From Department of Physiology and Pharmacology
Karolinska Institutet, Stockholm, Sweden

PAIN IN RHEUMATOID ARTHRITIS

BONE AND NEUROINFLAMMATION-ASSOCIATED MECHANISMS

Resti Rudjito

Stockholm 2021

All previously published papers were reproduced with permission from the publisher.
Published by Karolinska Institutet.
Printed by Universitetsservice US-AB, 2021
© Resti Rudjito, 2021
ISBN 978-91-8016-232-6
Cover illustration: “Joint Pain” is the author’s artistic interpretation of pain in the joint

Pain in rheumatoid arthritis
Bone and neuroinflammation-associated mechanisms

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

Resti Rudjito

The thesis will be defended in public at CMM Lecture Hall (L8:00, Karolinska University Hospital)

Friday 18th June 2021, 13:00

Principal Supervisor:
Professor Camilla Svensson
Karolinska Institutet
Dept. of Physiology and Pharmacology

Co-supervisor(s):
Professor Chantal Chenu
Royal Veterinary College
Dept. of Comparative Biomedical Sciences

Professor Helena Erlandsson Harris
Karolinska Institutet
Dept. of Medicine

Professor Juan Miguel Jimenez Andrade
Autonomous University of Tamaulipas
Multidisciplinary Academic Unit
Reynoza-Aztlan

Opponent:
Professor Jason McDougall
Dalhousie University
Dept. of Pharmacology

Examination Board:
Professor Hermona Soreq
Hebrew University of Jerusalem
Dept. of Biological Chemistry

Associate Professor Andrei Chagin
Karolinska Institutet
Dept. of Physiology and Pharmacology

Assistant Professor Shafaq Sikandar
Queen Mary University of London
William Harvey Research Institute

To my parents

"The cure for pain is in the pain" - Rumi

POPULAR SCIENCE SUMMARY OF THE THESIS

Hitting your elbow against a wall. Touching a hot stove with your hands. Sitting in front of the computer all day. What do they all have in common? These situations cause pain. Pain is a signal in your body that something is wrong. The capacity to feel pain serves as a guide to avoid danger and to allow recovery if injury or inflammation has already occurred.

Imagine, however, if pain still exists even if the damage has been fixed. This type of pain no longer provides useful function and instead creates health concerns. In rheumatoid arthritis (RA), which is commonly presented as a disease of joint inflammation, a considerable number of patients still report pain despite responding well to drugs that aim to reduce inflammation. It is rather baffling that inflammation in RA do not always equal to pain and this phenomenon is still a mystery to scientists. Extensive research is now ongoing to develop more effective painkillers for these patients. Using an animal model of RA, this thesis explores additional mechanisms that can explain persistent pain in RA with the hope of finding new targets for treatments.

First, the important contribution of the bone environment, especially cells called the osteoclasts to pain, were demonstrated. The main function of osteoclasts are to degrade bones and while there are reports showing that this action can lead to pain, the work presented here revealed that osteoclasts can also induce pain by regulating blood vessels and nerve fibers in the bone. Importantly, through this mechanism drugs that inhibit osteoclast activity can reduce pain.

Second, the involvement of molecules that can maintain the system that drives pain in RA was determined. The antirheumatic drug baricitinib is known to dampen joint inflammation by targeting a signaling pathway called the JAK/STAT pathway. Instead, it was revealed here that this drug reduces pain by targeting other molecular interactions, known as AAK1/AP2M1 axis. In addition to this, the role of another inflammatory molecule called HMGB1 in regulating pain in RA was carefully characterized. Interestingly, the actions of this molecule was observed to be different in male versus female mice, which indicate sex differences in pain mechanisms.

Together, three alternative mechanisms for chronic pain in RA have been proposed in this thesis, with the added message that these mechanisms may work differently in males and females.

RINGKASAN ILMIAH POPULER DISERTASI

Apa kesamaan diantara siku yang terbentur dinding, memegang kompor panas, dan duduk di depan komputer seharian? Semua kejadian tersebut membuat kita merasa nyeri. Rasa nyeri adalah tanda yang dikirim tubuh kita atas sesuatu yang tidak lazim. Kemampuan untuk merasakan nyeri sangat penting agar tubuh kita dapat menghindari bahaya dan memfasilitasi pemulihan saat terjadi cedera atau inflamasi.

Namun bayangkan, jika kita masih merasa nyeri walau cedera yang diderita telah lama sembuh. Alih-alih berguna, rasa nyeri semacam ini malah menjadi gangguan kesehatan. Contohnya, para penderita penyakit rematik atau artritis reumatoide. Keluhan rasa nyeri seringkali ditemukan pada pasien walaupun mereka telah merespon obat anti-inflamasi dengan baik. Inflamasi pada para penderita artritis tidak serta merta menyebabkan rasa nyeri, sehingga ini masih membuat para saintis kebingungan. Saat ini, para saintis tengah berusaha keras untuk mengembangkan obat pereda nyeri yang lebih efektif bagi penderita artritis. Dengan menggunakan hewan uji coba, disertasi ini berusaha menguak mekanisme dibalik nyeri tersebut, dan menemukan target baru untuk pengobatan.

Pertama, disertasi ini mendemonstrasikan peran tulang, terutama sel osteoklas, pada kemunculan rasa nyeri. Penelitian sebelumnya menunjukkan bahwa osteoklas dapat menyebabkan rasa nyeri pada saat melakukan proses peremajaan tulang. Disertasi ini menemukan bahwa rasa nyeri tersebut dapat ditimbulkan oleh osteoklas dengan cara mengatur kinerja sel-sel syaraf dan pembuluh darah di sekitar tulang. Penemuan ini berpotensi menjadi mekanisme baru bagi obat pereda nyeri melalui inhibisi aktivitas osteoklas.

Kedua, disertasi ini mengeksplorasi keterlibatan molekul-molekul yang umunya berfungsi untuk mengatur proses peradangan dalam mempertahankan rasa nyeri. Obat anti-rematik barictinib telah diketahui dapat menekan inflamasi melalui jalur sinyal JAK/STAT. Disertasi ini menunjukkan bahwa sebenarnya barictinib menyasar interaksi molekuler lain, yang dikenal sebagai AAK1/AP2M1. Selain itu, peran molekul lain, HMGB1, dalam regulasi rasa nyeri pada penderita penyakit rematik juga digambarkan dalam disertasi ini. Menariknya, peran molekul ini dapat berbeda pada pria dan wanita.

Secara keseluruhan, disertasi ini membahas tiga mekanisme alternatif penyebab nyeri kronis pada penderita artritis reumatoide, dimana mekanisme tersebut dapat berbeda bagi pria dan wanita.

ABSTRACT

Pain is often the primary reason patients with rheumatoid arthritis (RA) seek medical care. Despite effective disease control with currently available disease modifying antirheumatic drugs (DMARDs), there are still hurdles to overcome as a significant proportion of patients still report continuous pain. This suggests that the relationship between joint inflammation and pain severity is not linear. Currently few effective pain treatments for RA are available, which leads to individual and societal burden. Understanding the regulation of chronic pain in RA is thus vital to identify new drug targets and improve therapeutical strategies. This thesis explores alternative mechanisms of pain in RA with a specific focus on bone and neuroinflammation-associated mechanisms.

In **Paper I**, the contribution of osteoclasts to pain mechanisms was characterized in the collagen antibody-induced arthritis (CAIA) model. As previously reported, CAIA induces transient joint inflammation and persistent mechanical hypersensitivity that outlasts active inflammatory state. Herein, local bone erosion was detected in the calcaneus during both inflammatory and late phases of the CAIA model. Interestingly, while osteoclast activity was prominently increased during the inflammatory phase, pain-like behavior was reversed by two different osteoclast inhibitors in the late phase. In order to understand the contribution of osteoclast activity in nociceptive mechanisms, bone vascularization and innervation were examined. Both vascular and nerve densities were increased in the calcaneus during inflammation, but surprisingly remained elevated in the late phase despite resolution of joint inflammation. Notably, the CAIA-induced changes in bone, vascular and nerve densities in the late phase were attenuated by osteoclast-blocking agents correlating with suppression of osteoclast-derived angiogenic and neurogenic factors, such as netrin-1. Blockade of netrin-1 activity reversed CAIA-induced hypersensitivity in the late phase. Collectively, these findings suggest that the pronociceptive role of osteoclasts is not entirely dependent of their resorbing actions and that osteoclast inhibitors are effective in alleviating pain during the refractive phase of RA.

Janus kinase/signal transducers and activators of transcription (JAK/STAT) inhibitors represent a new class of DMARDs. In **Paper II**, the antinociceptive effects of the JAK1/2 inhibitor baricitinib on the CAIA model as well as the underlying mechanisms were determined. In this study, baricitinib produced reversal effects on CAIA-induced pain-like behavior, which was more pronounced in the late phase of the model. Importantly, the antinociceptive properties of baricitinib in the CAIA model do not completely covary with its anti-inflammatory effects. Intriguingly, no sign of JAK/STAT activation was detected in the dorsal root ganglia (DRGs) or spinal cords of CAIA-subjected mice, thus prompting other signaling pathways targeted by baricitinib to be explored. The effect of baricitinib on AAK1 activity was examined as this pathway was recently identified to be an additional target of baricitinib. mRNA levels of *Aak1* and its downstream target *Ap2m1* as well as phosphorylation and total protein of AP2M1 were upregulated in DRGs from the late phase of the CAIA model.

Baricitinib treatment was able to normalize phosphorylation and total protein levels of AP2M1. Taken together, our data suggest that baricitinib may exert its antinociceptive effects through modulation of AAK1 rather than JAK/STAT signaling in the phase of refractive arthritis-induced pain.

Paper III and **IV** delineated the role of peripheral and spinal high mobility group box 1 (HMGB1) in arthritis-induced pain and if the pronociceptive actions of HMGB1 is sex-dependent. In **Paper III**, blocking the activity of HMGB1 in the periphery was shown to alleviate CAIA-induced pain-like behavior in male but not female mice. Interestingly, local injection of Toll-like receptor (TLR)4-activating disulfide HMGB1 induced mechanical hypersensitivity in both sexes, but was associated with more pronounced contribution of immune cells in males compared to females. CAIA induction has been associated with activation of microglia in the spinal cord. In **Paper IV**, disrupting microglial activity was shown to prevent development of disulfide HMGB1-induced pain-like behavior in male but not female mice. To further explore sex-specific differences, global spinal protein expression was examined using liquid chromatography-mass spectrometry. Surprisingly, several antinociceptive and anti-inflammatory proteins were elevated in only male mice that received the microglial inhibitor minocycline, some of which modulate protein activation cascade that converges on proteinase-activated receptor (PAR)2. Targeting the identified proteins individually, however, did not produce robust antinociceptive effects as minocycline. Overall, these studies demonstrate the important aspects of sex and cellular location in the contribution of peripheral and spinal HMGB1 and TLR4 to arthritis-induced pain.

In summary, this thesis has described three additional mechanisms of RA-induced pain. The findings suggest the involvement of osteoclasts, AAK1/AP2M1 and HMGB1 in mediating CAIA-induced hypersensitivity, particularly in the refractive state of the model. In addition, this work has highlighted the importance of mapping sex dimorphism and the prospective that pain relief is achieved differently in different sexes. Although more research are warranted in order to decipher the exact mechanisms that drive and maintain chronic pain in RA, this thesis has provided interesting mechanistic insights with respect to the bone environment and neuroinflammatory factors.

LIST OF SCIENTIFIC PAPERS

I. **Bone vascularization and innervation regulated by osteoclasts contribute to refractive pain-like behavior in the collagen antibody-induced arthritis model.** Resti Rudjito, Nilesh M Agalave, Alex Bersellini Farinotti, Azar Baharpoor, Arisai Martínez Martínez, Enriqueta Muñoz Islas, Preety Panwar, Dieter Brömme, Julie Barbier, Fabien Marchand, Patrick Mehlen, Thomas Levin Andersen, Juan Miguel Jimenez Andrade, Camilla I Svensson. *Submitted*

II. **Baricitinib reduces refractive mechanical hypersensitivity in the collagen antibody-induced arthritis model.** Resti Rudjito, Nils Simon, Katalin Sandor, Camilla I Svensson. *Manuscript*

III. **Sex- and cell-dependent contribution of high mobility group box 1 protein and TLR4 in arthritis-induced pain.** Resti Rudjito*, Nilesh M Agalave,* Alex Bersellini Farinotti, Peter Lundbäck, Thomas A Szabo-Pardi, Theodore J Price, Helena Erlandsson Harris, Michael D Burton#, Camilla I Svensson#. *Pain.* 2021 Feb 1;162(2):459-470

IV. **Sex-dependent role of microglia in disulfide high mobility group box 1 protein-mediated mechanical hypersensitivity.** Nilesh M Agalave*, Resti Rudjito*, Alex Bersellini Farinotti, Payam Emami Khoonsari, Yuki Nomura, Thomas A Szabo-Pardi, Carlos Morado Urbina, Vinko Palada, Theodore J Price, Helena Erlandsson Harris, Michael D Burton, Kim Kultima#, Camilla I Svensson#. *Pain.* 2021 Feb 1;162(2):446-458

PUBLICATIONS NOT INCLUDED IN THIS THESIS

I. **Antibody-induced pain-like behavior and bone erosion – links to subclinical inflammation, osteoclast activity and ASIC3-dependent sensitization.** Alexandra Jurczak*, Lauriane Delay*, Julie Barbier, Nils Simon, Emerson Krock, Katalin Sandor, Nilesh Agalave, Resti Rudjito, Gustaf Wigerblad, Katarzyna Rogóż, Arnaud Briat, Elisabeth Miot-Noirault, Arisai Martínez, Dieter Brömme, Caroline Grönwall, Vivianne Malmström, Lars Klareskog, Spiro Khoury, Thiery Ferreira, Bonnie Labrum, Emmanuel Deval, Juan Miguel Jimenez Andrade, Fabien Marchand#, Camilla I Svensson#. *Manuscript*

II. **Involvement of inflammatory mediators in arthritis-induced pain: what do experimental models tell us?** Duygu B Bas, Resti Rudjito, Jie Su, Gustaf Wigerblad, Camilla I Svensson#. *Book chapter in Pain 2016: Refresher Course, 16th World Congress on Pain.* 2016. Washington: IASP Press.

*,# Contributed equally

CONTENTS

1 INTRODUCTION 1
2 LITERATURE REVIEW 3
2.1 The biology of pain 3
2.1.1 The nociceptive system 3
2.1.2 Peripheral and central sensitization 4
2.1.3 Bone pain 5
2.1.4 Sex differences in pain 7
2.2 Rheumatoid Arthritis 9
2.2.1 Pathogenesis of rheumatoid arthritis 9
2.2.2 Pharmacological pain treatment in rheumatoid arthritis 11
2.2.3 JAK/STAT signaling and rheumatoid arthritis 11
2.2.4 Rheumatoid arthritis-induced pain models 13
2.3 High mobility group box 1 14
2.3.1 HMGB1: characteristics and functions 14
2.3.2 HMGB1 and rheumatoid arthritis 17
2.3.3 HMGB1 and pain 18
2.4 Summary 20
3 RESEARCH AIMS 21
4 MATERIALS AND METHODS 23
4.1 Animal studies 23
4.1.1 Animals 23
4.1.2 Animal models 23
4.1.3 Drugs and drug delivery 24
4.1.4 Assessment of pain-like behavior 26
4.1.5 Ethical considerations 27
4.2 Cell culture 28
4.2.1 Osteoclast culture 28
4.2.2 Dorsal root ganglia (DRGs) culture 29
4.2.3 Macrophage culture 29
4.2.4 Microglia culture 29
4.3 Imaging analyses 30
4.3.1 Micro-computed tomography imaging 30
4.3.2 ^{99m}Tc-HMDP scintigraphic imaging 30
4.3.3 Calcium imaging 30
4.4 Molecular analyses 31
4.4.1 Quantitative polymerase chain reaction 31
4.4.2 Immunohistochemistry 32
4.4.3 *In situ* hybridization 32
4.4.4 Western blot 33
4.4.5 Immunoassay 33
4.4.6 Liquid chromatography-mass spectrometry 33

4.5 Statistical analyses 34
5 RESULTS AND DISCUSSION 35
5.1 Contribution of osteoclasts to refractive pain in the collagen antibody-induced arthritis model 35
5.2 Baricitinib reduces refractive pain in the collagen antibody-induced arthritis model through AAK1 inhibition 39
5.3 Sex- and cell-dependent contribution of high mobility group box 1 protein and TLR4 in arthritis-induced pain 41
5.4 Sex-dependent role of microglia in disulfide high mobility group box 1 protein-mediated mechanical hypersensitivity 44
6 CONCLUSIONS AND FUTURE PERSPECTIVES 47
7 ACKNOWLEDGEMENTS 51
8 REFERENCES 55

LIST OF SELECTED ABBREVIATIONS

A1AT	Alpha-1-antitrypsin
AAK1	AP2-associated protein kinase 1
Ab	Antibody
ACPA	Anti-citrullinated protein antibodies
AIA	Antigen-induced arthritis
ASIC3	Acid sensing ion channel 3
CAIA	Collagen antibody-induced arthritis
CCI	Chronic constriction injury
CFA	Complete Freund's adjuvant
CGRP	Calcitonin gene-related peptide
CIA	Collagen-induced arthritis
CIBP	Cancer-induced bone pain
CIPN	Chemotherapy-induced peripheral neuropathy
CRPS	Complex Regional Pain Syndrome
CTSK	Cathepsin K
CXCL	Chemokine CXC motif ligand
CXCR	CXC chemokine receptor
DAMP	Damage-associated molecular pattern
DMARD	Disease modifying antirheumatic drug
DRG	Dorsal root ganglion
G6PI	Glucose-6-phosphaste isomerase
HMGB1	High mobility group box 1
i.a.	Intra-articular
i.p.	Intraperitoneal
i.pl	Intraplantar
i.t.	Intrathecal
i.v.	Intravenous
IASP	International Association for the Study of Pain
Iba-1	Ionized calcium binding adaptor molecule 1
IB4	Isolectin B4

JAK/STAT	Janus kinase/signal transducer and activator of transcription proteins
LPS	Lipopolysaccharide
M-CSF	Macrophage colony-stimulating factor
MD-2	Myeloid differentiation factor-2
NGF	Nuclear growth factor
NLS	Nuclear localization signal
NSAID	Non-steroid anti-inflammatory drugs
NRG	Neuregulin
NRP1	Neuropilin-1 receptor
OPG	Osteoprotegerin
p.o.	Peroral
PAMP	Pathogen-associated molecular pattern
PDGF-BB	Platelet-derived growth factor BB
PAR2	Proteinase-activated receptor 2
RA	Rheumatoid arthritis
RAGE	Receptor for advanced glycation products
RANKL	Receptor activator of nuclear factor-kappa B ligand
SEMA4D	Semaphorin 4D
s.c.	Subcutaneous
SNI	Spared nerve injury
TLR	Toll-like receptor
TM	Thrombomodulin
TNF	Tumor necrosis factor
TrkA	Tropomyosin kinase receptor A
TRAP	Tartrate-resistant acid phosphatase
TRPV1	Transient receptor potential channel vanilloid subfamily member 1
V-ATPase	Vacuolar-H^+-ATPase a3 isoform
VEGF	Vascular endothelial growth factor

1 INTRODUCTION

Chronic pain is one of the most under-recognized and under-treated conditions in modern medicine. It is associated with high healthcare costs and societal burden compounded by the loss in productivity due to reduced activity, sick leave, fatigue, anxiety and depression (1, 2). Failure to address that chronic pain is a serious health concern has resulted in poor pain management, substance abuse and inadequate drug development. A large-scale survey on chronic pain conducted in Europe showed that around 20% of the adult populations suffer from chronic pain, with higher prevalence in women and in which 50% of the cases are related to musculoskeletal diseases, such as fibromyalgia, low-back pain, osteoarthritis and rheumatoid arthritis (RA) (3). A follow-up survey reported of a huge treatment gap for chronic pain in many European countries, largely due to lack of early diagnosis and timely intervention (4). Chronic pain has thus been widely acknowledged by the scientific community as not merely a symptom of a disease, but as a disease itself, which requires unique treatment strategies (1). Therefore, there is an urgent need to advance our understanding of mechanisms regulating chronic pain to underpin novel drug targets and improve therapies.

RA is a chronic autoimmune disease marked by symptoms of joint inflammation and pain, which adversely affect physical and social function (5). In fact, patients consistently rate pain relief as one of their higher priorities. Although pain in RA is classically viewed as inflammatory, increasing evidence shows that pain can be refractive and constantly present even after optimal control of the disease activity with antirheumatic drugs. This is likely due to the multifactorial nature of pain that is only partly dependent on inflammation (6). For example, the contribution of structural changes to the joint environment has been suggested in non-inflammatory RA pain. Though the underlying mechanisms are still not clear, mounting data point to the important role of the bone-resorbing cells known as osteoclasts in nociception and the possibility that these cells can be targets for pain therapy (7, 8).

Neurochemical changes in the joint and the sensory nervous system are also important aspects of RA that may underlie non-inflammatory pain mechanisms. Neuroinflammatory factors, such as cytokines, mediate peripheral sensitization that leads to increased pain. Persistent afferent input from these factors can also lead to altered central pain processing thus promoting enhanced pain sensitivity (9, 10). Therefore, targeting upstream and downstream signaling pathways in specific cell types or factors that regulate cytokine production and activity may therefore provide beneficial effects for pain.

In recent years, there has been a growing number of preclinical studies showing sex differences in pain mechanisms, including RA-induced pain. While this is not surprising as it is recognized that sex differences exist in human pain conditions (11), it has significantly challenged the field as the use of male rodents is overrepresented compared to females in preclinical research. Much of the sex-specific studies on pain have been focused on the interplay with the immune system. For instance, it has been shown that male and female rodents utilize different immune cells to

modulate pain (12, 13), though some argue that pain regulation does not always depend on one cell type for each sex but may be related to the pain condition in question (14). Together, this shows that more studies are needed to uncover the biological basis for sex differences in pain.

Pain in RA can arise from multiple mechanisms. This thesis explores alternative mechanisms of pain in RA using experimental models that mimic clinical features of RA. The works presented here are focused on the role of the osteoclasts as well as neuroinflammation-associated mechanisms potentially driven by either the Janus kinase/signal transducers and activators of transcription factor (JAK/STAT) signaling pathway or high mobility group box 1 (HMGB1) protein with the goal of finding potential new therapeutical targets. In addition, sex differences in pain are examined in order to gain more insights whether there is a need for sex-specific treatment to reduce sexual disparities in pain management.

2 LITERATURE REVIEW

2.1 THE BIOLOGY OF PAIN

According to the International Association for the Study of Pain (IASP), pain is defined as "an unpleasant sensory and emotional experience associated with, or resembling with, actual or potential tissue damage" (15). The sensation of pain, often referred as acute or adaptive pain, serves as the body's warning system to sickness and injury, and promotes healing responses in injured tissues. However, under some circumstances, pain can persist or reoccur, outlasting its protective function and becomes debilitating, and this is defined as chronic or maladaptive pain (16).

2.1.1 The nociceptive system

Nociception is the process by which painful stimuli are detected by a subpopulation of peripheral nerve fibers called nociceptors (17). Electrophysiological recordings show that nociceptors can be activated by painful noxious stimuli, such as pressure, heat, or chemical irritants, but not by innocuous stimuli, such as warmth or light touch (18). Nociceptors can be classified into two major groups based on their diameter, degree of myelination and conduction velocity. Small sized, unmyelinated C fibers are 0.2-1.5 μm in diameter and trigger unlocalized, slow pain at a conduction velocity of 0.5-2 m/s. In contrast, medium sized, myelinated Aδ fibers, which are 1-5 μm in diameter, elicit localized, fast pain at a higher conduction velocity of 5-35 m/s due to the myelination of the fibers. As a result, pain comes in two phases: a rapid, acute, sharp pain followed by a delayed, more diffused, dull pain, consequent to the activation of Aδ and C fibers, respectively (16). Aδ fibers are subdivided into two main classes: Type I responds to both mechanical and chemical stimuli with a relatively high heat threshold (>50°C) response, while Type II has a much higher mechanical threshold but lower heat threshold than Type I. In addition, C fibers are also heterogenous, in that they include a population sensitive to both thermal and mechanical stimuli. C fibers can also be categorized based on their molecular properties, such as the so-called peptidergic C fibers that release neuropeptides, calcitonin-gene related peptide (CGRP), substance P and express tropomyosin kinase receptor A (TrkA) for nerve growth factor (NGF). The non-peptidergic population of C fibers notably stain positive for isolectin B4 (IB4) and express the purinergic receptor subtype for ATP, P2X3 (9). With recent advances in single cell sequencing technology, it has been demonstrated that nociceptors display distinct transcriptional states that may be altered upon tissue injury (19, 20).

Primary afferent fibers are pseudo-unipolar, in which a common axon that connects to the cell body branches into central and peripheral terminals. The cell bodies of nociceptors are located in the dorsal root ganglia (DRGs), except for sensory nerves innervating the face, which reside

in the trigeminal ganglion (9). When nociceptors are activated by noxious stimuli, the stimulus is converted into action potentials, which is conducted from the periphery to the central terminal. Primary afferent fibers project into the dorsal horn of the spinal cord, wherein different subtypes project into distinct laminae. Aδ fibers project to laminae I and V, while C fibers project more superficially to laminae I and II (9). C fibers exhibit unique projection patterns with peptidergic C fibers terminating within lamina I and the most dorsal part of lamina II and non-peptidergic C fibers projecting into the deeper, mid-region of lamina II (21). These fibers make synaptic contact with interneurons and projection neurons in the spinal cord, which then convey the information to the centers in the brain. Projection neurons from lamina I innervate the parabrachial nucleus (22) and periaqueductal grey (23) in the brainstem, and thus engage in the descending pain pathway. By contrast, projection neurons from lamina V mainly project to the thalamus and various cortical regions, which attribute to the affective component of the pain experience (24).

2.1.2 Peripheral and central sensitization

While nociception describes the neural process of encoding noxious stimuli, sensitization refers to the condition in which there is an enhancement of neuronal function and is a common feature of chronic pain. This can result in hyperalgesia, which is an exaggerated reaction to a stimulus that is normally painful and allodynia, which is pain response to non-noxious stimuli. Typically, sensitization is divided into two different types, peripheral and central sensitization (9, 10).

Peripheral sensitization is defined as a heightened sensitivity at the peripheral nerve endings. This usually occurs after peripheral tissue inflammation and injury due to pain-inducing factors being released by nociceptors and non-neuronal cells (*e.g.* immune cells, endothelial cells, fibroblasts and keratinocytes). Common examples of factors that activate peripheral sensitization are cytokines and chemokines, which subsequently stimulate nociceptors since it has been reported that nociceptors express various receptors for cytokines and chemokines (25). Indeed, intra-articular injection of tumor necrosis factor (TNF) (26), interleukin (IL)-1β (27), IL-6 (28) or IL-17 (29) led to an increased responses of joint nociceptors in rodents. Other chemical mediators of peripheral sensitization include growth factors such as NGF, prostaglandins, neuropeptides, substance P and lipids. In response to these algesic mediators, the threshold for nociceptors to generate action potentials are reduced, and this leads to enhanced pain sensitivity (25).

Central sensitization describes the circumstance in which there is amplification of pain by the central nervous system (CNS) (30). While originally thought to be a consequence of ongoing nociceptive signaling in the periphery, it is increasingly known that central sensitization may occur without tissue injury or inflammation. Central sensitization has been identified in most pain conditions, and may play a fundamental role in the widespread pain observed in patients with fibromyalgia (31). It occurs mainly in the dorsal horn of the spinal cord, whereby the

spinal neurons exhibit a reduction in the threshold for activation and an augmented pain response. Different processes facilitate central sensitization. For example, persistent nociceptive inputs from the periphery induces activation of glutamatergic signaling and spinal glial cells, microglia and astrocytes (31). Spinal microglia and astrocytes monitor the CNS parenchyma to maintain homeostasis, but in response to stress or injury these cells are activated and secrete factors that contribute to central sensitization by amplifying excitatory and decreasing inhibitory currents (32). The majority of these findings is, however, based on animal studies and it is important to take into account the difficulties of translating them into different human pain conditions.

2.1.3 Bone pain

2.1.3.1 Osteoclasts and pain

Recent advances have focused on delineating potential mechanisms driving pain that may arise from the skeletal structure, often referred to as bone pain, such as over-activation of bone remodeling or changes in innervation of the skeleton. The majority of musculoskeletal disorders that exhibit bone pain often show enhanced bone resorption. This notion led many to hypothesize that osteoclasts, which are the cells that degrade bones, play a role in regulating pain mechanisms. Indeed, osteoclasts have been shown to be significantly increased in number and are over-activated to destroy bones in several bone pain conditions (7, 33, 34). In addition, clinical evidence shows that several osteoclast inhibitors such as bisphosphonates (35) and calcitonin (36) are able to reduce pain in patients with cancer and osteoporosis. Increasing data in experimental animal studies have also shown the analgesic capabilities of various osteoclast inhibitors (Table 1). Interestingly, several studies revealed that osteoclast inhibition led to antinociception in pain models without overt bone pathology (37-39). This suggests that pronociceptive properties of osteoclasts are not necessarily coupled to bone erosion or that the osteoclast inhibitors have off-target effects.

Osteoclast differentiation is triggered by osteoblasts' surface-bound receptor activator of nuclear factor-kappa B ligand (RANKL), which binds to its receptor on osteoclast precursor cells. Mature osteoclasts resorb bones by attaching to the surface and then secreting protons through the plasma membrane a3 isoform vacuolar-H^+-ATPase (V-ATPase), which creates an acidic environment (pH range = 4.0-6.0) (40, 41). Acidification is necessary for bone mineral solubilization and matrix degradation, however protons are also known to be potent pain-inducing mediators (9). Previous studies reported that acid-sensing receptors, the transient receptor potential channel vanilloid subfamily member 1 (TRPV1) and the acid sensing ion channel 3 (ASIC3), are found to be expressed in the sensory neurons innervating bones (34). TRPV1 is activated by pH values <6.0 (42), which is similar to the pH under the ruffled border (40). In contrast, ASIC3 is sensitive to milder pH between 6.5 and 7.3 (43), which is equivalent to the pH of the bone environment affected by metastatic cancer (44). In accordance, inhibition

of V-ATPase (bafilomycin A1) and/or ASIC3 (APETx2) abrogated pain-related behavior in experimental models of bone pain (33, 45).

Table 1 List of osteoclast inhibitors that show antinociceptive effects in experimental models of pain

Animal model	Osteoclast inhibitors	Route of administration	Pain assessment	References
Acute pain models				
-i.pl. α,β-methylene ATP	Minodrate	s.c.	Spontaneous	(37)
-i.p. acetic acid	Minodrate	s.c.	Writhing	(37)
-i.pl. formalin	Minodrate	s.c.	Spontaneous	(37)
G6PI-induced arthritis	Zoledronate	i.p.	Thermal	(46)
Zymosan-induced arthritis	Risedronate	s.c.	Weight bearing	(47)
CFA-induced monoarthritis	Zoledronate	i.p./i.v.	Spontaneous, thermal	(45, 48)
	Alendronate	i.v.		
	OPG-Fc	s.c.		
	Bafilomycin A1	s.c.		
Osteoarthritis	OPG-Fc	s.c.	Weight bearing, mechanical	(49-51)
	Zoledronate	s.c.		
	Cathepsin K inhibitor	p.o.		
CRPS	Alendronate	s.c.	Mechanical, unweighting	(52)
	Zoledronate	p.o.		
Bone metastasis	OPG-Fc	s.c.	Mechanical	(53)
Osteoporosis	Alendronate	s.c.	Mechanical, thermal, locomotion	(54-56)
Multiple myeloma	Bafilomycin A1	i.p	Mechanical, thermal	(33)
	Zoledronate	i.p.		
CCI	Minodronate	s.c.	Mechanical, thermal	(38)
	Alendronate	i.t./i,p.	Mechanical, thermal	(39)

G6PI: glucose-6-phosphate isomerase; CFA: Complete Freund's Adjuvant; OPG-Fc: Osteoprotegerin-Fc; CRPS: Complex Regional Pain Syndrome; CCI: chronic constriction injury; i.pl.: intraplantar; s.c.: subcutaneous; i.p.: intraperitoneal; p.o.: peroral

While osteoclasts are mainly associated with bone resorption, emerging reports show a multifunctional role of osteoclasts in pain signaling. This includes the ability of osteoclasts to secrete inflammatory factors (*e.g.* IL-8, TNF), which induces nociception by binding to their corresponding receptors on sensory neurons (7, 57). In addition, osteoclasts release axonal guidance molecules such as semaphorin 4D (SEMA4D) and netrin-1 (8, 58). Although these factors are known to be important for bone remodeling, netrin-1 was reported to induce bone innervation and hypersensitivity in models of osteoarthritis and lumbar spine instability (8, 59). Furthermore, osteoclasts are also known to secrete angiogenic factors, for example platelet-derived growth factor BB (PDGF-BB) (60). While the capability of osteoclasts to modulate angiogenesis has not been associated with nociceptive mechanisms, the presence of blood vessels is crucial for neuronal growth and may thus indirectly contribute to innervation and pain. Interestingly, application of exogenous RANKL did not induce pain-like behavior in mice indicating that activation of osteoclasts alone does not evoke nociception (61). Hence, the exact

mechanism as to how osteoclasts mediate bone pain is still not clear and warrants further investigation.

2.1.3.2 Bone and joint innervation

Both Aδ and C fibers innervate the bone and different joint compartments, such as ligaments, fibrous capsules, synovia and menisci (62). As bones and joints are deep structures, most of the innervating sensory nerve fibers are "silent nociceptors" that are only activated by inflammation, injury or damage to the surrounding structures. Upon activation, silent nociceptors can either be spontaneously active or elicit decreased threshold to mechanical and chemical stimulations (63). In stark contrast to the skin, bones and joints are largely innervated by thinly myelinated $TrkA^{+}$ Aδ fibers and peptidergic $TrkA^{+}$ C fibers and, if any, minor innervation by non-peptidergic C fibers (64). The reason for this is not yet completely clear.

Although it appears that the same population of nerve fibers innervate both bones and joints, the density, pattern and morphology of innervation are remarkably distinct in these two structures. The periosteum, which is a thin layer of connective tissue that covers the outer surface of bones except at joint sites, is the most densely innervated compartment. Here, Aδ and C fibers are organized in a fishnet-like structure, which appears to detect mechanical injury and protect the underlying cortical bone (65). The same population of Aδ and C fibers also innervate the cortical bone and bone marrow, although the relative density of fibers per area is considerably lower when compared to the periosteum (63). In a normal joint, the articular cartilage lacks any detectable innervation and vascular structures. On the other hand, neighboring synovial membrane and subchondral bone are both innervated and vascularized (66). Following injury or inflammation, sensory nerve fibers in these compartments may undergo sprouting, and thus serve as generator of pain in arthritic conditions (67). Previous work also shows that sprouting of $TrkA^{+}$ sensory nerve fibers in damaged bones are often observed with sprouting of $TrkA^{+}$, tyrosine hydroxylase^{+} nerve fibers located nearby (68). The sympathetic nerve fibers are thought to mediate sensory nerve fiber function and modulate hypersensitivity in an experimental model of arthritis (69).

2.1.4 Sex differences in pain

In the field of pain research, the importance of comparing nociceptive mechanisms in male and female subjects has been widely acknowledged. However, before the discovery made by Sorge and colleagues that pain in rodents displays sex dimorphism (70), the idea of sex difference in pain mechanism was often neglected. Women have long been recognized to outnumber men as sufferers of chronic pain (11). Similarly, mounting evidence shows that several animal models of pain demonstrate females to be more sensitive to nociceptive stimuli. Among these reports, females show earlier emergence of pain in the femoral cancer pain model (71), and more allodynia following chronic constriction injury (72) and fracture in the CRPS model (73).

Studies on the biological basis of sex dimorphism in pain have been focused on immune system differences between male and female subjects. The immune system relies on pattern recognition receptors, such as toll like receptors (TLRs), to induce a response. Multiple TLRs have been associated with pain, including TLR2 (74), TLR4 (75), TLR5 (76), and TLR7 (77). To date, sex dimorphism studies in pain processing have been centered on spinal TLR4. This specific interest derived from the finding that two mouse substrains known to have aberrant TLR4 functions (C3H/Hej, known to have a point mutation in *Tlr4* gene; B10ScNJ, known to have recessive null mutation of Tlr4 gene and thus equivalent to *Tlr4* knockout mouse) showed reduced hypersensitivity in males but not females following nerve injury (70). In support of this, intrathecal injection lipopolysaccharide (LPS), which is a TLR4 ligand, evoked mechanical hypersensitivity only in male mice. Furthermore, blockade of spinal TLR4 with the TLR4 antagonist LPS-RS reversed pain-like behavior in male but not female mice subjected to local injections of algogenic agents or nerve injury (70). However, others have shown contradicting data with regards to sex-dependent pronociceptive actions of TLR4. For instance, activation of spinal TLR4 induced pain-related behavior in both males and females (75, 78), although to a greater degree in male mice (78). Administration of TLR4 ligands into the brain, hind paw and ankle joint also induced hypersensitivity in both sexes (78).

As TLR4 is expressed primarily by glial cells in the CNS (32), it is thought that sex-dependent responses to TLR4 ligands are differentially regulated at the immune system level. This idea prompted researchers to explore if male and female rodents employ different immune cells to modulate pain. Indeed, in the CNS, it has been demonstrated that males use microglia, while females use the adaptive immune cells, such as T lymphocytes, to regulate hypersensitivity (12). In support of this, several studies show that blockade of microglial activity using minocycline (a tetracycline antibiotic) led to reversal of pain-associated behavior only in male rodents (13, 79, 80). These sex differences are, however, not so clear-cut as microglial inhibition has been shown to alleviate pain-related behavior in female rats subjected to bone cancer (14). Recently, it was also shown that there is a sex-dependent and temporal contribution of microglial TLR4 in nociception in the CRPS model. Microglial TLR4 contributed to nociception in the early phase of the model in males, and to a lesser extent in females, while it was important for maintenance of hypersensitivity in the late phase in both sexes (81). Compared to microglia, there is limited information on astrocytes and sex-dependent mechanisms with one only study reporting so far that astroglial signaling is sex-independent in both inflammatory and neuropathic pain models (80). Interestingly, reversal of hypersensitivity due to common glial inhibitors (*e.g.* pentoxyfilline, which also inhibits astroglial activity) is male-specific in mice (13). In the periphery, reports show that resident immune cells such as macrophages play a greater involvement in pain signaling in male rodents compared females (82). Thus, more studies are needed to uncover the underlying mechanism of sex differences in pain mechanisms.

2.2 RHEUMATOID ARTHRITIS

2.2.1 Pathogenesis of rheumatoid arthritis

Rheumatoid arthritis (RA) is a chronic and systemic autoimmune disease, typically presented with symmetrical synovial inflammation, cartilage serration, bone erosion and joint pain (arthralgia), which lead to functional impairment and disability. This disease mainly manifests into the small joints of the hand and feet, but as the disease advances larger joints such as wrists, ankles, knees and hips are afflicted too. RA occurs approximately in 0.5-1% of the global population with higher prevalence in women than men (ratio 3:1), and it is predominantly observed in the elderly population (5). The etiology of RA remains unclear, although genetic and environmental factors have been associated with higher risk of developing RA, such as specific human leukocyte antigen (HLA) alleles and cigarette smoking (83). Pain is the predominant problem in RA patients and often the main reason for seeking medical help. Patients can experience different types of pain with descriptors such as "sharp" and "shooting" often used by patients with active disease and "tender" and "gnawing" used by patients with low disease activity. Many patients also report neuropathic pain qualities such as "burning" or "prickling", and widespread pain that is usually accompanied with sleep disturbances and low mood (6, 84, 85). In addition, arthralgia may prelude signs of inflammation (86, 87), and persists even after disease activity is controlled by pharmacological treatments (88, 89). Thus, there is poor correlation between inflammation and pain in RA.

RA is characterized by the presence of autoantibodies in the serum, such as antibodies against IgG (rheumatoid factor, RF), anti-citrullinated protein (ACPA), anti-collagen type II (anti-CII), anti-carbamylated proteins (anti-CarP) and anti-glucose-6-phosphaste isomerase (anti-GPI). Notably, ACPA can be found in approximately 70% of RA patients and frequently used as a diagnostic marker. Studies have shown the presence of these autoantibodies up to 10 years before the onset of disease (90). Even though there is no detectable inflammation during this phase, also known as preclinical phase of RA or "pre-RA", arthralgia is reported in many individuals. The indication of autoantibodies and arthralgia in pre-RA is associated with a higher risk for developing RA (91, 92). In addition to this, bone loss is observed at this pre-RA stage (93, 94), thus suggesting a potential link between autoantibodies, bone erosion and arthralgia. Indeed, prior works have shown that activation of osteoclasts by ACPA leads to the secretion of IL-8 and murine homologue chemokine CXC motif ligand (CXCL)1 and CXCL2, which may potentially induce nociception by binding to CXC chemokine receptors (CXCRs) 1 and 2 expressed on sensory neurons (7, 95, 96). This evidence shows an intriguing possibility that systemic autoimmunity in RA can mediate arthralgia via effects on bone metabolism independent of joint inflammation. Moreover, autoantibodies can form immune complexes and directly activate nociceptors by binding to Fc-gamma receptors expressed on neuronal cells (97) (Figure 1, right panel).

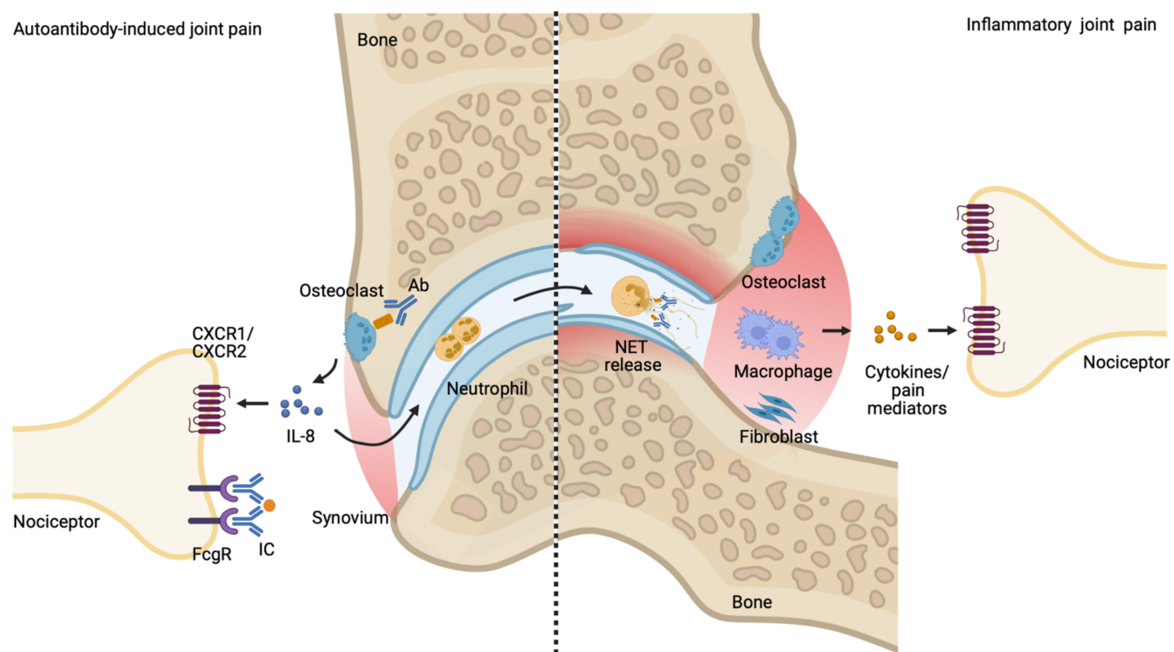

Figure 1. Autoantibodies mediate nociception via direct/indirect stimulation of nociceptors. In pre-RA, autoantibodies (Ab) activate osteoclasts and induce release of IL-8, which may induce nociception by activating sensory neurons. Additionally, autoantibodies can form immune complexes (ICs) and can directly activate nociceptors by binding to Fc-gamma receptors (FcgR) (right panel). In established RA, pain can be induced by activation of different immune cells through release of inflammatory cytokines and pain-inducing factors at the inflamed joint site (left panel). Created in Biorender.com.

In addition to the development of RA, autoantibodies are also involved in the active disease state. It is thought that an increase in titer, epitope spreading, isotype switching and changes in the glycosylation state of autoantibodies results in the pathophysiology of RA (98, 99). Autoantibodies bind epitopes in the joint causing an inflammatory cascade via activation and infiltration of immune cells, such as monocytes, lymphocytes and fibroblasts. Cytokines released by these cells (*e.g.* TNF, IL-1β, IL-6, IL-15 and IL-17) drive many of the pro-inflammatory and nociceptive pathways in the synovium (100). RA synovium is also a source for essential factors that regulate osteoclast activation and maturation, including RANKL and osteoprotegrin ligand derived from either T cells or fibroblasts (101). Consequently, an invasive synovial tissue called pannus is formed, which causes further destruction of the cartilage, bone and soft tissues of the joints, leading to joint pain and disability (86) (Figure 1, left panel).

As of now, it is still not clear how antibody-induced bone loss, which is present in pre-RA conditions, develops into chronic synovitis. One theory suggests that there is diffusion of soluble molecules through a communication system between the bone and synovium. Furthermore, it has been suggested that bone damage is progressive, initially observed at juxta-articular sites, but eventually spreading into the cortical bones. During this process, soluble molecules in the bones may diffuse along a concentration gradient into the synovium, and thereby causing synovial inflammation (86).

2.2.2 Pharmacological pain treatment in rheumatoid arthritis

Current guidelines to treat RA-associated pain include direct acting analgesics and indirect acting disease-modifying antirheumatic drugs (DMARDs). Several types of analgesic drugs used to treat RA-induced pain include non-steroid anti-inflammatory drugs (NSAIDs), paracetamol, opioids, tricyclic antidepressants (TCA) and neuromodulators (*e.g.* pregabalin, gabapentin, capsaicin). TCA and neuromodulators are often used as adjuvant therapies (102). However, the efficacy of these pain medications is typically short-term (less than 6 weeks) (6). Prolonged use of these drugs is often limited by adverse effect, for example gastrointestinal toxicity, renal complications and cardiovascular events with NSAIDs (103). Despite these limitations, analgesics provide rapid benefits for RA patients suffering from pain.

Glucocorticoids and conventional, non-biologic DMARDs such as methotrexate reduce joint swelling and inflammation as well as suppress tissue damage within weeks. These may provide pain relief up to 12 months with systemic treatment (104, 105). Biologic DMARDs, including TNF blockers (etanercept, adalimumab, infliximab), IL-1R antagonist (anakinra) and CD20 antibody (rituximab), alleviate pain within 2-4 weeks, in conjunction with reductions in joint inflammation. Combination therapies, such as methotrexate and glucocorticoids, have been shown to provide higher pain reduction (106). In addition, there is a growing popularity of combination therapies with methotrexate and biologics, which were shown to be more efficacious than methotrexate alone in inhibiting disease progression (107), but there are no reports to date showing if these combinations enhance their pain-relieving properties. Still, approximately 30% of RA patients continue to suffer from pain, ranging from moderate to severe, even though the disease is medically controlled or in remission (108), indicating that alternative treatments to anti-inflammatory drugs should be considered for effective pain relief.

2.2.3 JAK/STAT signaling and rheumatoid arthritis

The Janus kinase/signal transducers and activators of transcription factor (JAK/STAT) is the principal signaling mechanism for numerous cytokines and growth factors, many of which are involved in the pathogenesis of RA (*e.g.* IL-6, IL-10, IL-15 and interferons). The JAK family consists of 4 tyrosine kinases: JAK1, JAK2, JAK3 and tyrosine kinase (TYK)2, all of which function as dimers when phosphorylated by the binding of a specific ligand to its receptor. Once phosphorylated, JAKs phosphorylate and activate signaling peptides from the STAT family. To date, 7 distinct members of the STAT family have been identified: STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b and STAT6. Upon activation, STAT peptides translocate into the nucleus and subsequently initiate gene transcription, which can lead to dysregulation of inflammatory and immune responses (109, 110). The broad action of JAK/STAT signaling makes it an attractive target for drug development for RA. In fact, three JAK inhibitors are clinically licensed by the US Food and Drug Administration (FDA) and the European Medicines Agency (EMA) for treatments of moderate to severe RA: tofacitinib (pan-JAK inhibitor with high activity against JAK1 and JAK3), baricitinib (selective JAK1 and JAK2

inhibitor) and upadacitinib (selective JAK1 inhibitor) (111). The use of filgotinib, which is another selective JAK1 inhibitor, as a treatment of RA has also been recently approved by the EMA but it is still yet waiting for approval from the FDA (112). Recently, it was shown that tofacitinib and baricitinib can directly affect bone homeostasis and suppress pathological bone loss in a mouse model of RA as well as in RA patients by stimulating osteoblast function (113).

Accumulating clinical data are showing robust pain-relieving effects of tofacitinib and baricitinib in RA patients. The efficacy of tofacitinib in improving patient reported outcomes (PRO), including pain, has been reported in several phase III clinical trials (ORAL studies). These studies reveal that patients with inadequate response to conventional DMARDs report significant pain improvements following tofacitinib treatment (114-116). In certain cases, pain relief was evident within days of tofacitinib therapy (114). Pain has also been evaluated in the recent phase III clinical trial RA-BEAM that compared the effect of baricitinib, adalimumab and placebo plus methotrexate. Baricitinib provided greater and faster improvements in terms of pain, joint stiffness and fatigue compared to adalimumab (117-119). Intriguingly, the antinociceptive actions of baricitinib were not correlated with levels of inflammation in patients (119, 120). A more recent clinical study compared the effect of baricitinib, tofacitinib, adalimumab and tocilizumab (IL-6 receptor inhibitor) monotherapy on pain in RA patients. Baricitinib resulted in greater pain reduction compared to tocilizumab and adalimumab. Of interest, no differences in pain improvement were observed between baricitinib and tofacitinib treatments (121).

While clinical evidence clearly demonstrates the pain relieving actions of JAK inhibitors, the underlying mechanisms by which these drugs disrupt pain signaling are not fully understood. An obvious mechanism is the effect of JAK inhibition on multiples cytokines involved in inflammation and pain regulation in RA (122) (Figure 2). This may also explain why JAK inhibition leads to greater pain improvements compared to single cytokine inhibitors such as adalimumab. Specific cytokines, such as IL-2, IL-6, IL-10, directly activate the JAK/STAT pathway. Moreover, even if other cytokines do not signal directly via JAK/STAT, they are indirectly affected by its inhibition (*e.g.* IL-1β and TNF) as production of these cytokines are regulated by JAK/STAT activating cytokines. In addition, most of these cytokines are found throughout the peripheral and central nervous system, except for a few that are found in specific locations. For instance, the action of IL-18 are mostly observed in the periphery close to the joint (122). Several studies, however, suggest that the JAK/STAT pathway may be directly involved in pain mechanisms. For example, alterations in STAT3 signaling have been reported in nerve injury-, chemotherapy- and arthritis-induced pain models (123). In addition to JAKs, baricitinib was recently identified as an inhibitor for the adaptor protein-2 (AP-2) associated kinase 1 (AAK1), which is an important regulator of clathrin-mediated endocytosis. This discovery has been monumental for the current COVID-19 pandemic as AAK1 serves pivotal role for viral entry, and therefore baricitinib can be used as an alternative COVID-19 therapy (123). The binding affinity to AAK1 is exclusive for baricitinib and was not observed in other JAK inhibitors, such as tofacitinib and upadacitinib (124). Interestingly, AAK1 blockade also leads to alleviation of pain-like behavior in neuropathic pain models suggesting a role of AAK1

in nociception (125), and this also raises the question if baricitinib reduces pain through actions on AAK1.

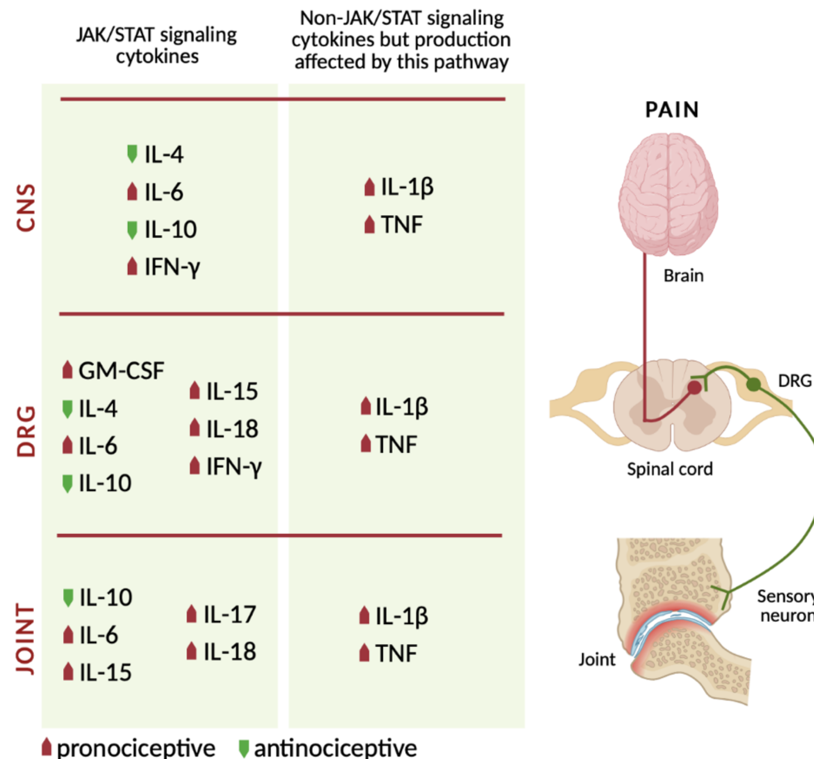

Figure 2. The involvement of the Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway in cytokine signaling that drives central and peripheral mechanisms of pain in rheumatoid arthritis. Certain cytokines such as IL-6 and IL-15 signal directly through the JAK/STAT pathway, however other cytokines such as IL-1β and TNF do not directly activate JAK/STAT signaling but may be indirectly affected by JAK inhibitors. Created with Biorender.com.

2.2.4 Rheumatoid arthritis-induced pain models

Over the years, studies using preclinical models have provided important information about the pathology that drives and maintains pain in RA. Surprisingly, there has been minimal overlap between the animal models used in RA and pain fields. This is because models of monoarthritis induced by injections of either complete Freund's adjuvant (CFA) (126) or carrageenan into the paw or joint are more frequently used to study pain. Although these models develop robust inflammation and pain-related behaviors, they do not recapitulate the complexity of RA as they are relatively short lasting and largely dependent on only the innate immune system. Besides, these models are not suitable to conduct studies on the uncoupling between inflammation and pain. At present, there has been a growing number of more disease-relevant RA models used for pain studies, including the collagen-induced arthritis (CIA) (127), antigen-induced arthritis (AIA) (128), collagen antibody-induced arthritis (CAIA) (129, 130) and K/BxN serum transfer models (131-133). These preclinical models have enabled researchers to dissect the multifactorial nature of pain pre-, during, and post-inflammation. Furthermore, transfer of RA

patient-derived autoantibodies such as ACPA into naïve mice produces long-term behavioral nociception and bone erosion despite not generating visual or histological signs of inflammation (7, 96). Although not yet an established RA model, this model is useful to study “pre-RA” arthralgia and decipher the missing link between inflammation and pain.

This thesis work is focused on studying pain in the CAIA model, which is induced by intravenous (i.v.) or intraperitoneal (i.p.) injection of a mixture of CII antibodies (IgG2a and IgG2b) followed by i.p injection of LPS. Consequently, this induces transient polyarthritis that lasts around 3-4 weeks as the antibodies are cleared (129, 130). The occurrence of transient inflammation provides a good opportunity to study pain in different phases of RA flares. Indeed, mechanical hypersensitivity has been reported pre-, during and post-inflammation in this model (75, 97, 134, 135). In addition, mice exhibit thermal hypersensitivity (134, 136) and reduced locomotor activity (137). Another clinically relevant feature of this model is that female mice are more susceptible in developing joint inflammation compared to male mice (13), thus representative of the fact that RA is more prevalent in women than men. Although the CAIA model has now been utilized for several pain-related studies, there are certain aspects of this model that remain to be elucidated. For instance, damages to the bone remain several weeks after resolution of inflammation (134), and this raises the question whether changes in the bone environment mediate persistent pain in RA.

2.3 HIGH MOBILITY GROUP BOX 1

Alarmins or damage-associated molecular pattern molecules (DAMPs) are endogenous molecules that form an important component of the innate immune system as their release serves as a signal for cell or tissue damage. High mobility group box 1 (HMGB1) protein, previously known as amphoterin or HMG-1, is a chromatin-binding protein that modulates key DNA events inside the nucleus, but also operates as a critical DAMP upon tissue injury or inflammation. Excessive amount of extracellular HMGB1 is implicated in the pathogenesis of various inflammatory and autoimmune diseases, including RA, and it is now recognized that HMGB1 also plays important roles in nociceptive signaling (138). Given such diverse roles, HMGB1 represents an interesting molecule to study in the context of RA and pain.

2.3.1 HMGB1: characteristics and functions

HMGB1 is a ~ 25-kDa protein of 214 amino acids that consists of two positively charged DNA binding motifs (A box and B box) and a negatively charged acidic C terminal. This protein also contain two nuclear localization signal (NLS), NLS1 and NLS2, for controlled nuclear transport (139). The amino acid sequence is highly conserved, with over 98% homology between humans and rodents. Studies have shown that the activities of secreted HMGB1 are largely dependent on the redox state of three cysteines at amino acid positions 23, 45 and 106 (140) (Figure 3). Reduction of these three cysteines results in fully reduced or all-thiol HMGB1

that acts as a ligand for receptor for advanced glycation products (RAGE) and promotes chemotactic activity via CXCR4 by forming a heterocomplex with CXCL12 (140). Nuclear and cytoplasmic HMGB1 is thought to exist in all-thiol form and most likely also initially upon release. As the extracellular environment is more oxidizing than the intracellular compartment, HMGB1 can undergo redox modifications after being released. If the cysteine thiol groups at positions 23 and 45 are oxidized, leaving the cysteine at position 106 in its thiol form, a disulfide bridge between C23 and C45 is formed. This modification converts HMGB1 into the disulfide form that induces nuclear factor-kappa B (NF-κB) activation and cytokine production by binding to TLR4 through its extracellular adaptor, myeloid differentiation factor-2 (MD-2) (141). Oxidation of the three cysteines leads to formation of fully oxidized HMGB1, which is considered to be the inactive form of HMGB1 as it has not been associated with a pro-immunogenic function (142). The conversion between all-thiol and disulfide redox forms is reversible, whereas modification into the fully oxidized isoform is irreversible (143).

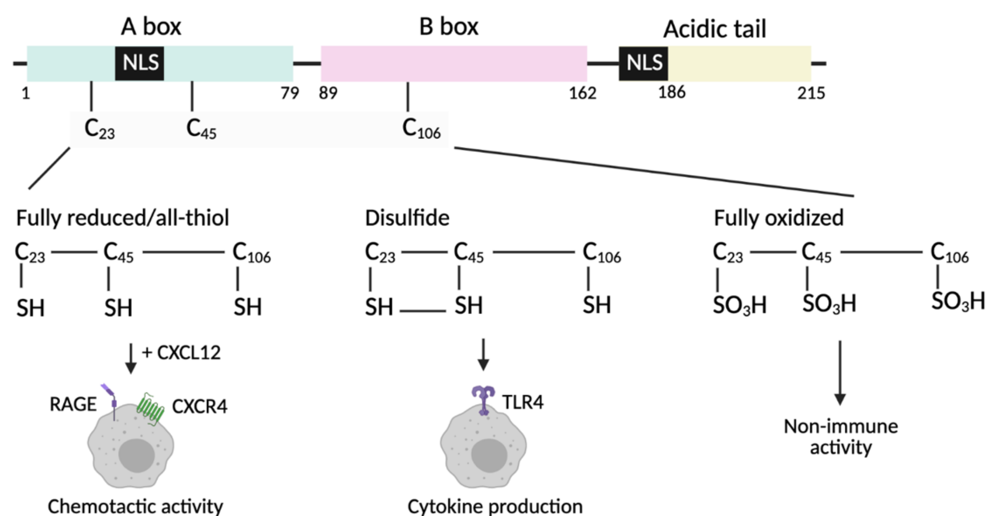

Figure 3. The structure and redox regulation of HMGB1 activity. The redox state of three cysteines located at amino acid 23, 45 and 106 within the protein regulates which receptor HMGB1 binds to and the functional consequence of its release. Created with Biorender.com.

HMGB1 release into the extracellular space involves passive and active cellular mechanisms. Passive release of HMGB1 is caused by necrotic cell death and the process is spontaneous. On the other hand, active HMGB1 secretion takes place as a result of tissue injury or inflammation, and is a relatively slower process than passive discharge. This process involves membrane receptor activation, such as binding of pathogen-associated molecular patterns (PAMPs; *e.g.* LPS, IL-1 and TNF) to their corresponding receptors, and activation of intracellular signal transduction (144-146). In general, active release of HMGB1 requires two crucial steps. The first step is the initial nucleus to cytosol shuttling of HMGB1 that involves JAK/STAT1-mediated acetylation of lysine residues located in NLS1 and NLS2 that prevents HMGB1 transport back to the nucleus (139, 145). The second step is the extracellular release of HMGB1

that could either involve inflammasome-controlled pyrotopsis or vesicle-mediated secretory pathway (147, 148).

Once released extracellularly, HMGB1 regulates various immunological functions such as activation of immune cells, production of cytokines, chemotaxis, and cell proliferation and differentiation. The broad repertoire of HMGB1's actions is mediated by the fact that this protein can interact with different receptor systems. As of now, at least 14 receptors have been reported to be targets for extracellular HMGB1 (149). In addition to TLR4 and RAGE that are mentioned above, HMGB1 has been reported to act as a ligand for other TLRs such as TLR2 and TLR5, although the precise molecular interactions between HMGB1 and these receptors are still unknown (76, 150). HMGB1 can also activate other receptor systems such as IL-1R, CD24 and Siglec-10 (Siglec-G in mice). However, these receptors are most likely receptors for molecules that can form complexes with HMGB1 (*e.g.* LPS, IL-1β, viral RNA, nucleosomes). Thus so far, direct interactions of HMGB1 with receptors are only clarified for TLR4 and RAGE. Nevertheless, the ability of HMGB1 to form complexes is an important mechanism for HMGB1-mediated inflammation since this elevates the responses as compared to induction by the ligand alone (151, 152).

Knowledge on HMGB1 and its receptor interactions originated from preclinical studies that primarily used male subjects, however increasing data show that similar HMGB1 activities are detected in females (Table 2). In preclinical studies, disulfide HMGB1 has been shown to induce muscle fatigue in both male and female mice, which is dependent on TLR4 but not RAGE signaling (153). HMGB1 signaling via RAGE and TLR2 has also been reported in several experimental models associated with cancer (154), lupus (155) and heart disorders (156) in female mice. There is also a growing number of preclinical pain studies that demonstrate these types of interactions in both male and female mice, and this will be further discussed in part 2.3.3. In clinical studies, HMGB1-TLR4 interactions are strongly implied in men and women infected with HIV (157), and in women with ovarian cancer (158).

Table 2. Evidence for HMGB1 and receptor interactions in either female or both male/female subjects

HMGB1 isoform	Target receptors	Pathological conditions	Species	Sex/gender	Refer-ences
Disulfide	TLR4	Myositis	Mouse	Male, female	(153)
Disulfide	TLR4	Osteoarthritis	Mouse	Female	(159)
-	RAGE	Myocarditis	Mouse	Female	(156)
-	TLR2	Brain cancer	Mouse	Female	(154)
-	TLR2	Lupus nephritis	Mouse	Female	(155)
-	TLR4/MD-2	HIV infection	Human	Male, female	(157)
-	TLR4	Ovarian cancer	Human	Female	(158)

To maintain homeostasis, pro-inflammatory factors, including HMGB1, are subject to downregulation. Several endogenous molecules have been identified to sequester and neutralize HMGB1. Haptoglobin, a protein that binds free hemoglobin, is an example of such

factors that can dampen pro-inflammatory activity of HMGB1. This protein forms a complex with either all-thiol or disulfide HMGB1, which are then taken up by macrophages via the scavenger receptor CD163 and this results in the release of anti-inflammatory mediators, such as heme oxygenase-1 and IL-10 (160). Another example is the endothelial thrombin-binding protein thrombomodulin that can interact with HMGB1 and promotes proteolytic cleavage by thrombin (161). The complement protein C1q can also interact with HMGB1 and shifts macrophage polarization into anti-inflammatory M2-like macrophages (162). Furthermore, the HMGB1 receptor RAGE exists in a soluble form (sRAGE) and can act as a decoy receptor that neutralizes the pro-inflammatory actions of HMGB1 (163).

2.3.2 HMGB1 and rheumatoid arthritis

Accumulating evidence shows a strong connection between HMGB1 and RA, with several studies suggesting that HMGB1 is released at the site of joint inflammation. Indeed, enhanced levels of extranuclear HMGB1 are detected in synovial fluid and synovial biopsies from RA patients (164-166). HMGB1 levels in RA synovial fluid are markedly higher when compared with samples derived from osteoarthritis patients (166). In addition, HMGB1 expression in inflamed synovium is predominantly observed in macrophages and vascular endothelial cells (166). HMGB1 levels are also increased in sera of RA patients and this correlates with higher disease activity (167).

Important discoveries regarding the role of HMGB1 in RA also comes from animal studies. In synovial tissues of the CIA model, HMGB1 expression was increased in the extracellular space and cytoplasm of macrophages, fibroblasts and endothelial cells, while it was restricted to the nucleus in healthy animals (165, 168). HMGB1, in synergy with vascular endothelial growth factor (VEGF), also induced neovascularization in the synovium and contributed to pannus formation in the CAIA model (169). Moreover, animal studies have shown the important contribution of HMGB1 in osteoclastogenesis (170, 171), and thus may suggest a role of HMGB1 in RA-associated bone loss. Another key observation pointing to the role of HMGB1 in RA is that injection of recombinant HMGB1 into murine knee joints triggered long-term arthritic symptoms (172). Consistently, inhibition of HMGB1 with specific antagonists reduced clinical signs of arthritis and conferred significant protection against structural damage to the joint in several experimental models of RA (173-176). Of relevance to the antagonist used in this thesis work, the monoclonal HMGB1 antibody (2G7) alleviated joint inflammation, but only partially prevented cartilage and bone destruction in two arthritis model: CIA and a spontaneous RA model induced by deficiencies in genes encoding enzyme DNase type II and interferon type I receptor (177). To date, there is no anti-HMGB1 therapy approved for clinical use, but animal studies show potential benefits of targeting HMGB1 in RA. Currently, two different humanized anti-HMGB1 monoclonal antibodies have been identified (149), but further investigations are still required to be able to introduce these agents for patient treatments.

2.3.3 HMGB1 and pain

It is now established that HMGB1 mediates the molecular pathways that drive and maintain nociception with mounting evidence showing that local administration of HMGB1 cause pain-like behavior in rodents. The earliest indication of this observation was reported 20 years ago whereby HMGB1 delivery onto rat sciatic nerve via a preimplanted indwelling perisciatic catheter was shown to evoke hypersensitivity to mechanical stimulation in a dose-dependent fashion (178). Similar observation was reported following the delivery of HMGB1 into sciatic nerves exposed by blunt dissection (179). Subsequently, it was shown that intrathecal injection of HMGB1 in naïve rats promoted lower threshold responses to calibrated touch/pressure stimuli (180). In mice, intrathecal injection of HMGB1 induced similar behavioral outcomes that last up to 5 days post-injection (75). Collectively, these studies establish the peripheral and central actions of HMGB1 in nociceptive signaling.

Since the discovery of the different HMGB1 isoforms and the importance of this structural change for HMGB1 functions, emerging evidence shows that the pronociceptive role of HMGB1 is also redox-dependent. In addition to this, the pronociceptive actions of HMGB1 and its interplay with different receptors may differ between sites (Table 3). Studies show that robust pain-like responses were elicited in mice following injection of disulfide, but not all-thiol, HMGB1 into the ankle joint (138), bladder (181, 182), spinal cord (75) or brain (183), via TLR4 signaling. Both isoforms of HMGB1 were, however, pronociceptive when injected intraplantarly. However, it should be taken into account that a 10-fold higher dose of all-thiol HMGB1 in comparison to disulfide HMGB1 was needed to produce this effect (184). Interestingly, it was shown that both HMGB1 isoforms promoted excitation of primary DRG neurons, but a slightly higher percentage of cells responded to stimulation with all-thiol compared to disulfide HMGB1 (185).

Table 3. Pain-inducing effects of different HMGB1 isoforms administered via several routes in rodents

Route of administration	HMGB1 isoform	Target receptors	Pain assessment	References
Intraplantar	Disulfide, all- thiol	TLR4, RAGE	Mechanical	(184)
Intra-articular	Disulfide	TLR4	Mechanical	(138)
Intravesical	Disulfide	TLR4	Mechanical	(181, 182)
Intrathecal	Disulfide	TLR4	Mechanical	(75)
Intracisternal	Disulfide	TLR4	Mechanical, thermal	(183)

Other key observations that indicate important roles of HMGB1 in nociception include elevated levels of extranuclear HMGB1 found in the DRGs, spinal cords and sciatic nerves in models of neuropathic and RA-induced pain. These changes often occur in concomitance with pain-associated behavior in the animals (75, 179, 186, 187). As the presence of extranuclear HMGB1 indicates translocation out of the nucleus and may be interpreted as release, extracellular HMGB1 in the nervous system can thus originate from neurons, glial cells and immune cells. Multiple TLRs and RAGE are expressed by these cells (188), and therefore

HMGB1 could directly affects nociceptor activity as well as induce an indirect effect by promoting the release of algesic factors from cells in the proximity.

Mounting data further supporting the role of HMGB1 in pain mechanisms show that blockade of endogenous HMGB1 led to attenuation of nociceptive in several experimental models. Global HMGB1 knockout mice has not been possible to use as this genetic depletion is lethal in newborn mice (189), and there are no reports of the use of conditional HMGB1 knockout for pain studies. Instead, several pharmacological agents that suppress HMGB1 activity have been used (Table 4). The reversal effects of neutralizing HMGB1 antibodies have been shown in numerous models of pain including those subjected to nerve injury (179, 187, 190) or arthritis (75). The truncated HMGB1 A Box domain, which inhibits HMGB1 activity in a yet unresolved mode of action, has also been reported to be antinociceptive in the CAIA model (75). Moreover, the antinociceptive effects of glycyrrhizin, a natural triterpene glycoside that has anti-inflammatory and antiviral properties, and thrombin-binding TM have been demonstrated in various pain models such as models of visceral pain (191-195). Together, this points to the potential use of HMGB1 blocking agents to treat different painful conditions.

Table 4. The antinociceptive effects of HMGB1 antagonists in experimental models of pain

HMGB1 antagonist	Pain model	Route of administration	Pain assessment	References
HMGB1 Ab	Nerve injury	Perisciatic, i.p., i.t.	Mechanical, thermal	(179, 187, 190)
	CIPN	i.p.	Mechanical, paw pressure, thermal	(196, 197)
	Diabetic neuropathy	i.t.	Mechanical	(198)
	Arthritis/joint pain	i.pl., s.c., i.t.	Mechanical, paw pressure, thermal	(75, 199)
	Visceral pain	i.p., i.t.	Mechanical, licking/biting lower abdomen	(193-195)
	CIBP	i.p., i.t.	Mechanical	(200-202)
	Orofacial pain	i.p., i.t.	Mechanical, thermal	(183)
Box-A peptide	Arthritis/joint pain	i.t.	Mechanical	(75)
Glycyrrhizin	Nerve injury	i.p.	Mechanical	(186)
	Diabetic neuropathy	i.p.	Paw pressure, thermal	(203)
	Arthritis/joint pain	i.p.	Mechanical, thermal	(204)
	Visceral pain	i.p., i.t.	Mechanical	(191, 192)
TM	CIPN	i.p.	Mechanical, paw pressure, thermal	(196, 197)
	Visceral pain	i.p.	Mechanical, licking/biting lower abdomen	(193-195)

CIPN: chemotherapy-induced painful neuropathy; CIBP: cancer-induced bone pain

Given that TLR4 is a receptor of HMGB1, one may assume that there is a general interest in examining if the role of HMGB1 in nociception is sex-dependent. Surprisingly, there is a limited number of studies that perform direct comparisons between males and females when it comes to examining the nociceptive roles of HMGB1 in RA. It has been shown that spinal delivery of disulfide HMGB1 lowers mechanical withdrawal thresholds in both male and

female mice, and that blocking the action of spinal HMGB1 provides pain-relieving effects in the CAIA model in both sexes (75). Even though it has been shown that HMGB1 evokes pain-like responses when administered into the ankle joint (138), the sex-dependent role of HMGB1 in the periphery was not explored. Previous work also shows that spinal glial inhibition led to reversal of mechanical hypersensitivity in male but not female mice subjected to late phase CAIA (13). However, it is not known if the observed sex differences are attributed to HMGB1. Though it has been reported that intrathecal injection of HMGB1 induced microglial activation in the spinal cord (75), this observation has not been linked to sex differences. Further studies are thus warranted to understand if the role of peripheral and spinal HMGB1 in nociception is sex-specific.

2.4 SUMMARY

Treatment for RA has changed profoundly over the past decade but despite adequate disease improvements with current antirheumatic drugs, pain is still a significant problem for patients. Patients with RA are found to have persistent pain despite well-controlled inflammation. A growing body of evidence suggests that that specific components of pain in RA are not coupled to inflammation and that patients may benefit from alternative therapies to anti-inflammatory drugs. One could argue that a possible strategy to circumvent this is to target the bone microenvironment since RA-affected joints exhibit increased bone erosion. However, it is still not known how cells in the bone mediate pain in RA and if inhibitors that could alter bone metabolism (*e.g.* bisphosphonates) may provide beneficial effects on pain. RA is also known as a complex disease involving different inflammatory factors, many of which have been suggested to cause a chronic neuroinflammatory state that may maintain pain in patients. Accumulating evidence shows that this condition may be mediated by the JAK/STAT signaling pathway or the DAMP molecule HMGB1. HMGB1 being an endogenous ligand of TLR4 is also an interesting molecule to explore with regards to sex differences in pain mechanism. Taken together, unraveling these alternative mechanisms may open new perspectives on novel drug targets for treatments of chronic pain in RA.

3 RESEARCH AIMS

This thesis has the overall aim to explore novel mechanisms that drive persistent pain in rheumatoid arthritis, focusing on the role of the bone environment and neuroinflammatory factors, and if specific mechanisms are sex-dependent. Thus, this thesis has three specific aims:

1. To elucidate the mechanisms by which osteoclasts mediate long-lasting hypersensitivity in the collagen antibody-induced arthritis model

2. To identify the effect of the JAK/STAT inhibitor baricitinib on pain-related behavior and its antinociceptive mechanism in the collagen antibody-induced arthritis model

3. To understand the role of peripheral and central HMGB1 in arthritis-induced pain and if its action is sex-dependent

4 MATERIALS AND METHODS

4.1 ANIMAL STUDIES

4.1.1 Animals

All animal works were approved by the local ethical committees for animal experiments in Sweden (Stockholm Norra djurförsöksetiska nämnd), France (Comité Régional d'Éthique en Matière d'Expérimentation Animale Auvergne) and USA (Institutional Animal Care and Use Committee of The University of Texas at Dallas). This thesis works also conforms to the Animal Research: Reporting of In Vivo Experiments (ARRIVE) and IASP guidelines. Animals were housed in standard cages (4-5 mice/cage) in a temperature-controlled environment with 12 h light/dark cycle and access to food and water *ad libitum*.

Different mouse strains and genetically modified mice were used across different studies. For **Paper I** and **II**, BALB/c female mice were obtained from Janvier Laboratories. For **Paper III**, BALB/c and C57BL/6 male and female were purchased from Janvier Laboratories and Charles River. In addition, male and female mice with specific TLR4 deletion in myeloid cells or nociceptors were used in this study. To generate cell-specific TLR4 depletion, $TLR^{fl/fl}$ mice were used as previously described (205). $TLR4^{fl/fl}$ mice were then crossed with mice expressing Cre under the control of the Lysozyme M (LysM) or Nav1.8 promoters to breed mice having TLR4 deletion in myeloid cells peripheral or nociceptors, respectively. The resulting LysM-$TLR4^{fl/fl}$, Nav1.8-$TLR4^{fl/fl}$, and $TLR4^{fl/fl}$ (control mice) were backcrossed and bred for 8 generations to a C57BL/6 background at the University of Texas at Dallas. **Paper IV** used C57BL/6 male and female mice, purchased from Janvier Laboratories, as well as the two cell-specific TLR4 depleted mice (LysM-$TLR4^{fl/fl}$ and Nav1.8-$TLR4^{fl/fl}$) described above. Proteinase-activated receptor (PAR)2-deficient ($PAR2^{-/-}$) mice obtained from Jackson Laboratory and bred at the University of Texas at Dallas were also used in **Paper IV**.

4.1.2 Animal models

4.1.2.1 Collagen antibody-induced arthritis model

For **Paper I-III,** the CAIA model was achieved as previously described (134). BALB/c male and female mice were injected i.v. with anti-collagen type II (CII) arthritogenic antibody cocktail (1.25-1.5 mg/mouse, purchased from Chondrex) on day 0 followed by i.p. injection of LPS (5-25 μg/mouse) on day 3 or 5 to boost and synchronize joint inflammation. A control group receiving saline solution i.v. on day 0 and i.p. on day 3 or 5 was included in all studies.

The degree of inflammation was assessed by visual inspection of the fore and hind paws. A score of 1 point was given for each inflamed toe or knuckle and a score of 2.5 or 5 points was given for each inflamed wrist or ankle for either moderate or severe inflammation. This results in maximum possible scores of 15 points per paw and 60 points per mouse. Incidence of arthritis refers to the percentage of animals that developed signs of joint inflammation. BALB/c female mice developed higher arthritis incidence than BALB/c male mice, as also reported previously (13). For **Paper I-III**, CAIA mice that did not receive any drug treatments were excluded from the study if they did not reach a score of 12 points.

4.1.2.2 Complete Freund's adjuvant-induced monoarthritis

For **Paper II**, monoarthritis was evoked in mice by unilateral intra-articular injection of complete Freund's adjuvant (CFA, 10 mg/mL) into the ankle joint in 2.5 µL volume, which is equivalent to 10 µg CFA per mouse. The procedure was performed under isoflurane anesthesia.

4.1.2.3 Spared nerve injury model

For **Paper II**, the spared nerve injury (SNI) model of neuropathic pain was performed on mice as previously described (206). Briefly, SNI was achieved by ligation (with 6-0-silk suture) and transection of tibial and common peroneal nerves under isoflurane anesthesia leaving the sural nerve intact. Control mice were sham-operated by exposing the sciatic nerves, without performing nerve ligation and transection. Subsequent to surgery, mice received subcutaneous (s.c.) injection of buprenorphine (0.1 mg/kg) every 12 h for 48 h.

4.1.3 Drugs and drug delivery

Different categories of drugs/reagents, including osteoclast and HMGB1 inhibitors, were used across **Paper I-IV**. All drugs, with their route of administration and doses, used for pharmacological experiments are summarized in Table 5.

4.1.3.1 Osteoclast inhibitor

For **Paper I**, two different osteoclast inhibitors were tested, the bisphosphonate zoledronate and the cathepsin K inhibitor tanshinone IIA sulfonic sodium (T06). Zoledronate is a third generation, nitrogen containing bisphosphonate that is used to treat and prevent osteoporosis, bone complications due to cancer, osteogenesis imperfecta and Paget's disease (207). As other bisphosphonates, zoledronate preferentially binds to calcium ions and accumulates in the bone. During the process of bone degradation mediated by osteoclast, zoledronate can enter osteoclasts and affects their differentiation, cell survival and cytoskeletal dynamics. On the other hand, T06 is a derivative of Tanshinone IIA, a natural component of the herb *Salvia miltiorrhiza*, which is used in traditional Chinese medicine for cardiovascular disorders. It binds to an ectosteric site of cathepsin K remote from its active site and subsequently suppresses the ability of osteoclasts to degrade collagen (208). For **Paper I**, zoledronate (100 µg/kg) was

injected subcutaneously (s.c.) every 3 days and phosphate-buffered saline (PBS) was used as vehicle control. T06 (40 mg/kg, q.d.), which was kindly provided by Dr. Dieter Brömme (University of British Columbia, Vancouver, Canada), was administered per oral gavage and distilled water was used as vehicle control.

Table 5. List of drugs/reagents used in animal studies

Drug/reagents	Category	Paper	Route	Dose	Dosing regimen
2G7	Anti-HMGB1 mAb	III	s.c.	100 μg/mouse	q.d.
Alpha-1-antitrypsin	Protease inhibitor	IV	i.t.	15-30 ng/mouse	once/twice inj.
All-thiol HMGB1	Recombinant protein	III-IV	i.a.	1 μg/mouse	once inj.
Baricitinib	JAK inhibitor	II	p.o.	20-40 mg/kg	q.d. or b.i.d
Disulfide HMGB1	Recombinant protein	III-IV	i.a. or i.t.	1 μg/mouse	once inj.
Haptoglobin	HMGB1 sequester	IV	i.t.	15 μg/mouse	once inj.
Minocycline	Macrophage/microglia inhibitor	III-IV	i.a. or i.t.	30-100 μg/mouse	once/twice inj.
NP137	Anti-netrin-1 Ab	I	i.p.	10 mg/kg	3 times/week
Sivelestat	Elastase inhibitor	IV	i.t.	0.5-1 ng/mouse	once/twice inj.
T06	CatK inhibitor	I	p.o.	40 mg/kg	q.d.
Zoledronate	Bisphosphonate	I	s.c.	100 μg/kg	every 3 days

mAb: monoclonal antibody; q.d.: once daily; b.i.d.: twice daily; inj.: injection

4.1.3.2 Netrin-1 inhibitor

The humanized monoclonal antibody of IgG1 isotype targeting netrin-1 (NP137) developed by Netris Pharma (Lyon, France) (209) was used in **Paper I** to inhibit netrin-1 signaling. NP137 (10 mg/kg) was administered via i.p. injection three times a week. For vehicle control, a control buffer (containing 20 mM Tris, 5% sucrose and 0.01% Tween 20) was used.

4.1.3.3 JAK inhibitor

Baricitinib is a selective JAK1/2 inhibitor, which has been approved by both FDA and EMA for treatment of RA (111). For **Paper II**, baricitinib was kindly provided by Eli Lilly (Indianapolis, IN, USA). Baricitinib (20-40 mg/kg, q.d. or b.i.d) was administered per oral gavage and for vehicle control, 0.5% methylcellulose was used.

4.1.3.4 HMGB1 inhibitor

The anti-HMGB1 non-commercial monoclonal antibody (2G7) was produced in house (210), and used in **Paper III** to suppress the activity of endogenous peripheral HMGB1. This antibody binds to an epitope within the amino acid region at position 53 to 65 of the A box unit. 2G7 (100 μg/mouse, q.d.) was administered via s.c. injection and PBS was used as vehicle.

4.1.3.5 Microglia/macrophage inhibitor

Minocycline is a tetracycline antibiotic used to treat various bacterial infections, which has also previously been used in preclinical studies as microglia/glia inhibitor (13). For **Paper IV**, mice received intrathecal (i.t.) injection minocycline (30 μg/mouse), whereas control mice received intrathecal injection of saline, all in 5 μL volume. As minocycline also exhibits inhibitory effects on macrophages (211), it was also used as a macrophage inhibitor for **Paper III**. Mice were given intra-articular (i.a.) injection of minocycline (30-100 μg/mouse), while control mice received i.a. injection of saline, all in 2.5 μL volume.

4.1.3.6 HMGB1 (different redox forms)

Different redox forms of HMGB1 were used in **Paper III** and **IV**; all-thiol HMGB1 (RAGE ligand, chemoattractant form) and disulfide HMGB1 (TLR4 ligand, cytokine-inducing form) (75). Both isoforms of HMGB1 were kindly provided by Dr. Huan Yang (Feinstein Institute for Medical Research, Manhasset, NY, USA) or purchased from HMGBiotech (Milan, Italy). In **Paper III**, i.a. injection of both all-thiol and disulfide HMGB1 (1 μg/mouse) was performed to investigate the redox-dependent nociceptive effects of peripheral HMGB1. For **Paper IV,** only disulfide HMGB1 (1 μg/mouse) was used for i.t. injection. For all studies, either i.a. or i.t. injection of PBS was used as vehicle control.

4.1.3.7 Alpha-1-antitrypsin

Alpha-1-antitrypsin (A1AT) is an inhibitor of serine proteases, primarily elastase but also other proteases such as proteinase 3 and cathepsin G (212). A1AT (15-30 ng/mouse) was either co-injected with disulfide HMGB1 or injected alone into the spinal cord in **Paper IV**.

4.1.3.8 Haptoglobin

Haptoglobin is a protein produced by the liver that clears free hemoglobin, which can induce deleterious oxidative activity. It has been shown that haptoglobin sequesters HMGB1 via CD163 and this elicits release of anti-inflammatory factors (160). In **Paper IV**, haptoglobin (15 μg/mouse) was injected i.t. in combination with disulfide HMGB1.

4.1.3.9 Sivelestat

Sivelestat is a selective leukocyte elastase inhibitor (213). Sivelestat (0.5-1 ng/mouse) was either co-injected with disulfide HMGB1 or injected alone into the spinal cord in **Study IV**.

4.1.4 Assessment of pain-like behavior

In **Paper I-IV**, mechanical hypersensitivity was examined as a measure of evoked pain-like behavior. Mechanical hypersensivity in the hind paws was assessed by von Frey OptiHair filaments (Marstock). Briefly, animals were habituated to the testing environment, which are

single units on an elevated mesh platform, before baseline measurements (3-5 measurements) and randomization into experimental groups. On indicated test days, animals were acclimatized to the testing apparatus for 30-45 prior to measurements. A series of filaments with a logarithmically incremental stiffness of 0.5, 1, 2, 4, 8, 16 and 32 mN (corresponding to 0.051 g, 0.102 g, 0.204 g, 0.408 g, 0.815 g, 1.63 g, and 3.26 g, respectively) were applied to the plantar surface of the hind paw and held for 2-3 seconds. A cut-off of 4 g was applied to avoid tissue damage. A brisk withdrawal of the paw was noted as a positive response. Values were calculated as 50% withdrawal thresholds (*i.e.* the thresholds of mechanical hypersensitivity at which there was a 50% probability of paw withdrawal) using the Dixon up-down method (214). Withdrawal thresholds from both hind paws were averaged, except in unilateral experiment such as i.a. injection of HMGB1 where only the ipsilateral paw was considered for analysis. Data are presented in grams or as percentage change to the mean baseline values within each treatment groups. However, when there was a great variation of baselines between groups, withdrawal thresholds are presented as percentage change to individual baseline values. For certain experiments, mechanical hypersensitivity was presented as a hyperalgesic index (HI index), which is calculated from the area between the extrapolated baseline and the time-response curve following nociceptive procedure (*i.e.* HMGB1 i.a. injection), or calculated as area under the curve (AUC) during specific treatment periods (i.e. baricitinib treatment). Increasing HI index indicates increasing hypersensitivity, while it is the opposite for AUC assessment. All behavioral experiments were performed in a blinded fashion throughout the experiment and data analysis to avoid bias.

4.1.5 Ethical considerations

Throughout this thesis work, strict policies were followed when it comes to the welfare and well-being of the animals. All procedures conformed to the regulations of the approved ethical permits as well as the ARRIVE and IASP guidelines stated above. Apart from this, the Russell and Burch 3R's principle (Replacement, Reduction and Refinement) of human experimental techniques were carefully implemented.

One would argue if there are ethical debates about using animals, one should turn to alternatives (*Replacement*). Although a great amount of work has been done in our laboratory, we were unsuccessful in identifying cell lines that would mimic their physiological properties *in vivo*, and this why primary cell cultures are still used for this thesis work. Human patients and cadaveric samples have also been used in the past to replace animal studies as well as sidestepping the issue of translation between species, however the data generated are often needing to be complemented by animal studies and thus animal research cannot be completely eliminated.

Careful decision is ensured on how many animals are included for each experiments (*Reduction*). No statistical tests were used to predetermine sample size. Instead, the general consensus implemented here is that no more than 10 mice per group are included, but if this

number does not give a statistically significant outcome, more mice included would be justifiable to detect small changes and give us more information. It should also be considered if reducing the number of animals would lead to heavier burden on those animals included in the study. For instance, repeated behavioral experiments or injections/surgical intervention in one animal as compared to spreading these procedures on several animals. The issue about animal numbers has become more of a problem since the discovery that there are sex differences in pain mechanisms. Traditionally researchers tend to use either males or females for practicality and by including both sexes the number of subjects would double substantially. One of the aims of this thesis is to understand if the regulation of pain processing is sex-dependent and therefore this factor is unavoidable for this work.

In terms of animal welfare, a close contact with veterinarians and animal technicians was maintained before the experiment as evident by project plans being made as well as throughout the course of experiment (*Refinement*). When inducing a severe model for example the CAIA model, it is the responsibility of the researcher to take daily care of the animals as well as to communicate with the animal technicians all of their needs and to take immediate actions if humane endpoints are reached. Enriched and stable environment was carefully monitored to reduce stress for animals. Furthermore, refinement was also applied for the experiment protocols themselves, as such that the intensity and duration of pain are minimized. In the classical von Frey test in which filaments with incremental force are applied on the plantar side of the paw, the experimenter decides the maximum force that could be applied based on the animal's withdrawal response in order to prevent intensified pain and injury.

4.2 CELL CULTURE

4.2.1 Osteoclast culture

In **Paper I**, primary osteoclast cultures were generated from mouse tibial and femoral bone marrow. Cells were isolated by centrifugation at 300*g* for 5 min and resuspended in Eagle's Minimum Essential Medium - alpha modification (αMEM) supplemented with 10% fetal bovine serum (FBS), 50 U/mL penicillin, 50 μg/mL streptomycin, 30 ng/mL macrophage colony-stimulating factor (M-CSF) and 50 ng/mL receptor activator of nuclear-kappa B ligand (RANKL). Cells were then seeded onto appropriate culture plates at a density of 1.25×10^5 cells/well and cultured for 6 days in a humidified 37°C incubator with 5% CO_2. Medium was refreshed every 2 days and starting on day 2 cells were stimulated with either 600 nM zoledronate, 1 μM T06 or vehicle (PBS). On the day of medium change, culture supernatant was collected and stored at -80°C. At the end of experiment, cells were either fixed in 4% paraformaldehyde (PFA) and stained for tartrate resistant acid phosphatase (TRAP) to validate differentiated osteoclasts.

4.2.2 Dorsal root ganglia (DRGs) culture

Mouse DRGs (C1-L6) were isolated to generate primary neuronal cultures in **Paper I**. Dissected DRGs were kept in cold PBS prior to enzymatic digestion initially with papain (0.8 mg/mL; 30 min at 37°C) followed by collagenase I and dispase II (12 and 14 mg/mL; 30 min at 37°C). Cells were then triturated in Ham's F12 Nutrient Mixture medium supplemented with 10% FBS, 50 U/mL penicillin and 50 μg/mL streptomycin, and seeded on glass bottom dishes pre-coated with poly-D-lysine and laminin. Dissociated cells were kept in a humidified 37°C incubator with 5% CO_2 for 24 h. The following day, cells were used for calcium imaging to determine the effect of netrin-1 application on neuronal function.

4.2.3 Macrophage culture

For **Paper III**, bone marrow cells were isolated from mouse tibia and femur to generate primary macrophage culture. Cells were cultured in low adherence flasks in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, 2mM GlutaMAX, 50 U/mL penicillin, 50 μg/mL streptomycin and 10 ng/mL M-CSF in a humidified 37°C incubator with 5% CO_2. Upon reaching 75-80% confluency, cells were dissociated and seeded onto 96-well plates in serum starved condition (medium containing 0.5% FBS). Cells were stimulated with 1 μg/mL disulfide HMGB1 for 24 h and afterwards culture supernatants were harvested for measurements of inflammatory factors.

4.2.4 Microglia culture

For **Study IV**, both cell line and primary cultures were used for *in vitro* assays. The murine microglial cell line N13 (CD1 strain) was kindly provided by Prof. Gunnar Schulte (Karolinska Institutet, Stockholm, Sweden). Cells were expanded in DMEM supplemented with 10% FBS, 100 U/mL penicillin, 50 μg/mL streptomycin and 2 mM glutamine until confluent, stimulated with 1 μg/mL disulfide HMGB1 in serum starved condition (0.5% FBS) and collected 6 h later for further analysis.

Primary microglial cultures were generated from mouse brain and spinal cord tissues. Microglia were isolated and cultured using a modified version of previously described protocols [2; 53]. In short, tissues were mechanically dissociated followed by enzymatic digestion with papain for 30 min at 37°C and isolation using Percoll density gradient. Cells were then seeded onto culture plates precoated with poly-D-lysine and incubated for 2 h at 37°C in microglial culture medium containing Dulbecco's modified Eagle's medium/nutrient mixture F-12 (DME/F12) supplemented with 10% FBS, 100 U/mL penicillin, 50 μg/mL streptomycin and 2 mM glutamine to allow for cell attachment. Subsequently, cells were serum starved (2% FBS) and maintained for 24 h in a humidified 37°C incubator with 5% CO_2. The

next day, cells were stimulated with 1 μg/mL disulfide HMGB1 and collected 6 h later for further analysis.

4.3 IMAGING ANALYSES

4.3.1 Micro-computed tomography imaging

For **Paper I** and **II**, bone erosion was evaluated using a micro-computed tomography (micro-CT) imaging system. Prior to imaging, hind ankle joints were harvested from euthanized mice, post-fixed in 4% PFA for 48 h and transferred to 0.01 M phosphate buffered saline (PBS). Distal tibia, talus and calcaneus were scanned using Skyscan 1272 (Bruker) at 10 μm voxel size, 60 kVp/166 μA X-Ray power and 627 ms integration time. Acquired images were reconstructed using NRecon software (Bruker) and analyzed using CT analyzer program (Bruker).

4.3.2 ^{99m}Tc-HMDP scintigraphic imaging

To evaluate bone remodelling in **Paper I**, mice were injected i.p. with ^{99m}Tc-HMDP radiotracer (10 MBq/mouse, Osteocys, IBA) and imaging was acquired 2.5 h post-injection. Planar acquisition was performed for 5 min (15% window at 140 keV) on the animal positioned in ventral decubitus on a parallel collimator (20mm/1.8/0.2) of a gamma camera for small animal (γImager, Biospace) under isoflurane anaesthesia. Quantitative analysis of bone scintigrams was performed with Gammavision+ software (Biospace).

4.3.3 Calcium imaging

Prior to imaging, DRG neurons were incubated in Fluo-3AM (4.4 μM, calcium indicator) for 30 min. Cells were then washed in modified HEPES buffer (10 mM HEPES, 2mM $CaCl_2$, 3mM KCl, 145 mM NaCl, 2mM $MgCl_2$, 10 mM glucose, pH 7.4, referred as EC solution) and placed in a recording station on Zeiss LSM800 confocal microscope. Calcium imaging was performed with argon laser (488 nm excitation) and 20x objective lens. The change in emission (560 nm), *i.e.* binding of intracellular calcium to Fluo-3 AM was measured every 2.5 sec using a photomultiplier tube. Mouse recombinant netrin-1 (0.2, 2 and 8 μg/mL) was directly applied using a pipette to the cells and imaging was performed for 16 min. Application of EC solution alone was used as control. KCl (50 mM) was applied at the end of each experiment to detect functional neurons. Acquired images were analyzed by ImageJ. Image analysis is described in further details in **Paper I**.

4.4 MOLECULAR ANALYSES

4.4.1 Quantitative polymerase chain reaction

Flash frozen tissues (ankle joints, L3-L5 DRGs and L3-L5 spinal cords) and cultured cells (osteoclasts and microglia) were homogenized in TRIzol reagent. mRNA was extracted followed by cDNA synthesis according to the manufacturer's protocol. Quantitative polymerase chain reaction (qPCR) was performed using predeveloped Taqman primers listed in Table 6. For **Paper I** and **III**, threshold cycle values for each sample were used to calculate the number of cell equivalents in the test samples using the standard curve method (215). The housekeeping genes *Hprt1* or *Rplp2* values were used to normalize the data, which were then plotted as relative expression units or percentage change to the control group. In **Paper II**, gene expression was analyzed according to the ΔΔCt method and data are presented as fold change to the housekeeping gene *Hprt1*.

Table 6. List of Taqman primers

Gene		Taqman primer	Paper
Aak1	AP2-associated protein kinase 1	Mm01183675_m1	II
Acp5	Acid phosphatase 5, tartrate resistant	Mm00475698_m1	I
Ap2m1	Adaptor related protein complex 2 subunit mu 1	Mm05884025_g1	II
Asic3	Acid-sensing ion channel 3	Mm00805460_m1	I
Ccl2	C-C motif chemokine ligand 2	Mm00441242_m1	III, IV
Cd11b (Itgam)	Cluster of differentiation molecule 11B	Mm00434455_m1	III
Cd34	Cluster of differentiation molecule 34	Mm00519283_m1	III
Clcn7	Chloride channel 7	Mm00442400_m1	I
Cox2	Cyclooxygenase 2	Mm00478374_m1	III
Ctsk	Cathepsin K	Mm00484039_m1	I
Cxcl1	Chemokine (C-X-C motif) ligand 1	Mm04207460_m1	III
Cxcl2	Chemokine (C-X-C motif) ligand 2	Mm00436450_m1	III
Hmgb1	High mobility group box 1	Mm00849805_gH	III
Hprt1	Hypoxanthine phosphoribosyl transferase 1	Mm03024075_m1	I-IV
Il1b	Interleukin 1β	Mm00434228_m1	III-IV
Il6	Interleukin-6	Mm00446190_m1	III-IV
Mpo	Myeloperoxidase	Mm01298424_m1	III
Ngf	Nerve growth factor	Mm00443039_m1	III
Ntn1	Netrin-1	Mm00500896_m1	I
Pecam1	Platelet endothelial cell adhesion molecule 1	Mm01242576_m1	I
Rplp2	60S acidic ribosomal protein P2	Mm00782638_s1	I-III
Sema4d	Semaphorin 4D	Mm00443147_m1	I
Tcirg1	V-type proton ATPase 116 kDa subunit a isoform 3	Mm00469406_m1	I
Tlr4	Toll-like receptor 4	Mm00445273_m1	III
Tnf	Tumor necrosis factor	Mm00434258_m1	III-IV
Trpv1	The transient receptor potential cation channel sul V member 1	Mm01246302_m1	I

4.4.2 Immunohistochemistry

Mice were deeply anesthetized in isoflurane and transcardially perfused with PBS followed by 4% PFA. For **Paper I**, hind ankle joints were harvested, postfixed 48 h in 4% PFA and decalcified 4-5 weeks in 10% ethylenediaminetetraacetate (EDTA) decalcification solution. The decalcified joints were dehydrated using 70% ethanol and xylene followed by embedding in paraffin. Sagittal sections (5 µm) were cut using a rotary microtome. Prior to labelling, sections were deparaffinized, blocked for endogenous peroxidase and subjected to overnight antigen retrieval in Tris-acetate buffer, pH 6 at 60°C. Sections were then incubated with 5% normal goat serum in 0.2% Triton X-100 PBS to reduce non-specific binding and labelled with the appropriate primary antibodies listed in Table 7. Alkaline phosphatase-conjugated anti-rabbit IgG (1:500) was used for secondary labelling followed by visualization using liquid fast-red substrate kit. Sections were counterstained with Mayer's hematoxylin and mounted in Aquatex mounting medium. Histomophometrical analyses are outlined in details in **Paper I**.

In **Paper IV**, lumbar spinal cord (L1-L6) tissues were dissected, postfixed in 4% PFA for 12-20 h and cryoprotected in 20% sucrose. Tissues were then embedded in OCT and cut into 20 µm sections. Sections were incubated in 5% normal goat serum in 0.2% Triton X-100 PBS to block non-specific bindings, followed by primary antibodies listed in Table 7. Afterwards, sections were labelled with the respective secondary antibodies. Tyramide signal amplification (TSA) was performed to enhance visualization. Stained sections were then mounted in Prolong Gold antifade mountant.

Table 7. List of primary antibodies used for immunohistochemistry

Primary antibody	Vendor/catalog	Dilution	Paper
Rabbit anti-cathepsin K	OriGene, TA318059	1:100	I
Rabbit anti-CD31	Abcam, ab124432	1:250	I
Rabbit anti-PGP9.5	Cedarlane, 184	1:500	I
Rabbit anti-Iba1	Wako, 019-19741	1:2000	IV

4.4.3 *In situ* hybridization

Paraffin-embedded sections were *in situ* hybridized using a modified version of the ACD RNAScope 2.0 high-definition procedure to detect mRNA expression of *Acp5* in **Paper I**. In brief, sections were incubated with pretreatment 1 for 10 min at room temperature, pretreatment 2 for 15 min at 85°C and pretreatment 3 using a pepsin treatment (10% diluted in RNase-free water) for 20 min at 40°C. The subsequent hybridization and amplification were performed according to the manufacturer's protocols, followed by incubation in digoxigenin (DIG)-labeled tyramide (1:200, Perkin Elmer) and labelling with alkaline phosphatase-conjugated anti-DIG FAB fragments (1:1000, Roche). Sections were then visualized with liquid permanent red, counterstained with Mayer's hematoxylin and mounted in Aquatex mounting medium. Histomophometrical analyses are outlined in **Paper I**.

4.4.4 Western blot

Proteins from cell culture in **Paper I** and DRG or spinal cord tissues in **Paper II** and **IV** were separated using gel electrophoresis and transferred to nitrocellulose membrane. Non-specific binding sites were blocked with 5% non-fat milk in Tris based-buffer (50 mmol/L Tris-HCl and 6 mmol/L NaCl with 0.1% Tween 20). Consequently, membranes were incubated overnight with primary antibodies (Table 8) followed by the appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies. Chemiluminescent reagent was used to visualize immunopositive bands detected on ChemiDoc Imaging System. Immunopositive bands were normalized to the bands of the protein reference (GAPDH or β-actin), and results are presented as percentage change to the respective control group.

Table 8. List of primary antibodies used for Western Blot

Primary antibody	Vendor/catalog	Dilution	Paper
Goat anti-A1AT	R&D systems, AF2979	1:2000	IV
Rabbit anti-AAK1	Abcam, ab59740	1:1000	II
Rabbit anti-AP2M1	Abcam, ab75995	1:1000	II
Rabbit anti-pAP2M1 (T156)	Abcam, ab109397	1:1000	II
Mouse anti-β-actin	Cell Signaling, 3700S	1:10000	I
Sheep anti-ELA2	R&D systems, AF4517	1:1000	IV
Mouse anti-GAPDH	Abcam, ab8245	1:10000	II, IV
Goat anti-haptoglobin	ICL, GHPT-90A-Z	1:1000	IV
Rabbit anti-netrin-1	Abcam, ab1267729	1:1000	I
Rabbit anti-PAR2	Abcam, ab180953	1:250	IV
Rabbit anti-STAT3	CST, 12640	1:1000	II
Rabbit anti-pSTAT3 (Y705)	Millipore, 630098	1:1000	II
Rabbit anti-pSTAT3 (S727)	CST, 9134	1:1000	II

4.4.5 Immunoassay

For **Paper I,** serum was collected and levels of C-telopeptide of type I collagen (CTX-I) and propeptide of type I procollagen (PINP) were quantified using the RatLaps CTX-I and Rat/Mouse PINP EIA kits, respectively. Moreover for **Paper I**, platelet-derived growth factor BB (PDGF-BB) and semaphorin 4D (SEMA4D) were also quantified in osteoclast culture supernatant using Mouse/Rat PDGF-BB Quantikine ELISA and Mouse Semaphorin 4D/CD100 ELISA kits, respectively. For **Paper III**, levels of TNF, IL-6, CCL2 and CXCL1 were measured in macrophage culture supernatants using electrochemiluminescence (ECL) immunoassay kits following the manufacturer's instructions.

4.4.6 Liquid chromatography-mass spectrometry

Liquid chromatography-mass spectrometry (LC-MS/MS) was performed in **Paper IV** to explore differential protein profile in male and female mice following i.t. injection of disulfide

HMGB1 with or without minocycline. Total protein was extracted from the lumbar spinal cord (L3-L5) with repetitive freeze/thaw process and sonication in lysis buffer (PBS with 1% SDS and a cocktail of protease inhibitors). Protein samples were further alkylated, reduced and enzymatically digested in endoproteases Lys-C and trypsin. The resulting peptide samples were labelled using TMT6 plex isobaric label reagent set according to the manufacturer's instructions and fractionated using high pH reverse-phase liquid chromatography. Afterwards, the fractions were analyzed by high-resolution nano LC-MS/MS. The LC-MS/MS procedure are described in details in **Paper IV**.

4.5 STATISTICAL ANALYSES

Statistical tests were performed using GraphPad Prism. Results were analyzed using unpaired student's t-test (for differences between two groups), one-way analysis of variance (for multiple groups), and two-way analysis of variance (for multiple group time course experiments). For LC/MC-MS data, statistical analyses were performed on R. Specific details on the data analysis can be found in **Paper IV**. All data are presented as mean ± standard error of mean (SEM) and p-values less than 0.05 were considered statistically significant.

5 RESULTS AND DISCUSSION

5.1 CONTRIBUTION OF OSTEOCLASTS TO REFRACTIVE PAIN IN THE COLLAGEN ANTIBODY-INDUCED ARTHRITIS MODEL

Pain and inflammation in RA do not always coincide. Joint pain (arthralgia) may precede joint inflammation or persists even after successful anti-inflammatory treatments (86-88, 108). Clinical evidence shows that the presence of eroded bones in the joints can outlast disease activity (216, 217). However, it has not been thoroughly studied if these observations are coupled to persistent pain in RA. Thus, the aim of **Paper I** was to investigate the contribution of the bone degrading cells, osteoclasts, in maintaining long-lasting pain in experimental RA.

The CAIA model (described in 2.2.4) was utilized to investigate RA-associated nociception in this study. An important aspect of this model from a pain perspective is that mechanical hypersensitivity is detected before, during and after the CII antibody-induced flare of joint inflammation (referred to as the late phase) (13, 75, 97, 134, 135). Additionally, histological signs of bone loss in the ankle joint are still present several weeks after joint inflammation has resolved (134). Therefore, the mechanistic link between refractive bone loss and pain in RA can be studied in this model.

Micro-CT analyses confirmed that bone loss is present in the ankle joint in both inflammatory and late phases of the CAIA model. Intriguingly, erosive lesions were found to be most prominent and consistent in the calcaneus (**Paper I**, Figure 1). The reason for which erosions are inclined to certain bone compartments remains to be elucidated. A possible explanation is that CAIA induced changes in gait, which altered mechanical loading in a way that promoted bone loss prominently in the heel. Gait analyses are therefore warranted in future studies, though this may be difficult due to the polyarthritic feature of the model. Based on these observations, we focused on the calcaneus in subsequent studies.

To identify if persistent bone loss in the CAIA model is due to constant overactivation of osteoclasts or is a remnant of the inflammatory processes, several experiments were carried out (**Paper I**, Figure 2). First, osteoclast numbers were determined in whole ankle joint samples and calcanei, which indicated that there were higher osteoclast numbers in the inflammatory but not the late phase. Second, assessment of serum markers for bone resorption and formation only revealed enhanced resorptive activity during inflammation and no changes in bone formation. Third, higher bone turnover was observed only in the inflammatory phase of the CAIA model as measured by ^{99m}Tc-HMDP scintigraphic imaging. Fourth, mRNA levels of genes that encode for the acidification machinery (*Tcirg1* and *Clcn7*) were increased in the ankle joint in the inflammatory but not the late phase. Interestingly, there were no changes in expression of acid sensing receptors (*Asic3* and *Trpv1*) in the DRGs in both phases of the CAIA model. Collectively, these findings show that increased osteoclast activity only coincide with joint inflammation and that acidification is not likely involved in mediating long-lasting nociception in the CAIA model.

In order to delineate the link between osteoclast activity and pain in the CAIA model, the effect of two different osteoclast inhibitors were examined: zoledronate and the cathepsin K inhibitor T06. None of these inhibitors elicited anti-inflammatory effects in the CAIA model or antinociceptive effects during inflammation (**Paper I**, Figure 3). Interestingly, both drugs reversed pain-like behavior in the late phase. Systemic chronic or short treatment with zoledronate alleviated mechanical hypersensitivity in the late phase (Figure 4A, B). Similarly, oral administration of T06 in the late phase resulted in normalization of paw withdrawal thresholds (Figure 4C). These results show intriguingly that while resorption is elevated during inflammation, blocking the actions of osteoclasts was only antinociceptive in the late phase. We therefore speculate that osteoclasts can drive pain signaling without resorbing bones.

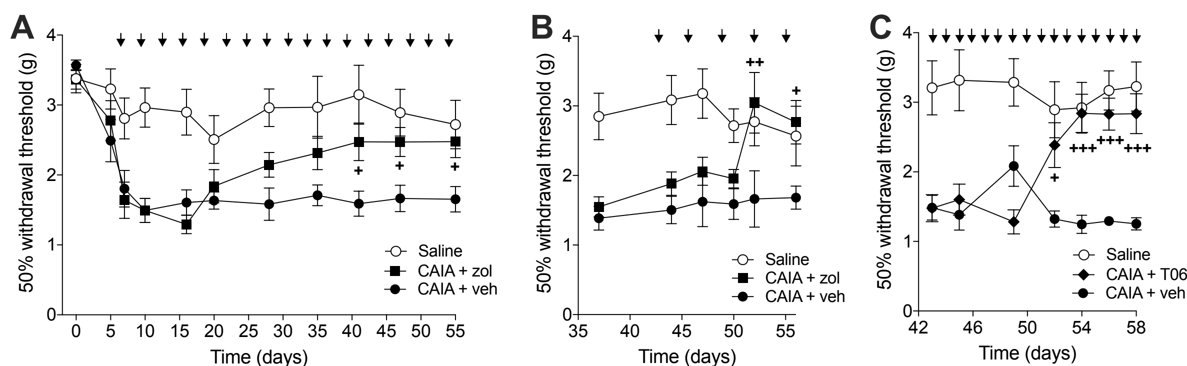

Figure 4. The osteoclast inhibitor zoledronate (zol) and T06 alleviate mechanical hypersensitivity in the late phase of the CAIA model. Paw withdrawal thresholds following s.c. injection of zoledronate (100 µg/kg) on days 6-56 (A) and 43-56 (B), and oral gavage of T06 (40 mg/kg) on days 43-56 (C). Data are presented as mean ± SEM, n=5-11 mice/group, $^{+}p<0.05$, $^{++}p<0.01$, $^{+++}p<0.001$ for CAIA + osteoclast inhibitor vs. CAIA + vehicle (veh). Arrows represent drug delivery. Adapted from **Paper I**, Figure 3.

Several reports show that both zoledronate and T06 do not selectively target osteoclasts when it comes to pain mechanisms. Bisphosphonates were reported to act on microglia, which led to attenuation of pain-like behavior in the chronic constriction nerve injury model (39). However, microglial inhibition only reverse CAIA-induced pain-like behavior in male mice (13) and as female mice were used in this study, it is unlikely that the behavioral effects of zoledronate are due to microglial inhibition. Studies also show that zoledronate can polarize macrophages towards the M1 pro-inflammatory phenotype in male rats, which if it also takes place in female mice, would promote nociceptor sensitization rather than reduce it (218). Furthermore, the pain-relieving effects of T06 have been associated with its actions in the spinal cord, though the cellular target was not identified (219). While it cannot be excluded that zoledronate and T06 have off-target effects contributing to their analgesic effects, it seems unlikely that two different drugs with different off-target effects would have similar antinociceptive profile. Both drugs lacked effect during the inflammatory phase and required 7-10 days exposure before reversing hypersensitivity in the late phase. In addition, as micro-CT analyses demonstrated that both drugs induce bone protective effects (**Paper I**, Figure S2), it is tempting to conclude that their antinociceptive effects are mediated via inhibition of osteoclasts.

Emerging data show that in addition to bone resorption, osteoclasts produce factors that regulate inflammation (*e.g.* IL-8), angiogenesis (*e.g.* PDGF-BB) and neurogenesis (*e.g.* Sema4D, netrin-1) (7, 58, 60, 96, 220). Some of these factors have been associated with nociceptive signaling (7, 220). As our data did not point to the involvement of erosive activity and acidification in late phase CAIA, the ability of osteoclasts to mediate vascularization and innervation and if these processes lead to nociception were explored in this study.

First, vascularization and innervation of the calcaneus were analyzed in the inflammatory and late phase of the CAIA model as well as the effect of both zoledronate and T06. Vascular and nerve profile densities were elevated during inflammation and remained increased in the late phase. Notably, these alterations were attenuated by both osteoclast inhibitors but only following resolution of joint inflammation (**Paper I**, Figure 4).

Second, a series of *in vitro* experiments were conducted using mouse bone marrow-derived primary osteoclast culture to identify if these cells are able to produce factors that can modulate bone vascularization and innervation. Mature osteoclasts release PDGF-BB, Sema4D and netrin-1. Exposure to either zoledronate or T06 resulted in decreased PDGF-BB levels due to both inhibitors, while the reduction of Sema4D and netrin-1 protein levels were only achieved by zoledronate and not T06 (**Paper I**, Figure 5).

Recent studies point to the crucial role of osteoclast-derived netrin-1 in modulating nociception (59, 220, 221). Thus, the involvement of this factor in CAIA-induced hypersensitivity was assessed. Repeated systemic injection of the anti-netrin-1 antibody (NP137) produced no effects on arthritis scores or pain-like behavior in the inflammatory phase (**Paper I**, Figure 6), but was antinociceptive in the late phase (Figure 5). Furthermore, *in vitro* calcium imaging experiments with primary DRG neurons demonstrated that direct netrin-1 application enhanced the intracellular Ca^{2+} signal response rate in a dose-dependent manner (**Paper I**, Figure 6).

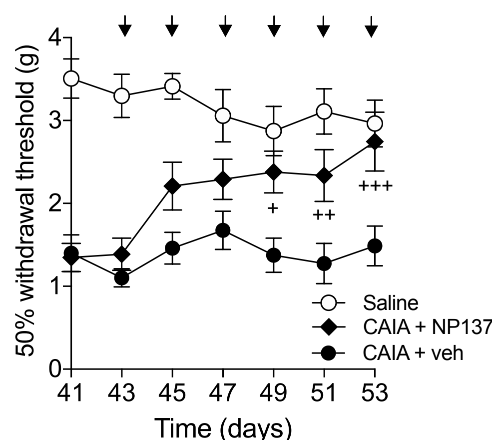

Figure 5. Netrin-1 induces pronociceptive effects in the CAIA model. Paw withdrawal thresholds following i.p. injection of the anti-netrin-1 antibody (NP137, 10 mg/kg) on days 43-53. Data are presented as mean ± SEM, n=9-10 mice/group, $^{+}p<0.05$, $^{++}p<0.01$, $^{+++}p<0.001$, where $^{+}$represents CAIA + NP137 vs. CAIA + vehicle (veh). Arrows represent drug delivery. Adapted from **Paper I**, Figure 6.

The findings in this study together allude to the divergent pronociceptive actions of osteoclasts throughout the different phases of the CAIA model (Figure 6). In addition to acidification that was found to be present during inflammation, the results show the possibility that osteoclasts exert different actions that may lead to nociception, particularly during a low bone resorptive state. For instance, they could i) directly activate sensory neurons through release of netrin-1 that may bind to one of its receptors, UNC5B, which was reported to be expressed on nociceptors in the publicly available Pain-seq dataset (20), ii) mediate neurite growth in the bone, possibly through production of Sema4D and netrin-1 and (iii) promote vasculogenesis by secreting PDGF-BB, which in turn supports neoinnervation (222).

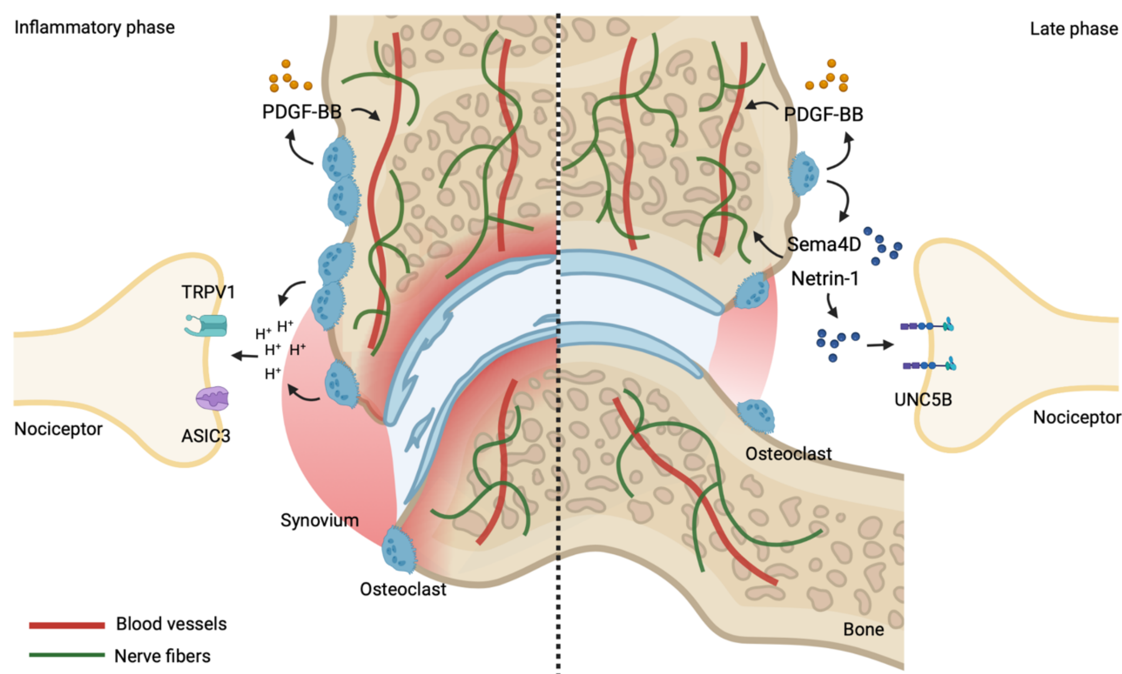

Figure 6. Osteoclasts modulate different pronociceptive actions in the inflammatory and late phase of the CAIA model. During inflammation, resorbing osteoclasts induce acidification through release of protons that may activate sensory neurons by binding to acid sensing nociceptors, ASIC3 and TRPV1. Increased bone vascularization and innervation are also observed during this phase (right panel). In the late phase, non-resorbing osteoclasts release netrin-1 that could directly activate sensory neurons by binding to its receptor, UNC5B expressed on nociceptors or by stimulating neurite outgrowth in the bone. Non-resorbing osteoclasts can also release PDGF-BB and Sema4d which can increase innervation and vascularization, which also supports neoinnervation (left panel). Created in Biorender.com

Overall, this study highlights the complexity of osteoclast activity in nociception and presents the concept that their pronociceptive actions are not completely coupled to resorptive activity. In addition, this study shows the beneficial effects of inhibiting osteoclast activity or nociceptive factors released by these cells for pain-like behavior in the CAIA model, especially in the refractive state. In a translational perspective, this study illustrates the potential use of osteoclast inhibitors to treat long-lasting pain in RA.

5.2 BARICITINIB REDUCES REFRACTIVE PAIN IN THE COLLAGEN ANTIBODY-INDUCED ARTHRITIS MODEL THROUGH AAK1 INHIBITION

JAK/STAT inhibitors such as tofacitinib and baricitinib are the latest disease-modifying drug class to emerge for the treatment of RA. A recent Phase III clinical trial showed that baricitinib significantly improved joint pain and physical function in RA patients (117-119). More interestingly, the pain-relieving property of baricitinib on these patients was not correlated with its anti-inflammatory effects (118). This suggests that baricitinib is also targeting non-inflammatory aspects of pain mechanisms and may therefore be beneficial for RA patients with refractive pain.

The aim of **Paper II** was to characterize the antinociceptive effect of baricitinib in the CAIA model as well as to delineate its underlying mechanism. Originally this study was intended to further explore the contribution of osteoclasts in RA-induced pain as baricitinib is known to reduce pathological bone loss (223, 224). However, it has been shown that baricitinib does not directly act on osteoclasts but rather inhibits bone loss via activation of osteoblast function (113). Since we did not find indications of altered bone formation in the CAIA model in **Paper I** and that baricitinib did not prevent CAIA-induced bone loss (data not shown), we decided to examine other mechanisms for this study.

Baricitinib significantly reduced joint inflammation in CAIA animals (**Paper II**, Figure 1), which is in accordance with animal studies showing clear anti-arthritic effects of this drug on rodents subjected to arthritis (113, 225). Surprisingly, baricitinib induced very modest effect on mechanical hypersensivity during inflammation (**Paper II**, Figure 1), but was considerably effective in reversing pain-like behavior in the late phase (Figure 7). These findings are in agreement with clinical evidence showing that the pain-relieving effects of baricitinib do not completely correlate with levels of inflammatory markers and that this drug is also beneficial for pain in RA patients with minimal disease activity (119, 120).

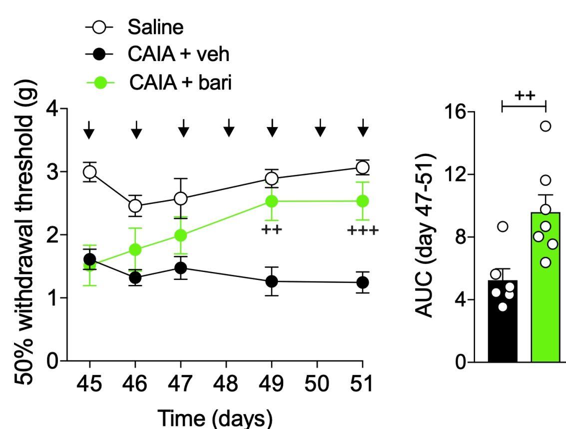

Figure 7. Baricitinib reverses mechanical hypersensitivity in the late phase of the CAIA model. Baricitinib (40 mg/kg, b.i.d) was administered per oral gavage on days 45-51. Paw withdrawal thresholds and quantification for area under the curve (AUC) for withdrawal thresholds of CAIA animals from days 47-51. Data are presented as mean ± SEM, n=5-7 mice/group, $^{++}p<0.01$, $^{+++}p<0.001$ for CAIA + vehicle (veh) vs. CAIA + baricitinib (bari). Arrows represent oral administration. Adapted from **Paper II**, Figure 1.

The JAK/STAT signaling regulates multiple cytokine pathways, many of which are implicated in pain (122). Thus, we speculate that baricitinib blocks pain signaling through interference with cytokine receptor signal transduction and production. Alterations in the downstream STAT3 signaling have been reported in models of inflammatory and neuropathic pain (226-228). Interestingly, we failed to find increased activation of STAT3 in DRGs or spinal cords in the late phase of the CAIA model, though baricitinib reduced basal levels of phospho-STAT3 level (**Paper II**, Figure 2). These findings suggest that the STAT3 pathway is not the main target of the antinociceptive action of baricitinib in the late phase CAIA.

In addition to JAKs, baricitinib was recently discovered as an inhibitor for the adaptor protein-2 (AP-2) associated kinase 1 (AAK1) (123). AAK1 regulates clathrin-mediated endocytosis through phosphorylation of the adaptor protein AP2 on the μ subunit (encoded by *Ap2m1* gene) (229). This finding was important for identification of potential drugs for COVID-19 as it is predicted that AAK1/clathrin-mediated endocytosis paves way for viral entry into the lung cells. Interestingly, AAK1 has also been implicated in pain and electrophysiological recordings showed the spinal cord as a possible site of pronociceptive action for this protein (125). Based on this, experiments were carried out to investigate if AAK1 is involved in the pain-modulating actions of baricitinib in the CAIA model.

First, evaluating mRNA levels of *Aak1* and its downstream target *Ap2m1* in ankle joints, DRGs and spinal cords from CAIA mice showed upregulation exclusively in DRGs from the late phase (**Paper II**, Figure 3). Second, there was a differential regulation of the AAK1/AP2M1 axis on protein levels in the late phase of the CAIA model, whereby it is induced in DRGs but inhibited in spinal cords. The presence of baricitinib normalized phosphorylation and protein expression of AP2M1 in DRGs but not in spinal cords (Table 9). While these findings indicate an involvement of the AAK1/AP2M1 axis in the regulation of pain in the CAIA model, the results point to a more prominent role of these proteins in DRGs rather than in spinal cords as previously suggested (125). Intriguingly, in the publicly available Pain-seq database both *Aak1* and *Ap2m1* mRNA levels are upregulated in DRG neurons from models of nerve injury or chemotherapy-induced pain (20). This supports the hypothesis that AAK1 and AP2M1 have pronociceptive roles in DRGs. Thus, we speculate that there is a temporal regulation and site specific function of AAK1 signaling at the level of DRG compared to the spinal cord in the CAIA model, which will be explored in future studies.

The downstream consequences of AAK1 activation in relation to pain remain to be elucidated. One group identified a possible interplay between AAK1 and the noradrenergic pain pathway since antagonism of α2-adrenergic signaling blocked the antinociceptive effects of AAK1 inhibition (125). Another group reported a potential role of AAK1/AP2M1 in regulation of neuronal excitability as endocytosis of sodium-activated potassium channels in DRG neuronal cultures and spinal cord slices is mediated by the AAK1-activated adaptor protein AP2 (230). Though further studies are required to better understand the role of AAK1 in pain, this work highlights the possibility that AAK1 and clathrin-mediated endocytosis play an important role

in non-inflammatory hypersensitivity and the mechanisms targeted by the antinociceptive actions of baricitinib.

Table 9. Phospho-AP2M1, APM1 and AAK1 protein levels in DRGs and spinal cords of CAIA mice treated with vehicle (veh) or baricitinib (bari) to saline control (adapted from **Paper II**, Figure 4)

	pAP2M1	AP2M1	AAK1
DRG			
CAIA + veh	↑	↑	=
CAIA + bari	=	=	=
Spinal cord			
CAIA + veh	↓	=	=
CAIA + bari	↓	=	=

The urgent need for identification of alternative drugs for COVID-19 has revealed baricitinib as not only a classic JAK/STAT inhibitor, but also an inhibitor of other kinases. This study provides insights that the antinociceptive effects of baricitinib are mediated by the AAK1/AP2M1 axis in the DRGs. More importantly, baricitinib was able to reverse hypersensitivity in post-inflammatory arthritic conditions thus indicating its therapeutical potential for pain management in RA patients with refractive pain.

5.3 SEX- AND CELL-DEPENDENT CONTRIBUTION OF HIGH MOBILITY GROUP BOX 1 PROTEIN AND TLR4 IN ARTHRITIS-INDUCED PAIN

Spinal high mobility group box 1 protein (HMGB1) serves important roles in pain processing in the CAIA model (75), however the involvement of peripheral HMGB1 has not yet been examined. **Paper III** aimed to investigate the role of peripheral HMGB1 in RA-induced pain and explore if sex contributes differentially to nociception in this context. We reasoned that examination of sex differences are especially important in studies exploring links between HMGB1 and pain as HMGB1 is a ligand for TLR4 and the role of TLR4 in pain has been associated with sex dimorphism in rodents (70).

In accordance with previous work (75), no significant differences were observed in terms of arthritis severity and mechanical hypersensitivity between male and female mice subjected to CAIA. Following CAIA induction, *Hmgb1* mRNA levels were increased in the ankle joints in both sexes (**Paper III**, Figure 1). Intriguingly, while inhibiting the action of peripheral HMGB1 with the neutralizing antibody 2G7 produced no anti-inflammatory effects in males and females (**Paper III**, Figure 1), pain-like behavior was reversed in only male mice (Figure 8). This result is rather surprising given that systemic TLR4 antagonist produced beneficial effects in both sexes of mice injected with formalin (78). One possible explanation is that HMGB1 may act through other receptor systems which have more prominent roles in females than males. Future studies are thus warranted to explore this hypothesis.

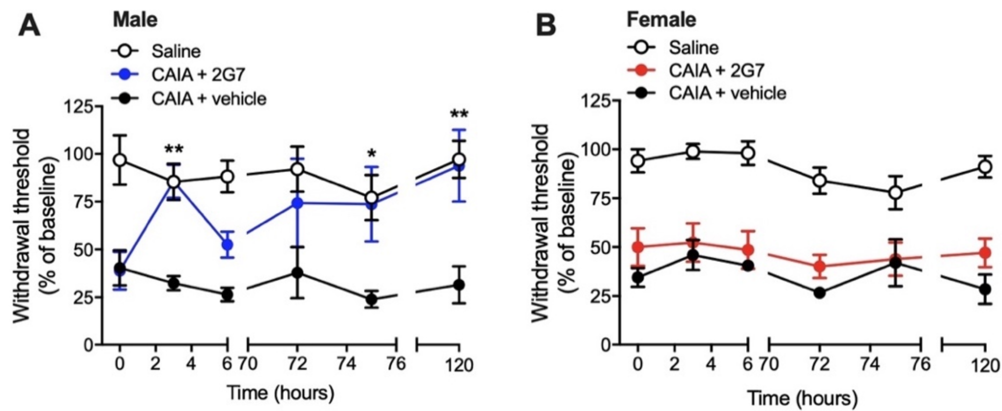

Figure 8. Anti-HMGB1 antibody (2G7) reverses CAIA- induced pain-like behavior in male but not in female mice. Mechanical hypersensitivity (A, B) following s.c. injection of 2G7 (100 μg/mouse) or vehicle once a day on day 12-16 in male and female mice. Data are presented as mean ± SEM, n=6 mice/group, *p<0.05, **p<0.01 for CAIA + 2G7 vs. CAIA + vehicle group. Adapted from **Paper III**, Figure 1.

The above results prompted us to further characterize the pronociceptive role of HMGB1 in the ankle joint. First, we observed injection of disulfide, but not all-thiol, HMGB1 into the ankle joint evoked mechanical hypersensitivity in male and female mice (**Paper III**, Figure 3). This indicates that the ability of local HMGB1 in the joint to produce nociception is redox-dependent rather than sex-dependent. Second, we showed that disulfide HMGB1 stimulation led to greater inflammatory response in male compared to female mice as observed by assessment of cytokine and chemokine expression in the joint following injection (**Paper III**, Figure 4) as well as release of these factors in cultured macrophages (**Paper III**, Figure 6). Third, we found indications that these processes could be mediated by resident macrophages in the joint, which appeared to be more strongly implicated in males than females. This is because blockade of macrophages with minocycline protected male but not female mice from developing disulfide HMGB1-induced hypersensitivity (**Paper III**, Figure 7). The results are aligned with previous studies showing that while TLR4 agonist in the periphery induced nociceptive behaviors in both sexes (70), TLR4 activation led to a more pronounced inflammatory response in males versus females (231, 232).

Having observed the involvement of immune cells in HMGB1-induced pain, we asked the question if HMGB1 can directly activate nociceptors since TLR4 is also expressed in these cells (25). Using mice with specific TLR4 deletion in myeloid cells including macrophages (LysM-TLR4$^{fl/fl}$) and nociceptors (Nav1.8-TLR4$^{fl/fl}$), we found that TLR4 ablation in myeloid cells induced greater protection against HMGB1-induced hypersensitivity in males compared to females (Figure 9A, B). However, deletion of TLR4 on sensory neurons prevented HMGB1-induced pain-like behavior in both sexes (Figure 9C, D). These results did not point to an exclusive cell-specificity of HMGB1 actions in the joint, but it demonstrated a greater contribution of myeloid cells possibly macrophages to nociceptive mechanisms in males compared to females.

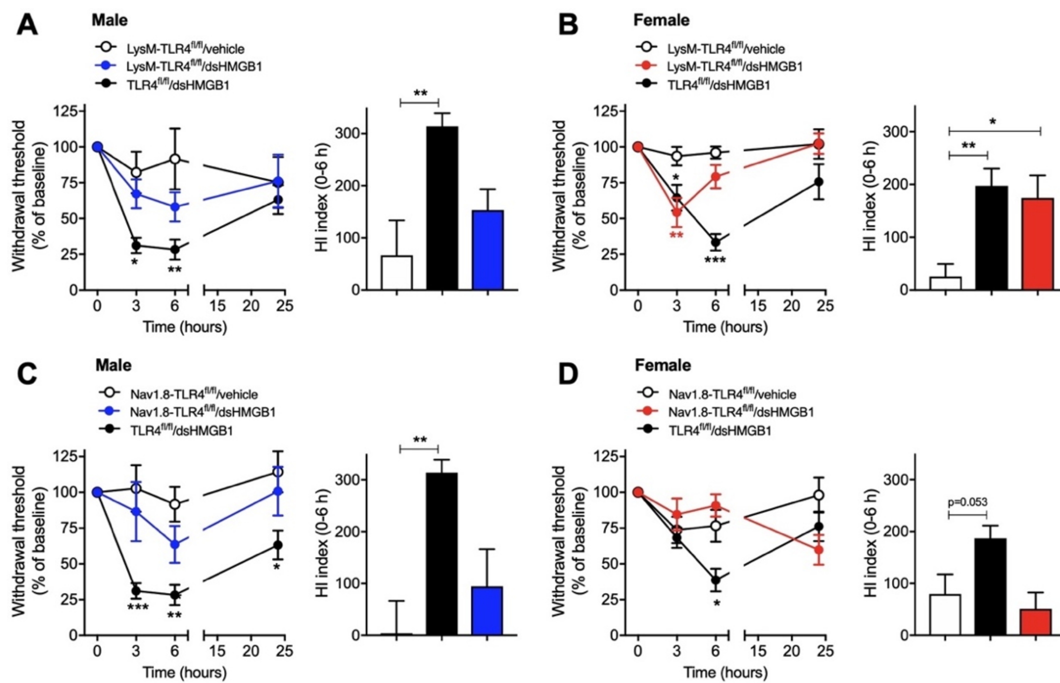

Figure 9. TLR4 deletion in nociceptors prevents HMGB1-induced hypersensitivity in both sexes while depletion in myeloid cells only protects males. Withdrawal response and hyperalgesic (HI) index (0-6h) after i.a. injection of disulfide HMGB1 (dsHMGB1, 1 μg/mouse) or PBS (vehicle) in mice with TLR4 deletion in nociceptors (Nav 1.8-TLR4$^{fl/fl}$) (A, B) or myeloid cells (LysM-TLR4$^{fl/fl}$) (C, D). Data are presented as mean ± SEM, n=5-8 mice/group, *p<0.05, **p<0.01, ***p<0.001 for comparisons with the vehicle group. Adapted from **Paper III**, Figure 8.

With respect to the involvement of macrophages in HMGB1-induced pain, we cannot exclude the contribution of other cells. This is because minocycline, which was used to block activation of resident macrophages in the periphery, is known to have inhibitory effects on T cells and neurons (22). In addition, the LysM promoter used to delete cell-specific TLR4 in mice are not exclusive to macrophages, but also expressed by other myeloid cells (233). Importantly, however, we show that disrupting the activation of immune cells was more effective in protecting males than females from developing HMGB1-induced pain-like behavior.

On the subject of RA-induced pain, only few studies have made side-by-side comparisons between male and female mice (13, 75, 234, 235). Herein, we demonstrate that while peripheral disulfide HMGB1 can induce joint hypersensitivity in both male and female mice, HMGB1-TLR4 interactions in immune cells contributes more prominently to this mechanism in male mice. Though more studies are needed to uncover the underlying mechanism, this work suggests that targeting the immune system will be more successful for treating pain in males with RA.

5.4 SEX-DEPENDENT ROLE OF MICROGLIA IN DISULFIDE HIGH MOBILITY GROUP BOX 1 PROTEIN-MEDIATED MECHANICAL HYPERSENSITIVITY

As stated above, HMGB1 is regarded as a crucial player in the spinal regulation of chronic pain. Earlier work showed that HMGB1-induced microglial activation in the spinal cord is associated with pain-like behavior observed in the animals (75). However, sex dimorphism was not addressed in this study. Since **Paper III** indicated a sex-dependent role of HMGB1 in the periphery and several studies showed the role microglia in pain to be sex-specific (12, 13), the aim of **Paper IV** was to investigate if disulfide HMGB1 induces nociception via microglial activation in a sex-dependent fashion.

Following spinal injection of disulfide HMGB1 in both male and female mice, microglia displayed signs of activation to the same extent in both sexes in the form enlarged cell bodies and Iba-1 immunoreactivity (**Paper IV**, Figure 1). This supports previous studies showing equal levels of microglial activation in both sexes subjected to nerve injury (70, 236). Another feature of activated microglia is the production of pro-inflammatory factors (237, 238). Intriguingly, direct stimulation of disulfide HMGB1 on cultured microglia induced more pronounced mRNA expression of inflammatory factors in male- versus female-derived cells (**Paper IV**, Figure 2). This shows that the proinflammatory response of microglia to HMGB1 stimulation is sex-dependent which is in congruent with previous work showing that TLR4 activation by LPS evokes higher mRNA levels of *Il1b* in male microglia (239).

To delineate if spinal microglia display a sex-dependent role in pain mechanisms mediated by HMGB1, microglia activation was inhibited by either genetic or pharmacological manipulations. First, specific TLR4 deletion in myeloid-derived cells including microglia (LysM-TLR4$^{fl/fl}$) in the spinal cord protected male but not female mice from developing hypersensitivity due to intrathecal disulfide HMGB1 (Figure 10A, B). Second, co-injection of minocycline (microglial inhibitor) with disulfide HMGB1 produced antinociceptive effects only in male mice (Figure 10C, D). Both of these results indicate a response that follows a sexually dimorphism pattern.

The use of LysM-Cre to target microglia has been debated since LysM is also expressed by other myeloid cells such as macrophages (205) and expressed only in a subpopulation of microglia (20-45% of total population) (240, 241). However, several studies including our own show that microglia are the predominant myeloid cells present in naïve spinal cord (13, 242, 243), and therefore TLR4 depletion in LysM-TLR4$^{fl/fl}$ mice is highly specific for microglia. Notably, targeting this subset of microglia was sufficient enough to reveal sex dimorphism in our study. Furthermore, results from the minocycline experiments have to be treated with cautions since this drug has well-documented anti-inflammatory actions that are not restricted to microglia, but also T cells, neutrophils, neurons and astrocytes (22). Accumulating evidence, however, shows that minocycline produces sex-dependent antinociceptive effects on different models of pain (12, 13, 79, 244-246) as presented here, which demonstrate that there are important sex differences between male and female pain mechanisms.

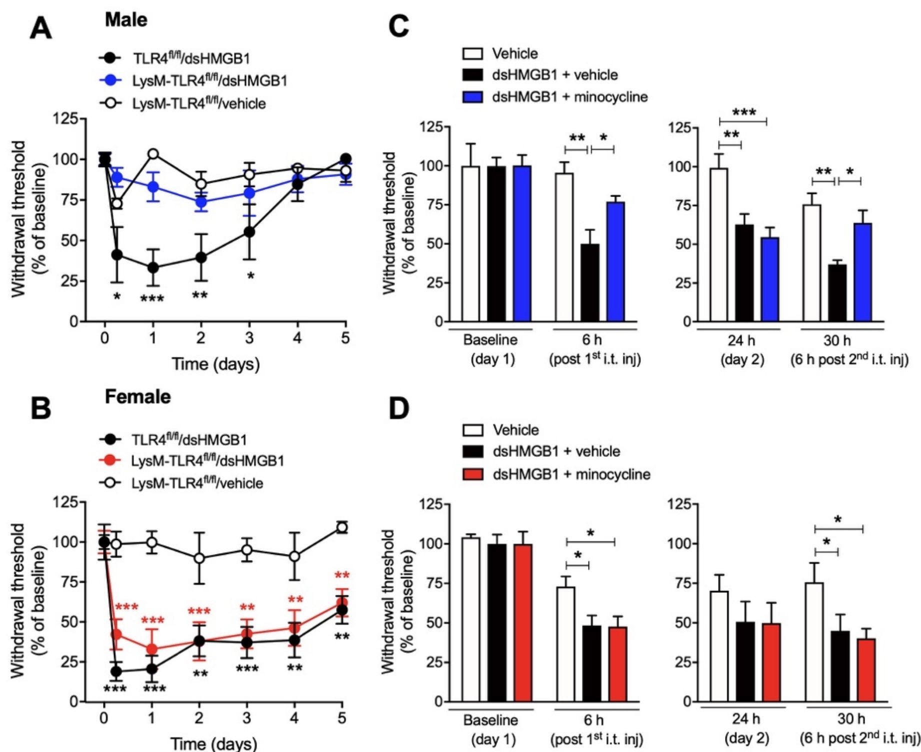

Figure 10. Blockade of microglial activity is antinociceptive for male but not female mice injected with disulfide HMGB1 (dsHMGB1) into the spinal cord. Withdrawal response after i.t. injection of dsHMGB1 (1 µg/mouse) in male and female mice lacking TLR4 in microglia (LysM-TLR4$^{fl/fl}$) (A, B). Withdrawal response before and after i.t. injection of of dsHMGB1 (1 µg/mouse) mixed with minocycline (30 µg/mouse, microglial inhibitor) or dsHMGB1 (1 µg/mouse) mixed with vehicle (PBS), as well as 6 h after the second day i.t. injection of minocycline (30 µg/mouse) or vehicle in male and female mice (C, D). Data are presented as mean ± SEM, n=4-8 mice/group, *p<0.05, **p<0.01, ***p<0.001 for comparisons with the vehicle group. Adapted from **Paper IV** Figure 3.

In-depth proteomics of the spinal cord with LC/MS-MS was therefore used to further explore the downstream proteins regulating sex differences in response to minocycline treatment. An interesting pattern observed among the proteins identified is the dominance of anti-inflammatory and antinociceptive factors, which were upregulated in the male mice group that received minocycline treatment, especially three isoforms of alpha-1-antitrypsin that were differentially expressed between sexes (**Paper IV**, Figure 5).

We were particularly interested in exploring the role of alpha-1-antitrypsin (A1AT) in nociception since an earlier report has indicated an antinociceptive role of A1AT in an osteoarthritis-induced pain model (247). Notably, alpha-1-antitrypsin partially protected male but not female mice from developing hypersensitivity following co-injection with HMGB1 (Table 10). Previous reports show that the antinociceptive effects of alpha-1-antitrypsin are mediated by inhibition of both neutrophil elastase and proteinase-activated receptor 2 (PAR2) (247, 248). Interestingly, our data do not completely support this linkage as suppression of neutrophil elastase did not block the development of HMGB1-induced hypersensitivity in both male and female mice (Table 10). On the other hand, global PAR2 deletion prevented disulfide HMGB1-induced pain in both sexes (Table 10). While the mechanism is still not clear, we hypothesize that since alpha-1-antitrypsin can inhibit other proteases (249), blocking elastase

was not sufficient to dampen the pronociceptive effects of HMGB1. Furthermore, we speculate that disulfide HMGB1 may activate different signaling axis in male and female mice which may converge in PAR2, and that this pathway was affected by minocycline treatment in male mice.

Table 10. The effect of alpha-1-antitrypsin, neutrophil elastase inhibition and PAR2 depletion on HMGB1-induced pain-like behavior in male and female mice (Adapted from **Paper IV**, Figures 6, 7**)**

	Male	Female
Alpha-1-antitrypsin	Partial protection	No protection
Sivelestat (neutrophil elastase inhibitor)	No protection	No protection
PAR2 knock-out	Full protection	Full protection

Taken together, the findings of this study support previous work showing a sexually dimorphic role of microglia in pain processing. Of importance, the proteomics approach has uncovered an interesting pattern of protein expression, which suggests differential microglial function between male and female mice in response to minocycline. That is, minocycline elevates anti-inflammatory and antinociceptive factors in only male mice, but that there may be points of convergence in both sexes. Most certainly, several of the identified proteins represent intriguing targets for future pain studies to further understand sex dimorphism.

6 CONCLUSIONS AND FUTURE PERSPECTIVES

Pain is a debilitating feature of RA and the multifactorial nature of its pathogenesis has hindered effective pain managements in patients. This thesis has focused on dissecting the underlying mechanisms of pain in RA, in particular the chronic state. Overall, this thesis proposed three novel mechanisms that may regulate nociception in RA (Figure 11).

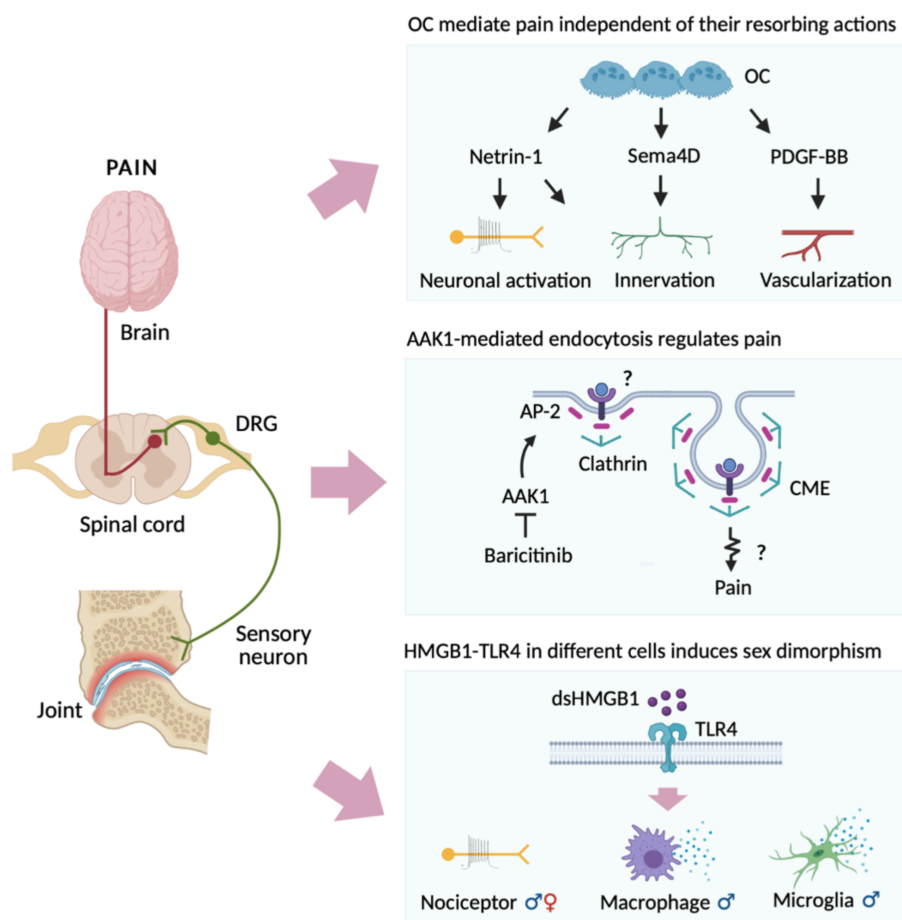

Figure 11. Bone and neuroinflammation-associated mechanisms mediate pain in RA. Using the disease-relevant CAIA model, three novel mechanisms driving long-lasting pain in RA are proposed in this thesis. These mechanisms include: (i) the role of osteoclasts (OC) in nociception that is independent of their resorptive activities; (ii) the role AAK1/AP2M1 in clathrin-mediated endocytosis (CME) that is inhibited by the JAK/STAT inhibitor baricitinib; (iii) the role of peripheral and spinal disulfide HMGB1 (dsHMGB1) that regulate sex-specific pain mechanisms. Created in Biorender.com.

The first mechanism proposed is the important contribution of osteoclasts in RA-induced pain, which is independent of their resorbing actions, as described in **Paper I**. In prospective, the findings in this study imply for the potential use of osteoclast-blocking as therapeutic intervention for RA patients with refractive pain. While this is possible with osteoclast inhibitors that have broad mechanism of actions, this study also investigated the possibility that similar outcomes can be achieved by inhibiting nociceptive factors specifically released by

osteoclast such as netrin-1. An anti-netrin-1 antibody is currently undergoing Phase II clinical trials for treatments of gynecological cancers (250, 251) and it has shown an excellent safety profile that may be compatible with examining the potential of netrin-1 inhibition in RA patients with chronic pain. In order to reach these goals, future studies are warranted to further explore the translational perspective of these findings. For instance, evaluating factors that we found to be produced by osteoclasts in human samples would be relevant.

Expanding on the knowledge of refractive pain in RA, **Paper II** proposed AAK1 and AP2M1 to be novel players in the scene. While more studies are needed to understand the downstream consequence of AAK1/AP2M1 activation, this study presents the important observation that baricitinib being an inhibitor for this pathway is more effective in counteracting pain sensitivity in a non-inflammatory state. As clinical evidence also points to the non-inflammatory actions of baricitinib as a pain-relieving drug in patients (118, 120), subsequent studies are required to understand if this drug can be used to treat patients with remaining pain. In addition, this study points to AAK1 and AP2M1 as potential targets for pain in RA, especially as the AAK1 inhibitor LX9211 is now currently clinically assessed for indications of neuropathic pain (252, 253). Whilst the role of clathrin-mediated endocytosis is relatively unexplored in pain studies, a recent report on primate sensory neurons shows that genes associated with this mechanism are identified in peptidergic neurons and highly correlates to different human chronic pain conditions (254). This may suggest that clathrin-mediated endocytosis is an integral aspect of pain signaling, which should be further dissected.

In **Paper III** and **IV**, it was proposed that disulfide HMGB1-TLR4 signaling in different cells may lead to sex-specific differences in pain mechanisms. In the periphery, **Paper III** showed the importance for this molecular interaction in nociceptors for both males and females, while interactions in immune cells were more important for males. In the spinal cord, **Paper IV** illustrated that activation of TLR4 by disulfide HMGB1 in microglia played greater roles for pain signaling in male versus female mice. Though this data is important from a translational perspective as it points to HMGB1 as a potential target for pain relief, it also brings light that such approach would require deepening our perception of sex differences associated with TLR4. Together, these findings highlight the importance of including both sexes and the perception that the effect of blocking HMGB1 on one sex does not always translate into the same effect on the other sex.

An important aspect of this thesis that remains to be elucidated is the notion if the proposed mechanisms in **Paper I-IV** are somewhat linked to one another. Thus, the following questions are relevant to address for future research:

Are there links between endocytosis, vascularization and innervation in the CAIA model?

We have preliminary data indicating that low dose baricitinib reduced *Sema4d* and *Ntn1* mRNA levels in ankle joints from late phase CAIA mice. Previous studies show that AAK1 inhibits neuregulin-1 (NRG1)-mediated neuritogenesis (255). While one could argue that this contradicts our findings, it is not known if NRG1 is involved in innervation in the CAIA model

and if AAK1 produces different effects on netrin-1 activity. Thus, exploring the connection between AAK1, netrin-1 and innervation may reveal if the antinociceptive actions of baricitinib is also through modulation of AAK1-mediated innervation.

Recent studies show that SARS-Cov-2 spike protein inhibits vascular endothelial growth factor-A/neuropilin-1-receptor (VEGF-A/NRP1) signaling to dampen pain and may therefore increase disease transmission in asymptomatic individuals (256). Besides being pronociceptive factors, VEGF-A/NRP1 are also angiogenic factors. Interestingly, it was shown that VEGF-A induces NRP1 endocytosis, which is essential for activation of nociceptive signaling (257). Therefore, it may be possible that in addition to supporting neoinnervation, angiogenic factors could also control endocytic trafficking of proteins that are involved in pain signal transmission, and this warrants further investigations.

Does HMGB1 regulate the pronociceptive actions of osteoclasts?

HMGB1 is known to promote osteoclast activity (170). Although not studied within this thesis work, our laboratory is currently investigating how osteoclasts and HMGB1 may be coupled to pain in arthritis conditions. Our preliminary data indicates that HMGB1 promotes the production of inflammatory mediators in differentiating osteoclasts, including IL-8. As HMGB1 is involved in angiogenesis and neurite growth (258, 259), an important question to be addressed is if HMGB1 modulates the capability of osteoclasts to induce these processes.

Are there sex-specific differences in the antinociceptive effects of osteoclast inhibitors or baricitinib?

While sex differences were not examined in **Paper I** and **II**, the CAIA model used for these studies shows higher arthritis incidence in females than males (13). As this reflects the clinical situation in which more women suffer from RA than men, the findings are important for a large proportion of RA patients. Future investigations are however still warranted to identify if similar mechanisms are present in the male counterparts.

In conclusion, as pain remains to be a significant problem for many patients with RA, it is imperative to increase efforts to improve pain management. The results presented in this thesis have shed new lights on the role of osteoclasts, AAK1/AP2M1 and HMGB1 that may be pertinent to long-lasting pain in RA. While more research is needed to grasp the exact mechanisms of pain in RA, this work is a step forward to advance more effective pain treatments for RA, and if needed sex-specific treatment approaches.

7 ACKNOWLEDGEMENTS

This thesis presents not only the final step of my education, but it is a milestone that represents my personal development over the past half decade. I would like to thank the many wonderful people who have helped me in any way possible during this time.

First and foremost, my sincere gratitude to my main supervisor **Camilla Svensson** who have given me the privilege to do my PhD studies in your group. It seems like it was only yesterday I stepped into your lab to have my interview only days after you have just given birth. You made this journey very exciting with your passion, enthusiasm and energy for science. I am forever grateful for your belief in me and for giving me the right balance of guidance and freedom to work on my projects, which has helped me evolved to who I am today. I could not imagine for a better supervisor!

I am also thankful for my co-supervisors who have facilitated ways so that I could learn and develop. Thank you to **Helena Erlandsson Harris** for your expertise in the HMGB1 and arthritis fields, and for your help and knowledgeable feedbacks at key moments. Many thanks for **Miguel Jimenez Andrade** for all your help with micro-CT imaging, and also for being so prompt in answering my questions regardless the time or day of the week. I definitely learned a lot from you. My appreciation also for **Chantal Chenu** for introducing me about osteoclasts and for taking care of me during my visit to London.

I could not be more grateful for being part of **Bonepain**. Thank you to all the **Bonepain ESRs** – **Alexandra**, **Freija**, **Anna**, **Marta**, **Manasi**, **Sonny**, **Ramin**, **Ran**, **Mateusz**, **Carlota**, **Larissa** - for sharing this adventure with me. Thank you to **Anne-Marie Heegaard** and **Ulla Kløve Jakobsen** for organizing all the meetings and taking care of all administrative works. A special thank you to **Thomas Levin Andersen** for hosting my secondment in Vejle and for teaching me everything about bone staining techniques, which are integral aspects of my osteoclast paper. I am honored to know that I am always welcomed back in your lab. Thank you also to **Birgit** and **Kaja** for all the technical assistance.

I would like to thank all the co-authors and collaborators who have provided experimental contributions and critical inputs for all my papers: **Arisai Martínez Martínez**, **Enriqueta Muños Islas**, **Dieter Brömme**, **Patrick Mehlen**, **Julie Barbier**, **Fabien Marchand**, **Ted Price**, **Michael Burton**, **Payam Emami Khoonsari**, **Kim Kultima**, **Eli Lilly**.

I would also like to acknowledge all the committee members for my half-time seminar (**Malin Ernberg**, **Andrei Chagin**, **Peder Olofsson**) as well as for my thesis defence (**Jason McDougall, Hermona Soreq**, **Andrei Chagin**, **Shafaq Sikandar**) for dedicating their time to review my work and give valuable suggestions.

Thank you to all the teachers and staff at **FyFa** who have provided support for me during these years. I am truly grateful to have **Kent Jardemark** as Director of Doctoral Studies (also my defence chairman), for all your help and guidance, especially during my defence application.

I would like to express my gratitude to my previous teachers and mentors who have motivated and encouraged me to continue my education at higher levels: **Ibu Marselina Tan**, **Bapak Indra Wibowo**, **Gerjo van Osch, Wu Wei, Marjolein van Driel**.

The most cherished moment of my PhD is that I got the chance to work with an amazing group of talented people that have helped, motivated and inspired me during the past years. I start with my HMGB1 buddy, **Nilesh**, who introduced me to the lab and the projects. It still amazes me how we manage to synchronize our working rhythms, even if you were far away in the US. I am happy that we managed to push the 2 HMGB1 projects to publications. I can still remember the time when I was helping you with your thesis and I am grateful that you can return the favor. My dear **Alex**, for being not just a wonderful colleague but also friend too. Thinking back all the time we worked together has always put a smile on my face. I am forever thankful that I can share almost anything with you and I am honored that you feel the same way too. I have missed our small chit-chats and fika breaks during this pandemic, but even so I know that I can always reach you with a simple message. **Alexandra**, I am happy to have shared this journey closely with you, from doing courses, teaching to travelling abroad for meetings together. Thank you for all the nice chats about science and life. I wish you all the best for your PhD defence! **Kata**, I cannot thank you enough for the countless hours you spent in the animal lab, always with impeccable patience. Your support during my PhD has been crucial, from your help with experiments and valuable advice. Your organization and attention to details, and of course your perfect ELN entries are truly inspiring. **Emerson**, for keeping me (and I guess everyone else) up to date with pain research. Even though we have never worked closely together, except perhaps lab cleaning, I know I can always turn to you for help. **Carlos**, my fellow commuter for all your help with animal experiments, especially for the early mornings and weekends. I commend your work ethics and I am sure you will shine with the rest of your PhD. **Nils**, for running essential experiments for my thesis and for being so organized and dedicated with your works. We will rock the bari project together! **Zeri**, your enthusiasm for science inside and outside the lab is admirable. I truly enjoy our discussions about the CAIA model and sex differences. **Joana**, for your kind and humble personality and always keen to help others whenever needed. **Harald**, I admire that you always ask out of the box questions, your passion for science is enviable. **Lydia**, our discussion about osteoclast and macrophage cultures has been interesting, good luck with these guys.

I have also been fortunate to have worked with amazing colleagues who I missed so much since they left the group. Special mention for **Azar** for being the core person that keeps the lab organized all these years and making things magically appear when I need them. **Yuki**, for helping me do critical experiments, for your kind and positive attitude, and for the endless supplies of Japanese sweets. **Freija**, for your assistance in experiments during the pandemic and for involving me in your sci-fi ion pump project. **Diana**, for your bubbly and funny personality, making our lab a more enjoyable place to work in. **Teresa,** for trusting me to do behavior experiments and for being an inspiration of a strong and confident person. **Gustaf**, for teaching me all about joints during the early days of my PhD. **Jungo**, for teaching me surgery, an important skill that I will take with me for the future. Thank you also to the other

past group members for the good moments that we spent together: **Jie**, **Duygu**, **Kasia**, **Jaira**, **Shibu, Sally**, **Vinko**, **Tinna**. Thank you to the new additions to the group, **Francisca**, **Zhenggang**, for helping out whenever possible, and a warm welcome back for **Matt**.

Furthermore, I want to thank all the people from **FyFa** and **CMM** for creating a collaborative and friendly environment to work in. Special mention to **Aisha**, **Chen** and **Hoda**, whom we share lab spaces together for being kind and considerate colleagues.

I would also like to acknowledge the HMGB1 group, especially **Henna**, **Lena**, and **Cecilia**, for providing me reagents for my experiments and all the interesting discussions.

During my PhD, I was lucky to have had the chance to do a company internship. Many thanks for my supervisor **Beata** for your valuable advice and guidance. Thank you also to **Dan**, **Connie**, **Nathalie**, **Vasiliki** and **Eleonora** for the chats and laughs during this month.

I would also like to thank all my friends in Sweden and around the globe for keeping me sane during this time. I am truly grateful for **PPI Stockholm/Uppsala** (Indonesian community) for making Sweden feels like home. Special thanks for **Jumpei** for letting me share your allotment so that I could practice my newfound love of gardening. Good luck with your PhD. Many thanks to syubidu: **Sara**, **Sisi**, **Aisha**, **Fitri**, **Via**, **Rinda** and **Ptonk**. Even though we are spread across different continents, I never felt that the distance has separated our friendship. Thank you also to **Erika**, **Aria**, **Lia**, **Vika**. I can still remember the days we daydream of studying and travelling abroad. I miss you all very much and I hope we can meet very soon!

I could not have achieved this far without the support of my beloved family and no words are enough to express my heartfelt appreciation for them. To my parents, **Ibu** and **Bapak**, for the endless encouragement and unconditional love. Thank you for showing faith in me and letting me choose my own path. To my sisters, **Lia**, **Dita** and **Mila**, my source of happiness, for making me always smile and laugh, and inspiring me to be the best. To the new addition to my family, **Agys**, for your help and generosity.

My deepest regard also goes for my second family. Thank you my parents-in-law, **Ibu** and **Ayah**, my siblings-in-law, **Bang Fiki**, **Kak Ira**, **Bang Faiz**, **Mbak Restu**, and my nieces/nephew, **Aliya**, **Kaelum**, **Millie**, for making me part of the family.

I owe thanks to a very special person, my husband **Fahri**, who embarked on this journey with me. We have had so much adventure trying to conquer our dreams together, even at times that means we have to be apart for a while. It still feels like a dream that we managed to choose Sweden to study and spend our lives together. Thank you is a small word to express my gratitude for your unfailing love and support. Without you beside me, none of this would be possible.

8 REFERENCES

1. Raffaeli W, Arnaudo E. (2017) Pain as a disease: an overview. *Journal of pain research* **10:** 2003-2008.
2. Apkarian AV, Baliki MN, Geha PY. (2009) Towards a theory of chronic pain. *Progress in neurobiology* **87:** 81-97.
3. Breivik H, Collett B, Ventafridda V, Cohen R, Gallacher D. (2006) Survey of chronic pain in Europe: prevalence, impact on daily life, and treatment. *European journal of pain (London, England)* **10:** 287-333.
4. Europe PA. (2017) Survey on chronic pain: diagnosis, treatment and impact of pain. *Pain Alliance Europe*.
5. McInnes IB, Schett G. (2011) The pathogenesis of rheumatoid arthritis. *The New England journal of medicine* **365:** 2205-2219.
6. Walsh DA, McWilliams DF. (2012) Pain in rheumatoid arthritis. *Curr Pain Headache Rep* **16:** 509-517.
7. Wigerblad G*, et al.* (2016) Autoantibodies to citrullinated proteins induce joint pain independent of inflammation via a chemokine-dependent mechanism. *Ann Rheum Dis* **75:** 730-738.
8. Zhu S*, et al.* (2018) Subchondral bone osteoclasts induce sensory innervation and osteoarthritis pain. *The Journal of clinical investigation*.
9. Basbaum AI, Bautista DM, Scherrer G, Julius D. (2009) Cellular and molecular mechanisms of pain. *Cell* **139:** 267-284.
10. Woolf CJ. (2010) What is this thing called pain? *The Journal of clinical investigation* **120:** 3742-3744.
11. Mogil JS. (2012) Sex differences in pain and pain inhibition: multiple explanations of a controversial phenomenon. *Nature reviews. Neuroscience* **13:** 859-866.
12. Sorge RE*, et al.* (2015) Different immune cells mediate mechanical pain hypersensitivity in male and female mice. *Nature neuroscience* **18:** 1081-1083.
13. Fernandez-Zafra T*, et al.* (2018) Exploring the transcriptome of resident spinal microglia after collagen antibody-induced arthritis. *Pain*.
14. Yang Y*, et al.* (2015) Delayed activation of spinal microglia contributes to the maintenance of bone cancer pain in female Wistar rats via P2X7 receptor and IL-18. *J Neurosci* **35:** 7950-7963.
15. Raja SN*, et al.* (2020) The revised International Association for the Study of Pain definition of pain: concepts, challenges, and compromises. *Pain* **161:** 1976-1982.
16. Julius D, Basbaum AI. (2001) Molecular mechanisms of nociception. *Nature* **413:** 203-210.
17. Burke RE. (2007) Sir Charles Sherrington's the integrative action of the nervous system: a centenary appreciation. *Brain : a journal of neurology* **130:** 887-894.
18. Burgess PR, Perl ER. (1967) Myelinated afferent fibres responding specifically to noxious stimulation of the skin. *The Journal of physiology* **190:** 541-562.
19. Usoskin D*, et al.* (2015) Unbiased classification of sensory neuron types by large-scale single-cell RNA sequencing. *Nature neuroscience* **18:** 145-153.
20. Renthal W*, et al.* (2020) Transcriptional Reprogramming of Distinct Peripheral Sensory Neuron Subtypes after Axonal Injury. *Neuron* **108:** 128-144.e129.
21. Braz JM, Nassar MA, Wood JN, Basbaum AI. (2005) Parallel "pain" pathways arise from subpopulations of primary afferent nociceptor. *Neuron* **47:** 787-793.
22. Moller T*, et al.* (2016) Critical data-based re-evaluation of minocycline as a putative specific microglia inhibitor. *Glia* **64:** 1788-1794.
23. Page GG, Opp MR, Kozachik SL. (2014) Reduced sleep, stress responsivity, and female sex contribute to persistent inflammation-induced mechanical hypersensitivity in rats. *Brain, behavior, and immunity* **40:** 244-251.
24. D'Mello R, Dickenson AH. (2008) Spinal cord mechanisms of pain. *British journal of anaesthesia* **101:** 8-16.
25. Schaible HG. (2014) Nociceptive neurons detect cytokines in arthritis. *Arthritis research & therapy* **16:** 470.

26. Richter F*, et al.* (2010) Tumor necrosis factor causes persistent sensitization of joint nociceptors to mechanical stimuli in rats. *Arthritis and rheumatism* **62:** 3806-3814.
27. Ebbinghaus M*, et al.* (2012) The role of interleukin-1beta in arthritic pain: main involvement in thermal, but not mechanical, hyperalgesia in rat antigen-induced arthritis. *Arthritis and rheumatism* **64:** 3897-3907.
28. Brenn D, Richter F, Schaible HG. (2007) Sensitization of unmyelinated sensory fibers of the joint nerve to mechanical stimuli by interleukin-6 in the rat: an inflammatory mechanism of joint pain. *Arthritis and rheumatism* **56:** 351-359.
29. Richter F*, et al.* (2012) Interleukin-17 sensitizes joint nociceptors to mechanical stimuli and contributes to arthritic pain through neuronal interleukin-17 receptors in rodents. *Arthritis and rheumatism* **64:** 4125-4134.
30. Boettger MK*, et al.* (2008) Antinociceptive effects of tumor necrosis factor alpha neutralization in a rat model of antigen-induced arthritis: evidence of a neuronal target. *Arthritis and rheumatism* **58:** 2368-2378.
31. Woolf CJ. (2007) Central sensitization: uncovering the relation between pain and plasticity. *Anesthesiology* **106:** 864-867.
32. Lehnardt S*, et al.* (2003) Activation of innate immunity in the CNS triggers neurodegeneration through a Toll-like receptor 4-dependent pathway. *Proceedings of the National Academy of Sciences of the United States of America* **100:** 8514-8519.
33. Hiasa M*, et al.* (2017) Bone Pain Induced by Multiple Myeloma Is Reduced by Targeting V-ATPase and ASIC3. *Cancer research* **77:** 1283-1295.
34. Yoneda T*, et al.* (2011) Involvement of acidic microenvironment in the pathophysiology of cancer-associated bone pain. *Bone* **48:** 100-105.
35. Kohno N*, et al.* (2005) Zoledronic acid significantly reduces skeletal complications compared with placebo in Japanese women with bone metastases from breast cancer: a randomized, placebo-controlled trial. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* **23:** 3314-3321.
36. Francis RM, Aspray TJ, Hide G, Sutcliffe AM, Wilkinson P. (2008) Back pain in osteoporotic vertebral fractures. *Osteoporosis international : a journal established as result of cooperation between the European Foundation for Osteoporosis and the National Osteoporosis Foundation of the USA* **19:** 895-903.
37. Kakimoto S*, et al.* (2008) Minodronic acid, a third-generation bisphosphonate, antagonizes purinergic P2X(2/3) receptor function and exerts an analgesic effect in pain models. *European journal of pharmacology* **589:** 98-101.
38. Kasukawa Y*, et al.* (2018) Analgesic effects of minodronate in a rat chronic pain model. *Biomedical research (Tokyo, Japan)* **39:** 261-268.
39. Yao Y*, et al.* (2016) Alendronate Attenuates Spinal Microglial Activation and Neuropathic Pain. *The journal of pain : official journal of the American Pain Society* **17:** 889-903.
40. Qin A*, et al.* (2012) V-ATPases in osteoclasts: structure, function and potential inhibitors of bone resorption. *The international journal of biochemistry & cell biology* **44:** 1422-1435.
41. Kikuta J*, et al.* (2013) Dynamic visualization of RANKL and Th17-mediated osteoclast function. *The Journal of clinical investigation* **123:** 866-873.
42. Caterina MJ*, et al.* (2000) Impaired nociception and pain sensation in mice lacking the capsaicin receptor. *Science (New York, N.Y.)* **288:** 306-313.
43. Deval E*, et al.* (2008) ASIC3, a sensor of acidic and primary inflammatory pain. *The EMBO journal* **27:** 3047-3055.
44. Gatenby RA, Gawlinski ET, Gmitro AF, Kaylor B, Gillies RJ. (2006) Acid-mediated tumor invasion: a multidisciplinary study. *Cancer research* **66:** 5216-5223.
45. Nagae M*, et al.* (2006) Osteoclasts play a part in pain due to the inflammation adjacent to bone. *Bone* **39:** 1107-1115.
46. Ebbinghaus M*, et al.* (2019) Contribution of Inflammation and Bone Destruction to Pain in Arthritis: A Study in Murine Glucose-6-Phosphate Isomerase-Induced Arthritis. *Arthritis & rheumatology (Hoboken, N.J.)* **71:** 2016-2026.
47. Carvalho AP, Bezerra MM, Girao VC, Cunha FQ, Rocha FA. (2006) Anti-inflammatory and anti-nociceptive activity of risedronate in experimental pain models in rats and mice. *Clinical and experimental pharmacology & physiology* **33:** 601-606.

48. Morado-Urbina CE*, et al.* (2014) Early, middle, or late administration of zoledronate alleviates spontaneous nociceptive behavior and restores functional outcomes in a mouse model of CFA-induced arthritis. *Drug development research* **75:** 438-448.
49. Sagar DR*, et al.* (2014) Osteoprotegerin reduces the development of pain behaviour and joint pathology in a model of osteoarthritis. *Annals of the rheumatic diseases* **73:** 1558-1565.
50. Strassle BW*, et al.* (2010) Inhibition of osteoclasts prevents cartilage loss and pain in a rat model of degenerative joint disease. *Osteoarthritis and cartilage* **18:** 1319-1328.
51. Nwosu LN*, et al.* (2018) Analgesic effects of the cathepsin K inhibitor L-006235 in the monosodium iodoacetate model of osteoarthritis pain. *Pain reports* **3:** e685.
52. Wang L*, et al.* (2016) Bisphosphonates Inhibit Pain, Bone Loss, and Inflammation in a Rat Tibia Fracture Model of Complex Regional Pain Syndrome. *Anesthesia and analgesia* **123:** 1033-1045.
53. Roudier MP, Bain SD, Dougall WC. (2006) Effects of the RANKL inhibitor, osteoprotegerin, on the pain and histopathology of bone cancer in rats. *Clinical & experimental metastasis* **23:** 167-175.
54. Naito Y*, et al.* (2017) Alendronate inhibits hyperalgesia and suppresses neuropeptide markers of pain in a mouse model of osteoporosis. *Journal of orthopaedic science : official journal of the Japanese Orthopaedic Association* **22:** 771-777.
55. Abe Y*, et al.* (2015) Inhibitory effect of bisphosphonate on osteoclast function contributes to improved skeletal pain in ovariectomized mice. *Journal of bone and mineral metabolism* **33:** 125-134.
56. Miyamura G*, et al.* (2020) Prevention of bone loss and improvement of pain-related behavior in hind limb-unloaded mice by administration of teriparatide and bisphosphonate. *Modern rheumatology***:** 1-10.
57. Mazaki A*, et al.* (2019) Tumor Necrosis Factor-α Produced by Osteoclasts Might Induce Intractable Pain in a Rat Spinal Metastasis Model of Breast Cancer. *Spine surgery and related research* **3:** 261-266.
58. Negishi-Koga T*, et al.* (2011) Suppression of bone formation by osteoclastic expression of semaphorin 4D. *Nature medicine* **17:** 1473-1480.
59. Ni S*, et al.* (2019) Sensory innervation in porous endplates by Netrin-1 from osteoclasts mediates PGE2-induced spinal hypersensitivity in mice. *Nature communications* **10:** 5643.
60. Gao SY*, et al.* (2017) Zoledronate suppressed angiogenesis and osteogenesis by inhibiting osteoclasts formation and secretion of PDGF-BB. *PloS one* **12:** e0179248.
61. de Clauser L, Santana-Varela S, Wood JN, Sikandar S. (2021) Physiologic osteoclasts are not sufficient to induce skeletal pain in mice. *European journal of pain (London, England)* **25:** 199-212.
62. Mach DB*, et al.* (2002) Origins of skeletal pain: sensory and sympathetic innervation of the mouse femur. *Neuroscience* **113:** 155-166.
63. Mantyh PW. (2014) The neurobiology of skeletal pain. *Eur J Neurosci* **39:** 508-519.
64. Jimenez-Andrade JM*, et al.* (2010) A phenotypically restricted set of primary afferent nerve fibers innervate the bone versus skin: therapeutic opportunity for treating skeletal pain. *Bone* **46:** 306-313.
65. Martin CD, Jimenez-Andrade JM, Ghilardi JR, Mantyh PW. (2007) Organization of a unique net-like meshwork of CGRP+ sensory fibers in the mouse periosteum: implications for the generation and maintenance of bone fracture pain. *Neuroscience letters* **427:** 148-152.
66. Kelly S*, et al.* (2012) Spontaneous firing in C-fibers and increased mechanical sensitivity in A-fibers of knee joint-associated mechanoreceptive primary afferent neurones during MIA-induced osteoarthritis in the rat. *Osteoarthritis and cartilage* **20:** 305-313.
67. Jimenez-Andrade JM, Mantyh PW. (2012) Sensory and sympathetic nerve fibers undergo sprouting and neuroma formation in the painful arthritic joint of geriatric mice. *Arthritis research & therapy* **14:** R101.
68. Ghilardi JR*, et al.* (2011) Sustained blockade of neurotrophin receptors TrkA, TrkB and TrkC reduces non-malignant skeletal pain but not the maintenance of sensory and sympathetic nerve fibers. *Bone* **48:** 389-398.

69. Longo G, Osikowicz M, Ribeiro-da-Silva A. (2013) Sympathetic fiber sprouting in inflamed joints and adjacent skin contributes to pain-related behavior in arthritis. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **33:** 10066-10074.
70. Sorge RE*, et al.* (2011) Spinal cord Toll-like receptor 4 mediates inflammatory and neuropathic hypersensitivity in male but not female mice. *J Neurosci* **31:** 15450-15454.
71. Falk S, Uldall M, Appel C, Ding M, Heegaard AM. (2013) Influence of sex differences on the progression of cancer-induced bone pain. *Anticancer research* **33:** 1963-1969.
72. Vacca V*, et al.* (2014) Higher pain perception and lack of recovery from neuropathic pain in females: a behavioural, immunohistochemical, and proteomic investigation on sex-related differences in mice. *Pain* **155:** 388-402.
73. Tajerian M*, et al.* (2015) Sex differences in a Murine Model of Complex Regional Pain Syndrome. *Neurobiology of learning and memory* **123:** 100-109.
74. Kim D*, et al.* (2007) A critical role of toll-like receptor 2 in nerve injury-induced spinal cord glial cell activation and pain hypersensitivity. *The Journal of biological chemistry* **282:** 14975-14983.
75. Agalave NM*, et al.* (2014) Spinal HMGB1 induces TLR4-mediated long-lasting hypersensitivity and glial activation and regulates pain-like behavior in experimental arthritis. *Pain* **155:** 1802-1813.
76. Das N*, et al.* (2016) HMGB1 Activates Proinflammatory Signaling via TLR5 Leading to Allodynia. *Cell Rep* **17:** 1128-1140.
77. Park CK*, et al.* (2014) Extracellular microRNAs activate nociceptor neurons to elicit pain via TLR7 and TRPA1. *Neuron* **82:** 47-54.
78. Woller SA*, et al.* (2016) Systemic TAK-242 prevents intrathecal LPS evoked hyperalgesia in male, but not female mice and prevents delayed allodynia following intraplantar formalin in both male and female mice: The role of TLR4 in the evolution of a persistent pain state. *Brain, behavior, and immunity* **56:** 271-280.
79. Hua XY*, et al.* (2005) Intrathecal minocycline attenuates peripheral inflammation-induced hyperalgesia by inhibiting p38 MAPK in spinal microglia. *Eur J Neurosci* **22:** 2431-2440.
80. Chen G, Luo X, Qadri MY, Berta T, Ji RR. (2017) Sex-Dependent Glial Signaling in Pathological Pain: Distinct Roles of Spinal Microglia and Astrocytes. *Neurosci Bull*.
81. Huck NA*, et al.* (2021) Temporal contribution of myeloid-lineage TLR4 to the transition to chronic pain: A focus on sex differences. *J Neurosci*.
82. Liu L*, et al.* (2020) Sex Differences Revealed in a Mouse CFA Inflammation Model with Macrophage Targeted Nanotheranostics. *Theranostics* **10:** 1694-1707.
83. Klareskog L*, et al.* (2006) A new model for an etiology of rheumatoid arthritis: smoking may trigger HLA-DR (shared epitope)-restricted immune reactions to autoantigens modified by citrullination. *Arthritis and rheumatism* **54:** 38-46.
84. Roche PA, Klestov AC, Heim HM. (2003) Description of stable pain in rheumatoid arthritis: a 6 year study. *The Journal of rheumatology* **30:** 1733-1738.
85. Ulus Y*, et al.* (2011) Sleep quality in fibromyalgia and rheumatoid arthritis: associations with pain, fatigue, depression, and disease activity. *Clinical and experimental rheumatology* **29:** S92-96.
86. Catrina AI, Svensson CI, Malmström V, Schett G, Klareskog L. (2017) Mechanisms leading from systemic autoimmunity to joint-specific disease in rheumatoid arthritis. *Nature reviews. Rheumatology* **13:** 79-86.
87. Ruta S*, et al.* (2020) EULAR definition of "arthralgia suspicious for progression to rheumatoid arthritis" in a large cohort of patients included in a program for rapid diagnosis: role of auto-antibodies and ultrasound. *Clinical rheumatology* **39:** 1493-1499.
88. Taylor P*, et al.* (2010) Patient perceptions concerning pain management in the treatment of rheumatoid arthritis. *The Journal of international medical research* **38:** 1213-1224.
89. Lee YC*, et al.* (2011) Pain persists in DAS28 rheumatoid arthritis remission but not in ACR/EULAR remission: a longitudinal observational study. *Arthritis research & therapy* **13:** R83.
90. Rantapaa-Dahlqvist S*, et al.* (2003) Antibodies against cyclic citrullinated peptide and IgA rheumatoid factor predict the development of rheumatoid arthritis. *Arthritis and rheumatism* **48:** 2741-2749.

91. van Steenbergen HW, Mangnus L, Reijnierse M, Huizinga TW, van der Helm-van Mil AH. (2016) Clinical factors, anticitrullinated peptide antibodies and MRI-detected subclinical inflammation in relation to progression from clinically suspect arthralgia to arthritis. *Annals of the rheumatic diseases* **75:** 1824-1830.
92. van de Stadt LA*, et al.* (2011) The extent of the anti-citrullinated protein antibody repertoire is associated with arthritis development in patients with seropositive arthralgia. *Annals of the rheumatic diseases* **70:** 128-133.
93. Kleyer A*, et al.* (2014) Bone loss before the clinical onset of rheumatoid arthritis in subjects with anticitrullinated protein antibodies. *Annals of the rheumatic diseases* **73:** 854-860.
94. van Steenbergen HW, Ajeganova S, Forslind K, Svensson B, van der Helm-van Mil AH. (2015) The effects of rheumatoid factor and anticitrullinated peptide antibodies on bone erosions in rheumatoid arthritis. *Annals of the rheumatic diseases* **74:** e3.
95. Zhang ZJ, Cao DL, Zhang X, Ji RR, Gao YJ. (2013) Chemokine contribution to neuropathic pain: respective induction of CXCL1 and CXCR2 in spinal cord astrocytes and neurons. *Pain* **154:** 2185-2197.
96. Krishnamurthy A*, et al.* (2016) Identification of a novel chemokine-dependent molecular mechanism underlying rheumatoid arthritis-associated autoantibody-mediated bone loss. *Annals of the rheumatic diseases* **75:** 721-729.
97. Bersellini Farinotti A*, et al.* (2019) Cartilage-binding antibodies induce pain through immune complex-mediated activation of neurons. *The Journal of experimental medicine* **216:** 1904-1924.
98. Verpoort KN*, et al.* (2006) Isotype distribution of anti-cyclic citrullinated peptide antibodies in undifferentiated arthritis and rheumatoid arthritis reflects an ongoing immune response. *Arthritis and rheumatism* **54:** 3799-3808.
99. van der Woude D*, et al.* (2010) Epitope spreading of the anti-citrullinated protein antibody response occurs before disease onset and is associated with the disease course of early arthritis. *Annals of the rheumatic diseases* **69:** 1554-1561.
100. McInnes IB, Schett G. (2007) Cytokines in the pathogenesis of rheumatoid arthritis. *Nat Rev Immunol* **7:** 429-442.
101. Gravallese EM*, et al.* (2000) Synovial tissue in rheumatoid arthritis is a source of osteoclast differentiation factor. *Arthritis and rheumatism* **43:** 250-258.
102. Lee YC. (2013) Effect and treatment of chronic pain in inflammatory arthritis. *Current rheumatology reports* **15:** 300.
103. Harirforoosh S, Asghar W, Jamali F. (2013) Adverse effects of nonsteroidal antiinflammatory drugs: an update of gastrointestinal, cardiovascular and renal complications. *Journal of pharmacy & pharmaceutical sciences : a publication of the Canadian Society for Pharmaceutical Sciences, Societe canadienne des sciences pharmaceutiques* **16:** 821-847.
104. Hickling P, Jacoby RK, Kirwan JR. (1998) Joint destruction after glucocorticoids are withdrawn in early rheumatoid arthritis. Arthritis and Rheumatism Council Low Dose Glucocorticoid Study Group. *British journal of rheumatology* **37:** 930-936.
105. Williams HJ*, et al.* (1985) Comparison of low-dose oral pulse methotrexate and placebo in the treatment of rheumatoid arthritis. A controlled clinical trial. *Arthritis and rheumatism* **28:** 721-730.
106. van der Kooij SM*, et al.* (2009) Patient-reported outcomes in a randomized trial comparing four different treatment strategies in recent-onset rheumatoid arthritis. *Arthritis and rheumatism* **61:** 4-12.
107. Hazlewood GS*, et al.* (2016) Methotrexate monotherapy and methotrexate combination therapy with traditional and biologic disease modifying antirheumatic drugs for rheumatoid arthritis: abridged Cochrane systematic review and network meta-analysis. *BMJ (Clinical research ed.)* **353:** i1777.
108. Altawil R*, et al.* (2016) Remaining Pain in Early Rheumatoid Arthritis Patients Treated With Methotrexate. *Arthritis Care Res (Hoboken)* **68:** 1061-1068.
109. Babon JJ, Lucet IS, Murphy JM, Nicola NA, Varghese LN. (2014) The molecular regulation of Janus kinase (JAK) activation. *The Biochemical journal* **462:** 1-13.
110. Ivashkiv LB, Hu X. (2004) Signaling by STATs. *Arthritis research & therapy* **6:** 159-168.

111. Pope J*, et al.* (2020) Comparative Efficacy of JAK Inhibitors for Moderate-To-Severe Rheumatoid Arthritis: A Network Meta-Analysis. *Advances in therapy* **37:** 2356-2372.
112. Dhillon S, Keam SJ. (2020) Filgotinib: First Approval. *Drugs* **80:** 1987-1997.
113. Adam S*, et al.* (2020) JAK inhibition increases bone mass in steady-state conditions and ameliorates pathological bone loss by stimulating osteoblast function. *Science translational medicine* **12**.
114. Strand V*, et al.* (2015) Effects of tofacitinib monotherapy on patient-reported outcomes in a randomized phase 3 study of patients with active rheumatoid arthritis and inadequate responses to DMARDs. *Arthritis research & therapy* **17:** 307.
115. Strand V*, et al.* (2016) Tofacitinib versus methotrexate in rheumatoid arthritis: patient-reported outcomes from the randomised phase III ORAL Start trial. *RMD open* **2:** e000308.
116. Strand V*, et al.* (2020) Tofacitinib in combination with methotrexate in patients with rheumatoid arthritis: patient-reported outcomes from the 24-month Phase 3 ORAL Scan study. *Clinical and experimental rheumatology* **38:** 848-857.
117. Michaud K*, et al.* (2019) Relative Impact of Pain and Fatigue on Work Productivity in Patients with Rheumatoid Arthritis from the RA-BEAM Baricitinib Trial. *Rheumatology and therapy* **6:** 409-419.
118. Keystone EC*, et al.* (2017) Patient-reported outcomes from a phase 3 study of baricitinib versus placebo or adalimumab in rheumatoid arthritis: secondary analyses from the RA-BEAM study. *Annals of the rheumatic diseases* **76:** 1853-1861.
119. Taylor PC*, et al.* (2019) Achieving Pain Control in Rheumatoid Arthritis with Baricitinib or Adalimumab Plus Methotrexate: Results from the RA-BEAM Trial. *Journal of clinical medicine* **8**.
120. Fautrel B*, et al.* (2019) Effect of Baricitinib and Adalimumab in Reducing Pain and Improving Function in Patients with Rheumatoid Arthritis in Low Disease Activity: Exploratory Analyses from RA-BEAM. *Journal of clinical medicine* **8**.
121. Fautrel B*, et al.* (2020) Comparative effectiveness of improvement in pain and physical function for baricitinib versus adalimumab, tocilizumab and tofacitinib monotherapies in rheumatoid arthritis patients who are naïve to treatment with biologic or conventional synthetic disease-modifying antirheumatic drugs: a matching-adjusted indirect comparison. *RMD open* **6**.
122. Simon LS*, et al.* (2020) The Jak/STAT pathway: A focus on pain in rheumatoid arthritis. *Seminars in arthritis and rheumatism* **51:** 278-284.
123. Richardson P*, et al.* (2020) Baricitinib as potential treatment for 2019-nCoV acute respiratory disease. *Lancet (London, England)* **395:** e30-e31.
124. Stebbing J*, et al.* (2020) Mechanism of baricitinib supports artificial intelligence-predicted testing in COVID-19 patients. *EMBO molecular medicine* **12:** e12697.
125. Kostich W*, et al.* (2016) Inhibition of AAK1 Kinase as a Novel Therapeutic Approach to Treat Neuropathic Pain. *The Journal of pharmacology and experimental therapeutics* **358:** 371-386.
126. Macfarlane GJ. (2016) The epidemiology of chronic pain. *PAIN* **157:** 2158-2159.
127. Brackertz D, Mitchell GF, Vadas MA, Mackay IR. (1977) Studies on Antigen-Induced Arthritis in Mice. *The Journal of Immunology* **118:** 1645.
128. Brackertz D, Mitchell GF, Vadas MA, Mackay IR. (1977) Studies on antigen-induced arthritis in mice. III. Cell and serum transfer experiments. *Journal of immunology (Baltimore, Md. : 1950)* **118:** 1645-1648.
129. Terato K*, et al.* (1992) Induction of arthritis with monoclonal antibodies to collagen. *Journal of immunology (Baltimore, Md. : 1950)* **148:** 2103-2108.
130. Nandakumar KS, Svensson L, Holmdahl R. (2003) Collagen type II-specific monoclonal antibody-induced arthritis in mice: description of the disease and the influence of age, sex, and genes. *The American journal of pathology* **163:** 1827-1837.
131. Kouskoff V*, et al.* (1996) Organ-specific disease provoked by systemic autoimmunity. *Cell* **87:** 811-822.
132. Matsumoto I, Staub A, Benoist C, Mathis D. (1999) Arthritis provoked by linked T and B cell recognition of a glycolytic enzyme. *Science (New York, N.Y.)* **286:** 1732-1735.
133. Ji H*, et al.* (2001) Genetic influences on the end-stage effector phase of arthritis. *The Journal of experimental medicine* **194:** 321-330.

134. Bas DB*, et al.* (2012) Collagen antibody-induced arthritis evokes persistent pain with spinal glial involvement and transient prostaglandin dependency. *Arthritis and rheumatism* **64:** 3886-3896.
135. Su J*, et al.* (2015) Phenotypic changes in dorsal root ganglion and spinal cord in the collagen antibody-induced arthritis mouse model. *J Comp Neurol* **523:** 1505-1528.
136. Fujita M*, et al.* (2020) Sepiapterin Reductase Inhibition Leading to Selective Reduction of Inflammatory Joint Pain in Mice and Increased Urinary Sepiapterin Levels in Humans and Mice. *Arthritis & rheumatology (Hoboken, N.J.)* **72:** 57-66.
137. Sluka KA*, et al.* (2013) Acid-sensing ion channel 3 deficiency increases inflammation but decreases pain behavior in murine arthritis. *Arthritis Rheum* **65:** 1194-1202.
138. Agalave NM, Svensson CI. (2015) Extracellular high-mobility group box 1 protein (HMGB1) as a mediator of persistent pain. *Molecular medicine (Cambridge, Mass.)* **20:** 569-578.
139. Bonaldi T*, et al.* (2003) Monocytic cells hyperacetylate chromatin protein HMGB1 to redirect it towards secretion. *The EMBO journal* **22:** 5551-5560.
140. Venereau E*, et al.* (2012) Mutually exclusive redox forms of HMGB1 promote cell recruitment or proinflammatory cytokine release. *The Journal of experimental medicine* **209:** 1519-1528.
141. Yang H*, et al.* (2015) MD-2 is required for disulfide HMGB1-dependent TLR4 signaling. *The Journal of experimental medicine* **212:** 5-14.
142. Antoine DJ, Harris HE, Andersson U, Tracey KJ, Bianchi ME. (2014) A systematic nomenclature for the redox states of high mobility group box (HMGB) proteins. *Molecular medicine (Cambridge, Mass.)* **20:** 135-137.
143. Yang H, Wang H, Andersson U. (2020) Targeting Inflammation Driven by HMGB1. *Frontiers in immunology* **11:** 484.
144. Rendon-Mitchell B*, et al.* (2003) IFN-gamma induces high mobility group box 1 protein release partly through a TNF-dependent mechanism. *Journal of immunology (Baltimore, Md. : 1950)* **170:** 3890-3897.
145. Lu B*, et al.* (2014) JAK/STAT1 signaling promotes HMGB1 hyperacetylation and nuclear translocation. *Proceedings of the National Academy of Sciences of the United States of America* **111:** 3068-3073.
146. Jiang W, Pisetsky DS. (2006) The role of IFN-alpha and nitric oxide in the release of HMGB1 by RAW 264.7 cells stimulated with polyinosinic-polycytidylic acid or lipopolysaccharide. *Journal of immunology (Baltimore, Md. : 1950)* **177:** 3337-3343.
147. Lamkanfi M*, et al.* (2010) Inflammasome-dependent release of the alarmin HMGB1 in endotoxemia. *Journal of immunology (Baltimore, Md. : 1950)* **185:** 4385-4392.
148. Gardella S*, et al.* (2002) The nuclear protein HMGB1 is secreted by monocytes via a non-classical, vesicle-mediated secretory pathway. *EMBO reports* **3:** 995-1001.
149. Andersson U, Yang H, Harris H. (2018) Extracellular HMGB1 as a therapeutic target in inflammatory diseases. *Expert opinion on therapeutic targets* **22:** 263-277.
150. Aucott H, Sowinska A, Harris HE, Lundback P. (2018) Ligation of free HMGB1 to TLR2 in the absence of ligand is negatively regulated by the C-terminal tail domain. *Molecular medicine (Cambridge, Mass.)* **24:** 19.
151. Hreggvidsdóttir HS*, et al.* (2012) High mobility group box protein 1 (HMGB1)-partner molecule complexes enhance cytokine production by signaling through the partner molecule receptor. *Molecular medicine (Cambridge, Mass.)* **18:** 224-230.
152. Sha Y, Zmijewski J, Xu Z, Abraham E. (2008) HMGB1 develops enhanced proinflammatory activity by binding to cytokines. *Journal of immunology (Baltimore, Md. : 1950)* **180:** 2531-2537.
153. Zong M*, et al.* (2013) TLR4 as receptor for HMGB1 induced muscle dysfunction in myositis. *Annals of the rheumatic diseases* **72:** 1390-1399.
154. Curtin JF*, et al.* (2009) HMGB1 mediates endogenous TLR2 activation and brain tumor regression. *PLoS medicine* **6:** e10.
155. Feng XJ*, et al.* (2016) TLR2 Plays a Critical Role in HMGB1-Induced Glomeruli Cell Proliferation Through the FoxO1 Signaling Pathway in Lupus Nephritis. *Journal of interferon & cytokine research : the official journal of the International Society for Interferon and Cytokine Research* **36:** 258-266.

156. Bangert A, *et al.* (2016) Critical role of RAGE and HMGB1 in inflammatory heart disease. *Proceedings of the National Academy of Sciences of the United States of America* **113:** E155-164.
157. Trøseid M, *et al.* (2013) Circulating levels of HMGB1 are correlated strongly with MD2 in HIV-infection: possible implication for TLR4-signalling and chronic immune activation. *Innate immunity* **19:** 290-297.
158. Jiang C, *et al.* (2018) Association between the HMGB1/TLR4 signaling pathway and the clinicopathological features of ovarian cancer. *Molecular medicine reports* **18:** 3093-3098.
159. Aulin C, Lassacher T, Palmblad K, Erlandsson Harris H. (2020) Early stage blockade of the alarmin HMGB1 reduces cartilage destruction in experimental OA. *Osteoarthritis and cartilage* **28:** 698-707.
160. Yang H, *et al.* (2016) Identification of CD163 as an antiinflammatory receptor for HMGB1-haptoglobin complexes. *JCI insight* **1**.
161. Ito T, *et al.* (2008) Proteolytic cleavage of high mobility group box 1 protein by thrombin-thrombomodulin complexes. *Arteriosclerosis, thrombosis, and vascular biology* **28:** 1825-1830.
162. Son M, *et al.* (2016) C1q and HMGB1 reciprocally regulate human macrophage polarization. *Blood* **128:** 2218-2228.
163. Maillard-Lefebvre H, *et al.* (2009) Soluble receptor for advanced glycation end products: a new biomarker in diagnosis and prognosis of chronic inflammatory diseases. *Rheumatology (Oxford, England)* **48:** 1190-1196.
164. Hamada T, *et al.* (2008) Extracellular high mobility group box chromosomal protein 1 is a coupling factor for hypoxia and inflammation in arthritis. *Arthritis and rheumatism* **58:** 2675-2685.
165. Kokkola R, *et al.* (2002) High mobility group box chromosomal protein 1: a novel proinflammatory mediator in synovitis. *Arthritis and rheumatism* **46:** 2598-2603.
166. Taniguchi N, *et al.* (2003) High mobility group box chromosomal protein 1 plays a role in the pathogenesis of rheumatoid arthritis as a novel cytokine. *Arthritis and rheumatism* **48:** 971-981.
167. Goldstein RS, *et al.* (2007) Cholinergic anti-inflammatory pathway activity and High Mobility Group Box-1 (HMGB1) serum levels in patients with rheumatoid arthritis. *Molecular medicine (Cambridge, Mass.)* **13:** 210-215.
168. Palmblad K, *et al.* (2007) Morphological characterization of intra-articular HMGB1 expression during the course of collagen-induced arthritis. *Arthritis research & therapy* **9:** R35.
169. Biscetti F, *et al.* (2016) The role of high-mobility group box protein 1 in collagen antibody-induced arthritis is dependent on vascular endothelial growth factor. *Clin Exp Immunol* **184:** 62-72.
170. Zhou Z, *et al.* (2008) HMGB1 regulates RANKL-induced osteoclastogenesis in a manner dependent on RAGE. *Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research* **23:** 1084-1096.
171. Yamoah K, *et al.* (2008) High-mobility group box proteins modulate tumor necrosis factor-alpha expression in osteoclastogenesis via a novel deoxyribonucleic acid sequence. *Mol Endocrinol* **22:** 1141-1153.
172. Pullerits R, *et al.* (2003) High mobility group box chromosomal protein 1, a DNA binding cytokine, induces arthritis. *Arthritis and rheumatism* **48:** 1693-1700.
173. Kokkola R, *et al.* (2003) Successful treatment of collagen-induced arthritis in mice and rats by targeting extracellular high mobility group box chromosomal protein 1 activity. *Arthritis and rheumatism* **48:** 2052-2058.
174. Ostberg T, *et al.* (2008) Oxaliplatin retains HMGB1 intranuclearly and ameliorates collagen type II-induced arthritis. *Arthritis research & therapy* **10:** R1.
175. Van de Wouwer M, *et al.* (2006) The lectin-like domain of thrombomodulin interferes with complement activation and protects against arthritis. *Journal of thrombosis and haemostasis : JTH* **4:** 1813-1824.
176. Hofmann MA, *et al.* (2002) RAGE and arthritis: the G82S polymorphism amplifies the inflammatory response. *Genes and immunity* **3:** 123-135.

177. Schierbeck H*, et al.* (2011) Monoclonal anti-HMGB1 (high mobility group box chromosomal protein 1) antibody protection in two experimental arthritis models. *Molecular medicine (Cambridge, Mass.)* **17:** 1039-1044.
178. Chacur M*, et al.* (2001) A new model of sciatic inflammatory neuritis (SIN): induction of unilateral and bilateral mechanical allodynia following acute unilateral peri-sciatic immune activation in rats. *Pain* **94:** 231-244.
179. Shibasaki M*, et al.* (2010) Induction of high mobility group box-1 in dorsal root ganglion contributes to pain hypersensitivity after peripheral nerve injury. *Pain* **149:** 514-521.
180. O'Connor KA*, et al.* (2003) Further characterization of high mobility group box 1 (HMGB1) as a proinflammatory cytokine: central nervous system effects. *Cytokine* **24:** 254-265.
181. Ma F*, et al.* (2017) Disulfide high mobility group box-1 causes bladder pain through bladder Toll-like receptor 4. *BMC Physiol* **17:** 6.
182. Ma F*, et al.* (2017) Macrophage migration inhibitory factor mediates protease-activated receptor 4-induced bladder pain through urothelial high mobility group box 1. *Physiological reports* **5**.
183. Hu TT*, et al.* (2020) A crucial role of HMGB1 in orofacial and widespread pain sensitization following partial infraorbital nerve transection. *Brain, behavior, and immunity*.
184. Yamasoba D*, et al.* (2016) Peripheral HMGB1-induced hyperalgesia in mice: Redox state-dependent distinct roles of RAGE and TLR4. *Journal of pharmacological sciences* **130:** 139-142.
185. Allette YM*, et al.* (2014) Identification of a functional interaction of HMGB1 with Receptor for Advanced Glycation End-products in a model of neuropathic pain. *Brain, behavior, and immunity* **42:** 169-177.
186. Feldman P, Due MR, Ripsch MS, Khanna R, White FA. (2012) The persistent release of HMGB1 contributes to tactile hyperalgesia in a rodent model of neuropathic pain. *Journal of neuroinflammation* **9:** 180.
187. Nakamura Y*, et al.* (2013) Neuropathic pain in rats with a partial sciatic nerve ligation is alleviated by intravenous injection of monoclonal antibody to high mobility group box-1. *PloS one* **8:** e73640.
188. Kato J, Agalave NM, Svensson CI. (2016) Pattern recognition receptors in chronic pain: Mechanisms and therapeutic implications. *European journal of pharmacology* **788:** 261-273.
189. Calogero S*, et al.* (1999) The lack of chromosomal protein Hmg1 does not disrupt cell growth but causes lethal hypoglycaemia in newborn mice. *Nature genetics* **22:** 276-280.
190. Otoshi K, Kikuchi S, Kato K, Sekiguchi M, Konno S. (2011) Anti-HMGB1 neutralization antibody improves pain-related behavior induced by application of autologous nucleus pulposus onto nerve roots in rats. *Spine (Phila Pa 1976)* **36:** E692-698.
191. Ma F*, et al.* (2018) Protease activated-receptor 4 activation as a model of persistent bladder pain: Essential role of macrophage migration inhibitory factor and high mobility group box 1. *International journal of urology : official journal of the Japanese Urological Association* **25:** 887-893.
192. Ma F, Meyer-Siegler KL, Leng L, Bucala R, Vera PL. (2019) Spinal macrophage migration inhibitory factor and high mobility group box 1 mediate persistent bladder pain. *Neuroscience letters* **699:** 54-58.
193. Tanaka J*, et al.* (2014) Bladder pain relief by HMGB1 neutralization and soluble thrombomodulin in mice with cyclophosphamide-induced cystitis. *Neuropharmacology* **79:** 112-118.
194. Irie Y*, et al.* (2020) HMGB1 and its membrane receptors as therapeutic targets in an intravesical substance P-induced bladder pain syndrome mouse model. *Journal of pharmacological sciences* **143:** 112-116.
195. Irie Y*, et al.* (2017) Macrophage-derived HMGB1 as a Pain Mediator in the Early Stage of Acute Pancreatitis in Mice: Targeting RAGE and CXCL12/CXCR4 Axis. *Journal of neuroimmune pharmacology : the official journal of the Society on NeuroImmune Pharmacology*.

196. Nishida T, *et al.* (2016) Involvement of high mobility group box 1 in the development and maintenance of chemotherapy-induced peripheral neuropathy in rats. *Toxicology* **365:** 48-58.
197. Sekiguchi F, *et al.* (2018) Paclitaxel-induced HMGB1 release from macrophages and its implication for peripheral neuropathy in mice: Evidence for a neuroimmune crosstalk. *Neuropharmacology* **141:** 201-213.
198. Ren PC, *et al.* (2012) High-mobility group box 1 contributes to mechanical allodynia and spinal astrocytic activation in a mouse model of type 2 diabetes. *Brain Res Bull* **88:** 332-337.
199. Tanaka J, *et al.* (2013) Recombinant human soluble thrombomodulin prevents peripheral HMGB1-dependent hyperalgesia in rats. *British journal of pharmacology* **170:** 1233-1241.
200. Nakamura T, *et al.* (2020) High mobility group box 1 induces bone pain associated with bone invasion in a mouse model of advanced head and neck cancer. *Oncology reports* **44:** 2547-2558.
201. Okui T, *et al.* (2021) The HMGB1/RAGE axis induces bone pain associated with colonization of 4T1 mouse breast cancer in bone. *Journal of Bone Oncology* **26:** 100330.
202. Tong W, *et al.* (2010) Spinal high-mobility group box 1 contributes to mechanical allodynia in a rat model of bone cancer pain. *Biochemical and biophysical research communications* **395:** 572-576.
203. Thakur V, Sadanandan J, Chattopadhyay M. (2020) High-Mobility Group Box 1 Protein Signaling in Painful Diabetic Neuropathy. *International journal of molecular sciences* **21**.
204. Sun X, *et al.* (2018) Glycyrrhizin ameliorates inflammatory pain by inhibiting microglial activation-mediated inflammatory response via blockage of the HMGB1-TLR4-NF-kB pathway. *Experimental cell research* **369:** 112-119.
205. Jia L, *et al.* (2014) Hepatocyte Toll-like receptor 4 regulates obesity-induced inflammation and insulin resistance. *Nature communications* **5:** 3878.
206. Decosterd I, Woolf CJ. (2000) Spared nerve injury: an animal model of persistent peripheral neuropathic pain. *Pain* **87:** 149-158.
207. Devogelaer JP. (1996) Clinical use of bisphosphonates. *Current opinion in rheumatology* **8:** 384-391.
208. Panwar P, *et al.* (2019) An Ectosteric Inhibitor of Cathepsin K Inhibits Bone Resorption in Ovariectomized Mice. *Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research* **34:** 777-778.
209. Grandin M, *et al.* (2016) Structural Decoding of the Netrin-1/UNC5 Interaction and its Therapeutical Implications in Cancers. *Cancer cell* **29:** 173-185.
210. Lundback P, *et al.* (2016) A novel high mobility group box 1 neutralizing chimeric antibody attenuates drug-induced liver injury and postinjury inflammation in mice. *Hepatology (Baltimore, Md.)* **64:** 1699-1710.
211. Moini-Zanjani T, *et al.* (2016) Minocycline Effects on IL-6 Concentration in Macrophage and Microglial Cells in a Rat Model of Neuropathic Pain. *Iranian biomedical journal* **20:** 273-279.
212. Dunlea DM, Fee LT, McEnery T, McElvaney NG, Reeves EP. (2018) The impact of alpha-1 antitrypsin augmentation therapy on neutrophil-driven respiratory disease in deficient individuals. *Journal of inflammation research* **11:** 123-134.
213. Vicuna L, *et al.* (2015) The serine protease inhibitor SerpinA3N attenuates neuropathic pain by inhibiting T cell-derived leukocyte elastase. *Nature medicine* **21:** 518-523.
214. Chaplan SR, Bach FW, Pogrel JW, Chung JM, Yaksh TL. (1994) Quantitative assessment of tactile allodynia in the rat paw. *Journal of neuroscience methods* **53:** 55-63.
215. Boyle DL, Rosengren S, Bugbee W, Kavanaugh A, Firestein GS. (2003) Quantitative biomarker analysis of synovial gene expression by real-time PCR. *Arthritis research & therapy* **5:** R352-360.
216. Møller Døhn U, *et al.* (2009) Erosive progression is minimal, but erosion healing rare, in patients with rheumatoid arthritis treated with adalimumab. A 1 year investigator-initiated follow-up study using high-resolution computed tomography as the primary outcome measure. *Annals of the rheumatic diseases* **68:** 1585-1590.

217. Finzel S*, et al.* (2013) Interleukin-6 receptor blockade induces limited repair of bone erosions in rheumatoid arthritis: a micro CT study. *Annals of the rheumatic diseases* **72:** 396-400.
218. Weber M*, et al.* (2021) Zoledronate Causes a Systemic Shift of Macrophage Polarization towards M1 In Vivo. *International journal of molecular sciences* **22**.
219. Ren BX*, et al.* (2015) Effect of Tanshinone IIA intrathecal injections on pain and spinal inflammation in mice with bone tumors. *Genetics and molecular research : GMR* **14:** 2133-2138.
220. Zhu S*, et al.* (2018) Subchondral bone osteoclasts induce sensory innervation and osteoarthritis pain. *J Clin Invest* **129:** 1076-1093.
221. Cui W*, et al.* (2021) Spinal caspase-6 regulates AMPA receptor trafficking and dendritic spine plasticity through netrin-1 in postoperative pain after orthopedic surgery for tibial fracture in mice. *Pain* **162:** 124-134.
222. Chartier SR, Mitchell SAT, Majuta LA, Mantyh PW. (2018) The Changing Sensory and Sympathetic Innervation of the Young, Adult and Aging Mouse Femur. *Neuroscience* **387:** 178-190.
223. Thudium CS*, et al.* (2020) The Janus kinase 1/2 inhibitor baricitinib reduces biomarkers of joint destruction in moderate to severe rheumatoid arthritis. *Arthritis research & therapy* **22:** 235.
224. Emery P*, et al.* (2021) Baricitinib inhibits structural joint damage progression in patients with rheumatoid arthritis-a comprehensive review. *Arthritis research & therapy* **23:** 3.
225. Fridman JS*, et al.* (2010) Selective inhibition of JAK1 and JAK2 is efficacious in rodent models of arthritis: preclinical characterization of INCB028050. *Journal of immunology (Baltimore, Md. : 1950)* **184:** 5298-5307.
226. Wang C, Song Y, Wang X, Mao R, Song L. (2018) Baicalin Ameliorates Collagen-Induced Arthritis Through the Suppression of Janus Kinase 1 (JAK1)/Signal Transducer and Activator of Transcription 3 (STAT3) Signaling in Mice. *Medical science monitor : international medical journal of experimental and clinical research* **24:** 9213-9222.
227. Li YY*, et al.* (2017) Activation of STAT3-mediated CXCL12 up-regulation in the dorsal root ganglion contributes to oxaliplatin-induced chronic pain. *Molecular pain* **13:** 1744806917747425.
228. Dominguez E, Mauborgne A, Mallet J, Desclaux M, Pohl M. (2010) SOCS3-mediated blockade of JAK/STAT3 signaling pathway reveals its major contribution to spinal cord neuroinflammation and mechanical allodynia after peripheral nerve injury. *J Neurosci* **30:** 5754-5766.
229. Ricotta D, Conner SD, Schmid SL, von Figura K, Honing S. (2002) Phosphorylation of the AP2 mu subunit by AAK1 mediates high affinity binding to membrane protein sorting signals. *The Journal of cell biology* **156:** 791-795.
230. Gururaj S*, et al.* (2017) Protein kinase A-induced internalization of Slack channels from the neuronal membrane occurs by adaptor protein-2/clathrin-mediated endocytosis. *The Journal of biological chemistry* **292:** 19304-19314.
231. Marriott I, Bost KL, Huet-Hudson YM. (2006) Sexual dimorphism in expression of receptors for bacterial lipopolysaccharides in murine macrophages: a possible mechanism for gender-based differences in endotoxic shock susceptibility. *J Reprod Immunol* **71:** 12-27.
232. Kahlke V*, et al.* (2000) Immune dysfunction following trauma-haemorrhage: influence of gender and age. *Cytokine* **12:** 69-77.
233. Zschiebsch K*, et al.* (2019) Mast cell tetrahydrobiopterin contributes to itch in mice. *Journal of cellular and molecular medicine* **23:** 985-1000.
234. Nieto FR*, et al.* (2016) Neuron-immune mechanisms contribute to pain in early stages of arthritis. *Journal of neuroinflammation* **13:** 96.
235. Quadros AU*, et al.* (2015) Dynamic weight bearing is an efficient and predictable method for evaluation of arthritic nociception and its pathophysiological mechanisms in mice. *Scientific reports* **5:** 14648.
236. Taves S*, et al.* (2016) Spinal inhibition of p38 MAP kinase reduces inflammatory and neuropathic pain in male but not female mice: Sex-dependent microglial signaling in the spinal cord. *Brain, behavior, and immunity* **55:** 70-81.

237. Beggs S, Salter MW. (2013) The known knowns of microglia-neuronal signalling in neuropathic pain. *Neuroscience letters* **557 Pt A:** 37-42.
238. Tsuda M, Beggs S, Salter MW, Inoue K. (2013) Microglia and intractable chronic pain. *Glia* **61:** 55-61.
239. Loram LC*, et al.* (2012) Sex and estradiol influence glial pro-inflammatory responses to lipopolysaccharide in rats. *Psychoneuroendocrinology* **37:** 1688-1699.
240. Wang J*, et al.* (2015) Wild-type microglia do not reverse pathology in mouse models of Rett syndrome. *Nature* **521:** E1-4.
241. Goldmann T*, et al.* (2013) A new type of microglia gene targeting shows TAK1 to be pivotal in CNS autoimmune inflammation. *Nature neuroscience* **16:** 1618-1626.
242. Goldmann T*, et al.* (2016) Origin, fate and dynamics of macrophages at central nervous system interfaces. *Nature immunology* **17:** 797-805.
243. Shemer A, Erny D, Jung S, Prinz M. (2015) Microglia Plasticity During Health and Disease: An Immunological Perspective. *Trends in immunology* **36:** 614-624.
244. Ledeboer A*, et al.* (2005) Minocycline attenuates mechanical allodynia and proinflammatory cytokine expression in rat models of pain facilitation. *Pain* **115:** 71-83.
245. Lin CS*, et al.* (2007) Chronic intrathecal infusion of minocycline prevents the development of spinal-nerve ligation-induced pain in rats. *Reg Anesth Pain Med* **32:** 209-216.
246. Mika J, Zychowska M, Popiolek-Barczyk K, Rojewska E, Przewlocka B. (2013) Importance of glial activation in neuropathic pain. *European journal of pharmacology* **716:** 106-119.
247. Muley MM, Krustev E, Reid AR, McDougall JJ. (2017) Prophylactic inhibition of neutrophil elastase prevents the development of chronic neuropathic pain in osteoarthritic mice. *Journal of neuroinflammation* **14:** 168.
248. Muley MM*, et al.* (2016) Neutrophil elastase induces inflammation and pain in mouse knee joints via activation of proteinase-activated receptor-2. *British journal of pharmacology* **173:** 766-777.
249. Mihara K, Ramachandran R, Renaux B, Saifeddine M, Hollenberg MD. (2013) Neutrophil elastase and proteinase-3 trigger G protein-biased signaling through proteinase-activated receptor-1 (PAR1). *The Journal of biological chemistry* **288:** 32979-32990.
250. First in Human Evaluation of Safety, Pharmacokinetics, and Clinical Activity of a Monoclonal Antibody Targeting Netrin 1 in Patients With Advanced/Metastatic Solid Tumors. In. https://ClinicalTrials.gov/show/NCT02977195.
251. GYNecological Cancers Treated With NETrin mAbs in Combination With Chemotherapy and or Pembrolizumab. In. https://ClinicalTrials.gov/show/NCT04652076.
252. Efficacy and Safety of LX9211 in Patients With Postherpetic Neuralgia. In. https://ClinicalTrials.gov/show/NCT04662281.
253. Efficacy, Safety, and PK of LX9211 in Patients With Diabetic Peripheral Neuropathic Pain. In. https://ClinicalTrials.gov/show/NCT04455633.
254. Kupari J*, et al.* (2021) Single cell transcriptomics of primate sensory neurons identifies cell types associated with chronic pain. *Nature communications* **12:** 1510.
255. Kuai L*, et al.* (2011) AAK1 identified as an inhibitor of neuregulin-1/ErbB4-dependent neurotrophic factor signaling using integrative chemical genomics and proteomics. *Chemistry & biology* **18:** 891-906.
256. Moutal A*, et al.* (2021) SARS-CoV-2 spike protein co-opts VEGF-A/neuropilin-1 receptor signaling to induce analgesia. *Pain* **162:** 243-252.
257. Salikhova A*, et al.* (2008) Vascular endothelial growth factor and semaphorin induce neuropilin-1 endocytosis via separate pathways. *Circulation research* **103:** e71-79.
258. Park SY*, et al.* (2015) HMGB1 induces angiogenesis in rheumatoid arthritis via HIF-1α activation. *European journal of immunology* **45:** 1216-1227.
259. Kigerl KA, Lai W, Wallace LM, Yang H, Popovich PG. (2018) High mobility group box-1 (HMGB1) is increased in injured mouse spinal cord and can elicit neurotoxic inflammation. *Brain, behavior, and immunity* **72:** 22-33.