf ændring af de nociceptive signalveje. Dette indikerer, at en øget tilstedeværelse af inflammatoriske markører præ-operativt kan fungere som mulige prognostiske biomarkører for udviklingen af kronisk post-operativ smerte efter TKR.

Det er påvist, at epigenetiske modifikationer, så som ikke-kodende RNA'er (fx mikroRNA'er, lncRNA'er, siRNA'er, circRNA'er) også kan have en mulig medvirken i udviklingen af slidgigt. Dette kan på sigt åbne op for nye alternative terapeutiske tilgange til at diagnosticere patienter, der kan være i risiko for at udvikle slidgigt. Bl.a. har flere studier demonstreret, at cirkulerende ikke-kodende RNA'er er tilstede i forskellige vævsvæsker (f.eks. serum) hos patienter med slidgigt, og deraf antydning, at denne familie af molekyler muligvis kunne fungere som potentielle biomarkører for udviklingen af knæ- og hofte-slidgigt.

Real-time poly chain reaction (RT-PCR) er en kvantitativ og kvalitativ metode, som gør det muligt at identificere og evaluere tilstedeværelsen af cirkulerende ikke-kodende RNA'er i serumprøver via isolering og derefter retro-transkription af den totale mængde RNA. Denne metode reducerer ikke-specifikke resultater såsom falsk-positive fund og reducerer den vanskelige håndtering af store mængde data, som man opnår gennem andre molekylærbiologiske teknikker. Endvidere, en ny proteomisk metode, Proximity Extension Array (PEA) anvendt til at identificere samt give et specifikt og standardiseret overblik over de inflammatoriske markører, der er involveret i patologien.

Dette Ph.d.-projekt inkluderer tre originale studier. Studie 1 og studie 2 undersøgte, om der var en sammenhæng mellem tilstedeværelsen af mulige epigenetiske modifikationer (ikke-kodende RNA'er) hos patienter med slidgigt præ-operativt og deres smerteintensitet post TKR. Studie 3 undersøgte desuden, om der er en

sammenhæng mellem niveauet af inflammatoriske stoffer præ-operativt og smerteintensiteten.

Studie 1 viste at patienter med kronisk smerte efter TKR (>1 år) havde en nedregulering i ekspressionen af 3 ikke-kodende RNA'er i serum præ-operativt sammenlignet med patienter, der ikke havde smerter efter TKR. I studie 2 var 21 miRNAs analyseret og ud af disse udviste 3 miRNAs en anderledes ekspression præ-operativt hos patienter med lav smertelindring efter TKR sammenlignet med patienter med høj smertelindring (ingen smerte) efter TKR. En sådan dysregulering i disse miRNA's kan om muligt have en potentialbetydning for at identificere patienter, der er i risiko for at udvikle smerter efter TKR. Studie 3 evaluerede ekspressionen af 92 inflammatoriske stoffer i serum fra patienter med slidgigt. Heraf var ekspressionen af 10 cytokiner signifikant nedreguleret, mens 5 andre var opreguleret i forhold til ekspressionen af samme cytokiner i serummet fra raske personer. Studiet 3 viste også at FGF-21 og 4E-BP1 især var associeret med smerteintensiteten hos patienter med slidgigt, hvorimod TWEAK, FGF-21, CSF-1 og IL6 var associeret med smerteintensiteten generelt.

Dette PhD-projekt fremviser signifikante detaljer omkring sammenhængen mellem tilstedeværelsen af forskellige post-transkriptionelle modifikationer og post-operativ smerte efter TKR og deres potentiale som mulige biomarkører hos patienter med slidgigt, hvilket er vigtigt i forhold til senere studier, der vil undersøge involvering af epigenetiske modifikationer indenfor smerteområdet.

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“The perfect being, huh? There is no such thing as perfect in this world. That may sound cliché, but it’s the truth. The average person admires perfection and seeks to obtain it. But, what’s the point of achieving perfection? There is none. Nothing. Not a single thing. I despise perfection! If something is truly perfect, then there is nothing left. There is no room for imagination. No place left for intelligence or for a person to gain additional knowledge or abilities. Do you know what that means? For scientists such as ourselves, perfection is a dead end a condition of hopelessness. It is our job to create things more wonderful than anything before them, but never to obtain perfection. A scientist must be a person who finds ecstasy while suffering from that antimony.”

Tite Kubo

PREFACE

The work for this Ph.d. thesis was performed between September 2016 and August 2019 at the center for neuroplasticity and pain (CNAP), SMI[®], department of Health Science and Technology, Aalborg University, Aalborg, Denmark. The Danish National Research Foundation (DNRF121) is gratefully acknowledged for stipend and project support.

The Ph.d. thesis aimed to evaluate the expression of potential circulating biomarkers and their association with the post-operative pain as well as the preoperative low systemic grade of inflammation in patients with knee osteoarthritis.

The first chapter gives an introduction to the burden of osteoarthritis, how the pathology is characterized at a clinical level and which treatments are available at present time. Moreover, an introduction about the characterization of painful knee osteoarthritis and how post-surgical chronic pain need biomarkers will be given. This is further sustained by the introduction of new targets represented by epigenetic modifications. The second chapter gives an overview of methodological procedures and presents the cohort of patients and healthy subjects recruited for the project. The third, fourth, and fifth chapters present, in details, the results obtained in the three studies; with main focus for the third chapter in the evaluation of lncRNA as signature for post-operative pain, for the fourth chapter in highlighting miRNA as potential circulating biomarkers for post-operative pain and the fifth chapter in the evaluation of a new inflammatory panel in serum of patient with osteoarthritis versus healthy subject.

The Ph.d. thesis is based on three original manuscripts two published and one submitted in international peer-reviewed journals.

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CHAPTER 1. INTRODUCTION

1.1. OSTEOARTHRITIS

Osteoarthritis (OA) is considered a degenerative and progressive disease affecting synovial joints of e.g. the knees, hips, hands, and spine ⁵⁵. Many factors can influence the outbreak and progression of OA such as joint alignment alteration, trauma, or obesity, biomechanical, biochemical, and genetic factors ^{25,49,68,82}. Specifically, obesity is associated with a higher risk of diabetes type-2, which is correlated with increased inflammation in the OA affected joint ⁶⁵. The result of these factors is manifested by alterations of the joints which includes cartilage disruption, sclerosis of subchondral bone, osteophyte formation, and high levels of synovitis ⁸⁸. Radiological evaluations are the most applied for the diagnosis of osteoarthritis, and the degeneration of the joint is evaluated by X-ray imaging using the Kellgren & Lawrence (KL) ¹³⁸ system.

1.1.1. PREVALENCE OF OSTEOARTHRITIS

OA is defined as one of the most frequent musculoskeletal diseases in older populations and the most common cause of impairment ¹¹. It mostly affects women (around 40%) and in a lower incidence men (around 25%) in the age range of 60-70 years ³. The future projection of a global increase in the elderly population and the higher rates of sedentary lifestyle means that the incidence of OA in the coming years is predicted to dramatically increase ^{18,23,27}. Within the highest contributor of global disability, OA holds the 11th place^{47,256}. The burden of OA is also most likely underestimated due to the ongoing methodological issues regarding a conservative case definition and the restriction of diagnosis which only includes the hip and the knee ⁴⁸. Moreover, OA of hip and knee are included together with some major pathologies in a group of conditions with a high risk of mortality ¹⁹⁵. This underlines the great impact of OA on present and future society. The incidence of symptomatic OA has increased exponentially in men and women in the past two decades ¹⁹², and is becoming even more clear a substantial increase of this pathological condition in the future ¹¹¹.

1.1.2. TREATMENT OF KNEE OSTEOARTHRITIS

In terms of treatment for knee OA (from here on KOA), no definitive cure has been found. For this reason, any kind of intervention aims to reduce the effects of characteristic symptoms and prevent an additional progression of the disease. Early

stages of KOA can be treated by several pharmacological and non-pharmacological remedies¹¹⁰ and exercise in association with weight loss has been shown to yield pain relief¹⁷⁸. Treatment for KOA patients can be subdivided into three major ways that are defined as first-line, second-line, and third-line of intervention^{71,173}. International organizations active in the OA's research (EULAR and OARSI) recommends as first-line of treatment a combination of exercises aimed to achieve weight loss that can be associated with a second-line of treatments, which includes non-surgical treatments, while only after unsuccessful results the third-line of treatment can be considered^{131,173}. At the moment, the current end-treatment for a late stage of KOA is the total knee replacement (TKR). TKR is considered as a surgical effective treatment for KOA³¹, which consists of replacing the bone terminations part of the knee joint with metal and polyethylene prosthetic implants^{31,168}. The rising number of incidences associated with more patients in need of treatment as well as the potential for the development of pain after surgery underscore the importance of new preoperative prediction methods for individual treatment.

1.1.3. PAIN AND SENSITIZATION IN KNEE OSTEOARTHRITIS

Specific pathways underlying pain mechanism in OA are unfortunately still poorly investigated⁹⁶, although many types of research have demonstrated that a large contributor is the sensitization of the pain system^{8,73,91,135,236}. The pathogenesis of OA includes all joint tissues⁵³ and although pain cannot arise from this structure due to the absence of nociceptive fibers in the cartilage^{60,70}, KOA is diagnosed by using radiological evaluation of the cartilage alterations. The nociceptive input is possibly started by the occurrence of intense inflammation at the synovial level, or other features such as increased pressure in the subchondral bone, osteophyte growth, and sensitization of the periarticular tissues with central sensitization of the nervous system^{54,70}. The inflammatory state leads to the sensitization of peripheral nociceptors²²⁹, and this sensitization in the specific synovitis (inflammation of the synovial membrane)²⁶⁴ could lead to sensitization of peripheral nociceptors in KOA⁷⁰, where factors such as sensitization in the periarticular tissues have been proved associated with maintenance of pain^{3,7,8,91}.

Previous studies suggest that musculoskeletal pain is influenced by the persistence of pain and its intensity, which finds a possible cause in the central sensitization^{7,109}. This indicates that a certain pain condition or damaged tissue moves from a localized area to a more expanded area and ends in a condition of chronicity and widespread pain⁷. A generalized sensitization (e.g the knee area) has been demonstrated to be associated with high pain^{8,67,73,122,265}, poor quality of life¹²², modest outcome after TKR surgery¹⁶⁶, and high concentration of proinflammatory mediators in the circulation of patients with KOA¹⁴⁷.

1.2. PAIN AFTER SURGERY

1.2.1. ACUTE AND CHRONIC POST-OPERATIVE PAIN

Acute post-operative pain and its severity are correlated with chronic post-operative pain ¹³⁶. For the treatment of acute pain, the last decade has produced several guidelines but with limited results^{57,170,246}. Earlier studies have shown that the 24% of patients of major surgery, such as urological, cardiovascular, plastic, and orthopaedical, reports at six months an incidence of pain defined as mild (1,2 out 10 of the VAS), and 16% of them reported moderate/severe pain (more than 3 out 10 of the VAS), values that after 1 year are still present with the incidence of mild pain at 23% and the for the severe pain to a 12% ⁴⁴. In the particular case of TKR, patients have reported pain for 3 months in around 5% and for 6 months in the 9% of the cases ²⁷. More studies highlighted how 58% of OA patients developed chronic post-operative pain, where 11% showed a neuropathic component, and 22% of those presented moderate/severe chronic post-operative pain ^{44,208}. At present, a definitive reason for the development of chronic post-operative pain in some of the patients, while others recover fully in absence of pain, is lacking. In this direction are also missing clear explanation regarding the pathways that lead the acute pain to become chronic post-operatively.

1.2.2. RISK FACTORS FOR CHRONIC POST-OPERATIVE PAIN DEVELOPMENT

Previous studies showed how circulating markers such as pro-inflammatory cytokines and products of inflammatory cascades can be pointed out as potential risk factors of post-operative pain ^{6,121}. Further, evidence concords that epigenetic modifications, like the actions of noncoding RNAs (e.g. microRNAs, lncRNAs, siRNAs, circRNAs), might be highly related to the pathophysiology of OA, and this may pave the way for future treatments; in this regard several studies have illustrated the possible diagnostic potential of circulating non-coding RNAs in OA, indicating that this family of molecules may act as potential predictors for the onset and the clinical evolution of knee and hip osteoarthritis ^{15,24}.

1.3. PROTEOMICS IN CHRONIC PAIN AND OA

The action of proteins has been long studied in relation to acute and chronic pain and molecular research has shown the complexity of chronic pain^{90,193}. Mechanisms and molecular pathways under several pain syndromes are most probably different in the individuals and involves also a high number of molecular changes^{217,240}. In the last twenty years, research has improved for the identification of receptors, signaling proteins, and ion channels involved in nociception and pain²¹⁷ but potential therapies that targeted single proteins showed low efficacy. For this reason, the upcoming clinical research studies focus on a molecular pathways based approach for pain therapy^{16,108,225}. Interesting insights into modifications related to painful pathologies in preclinical and clinical studies have been described by research that aimed to evaluate the biological systems behind the complex molecular pathways that contribute to chronic pain, such as next-generation sequencing genomics^{2,291} and epigenomic and transcriptomic studies^{127,235}. Preclinical proteomic studies have focused on different aspects such as mice and rat models, which includes spinal nerve injury (SNI), spinal cord injury (SCI), Complete Freund's Adjuvant (CFA), and spinal nerve ligation (SNL)^{142,146,224,244}, and investigated changes in proteins levels in response to drug administration¹⁸⁹, highlighting several proteins dysregulation involved in response mechanism after painful and stress stimuli and inflammatory pathways^{142,180}. In humans, only a few studies have focused on the proteomics approach to assess pain mechanisms and highlight potential biomarkers in tissue biopsies in order to study painful conditions like chronic widespread pain^{202,203}, Complex Regional Pain Syndrome¹⁹⁸, and myalgia^{97,201}. In line with this, a study conducted on trapezius muscle micro biopsies from female patients with chronic widespread pain compared to healthy subjects showed 17 proteins differently regulated in the two groups, involved in multiple cellular functions which includes muscle damage, stress, and inflammation²⁰². Moreover, proteome signatures in liquid biopsies such as saliva and CSF are becoming more relevant showing protein dysregulation in a pathological condition characterized by a painful state^{14,288}. Other sources that are also evaluated are blood, plasma and serum due to their easy obtainment and for evaluation of changes in lifestyle, disease and treatment^{20,84}. For instance, Ghafouri et al. evaluated plasma samples from subjects with work-related musculoskeletal disorders through mass spectrometry (MS) showing dysregulation of markers that can be used to prevent this kind of musculoskeletal pain⁸⁵. Moreover, a study conducted in serum and synovial fluid (SF) from patients affected by KOA undergoing TKR, highlighted the association between inflammatory markers such as TNF α and IL6 and a predisposition to ongoing pain after TKR⁷⁹.

1.4. EPIGENETIC MODIFICATIONS

Epigenetics is the study of alterations in the phenotype, understood as gene functions, which are heritable and perpetuate alternative functional states in the presence of the same genomic DNA sequence ²⁶⁷. In humans, some epigenetic changes are inherited by a new generation and can be detectable in it, but still, these changes could be phenotypically not expressed by the new generation ¹⁰⁷. Moreover, epigenetic changes, even though their potential persistent nature, can be a target of further modifications or be reversed at any time ¹⁹³. Generally, epigenetic changes and modifications processes include the action of transcription factors, chromatin modifying enzymes and chromatin-remodeling complexes and the action of non-coding RNAs ^{77,137,212,222,257}. These modifications that can activate or repress have been shown to be may also dependent on lifestyle and environmental factors ²²⁰. Life experiences, behavior, nutrition, and exposure to toxins and pollutants are among the environmental factors associated with epigenetic modifications; for instance, identical twins sharing the same genome, although superficially phenotypically similar, are unique individuals with specific differences ²⁵⁰.

1.4.1. EPIGENETIC MECHANISMS, POST-TRANSCRIPTIONAL AND TRANSLATIONAL MODIFICATIONS

Epigenetics modifications include actions of several transcription factors and enzymes that change the way of reading the DNA double strand causing different expression of specific genes. These mechanisms mainly include histone modifications, DNA methylation and non-coding RNA.

Histone modifications

In eukaryotic cells, the DNA is folded around structural proteins called Histones, which combine two copies of each protein (H2A, H2B, H3 and H4) to form a stable structure, i.e. the nucleosome. Several histone modifications are important for gene expression regulation and DNA repair and replication ^{144,281}. These dynamic and reversible modifications are changes that involve histone acetylation catalyzed by the enzymes histone acetyltransferases and histone deacetylases. Histone methylation is regulated by histone methyltransferases (HMTs) and histone demethylases (HDMs) and plays a key role in gene expression depending on amino acid positions, the aminoacidic type residue, and levels of methylation ^{9,144}.

DNA Methylation

Methylation of DNA is one of the principal epigenetic modifications of the DNA which consists of 5-methylation of cytosine at cytosine-guanine dinucleotides (CpG sites) by methyltransferases (DNMTs). Mostly, after methylation follows the inhibition and silencing of gene expression by physically blocking RNA polymerase II. Dysregulation of DNA methylation is a well know process associated with several pathophysiological conditions such as cancer, schizophrenia, addiction to opioids, and pain ^{56,92,194,232}.

Non-coding RNA

In addition to histone modifications and DNA methylation, gene expression is regulated at the level of transcription and translation by noncoding RNAs, including microRNAs and long noncoding RNAs. miRNAs are a group of developmentally conserved small non-coding RNAs, with more than 7000 precursors and mature miRNAs recognized in humans ¹⁶². Several studies have highlighted a part for miRNAs as a clinical signature and objectives for a wide array of diseases including lung malignancy, colorectal disease, and diabetes ³⁴. Despite many studies highlighting different functions of several miRNAs involved in various pain states ¹, lncRNAs (ncRNAs with nucleotide lengths >200) are still to be investigated for the development of painful conditions. Recent studies indicate that more than 50% of the lncRNAs are present in the central nervous system (CNS), suggesting a prominent functional central role ¹⁰². Consistent with this observation, several studies have documented the importance of the action of lncRNAs in CNS disorders, like Alzheimer's disease, brain tumors, and Multiple Sclerosis ²²⁷. Only a single pre-clinical study has addressed an association between lncRNA and nociceptive modulation, identifying a novel antisense (asRNA) lncRNA for the potassium channel Kcna2 in rat dorsal root ganglion (DRG)²⁸⁷. Fu and colleagues in 2015 showed variable expression of lncRNAs by comparing the cartilage of healthy subjects and patients with OA, demonstrating upregulation of specific a lncRNA, uc.343, that by acting in *cis* on genes implicated in the development of cartilage caused degeneration and therefore faster progression in structural OA ⁷⁸. In a previous study, the possibility to detect lncRNAs in several body fluids such as whole blood, plasma, serum, and urine has been shown ²¹⁸, and their levels have been evaluated in exosomes, structures that stabilize the half-life of these molecules in the circulation and make them potential molecular biomarkers ¹⁸⁷. For example, their stabilized structure and a good detectable amount in blood serum and plasma of patients affected by different kinds of cancer warrant the possibility of using them as a marker for cancer development ^{46,58,155}. Moreover, a recent study highlighted more than 1800 dysregulated lncRNA in patients with KOA with mild and severe pain; pointing at eight of the different

lncRNA identified as potential molecular biomarkers associated with the progression of KOA³⁶.

1.5. AIM OF PHD PROJECTS

Since these premises, which points clearly to a pivotal role for molecules such as non-coding RNA and inflammatory mediators in relation to inflammation and pain in knee osteoarthritis; the aim of this Ph.D. was to evaluate the expression of potential circulating biomarkers that could correlate with the post-operative painful state and the low systemic grade of inflammation in patients with knee osteoarthritis. The main aim was assessed through the achievement of three objectives:

1. Evaluation of circulating lncRNA, involved in several inflammation pathways and miRNA regulation, as a preoperative signature for the development of post-operative pain.
2. Evaluation of circulating miRNA, associated with pain, inflammation, and OA pathophysiology, as a preoperative signature and potential predictive biomarkers for post-operative pain.
3. Evaluation of serum inflammatory markers (e.g. interleukins, chemokines, etc.) as the signature for pain and evaluate their associations with pain intensity.

1.6. PAPERS AND DISSERTATION OVERVIEW

The current Ph.D. thesis includes two published manuscripts and one submitted manuscript to international peer-reviewed journals. The three papers had the aim to address the three objectives of the main project.

Study I: Giordano R, Petersen KK, Santoro M, Pazzaglia C, Simonsen O, Valeriani M, Arendt-Nielsen L. Circulating long non-coding RNA signature in knee osteoarthritis patients with post-operative pain 1-year after total knee replacement. *Scandinavian Journal of Pain* 2020. (Submitted)

Study II: Giordano R, Petersen KK, Andersen HH, Lichota J, Valeriani M, Simonsen O, Arendt-Nielsen L. Preoperative serum circulating microRNAs as predictive biomarkers of chronic post-operative pain after total knee replacement. *Molecular Pain* 2020 (published).

Study III: Giordano R, Petersen KK, Andersen HH, Simonsen O, Arendt-Nielsen L. Serum inflammatory markers in patients with knee osteoarthritis: a proteomic approach. *Clinical Journal of Pain* 2020 (published).

Figure 1 conceptualizes the three studies and their relation to post-surgical pain and preoperative expression of circulation markers.

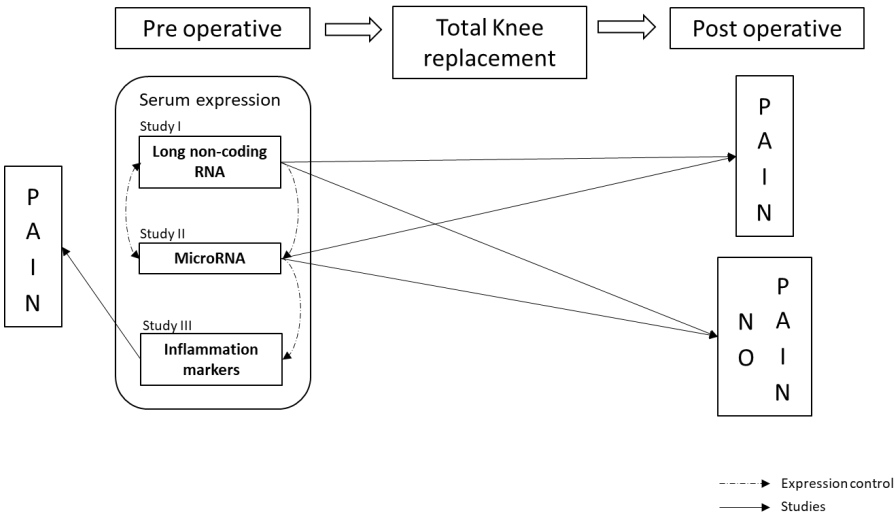


Figure 1. A conceptual overview of the dissertation studies. Study I, II aimed at the evaluation of non-coding RNA as potential biomarkers for post-operative pain. Study III evaluated inflammatory markers, potentially regulated by biomarkers from studies I and II, concerning knee OA and pain.

CHAPTER 2. METHODOLOGICAL CONSIDERATIONS

The following considerations regarding cohort and methodologies applied are shared part of the original research articles on which this dissertation is based (Study I, II, III).

2.1. COHORT

Two hundred and two KOA patients, scheduled for TKR were recruited from the clinic at Hospital Vendsyssel, Frederikshavn, Denmark (Study I, II, III). Patients were excluded from participating in the study if mental impairment or diagnosis for other pain conditions, such as hip OA, RA, neuropathic pain and fibromyalgia were present (Study I, II, III). The degree of OA severity was based on radiological evaluation, quantified through the scale of Kellgren and Lawrence (KL) (Study I, II, III). The study was approved by The North Denmark Region Committee on Health Research Ethics (N-20120015) and conducted in accordance with the Helsinki Declaration (Study I, II, III).

In study III as a control group of 39 healthy controls were recruited from a database at CCBP/C4Pain Aalborg (Study III). To be included in the study the healthy participants had to not present any sign of painful OA (evaluated radiologically), be pregnant, have any history of drugs and alcohol abuse, or present clinical records of neurological and musculoskeletal disorders (Study III). All patients and healthy participants in the studies were asked to not use analgesic medications at least 24h before participation (Study I, II, III). All patients and healthy participants read and signed an informed consent form prior to enrollment (Study I, II, III).

2.2. PAIN INTENSITY ASSESSMENT

In order to facilitate the rating of pain sensation by the participants, visual analog scale (VAS) was used in all three studies. Patients and healthy participants had to evaluate their feeling of pain on a scale from zero to ten, where zero indicated the absence of pain, whereas ten was referring to the worst pain experienced. The usage of the VAS has been extensively adopted in clinical trials and pain experiments¹²⁶. Associations between categorical pain ratings such as none, mild, moderate and severe, and VAS have been shown in previous studies, defining moderate pain to be above 3 and severe pain to be above 5 (Study I, II, III)^{42,160,258}. For Study I and III, the highest pain felt in the past 24 hours and sensitivity profiles of patients with KOA were chosen. In

Study II percentage of pain relief after surgery was used for evaluation of postoperative pain. All these ways of evaluation were used due to their previously well-established correlations ^{8,91}.

2.3. BLOOD AND SERUM COLLECTION

In order to isolate serum, whole blood was sampled by standard venipuncture procedures from patients and controls between 07:30-09:00 in a standard untreated tube of 9ml (Study I, II, III). Subsequently, the collected blood was left to clot at room temperature. Serum was isolated by centrifugation at 3000 RPM and stored in a -80°C refrigerator. The serum samples isolated were used in Study I, II, and III.

2.4. ARRAY BASED SERUM EVALUATION

In the three studies, an array evaluation approach was used. To date, specific arrays approaches have been used for the evaluation of nucleic acid or protein in a specific (customized array) or in a more broad way using a list of markers validated to be involved in specific pathways ^{14,227,239}. All the arrays used for Study I, II, III were based on the evaluation of double strand cDNA or oligonucleotides through real-time polymerase chain reaction (real-time PCR). Main outcome of this technique is to monitor the amplification of a specific segments of DNA or RNA, retro-transcribed in cDNA, during a PCR but with a visual result obtained in real-time. This technique has two main ways of applications depending on the quantification of targeted sequence (quantitative real-time PCR), and semi-quantitatively (semi-quantitative real-time PCR). In Study, I, II, III, the semi-quantitative method was performed. In study I, RT² lncRNA PCR Array Human Inflammatory Response & Autoimmunity (QIAGEN, Germany) was used to evaluate the serum expression of 84 validated lncRNAs involved in the expression and regulation of inflammation marker genes and miRNA (Study I). In study II Custom miScript miRNA PCR Array (QIAGEN, Germany) was used to evaluate the levels of miRNA associated previously with OA pain and inflammatory response (Study II). In study III, collected serum samples were analyzed for the levels of 92 proteins through a pre-customized array of proteins related to inflammation (Olink Bioscience, Sweden) (Study III).

2.5. COHORT CONSIDERATIONS

Serum samples were obtained from patients and controls enrolled for previous studies ^{208,209} and because of signed informed consent were stored in a biobank at Aalborg University, Department of health science and technology, Aalborg for future studies. Potential loss in sample number was due to alterations in the serum quality or

insufficient amount of sample. In study I, the number of patients was chosen because it was reflecting the previously reported risk for chronic post-operative pain following knee surgery ^{18,208,209}, and in order to express in the cohort the maximum effect of post-operative pain. In study III, a small number of samples was not included in the analysis due to internal standard settings at BioExpedia, Aarhus, Denmark facility who ran the laboratory tests.

CHAPTER 3. CIRCULATING LNCRNA AS SIGNATURE FOR POST-OPERATIVE PAIN IN KOA PATIENTS

The content of this chapter is the results of one of the original articles on which this dissertation is based (Study I).

Long non-coding RNA (lncRNA) are single strand macro molecules, that don't codify for protein, composed of more than 200 nucleotides (nt) involved in gene expression control regulation and other post- transcription modifications^{45,219}, with both nuclear and cytoplasmic localization, which can act on specific genes in *cis* and *trans*^{215,219}. Active functions of these molecules are well known to happen at a developmental level, e.g. the action of the gene XIST that encodes for a lncRNA critical for the inactivation of the X chromosome in eutherian mammals⁵⁹ or at neuronal level where animal studies showed that knocking out genes that encode for lncRNAs causes alteration in the normal development of the neural system^{22,251}. Levels of lncRNAs that are circulating intracellularly can also be evaluated, by the isolation of microvesicles in multiple biological fluids such as blood, cerebrospinal fluid, and others^{155,218}; increasing the discoveries linking lncRNAs to different pathologies and identifying in them novel targets to diagnose and treat human diseases¹⁹. Many of these lncRNA targets are still to be validated, and the clinical applications of lncRNA as a biomarker remain in their early stages. LncRNA has barely been explored in the pain field and therefore opens potential new avenues to approach post-operative pain. In this respect, few preclinical studies showed the involvement of these ncRNA in an experimental condition of neuropathic pain or correlating their dysregulation to a painful behavior^{175,278,287}.

3.1. STUDY I: SERUM CIRCULATING LNCRNA IN KOA PATIENTS

In study I, we combined the evaluation of circulating lncRNA in the serum of KOA patients and the expression of 84 lncRNA as a potential signature for post-operative pain (Study I). Twenty serum samples were chosen from KOA patients based on their post-operative pain intensity and divided into two groups "pain" and "no pain" in order to explore their preoperative expression of lncRNA (Study I). The two groups (both groups n = 10) did not differ on gender (5m;5f versus 6m; 4f); years of age (64.3 versus 61.8); Kellgren Lawrence (3.7 ± 0.48 versus 3.3 ± 0.69); BMI (kg/m^2) ($25.5 \pm$

3.23 versus 33.3 ± 6.52); preoperative VAS (8.0 ± 0.67 versus 6.9 ± 2.16); all p -values > 0.05 (Study I).

In Study I, 3 out of 84 lncRNAs were significantly downregulated in serum of patients who presented with post-operative pain higher than 5 on VAS, one year after surgery (figure 2.) (Study I).

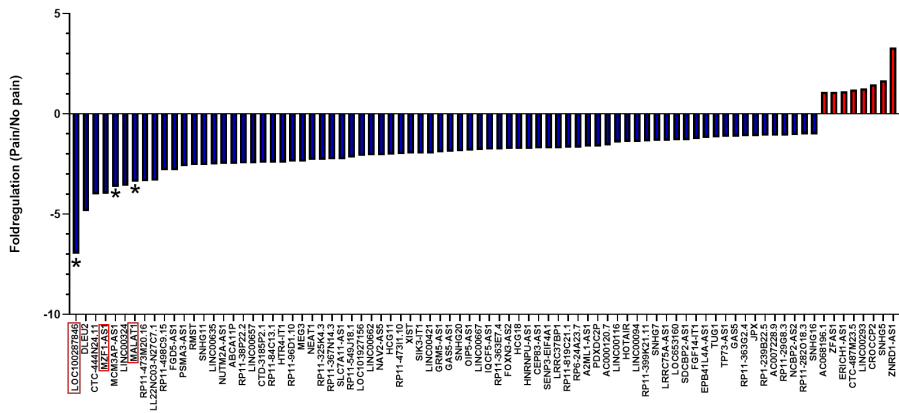


Figure 2. Bar chart lncRNA expression. Bar chart of upregulations (red bars) and downregulations (blue bars) of 84 lncRNA evaluated (Study I). Data expressed as fold regulation for “pain group” compared with the “no pain” group. Fold regulation reports the biological meaning of fold-change (Study I). Asterisks (*) indicate significance $p < 0.05$ from the “no pain” group (Study I).

Dysregulated lncRNAs included Patched 1 pseudogene (LOC100287846, ENST00000528139) with a -6.99 fold regulation (p -value: 0.029); myeloid Zinc Finger 1 Antisense RNA 1 (MZF1-AS1, ENST00000593642) with a -3.99 fold regulation (p -value: 0.038) and Metastasis associated lung adenocarcinoma transcript 1(MALAT1, NR_002819) with a -3.39 fold regulation (p -value: 0.044) (Study I). In study I, we demonstrated a preoperative downregulation of LOC100287846, an uncharacterized lncRNA, in patients with post-operative pain one year after TKR (Study I). No previous studies have demonstrated the involvement of this lncRNA in relation to OA pathophysiology (Study I). The lncRNA showing the highest downregulation was the myeloid Zinc Finger 1 Antisense RNA 1 (MZF1-AS1), which is a regulating factor of the transcription factor MZF1, a member of the SCAN-Zinc

Finger (SCAN-ZF) transcription factor family⁶³. Recent researches have highlighted the association of MZF1 in different types of cancers such as hematopoietic, breast, and lung cancers and its dysregulation in several myeloid lineages^{63,216}. Unfortunately, little is known about MZF1-AS1, but downregulation of this lncRNA could cause upregulation of MZF1, due to its role as antisense (Study I). Preclinical study have demonstrated action of MZF1 in the regulation of pathways involved in neuropathic pain genesis in rat with nerve injury²⁸⁷. In study I, down-regulation of MZF1-AS1 was found preoperatively in KOA patients with high pain intensity suggesting that MZF1-AS1 like its sense strand might be involved in processes that lead to altered regulation of various voltage ion channels causing the painful post-operative pain state (Study I). More research has been conducted on the lncRNA metastasis associated lung adenocarcinoma transcript 1 (MALAT1), which is a widely and stably expressed lncRNA, demonstrated to be associated with metastatic potential and cancer invasiveness in lung cancer¹²⁸. In many types of cancer, MALAT1 is upregulated, which enhances proliferation and inhibits apoptosis, conversely, a down-regulation of this lncRNA induces a reduction of cellular proliferation by promoting apoptosis^{157,268,289}. Few publications on the involvement of this lncRNA in relation to pain and pathways of osteoarthritis exist. A recent *in vitro* study showed the involvement of MALAT1 in OA progression²⁸⁵. These authors showed that the downregulation of MALAT1 increases the level of expression of metalloproteinase-13 (MMP-13) and ADAMTS-5 while decreasing collagen II and *aggrecan* in OA chondrocytes, indicates that down regulation of MALAT1 may contribute to extracellular matrix disruption and so to the pathogenesis of OA²⁸⁵. A preclinical study conducted in spinal cord neurons of a pain rat model showed that downregulation of MALAT1 could result in neuropathic pain by increasing the excitability of spinal cord dorsal horn neurons because of potential involvement in the regulation of the transmembrane flow of calcium ions¹⁷⁵. The data presented in this study on lncRNA expression and pain intensity corroborates those of others who reported that these non-coding macromolecules are involved in several regulatory pathways that might lead to pain and progression of OA, pointing at the potential use for some of them as a therapeutic target (Study I).

3.2. LNCRNA IN SILICO TARGET PREDICTION

Accurate characterization of lncRNA targets is considered fundamental to elucidate their regulatory roles. In study I, a prediction analysis was run using an on-line database that allows the alignment of the lncRNA's sequences based on their ID name with all potential miRNA (Study I). Different applications are available online and companies offer specific software that allows to highlight specific pathways in which the lncRNA could be involved. In study I, we decided to evaluate the potential targets

of the significant lncRNA through starBase v. 3.0 software (<http://starbase.sysu.edu.cn/>), a web-based tool that predicts miRNA-lncRNA and lncRNA-RNA alignment by imposing lncRNA sequences that could merge with specific RNA targets^{153,275} (Study I). Results of this in silico target prediction analyses were 357 miRNA targets for MALAT1 and 68 for MZF1-AS1. MiRNA included in the potential targets were e.g. hsa-miR-146-5p and hsa-miR-145-5p which are well known to be involved in the regulation of several pathways for pain and inflammation¹(Study I). LncRNA-RNA analysis showed 1637 genes regulated by MALAT1, 9 genes for MZF1-AS1. The functional enrichment analysis of GO biological processes, showed 26 significant pathways ($FDR \leq 0.05$) regulated by MALAT1, which revealed involvement of this lncRNA in the inflammation cascade and its activity in response to stimuli (Study I). Unfortunately, due to the uncharacterized nature of LOC100287846, it was not possible to evaluate any eventual alignment with a sequence of specific miRNA targets (Study I).

CHAPTER 4. MIRNA AS POTENTIAL BIOMARKERS FOR POST-OPERATIVE PAIN

The presented findings in this chapter are the results of one of the original articles on which this dissertation is based (Study II) ⁸⁶.

MicroRNAs (miRNAs) are a group of small noncoding RNA, with a peculiar length of 18 to 25 nucleotides ¹⁰⁰. Currently, the isoforms annotated in humans reaches numbers above 7000, and their main function involves the post-transcriptional regulation of several mRNAs ¹⁶². MiRNAs carry out their function thanks to their ability to target, by complementary sequence with the RNA messenger, around 30% of genes that codify for proteins ¹⁶². The action of targeting by miRNA results in translational suppression or degradation of the mRNA bind ¹⁸³. The majority of miRNAs are located intracellularly but many studies have investigated circulating or extracellular miRNA that can be detected in extracellular space, in cell media, and in biofluids like the serum, saliva, and cerebrospinal fluid^{10,35,119,182,253}. Moreover, studies show how through the embedding in microvesicles, or their binding with protein such as Argonate2, nucleophosmin or high density lipoprotein, circulating miRNA can evade the degradation process driven by RNase enzymes ^{10,253,255,260}. This structural protection, stabilize the miRNAs' half-life, allowing the evaluation of their levels that can be used as biomarkers in several conditions ²⁵⁹. Dysregulation of this non-coding RNA has been shown at the developmental level, in cancer pathogenesis, viral infection, and cardiovascular alterations ^{66,124,134,245}. The past few decades have seen an increase in investigating the multiple pathways, physiological and pathological, in which the miRNAs regulates several genes pain associated ¹⁹³. Direct miRNAs involvement in relation to pain was reported for the first time in 2010, when by using a knockdown model for the protein Dicer (crucial protein for miRNAs production) it was demonstrated a dysregulation in pain related gene expression and an alteration in the sensation of pain ²⁸⁶. Later more evidence showed altered expression of circulating miRNAs in patients affected by different pathologies characterized by the chronicization of pain, such as migraine, complex regional pain syndrome and fibromyalgia ^{148,204,247}. In recent years, more research has come up with evidence supporting the use of levels of circulating miRNAs as a diagnostic and predictive tool for different pain conditions ¹, but unfortunately, there are still missing studies that evaluate the use of circulating biomarkers for prediction of chronic post-operative pain.

4.1. STUDY II: PREOPERATIVE CIRCULATING MICRORNA SIGNATURE FOR POST- SURGICAL PAIN

In Study II the dysregulation of 21 serum circulating miRNA was evaluated in KOA patients in order to highlight their pre-operative profile and their predictive value for pain one year after TKR. Patients included in the study were assigned to two groups in relation to their percentage of post-operative pain relief (the difference between pre- and post-operative VAS scores divided by preoperative VAS score) (Study II). Patients were part of the group defined as high pain relief if their value of post-operative pain relief was above 30%, instead of patients with values of pain relief inferior to a cut-off of 30% were defined as low pain relief (Study II). The two groups did not differ on preoperative VAS (6.5 ± 1.8 versus 6.3 ± 1.8); years of age (69 ± 8.7 versus 68.00 ± 10.1); gender % of female (58.7 versus 68.1); Kellgren Lawrence (range) (3.7 (2-4) versus 3.6 (2-4)); all p -values > 0.05 , whereas the groups were significantly different on BMI (kg/m^2) (28.8 ± 4.6 versus 30.7 ± 4.6 , $p < 0.05$) (Study II).

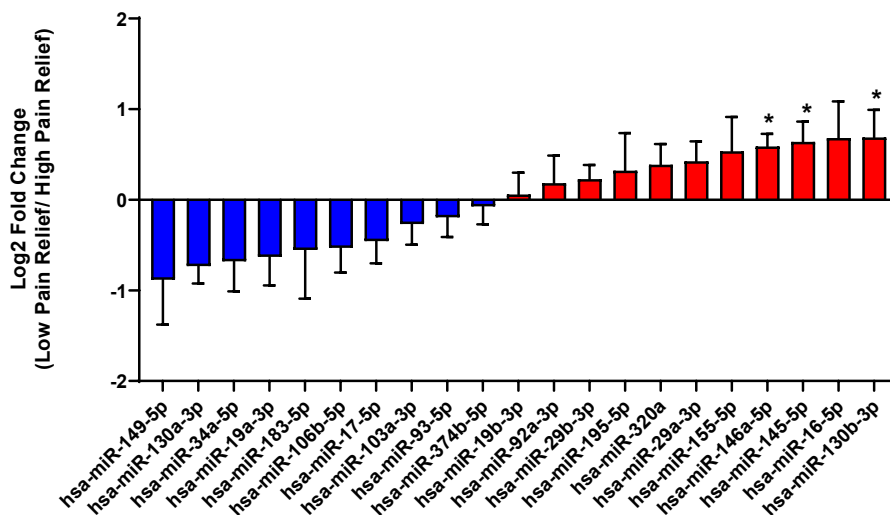


Figure 3. Bar chart miRNA expression. The bar chart represents the fold change of miRNAs evaluated in the two groups. Asterisk indicates the significant upregulation of three miRNAs ($p < 0.05$) (Study II).

Study II demonstrated that patients with low pain relief exhibited higher preoperative levels of hsa-miR-146a-5p, hsa-miR-145-5p and hsa-miR-130b-3p. Hsa-miR-146a-5p (fold change = 1.50 ± 0.86 SD, P-value = 0.021), hsa-miR-145-5p (fold change = 1.55 ± 1.24 SD, P-value = 0.037) and hsa-miR-130b-3p (fold change = 1.61 ± 1.4 SD, P-value = 0.039) (Figure 3.) (Study II). The current analysis corroborates previous studies which highlights the higher expression of hsa-miR-146a-5p, hsa-miR-145-5p and hsa-miR-130b-3p in relation to pain pathways, inflammation and OA ^{115,151,167} (Study II).

Study II showed dysregulation of three specific miRNA involved in regulating inflammatory processes, which is important since inflammation is participating in the peripheral sensitization which leads to pain onset (Study II) ³⁰. First miRNA (Hsa-miR-146a-5p) reported in Study II has been shown by earlier studies to be involved in relation to pain process, inflammatory response and pathophysiology of OA ^{156,273,276} (Study II). This miRNA, has is codifying gene situated on chromosome 5, and its mature sequence is shared by the other members of the miRNA-146 cluster ⁹³. Functions of this miRNA have been shown to be regulated by nuclear factor kappa B (NFκB), a factor involved in the inflammatory response which once activated by innate immune response induces the activity hsa-miR-146-5p ²⁴⁸. This miRNA is also involved in the regulatory expression of specific cytokines that leads to the enhanced action of enzymes (e.g. metalloproteinase), involved in cartilage degradation, one of the most common features of OA ¹⁵⁶. In addition, a preclinical study conducted on rats model showed how the negative regulation of miR-146-5p on TNF receptor-associated factor 6 (TRAF6), reduced neuropathic pain in the study animals ¹⁶⁵. The same authors suggest this miRNA as a future target as pharmaceutical therapy for neuropathic pain ¹⁶⁵. Involvement of this miRNA has been shown also in relation to arthritis, where in synovial tissue of patients with RA, up-regulation of it was induced by pro-inflammatory mediators like Interleukin-1β and TNF-α ¹⁹¹. In relation to OA, a study showed that miRNA 146-a-5p was over expressed in cartilage of OA patients when compared with normal cartilage ²⁷³. Moreover, increased expression of miR-146-5p has been shown in OA patients at an early stage of the pathology, suggesting the underlying role of this non-coding RNA in OA pathophysiology ²⁰⁰. The data presented in Study II highlights how circulating over-expression of hsa-miR-146a-5p in patients with pain after surgery is in line with previous findings regarding the involvement of this miRNA for pain process in OA, and give insight for the possibility

to detect its dysregulation at serum level that could lead to an easier pre-operative evaluation for pain after surgery (Study II).

Second miRNA found upregulated in Study II was hsa-miR-145-5p (Study II). Previous studies indicate this miRNA being induced by the high levels of inflammatory mediators such as TNF- α and IL-1 β , and to regulate the feedback levels of TNF- α and metalloproteinase, both involved in the alteration of the cartilage in OA^{114,274}. Previous findings, obtained from human chondrocytes of patients with KOA, found a higher regulation of miR-145-5p in the late stage of OA and after induction by IL-1 β ^{69,104,161}. Furthermore, the up-regulation of the same miRNA in the cerebrospinal fluid (CSF) correlated with high scores for pain and fatigue²¹. The data concerning the preoperative up-regulation of hsa-miR-145-5p, presented in Study II, corroborates previous findings, pointing out a possibility for high levels of this miRNA to promote and prolong the action of inflammatory response in KOA, which may lead to a bad recovery after surgery.

In regards to hsa-miR-130b-3p, there is still a lack of evidence concerning the action of it in relation to pain conditions, but established is its involvement at different levels in the pathophysiology of several types of tumors^{94,282}. Moreover, the dysregulation of this miRNA has been associated to several pathological conditions such as obesity, or resistance to insulin, but has been also found involved in physiological process which includes osteo and chondrogenesis^{101,169,171}. In Study II the results find hsa-miR-130b-3p positively dysregulated in the preoperative circulation of KOA patients with bad postoperative recovery, but more evidence is necessary to validate the involvement of this miRNA for post-operative pain conditions in patients with KOA and show whether it can serve as a potential biomarker (Study II).

4.2. MIRNA IN SILICO TARGET PREDICTION

In Study II the characterization of miRNA targets was run in order to elucidate their regulatory roles and highlight the specific pathways where the potential RNA messenger (mRNA), targeted by the significant miRNA, was involved. In Study II the between-group analysis was performed only for the dysregulated miRNA, with an opensource on-line software (DIANA-Tool TarBase v.8)¹³³ (Study II). The predicted targeted mRNA genes were chosen by their prediction score with a value between 0.5-1.0 and if they have been validated with a high/low throughput experiment (Study II). Moreover, a gene ontology analysis through two online software PANTHER and Reactome was run to discover the process in which the identified mRNA, potentially bind by the specific miRNAs (146, 145, 130b), are involved¹⁷⁹ (Study II). Detailed

analysis of genes involved with biological regulation, immune system, and cellular functionality and response to stimuli was performed (Study II). Study II showed that targets were 103 for hsa-miR-146a-5p, 35 for hsa-miR-145-5p and 337 for hsa-miR-130b-3p (Study II).

Most of the identified mRNA related genes were associated with to cells pathways, but Study II was focused on those associated with pathways in the inflammatory response (Study II).

One of the genes predicted to be targeted by hsa-miR-146a-5p, was interleukin-1 receptor-associated kinase 1 (IRAK1) gene which has been validated for being associated mostly with cytokine signaling and toll-like receptor signaling pathways^{112,276} (Study II). In addition, hsa-miR-145-5p was found to be involved in inflammatory pathways through the regulation of transcription factor JunB (JUNB) (Study II). However, as reported in Study II, evidence demonstrated its role in the regulation of IL-2, IL-4, IL-6, and TNF- α ^{80,89,152} and the promotion of its action due to external or endogenous stimuli leads to a cellular control, regulates the pathway of differentiation or cell death^{32,213,263} (Study II).

4.3. PREDICTIVE VALUE OF MIRNA FOR POST-OPERATIVE PAIN

In Study II several linear regressions were run to evaluate the predictive value of serum miRNA expression for post-operative pain relief. In Study II two models were established; model 1 which consisted of all significant miRNA and the preoperative VAS score as variables, showed a predictive value (R²) of 30% and identified preoperative pain intensity (p-value <0.001) as a significant factor (Study II). Model 2 was constructed with a backward identification of the parameters included in model 1 and showed preoperative pain intensity (p-value <0.001) as a significant independent parameter for post-operative pain relief prediction with a value of R² of 30%, and showed a trend of hsa-miR-146a-5p (p-value 0.06) (Study II).

Moreover, were highlighted significant correlations between pain relief and preoperative pain intensity (R=0.500, p-value <0.001) and hsa-miR-146a-5p (R=0.300, p-value=0.006) (Study II).

CHAPTER 5. INFLAMMATORY MARKERS IN SERUM OF KOA PATIENTS

Evidence reported in this chapter are results of one of the publications on which this dissertation is based (Study III)⁸⁷.

The inflammatory response in osteoarthritis is debilitating and intensively represented but is in most cases defined as sub-clinical⁶⁹, even though its actions in conjunction with process of synovial degradation, are well known factors at the base of progression of OA from the early stage of the disease^{69,230}.

Inflammation can occur locally at the synovium level due to disturbed metabolism and enhanced catabolism of joint tissue involved leading to the secretion of inflammatory factors such as proinflammatory cytokines. Several studies are showing the involvement of proinflammatory and anti-inflammatory markers in the onset and progression of OA in clinical and preclinical models, as well as contributor to the pathogenesis of OA pointing out the action of markers such as interleukin (IL)-6, IL-1 β , tumor necrosis factor (TNF)- α , IL-10, IL-13 and IL-4^{33,76,132,233}.

The local production of inflammatory mediators is the main actor for the cartilage disruption and alteration of the synovial membrane with the release of cells activated (e.g. neutrophils, lymphocytes, macrophages) in the synovial fluid. Additionally, the inflammatory response that occurs in the joint tissue can result in a systemic response in patients with OA^{221,238}. A systemic inflammation, defined as cold or low grade, has been found recently in patients affected by OA^{221,234}. Evidence showed serum levels of several inflammatory mediators are higher in OA than healthy subjects^{12,72,237}.

In this case, has been demonstrated an upregulation of IL-6 in patients which reported intensive synovitis⁶⁵, that is commonly related to increased pain intensity in these patients¹¹⁷ and is one of the few interleukins that correlates with OA pain⁴. Preclinical studies have found that both the central and peripheral sensory nervous systems can be sensitized by pro-inflammatory cytokines and several recent human studies have found that highly pain sensitive preoperative OA patients are at a greater risk of developing chronic post-operative pain^{208,209,211,252}. The origins of the increased inflammation are poorly understood but the associations between dysregulated serum inflammatory markers in OA and pain might be pivotal to increase our understanding of the pathways of this low-grade inflammation.

5.1. INFLAMMATION SYSTEMIC MARKERS IN KOA PATIENTS COMPARED WITH HEALTHY CONTROLS

In Study III in order to evaluate this systemic inflammation, 92 markers (List 3. Appendices) were assessed through multiplex OLIK inflammation panel in the serum of 127 KOA patients comparing them with 40 serum of healthy subjects (Study III). The two groups were no different on gender (% females) (57,8% versus 61,5%); years of age (68.82 ± 9.15 versus 68.46 ± 3.13); BMI (kg/m^2) (29.37 ± 4.79 versus 26.71 ± 4.21), whereas significant difference was present for the Osteoarthritis score Kellgren and Lawrence scale (1-4) (3.74 ± 0.49 versus 1.43 ± 0.59) (Study III).

In Study III 72 markers out of 92 were evaluated, and levels of 15 cytokines were found dysregulated in serum of KOA patients (Figure. 4) (Study III). The proteins are identified and listed in the table. 1 (Study III).

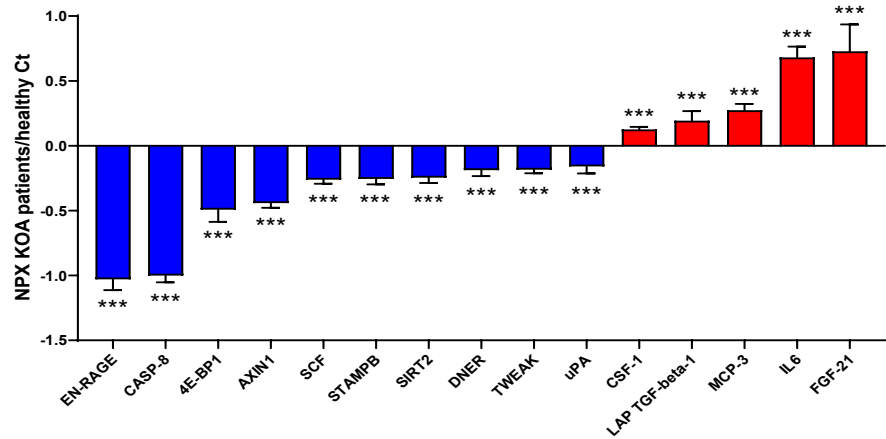


Figure 4. Bar chart of inflammatory markers expression. Bar chart of upregulations (red bars) and downregulations (blue bars) of significant markers evaluated in the PEA array (Study III). Data are expressed as Normalized Protein eXpression (NPX). Asterisks indicate significance $p < 0.001$ from controls (Study III). Error bars report SEM. All data were corrected for FDR (Study III).

<i>Downregulated markers</i>	<i>p-value</i>	<i>Upregulated markers</i>	<i>p-value</i>
(CASP-8)	< 0.001	(IL-6)	< 0.001
(EN-RAGE)	< 0.001	(CSF-1)	≤ 0.001
(DNER)	< 0.001	(FGF21)	≤ 0.001
(AXIN1)	< 0.001	(MCP-3)	< 0.01
(STAMBP)	< 0.001	(LAP TGF-beta-1)	< 0.01
(SIRT2)	< 0.001		
(SCF)	< 0.001		
(4E-BP1)	< 0.001		
(TWEAK)	< 0.01		
(uPA)	< 0.01		

Table 1. Inflammatory markers significant in patients with knee OA. The P-values showed are corrected using the False Discovery Rate (FDR) methods ²⁷².

5.1.1. CHEMOKINES

MPC-3

Chemokines are small cytokines that act as signaling proteins secreted by cells. Because of their ability to generate chemotaxis on the responsive closer cells. In Study III, monocyte chemotactic protein 3 (MCP-3, known as CCL7), members of chemokines CC subfamily, was upregulated in patients with painful KOA (Study III). An earlier animal study showed the involvement of this chemokine in neuropathic pain pathways and pain plasticity in a rat model of nerve ligation ¹²⁹. Its upregulation has been demonstrated to promote central sensitization and facilitate pain transmission in the dorsal root ganglion, in which MPC-3 codifying gene is upregulated by proinflammatory cytokines ¹²⁰. In Study III, the upregulation of MPC-3 agrees with previous literature indicating a cooperative action of proinflammatory cytokine and MPC-3 in pain sensation (Study III).

5.1.2. INTERLEUKINS

IL-6

Interleukin 6 (IL-6) is an interleukin, codified by the IL6 gene, which acts as both a pro-inflammatory cytokine and an anti-inflammatory myokine. In arthritic conditions, Interleukin-6 (IL-6) has been shown to be involved in innate immunity pathways and the general inflammation of the joint²⁸. This interleukin has been shown to activate and be involved in multiple signaling pathways and to have as targets several cell types^{13,28}. Preclinical studies show how IL-6 undertakes its function as pain regulator on nociceptive neurons^{50,197}. In relation to arthritis, preclinical and clinical studies find high expressed levels of this protein in painful KOA^{4,65,117}. Study III supported the evidence regarding higher levels of IL-6 in KOA patients, finding this dysregulation at systemic level which confirms the inflammation typical of KOA (Study III).

5.1.3. GROWTH FACTORS

FGF-21

Fibroblast growth factor-21 (FGF-21) part of FGF superfamily is a hormone with an endocrine function which regulates metabolic process¹⁶³. Previous research showed this growth factor to be present at systemic level and in synovial fluid (SF), and highlighted that its levels are lower in osteoarthritic cartilage in OA patients¹¹⁶. In this regard, a recent study demonstrated that high levels of FGF-21 in the circulation of patients with OA are correlated with severity of KOA¹⁵⁸. In Study III higher levels of FGF-21 were shown in serum of patients with KOA, adding evidence to previous studies and showing the association of this hormone in the pathophysiology of KOA and its correlation to pain (Study III).

LAP TGF-beta-1

The growth factor TGF-β1 is produced and released as a homodimer binding its latency-associated pro-peptide LAP. The connection with the factor LAP avoids the release of TGF-β1 defining the moment of secretion to the active function of the hormone²³¹. Recent evidence shows the involvement of LAP TGF-beta-1 in plasma of female patients with widespread pain and fibromyalgia with inflammatory levels of these factors and several pain outcomes^{14,83}. Study III shows high levels of LAP TGF-beta-1 at systemic level of KOA patients, suggesting that release of the active factor could happen in the knee area (Study III).

DNER

Delta/Notch-like EGF-related receptor (DNER) is a transmembrane protein that acts as a growth factor and is expressed in the central nervous system (CNS) in dendrites and cell bodies of CNS neurons ⁶⁴. It is heavily involved in several cancer types, in which regulation of certain pathways of the factor Notch leads to increased malignancy and metastasis formation ^{261,262}. Unfortunately, no evidence exists connecting the action of DNER to KOA pathways or pain perception but Study III, demonstrated a down-regulation of this receptor in KOA patients and warrants further consideration (Study III).

5.1.4. CYTOKINES

SCF

The stem cell factor (SCF) is produced by cells like fibroblasts and it is involved in cell differentiation and proliferation ²⁶⁶. Preclinical studies have shown SCF acting as a ligand for specific receptors, such as c-Kit, expressed on sensory neurons suggesting an involvement of this factor on pain and its transmission at central and peripheral level ^{181,249}. In Study III, SCF was found negatively expressed in KOA patients, supporting the evidence that sees this factor associated with pain sensitivity highly present in these patients (Study III).

TWEAK

Tumor necrosis factor-like weak inducer of apoptosis (TWEAK) is part of the TNF superfamily, expressed in epithelial cells with a pro-inflammatory function ³⁷. This factor has been shown also to induce proliferation of multiple types of cells, such as astrocytes and endothelial cells ^{52,271}. An *in vitro* study highlighted how high levels of TWEAK increase the release of pro-inflammatory markers (cytokines and chemokines) in culture media of fibroblasts and synoviocytes isolated from RA and OA patients³⁸. On the contrary with previous evidence, Study III found decreased levels of this factor in the circulation of KOA patients (Study III).

5.1.5. OTHER MARKERS

4E-BP1

The eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1) is a protein involved in the inhibition of the interaction between translation factor eIF4E and different elongation factors, which regulate protein production and regulation of cell homeostasis¹²⁵. This factor action is induced by several stimuli, including glutamate, growth factors, and cytokines, which leads to the proliferation and cellular differentiation but also synaptic plasticity^{105,150}. In this regard, authors have shown levels of 4E-BP1 in neurons, and downregulation of it which cause hypersensitivity due to external stimuli of mechanical, inflammatory, or chemical nature^{130,139,174}. Study III demonstrated that serum of KOA patients down-expressed 4E-BP1 suggesting that these patients with lower expression of this factor report more pain in phase of mechanical evaluation of pain intensity (Study III).

STAMPB

STAMPB is a deubiquitinase enzyme which through the binding with SRC Homology 3 domain of the signal-transducing adaptor molecule (STAM), activates the singling cascade of JAK-STAT and consequentially inflammatory response cytokine-mediated^{17,41}. Evidence shows the association of STAMPB for the modulation of differentiation and maturation of cytokines, and silencing of these factors leads to a decrease in the expression of cytokines such as IL-1b^{17,164}. Study III, highlighted that STAMPB was downregulated in serum of KOA patients.

AXIN1

Axis inhibition protein (AXIN1) is a protein express at cytoplasmic level of cells involved in the cartilage formation at the embryonic state and regulates negatively cell transduction pathways of Wnt factor^{140,280}. A previous animal study in an Axin1 KO mice model showed that depletion of this protein in the chondrocytes led to OA-like degeneration²⁹⁰. Study III showed that this protein was negatively expressed in the patients with KOA adding knowledge to the involvement of this protein in OA's pathogenesis (Study III).

CASP-8

Caspases are proteins usually involved in the process of cellular death, but previous evidence has proven the association of this protein in pathways leading to inflammation ⁷⁴, cell motility, T-cell activation, and osteoblast regulation ¹⁹⁰. Study III showed down regulation of CASP-8 pointing to a possible negative regulation of osteoblast activity (Study III).

SIRT2

Sirtuin2 is a member of the sirtuin family, group of nicotinamide adenine dinucleotide-dependent deacetylases ¹¹³, regulating cellular process which includes metabolism, proliferation, inflammation or cellular death ^{103,123}. Studies have reported the action of SIRT2 in the regulation of neuroinflammation and being involved in neurological diseases ^{277,279}. The SIRT2 pathway leads to inhibition of NF- κ B-dependent pro-inflammatory cytokine, through de-acetylation of the NF- κ Bp65 protein, consequentially reducing inflammation response ^{205,223}. Previous evidence has proven the function of SIRT2 as an arthritis blocker, demonstrating in mice model how severe collagen-induced arthritis is established when this protein is downregulated ¹⁵⁹. In addition, involvement of SIRT2 in neuropathic pain modulation has been proven in animal models, where up-regulation of this protein reduced pain sensation through the negative modulation of factors of NF- κ B-mediated neuroinflammation cascade ²⁸⁴. In Study III, significant downregulation of SIRT2 was showed in serum from patients compared with healthy controls, supporting the evidence that downregulation of SIRT2 might be associated with pain sensation and high levels of inflammatory markers in these patients (Study III).

EN-RAGE

Recent identified extracellular receptors involved in advanced glycation end-products (EN-RAGE or Protein S100-A12) is a protein highly represented in cells involved in the immune response, which recruits or stimulates inflammatory mediators, and cooperates in cell networks that influence immunological response ^{214,269}. Moreover, high levels of this protein are expressed in synovial tissue ²⁰⁷. EN-RAGE higher expression has been found in samples of rheumatoid arthritis patients, this receptor is secreted by granulocytes which promote localized inflammation resulting in chronic arthritis ⁷⁵. High levels of these proteins have been evaluated in synovial fluid OA patients, and have been correlated with the high disruption of the joint cartilage ¹⁴⁹. Conversely, Study III demonstrated downregulation of EN-RAGE in patients with KOA, taking into account that expression levels in two different body fluids could be sometimes inverse ²¹⁰. Nonetheless, this indicates that EN-RAGE may be involved in the low-grade systemic inflammation in KOA patients (Study III).

uPA

Urokinase plasminogen activator (uPA) is one of the two enzymes, that regulates the changes that lead the plasminogen to turn in plasmin, and is also involved in cell transduction signals that stimulate cellular proliferation and survival²⁵⁴. Animal studies highlighted an association of the action of uPA in the regeneration of axons in CNS and its release after ischemia^{176,177}. In relation to arthritis, authors suggested that levels of uPA are not evaluable in the synovium of OA patients when compared with RA patients and healthy controls²⁶. Study III reported significant downregulation of uPA in KOA patients, which may confirm previous evidence on this enzyme and its correlation with OA (Study III).

CSF-1

CSF-1 is a factor member of the hematopoietic CSF family involved in several biological functions of macrophages and osteoclasts such as differentiation, proliferation and survival^{40,141}. Several studies highlight the expression of CSF-1 receptor (CSF-1R) on microglia and neurons^{39,43,99} and signaling of CSF-1/CSF-1R is involved in several pathological conditions^{98,99} and active in an arthritis animal model⁸¹. Previous studies have demonstrated the critical role played by CSF-1 for arthritic diseases and pain intensity^{99,188} and have been shown the effect on microglia by the control of neuropathic pain due to nerve injury^{95,199}. Evidence demonstrated that acting peripherally CSF-1 regulates pain, through activation of macrophages and possibly in nerves¹⁷². Study III showed significantly higher CSF-1 presence in patients affected by KOA, supporting what has been shown in previous research²²⁶ (Study III).

5.2. INFLAMMATORY MARKERS ASSOCIATED WITH PAIN INTENSITY IN KOA PATIENTS

In study III associations between the significant markers highlighted in patients with KOA and pain intensity were evaluated. Markers such as FGF-21 and 4E-PB1 were positively associated with pain scores in patients in line with previous studies that look at these new markers as involved in the pathways of pain in preclinical and clinical models^{116,130,139,158,174}. Additionally, inflammatory markers such as IL-1b, IL-6, IL-8, TNF α and IL-10 have been shown to be associated with the severity of KOA and OA pain^{61,65,79,242}. The linear regression demonstrated that TWEAK (standard coefficient (SC) -0.355, p-value 0.001), FGF-21 (SC 0.264, p-value 0.002), CSF-1 (SC 0.264, p-value 0.015), and IL6 (SC -0.406, p-value <0.001) were independent factors predicting pain intensity in KOA patients with a predictive value (R^2) of 16% (Study

III). Although previous studies in synovial fluid and serum of KOA patients showed higher levels of IL-6 positively correlated with pain outcomes ^{79,121}, Study III showed a negative association of IL-6 serum levels with pain intensity, suggesting that IL-6 might act differentially at local site (the synovial fluid) and systemically (e.g. in serum) but confirming the low-grade inflammation present at systemic levels in patients with OA ²²¹ (Study III).

CHAPTER 6. GENERAL DISCUSSION

The need for finding biomarkers for OA is growing, due to the necessity to identify OA at an early stage, better definition of the clinical stages of the pathology and identify risk factors of progression that could be targeted for intervention ^{145,185}. Previous studies focused on the clinical features of the pathology looking at imaging outcomes in order to achieve an understanding of the natural history of the progression through the visualization of cartilage morphology and composition ¹⁰⁶. To date, research in OA is referring to the so-called “wet biomarkers”, molecules that can be easily accessible through blood, serum, plasma, synovial fluid (SF) and cerebral spinal fluid (CSF) ¹⁸⁶. A recent study showed how tumor necrosis factor-alpha (TNF α) in the serum of pre-OA patients is significantly different when compared with healthy participants ⁶². Moreover, Kosek et. al showed dysregulation of pro-inflammatory cytokines in CSF, serum and SF of patients with KOA indicating that IL-6 and IL-8 are associated with reduced symptom severity centrally and augmented peripherally ¹⁴³. Also, research on the action of miRNA and lncRNA is increasing which indicates a key role for these regulatory nucleic acids in the alteration and regulation of pathways related to OA progression and pathogenesis ^{78,241,270}. For example, studies have demonstrated that miRNAs, in both preclinical and clinical studies, are involved in processes of cartilage homeostasis ¹⁸⁴ or cartilage apoptosis-like the action of miR-145^{114,151,274}. Furthermore, studies showed their involvement in the regulation of the inflammatory response in OA that leads to a release of pro-inflammatory cytokines such as the action of has-miR-146 on the factor NF- κ B ^{154,283}. Regarding the lncRNA, few studies have explored these in relation to OA but emerging evidence has demonstrated that, in an OA animal model, MALAT1 is involved in the regulation of a specific miRNA (miR-19) which leads to inactivating Wnt/ β -catenin and NF- κ B pathways involved in inflammatory injuries ²⁰⁶. Moreover, the action of maternally expressed gene 3 (MEG3) has been proven to be downregulated in chondrocytes of KOA patients and its dysregulation is associated with higher levels of vascular endothelial growth factor (VEGF) suggesting a role that enhanced angiogenesis may be a mechanism by which inactivation of MEG3 contributes to OA development ²⁴³. Within this expanding body of research, the results presented in this thesis give more evidence regarding the relationship between the action of ncRNA and proteins with OA and increase our knowledge on the use of these molecules as future potential biomarkers for KOA. In study I, a signature of three lncRNA was found in the serum of patients who develop chronic pain after 1-year from the surgery. The results highlighted the downregulation of MALAT1, MZF1-AS1 and LOC100287846 involved at different levels in the pathogenesis of KOA (see *section 3.1*). Moreover, the significant lncRNA targets miRNA that we found dysregulated in serum of KOA

patients in study II such as hsa-miR-146a-5p, hsa-miR-145-5p and hsa-miR-130b-5p (see *section 3.2*); our data shows that these miRNAs are preoperatively upregulated in KOA patients with low pain relief after surgery and their involvement in many inflammatory pathways and chondrocyte homeostasis regulation suggests a possible use of these as potential intervention target (see *section 4.1*). Moreover, in study III, using a proteomic approach, we showed the link of several inflammatory markers with KOA, showing the higher expression levels of IL-6 and introducing new serum inflammatory mediators that could partly explain the low-grade inflammation present in osteoarthritic patients (see *section 5.1*).

Pain in OA has been the main focus of past research since it is the primary complaint from OA patients¹¹⁸. Previous studies have shown preoperative pain intensities^{208,209} and sensitization of central pain pathways⁵ as predictors for chronic postoperative pain following TKR. Besides a growing number of studies have explored epigenetics^{51,56,193} and other genes regulatory and transcriptomic changes in painful pathologies^{79,196}. In this thesis, the association between pain and the expression profile of ncRNA and inflammatory markers was shown. In study I lncRNA were dysregulated in patients who developed pain after 1 year from the surgery showing a signature for these molecules to be involved in pain process and corroborating data already present in the literature that indicates that these molecules take part in the regulation of neuropathic pain pathways (see *section 3.1, Study I*). More research is available on the relationship between miRNA and pain and our data show the expression of this short single stranded RNA in a pain condition, showing not only an upregulation in serum of patients that showed lower post-operative pain relief but also a trend in predicting the 30% of the post-operative pain relief in KOA patients (see *section 4.3, Study II*). Moreover, Study III indicated that markers found at serum level can be associated with high pain intensity in KOA patients and predicting 16% of the pain highlighting new inflammatory markers, like TWEAK, FGF-21, CSF-1, and IL6 as independent factors (see *section 5.2, Study III*).

CHAPTER 7. LIMITATIONS AND FUTURE PERSPECTIVES

7.1. LIMITATIONS

Due to the exploratory nature of the three studies is necessary to point out some limitations related to our approach. The type of sample, in which all three studies were conducted, has been chosen in order to evaluate systemic alterations in terms of ncRNA and proteins looking at the so-called “cold-inflammation”²⁹, knowing that the majority of the previous research has been conducted in cells and specific tissues probably closer related with the pathogenesis of KOA or the sensitization of peripheral nerves leading to pain process²²⁸. Moreover, it remains unknown which specific cell type is involved in the production and secretion of circulating free and exosomal lncRNA and miRNA, although several are involved in this process^{10,253,259}, making it difficult to define the origin tissue and also the cells that receive them. In this respect, more studies on the transporting mechanisms of circulating ncRNA are needed. In addition, all the data obtained and presented within the dissertation can be identified at the moment only as a signature feature of OA pathology and pain intensity; for this reason, validation of specific pathways, in which all the molecules presented are involved, through luciferase assays on suspected ncRNA-mRNA interactions of interest has to be focus of future studies.

7.2. FUTURE PERSPECTIVES

Confirmation of the action of all these post-transcription and translational modifications is needed before validating the pathways. Moreover, focus in the future should be on the evaluation of timing within all the modifications and action of ncRNA and mediators happening in order to highlight a potential intervention that could block the results of this modification or enhance the protective effect of them.

CHAPTER 8. CONCLUSION

The present Ph.D. project addressed three objectives: 1) Evaluation of circulating lncRNA, involved in several inflammation pathways and miRNA regulation, as a preoperative signature for development of postoperative pain; 2) Evaluation of circulating miRNA, associated with pain, inflammation and OA pathophysiology, as a preoperative signature and potential biomarkers for the development of postoperative pain; and 3) Evaluation of serum inflammatory markers (e.g. interleukins, chemokines, etc.) as a signature for pain and evaluate their associations with pain intensity.

Study I was the first study, to demonstrate an association between down-regulation of preoperative lncRNA in the specific case of MALAT1, MZF1-AS1 and LOC100287846 and chronic post-operative pain after total knee replacement, giving the first insight into the dysregulation of preoperative circulating lncRNAs, and how they can serve as a potential signature for post-operative pain condition due to their regulation of multiple pathways in relation to pain and inflammation (Study I).

Study II showed that postoperative pain is related with specific preoperative serum alteration in the expression of circulating miRNA (Study II). Patients with poor recovery after surgery showed positive increased levels of expression for hsa-miR-146a-5p, hsa-miR-145-5p and hsa-miR-130b-3p (Study II). Moreover, linear regression analysis highlighted preoperative pain intensity as an independent predictive factor for postoperative pain and demonstrated a trend for hsa-miR-146a-5p (Study II). Although explorative, Study II gives the first evidence into the preoperative alteration of miRNAs, and how those can be used as potential biomarkers for post-operative pain condition (Study II).

Study III found, a thorough evaluation of a new high throughput panel, serum of patients with painful KOA to have ten inflammatory markers downregulated and five markers with higher expression levels, when compared to healthy participants (Study III). In patients specifically, correlations were found between expression levels of FGF-21 and 4E-BP1 and pain intensity (Study III). A linear regression model showed that TWEAK, FGF-21, CSF-1 and IL6 were independent predictors for pain intensity showing a contribution of some of the involved inflammatory markers in OA and to the systemic low-grade inflammation in these patients that could be detected at serum level (Study III).

In summary, the studies presented in this dissertation have contributed to increasing the knowledge regarding the action of non-coding RNA and pain, giving proves that thorough evaluation of these type of epigenetic changes there could be a possibility to use such biomarkers for understanding pain in OA and for taking possible precautions to prevent the development of chronic postoperative pain after knee replacement.

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