DISSERTATION

The dual role of αCGRP in experimental arthritis/ „Die duale Rolle von αCGRP in experimenteller Arthritis“

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Vorwort

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Abbreviations

µCT micro-computed tomography
AC adenylate cyclase
ACPA anti-citrullinated protein antibody
ALPL alkaline phosphatase
ANOVA analysis of variance
AT1 angiotensin 1
ATP adenosine triphosphate
AvgPo.Dm average pore diameter
BGLAP bone gamma-carboxyglutamic acid-containing protein
BMD bone mineral density
Bo&Po density bone and pore density
BS bone surface
BV bone volume
BV/TV bone volume/ total volume
CAIA collagen antibody-induced arthritis
cAMP cyclic adenosine monophosphate
CCL2 chemokine ligand 2
CDAI Clinical Disease Activity Index
cDNA complementary DNA
cGMP cyclic guanosine monophosphate
CGRP calcitonin gene-related peptide
CIA collagen-induced arthritis
CLR CGRP receptor
CRLR calcitonin receptor-like receptor
CRP C-reactive protein
CT threshold cycle
Ct.V/TV cortical volume/ total volume
CTR calcitonin receptor
CTRL control
CTSK cathepsin K
DAMP danger-associated molecular patterns
DAPI 4',6-diamidino-2-phenylindole
DAS-28 disease activity score - 28
DKK1 dickkopf-1
DMARD disease-modifying anti-rheumatic drugs
DNA desoxyribonucleic acid
DRG dorsal root ganglia
ECE-1 endothelin-converting enzyme-1
FLS fibroblast-like synoviocytes
GIOP glucocorticoid-induced osteoporosis
GIT gastrointestinal tract
GPCR G-protein-coupled receptor
GPI glucose-6-phosphate isomerase
H&E hematoxylin and eosin
HA hydroxyapatite
HGNC Gene Nomenclature Committee of the Human Genome Organisation
HIF-1α hypoxia-inducible factor 1alpha
HLA humane leukocyte antigen
IL interleukin

IL-1ra _____ IL-1 receptor antagonist
JAK/STAT____ janus kinase/ signal transducer and activator of transcription proteins
K_{ATP}_____ ATP- sensitive potassium channels
LPR5/6 _____ LDL receptor–related protein 5/6
LPS_____lipopolysaccharides
mAb _____monoclonal antibody
MAP _____ mean arterial pressure
MCP _____ metacarpophalangeal
MHC _____major histocompatibility complex
miRNA _____ micro-RNA
MMP _____ metalloproteinases
mRNA_____messenger ribonucleic acid
MTP _____metatarsophalangeal
MTX _____methotrexate
MWU_____ Wilcoxon-Mann-Whitney-test
NF-κB _____ nuclear factor kappa B
NO _____nitric oxide
NSAID_____ nonsteroidal anti-inflammatory drugs
OA _____ osteoarthritis
OPG _____osteoprotegerin
OPGL _____ OPG ligand
PFA_____paraformaldehyde
PIP _____proximal interphalangeal
PKA _____ protein kinase A
qRT-PCR _____ quantitative realtime polymerase chain reaction
RA_____ rheumatoid arthritis
RAMP1 _____ receptor activity modifying protein one
RANKL _____the receptor activator of nuclear factor kappa-B ligand
RAS _____ renin-angiotensin system
RCP _____ receptor component protein
RF_____ rheumatoid factor
Runx2 _____ runt-related transcription factor 2
SP_____ substance P
TB _____toluidine blue
Tb. Nb. _____trabecular number
Tb. Sp. _____ trabecular separation
Tb. Th. _____ trabecular thickness
TNF-α _____tumour necrosis factor alpha
TRAP _____ tartate-resistant acid phosphatase
TRP _____ transient receptor potential cation channels
TRPV1 _____transient receptor potential cation channel vanilloid 1
vCN_____virtual copy number
VEGF _____vascular endothelial growth factor
VOI _____ volume of interest
WT _____ wild type

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Abstract

Introduction: Rheumatoid arthritis (RA), a chronic progressive joint disorder, is the most common autoimmune disease, affecting 1 % of the world population. Patients with RA suffer from cartilage degradation, bone erosions and inflammatory synovitis. Despite new targeted treatment options for RA including monoclonal antibody therapies, less than 50 % of patients show clinical remission under current treatments. Calcitonin gene-related peptide alpha (αCGRP) is a vasodilative neuropeptide with pro-inflammatory properties. It was shown to mediate neurogenic inflammation and pain perception. Patients with RA show elevated levels of CGRP in synovial fluid. Furthermore, monoclonal antibodies against αCGRP are already in clinical use for the prevention and treatment of migraine. To investigate the role of αCGRP in murine inflammatory arthritis, we exposed αCGRP-deficient mice to collagen antibody-induced arthritis (CAIA).

Materials and methods: To induce arthritis, αCGRP-deficient mice (αCGRP$^{-/-}$) and wild type (WT) C57BL/6J mice received ArthritoMab™, a reformulated cocktail of 4 arthritogenic monoclonal antibodies to collagen type II. Control (CTRL) animals of both genotypes received phosphate buffered saline (PBS). All animals were monitored daily over a period of 10 or 48 days to evaluate acute and long-term arthritis effects separately. Daily assessments included body weight, grip strength, clinical semi-quantitative arthritis score and ankle size measurements. Histological, immunohistochemical, micro-computed tomography (µ-CT) and gene expression analysis were performed at the endpoints of day 10 and day 48.

Results: αCGRP was intraarticularly increased in WT animals suffering from CAIA. WT mice additionally reached significantly higher arthritis scores and histological cartilage degradation scores than αCGRP$^{-/-}$ mice during the acute phase of arthritis. Furthermore, increased levels of the inflammatory markers *Tnfa, Cd80, Il1b, Ccl2* and *Mmp13* were present in the joints of WT CAIA but not αCGRP$^{-/-}$ CAIA mice. While enhanced bone resorption indicated by increased levels of *Trap/Acp5* and *Ctsk* was present in WT CAIA mice during the acute inflammation phase, significant bone loss was present in αCGRP$^{-/-}$ CAIA but not in WT CAIA after 48 days.

Conclusion: This study provides the first evidence for a dual pro-inflammatory and bone-protective role of αCGRP in experimental arthritis. CGRP inhibition could be a potential target for anti-inflammatory RA therapy, yet the bone-altering effects may compromise skeletal integrity during arthritis resolution. With an increased use of

CGRP antagonists in migraine therapy, careful consideration should be warranted in patients suffering from inflammatory bone diseases.

Keywords: rheumatoid arthritis, calcitonin gene-related peptide alpha (αCGRP), collagen antibody-induced arthritis (CAIA), bone erosions

Zusammenfassung

Einleitung: Die Rheumatoide Arthritis (RA) ist eine progressive Gelenkerkrankung und die häufigste Autoimmunerkrankung weltweit mit einer Prävalenz von etwa 1 %. Betroffene Gelenke zeigen Knorpelschäden, Knochenerosionen und Gelenkentzündung. Trotz neuer zielgerichteter Medikamente zeigen unter derzeitigen Therapieregimen weniger als 50 % der Patienten eine klinische Besserung. Calcitonin gene-related peptide alpha (αCGRP) ist ein vasodilatierendes Neuropeptid mit proinflammatorischen Eigenschaften. αCGRP konnte eine Vermittlerrolle in neurogener Entzündung nachgewiesen werden und Patienten mit entzündlicher Arthritis zeigen erhöhte Konzentrationen von CGRP in der Synovialflüssigkeit. Monoklonale Antikörper gegen αCGRP sind bereits zur Therapie und Prävention der Migräne im klinischen Einsatz. Um die Rolle von αCGRP in muriner Gelenkentzündung zu untersuchen, haben wir ein Antikörper-induziertes Arthritismodell in αCGRP-defizienten Mäusen etabliert und diese mit Wildtyptieren verglichen.

Material und Methoden: αCGRP-defizienten Mäusen (αCGRP$^{-/-}$) und Wildtypmäusen (WT) der Gattung C57BL/6J wurde ArthritoMab™, ein Cocktail aus 4 arthritogenen monoklonalen Antikörpern gegen Kollagen Typ II, der eine Gelenkentzündung auslöst, bzw. phosphate buffered saline (PBS) als Negativkontrolle injiziert. Alle Tiere wurden über einen Zeitraum von 10 oder 48 Tagen täglich überwacht, um sowohl Daten über Kurzzeit- als auch Langzeitauswirkungen zu gewinnen. Die täglichen Messungen beinhalteten Gewicht, Griffkraft, einen semi-quantitativen Arthritis Score sowie den Knöchelumfang. *Post mortem* wurden an den Endpunkten Tag 10 und Tag 48 eine histologische Auswertung, immunhistochemische Darstellungen der CGRP-Reaktivität in den Sprunggelenken, radiologische Messungen mittels micro-Computertomographie (µCT) sowie eine Analyse der Genexpression vorgenommen.

Ergebnisse: WT CAIA Tiere zeigten eine erhöhte intraartikuläre Expression von αCGRP. WT CAIA Tiere wiesen in der akuten Phase der Arthritis darüber hinaus sowohl höhere Arthritis-Scores als auch eine stärkere Knorpelschädigung als αCGRP$^{-/-}$ CAIA Mäuse auf, was zu den erhöhten Entzündungsmarkern *Tnfa*, *Cd80*, *Il1b*, *Ccl2* und *Mmp13*, die in αCGRP$^{-/-}$ CAIA Tieren nicht erhöht waren, passt. Während in der akuten Phase WT CAIA Mäuse die erhöhten Knochenresorptionsmarker *Trap* und *Ctsk* aufwiesen, zeigte die Erholungsphase nach 48 Tagen einen signifikanten Knochensubstanzverlust bei αCGRP$^{-/-}$ CAIA aber nicht bei WT CAIA Tieren.

Schlussfolgerung: Diese Studie zeigt erstmalig eine duale sowohl entzündungsfördernde als auch knochenprotektive Rolle von αCGRP in experimenteller Arthritis. Die medikamentöse CGRP-Blockade könnte zukünftig einen potentiellen Ansatz für anti-entzündliche Therapien in der RA darstellen, gleichzeitig aber langfristig die Knochenqualität beeinträchtigen. Darüber hinaus sollte der zunehmende Gebrauch von CGRP-blockierenden Medikamenten in der Migränetherapie bei Patienten im Rahmen der Pharmakovigilanz kritisch beobachtet werden.

1 Introduction

1.1 Rheumatoid Arthritis

1.1.1 Epidemiology and Clinical Appearance

Rheumatoid arthritis (RA), a progressive joint disorder, is the most common autoimmune disease, affecting approximately 1 % of the world population (2), with an increasing prevalence in recent years (3). The sex-dependent epidemiological distribution is asymmetrical, showing a fivefold higher prevalence in female patients especially in under 50 year-olds (4). RA has been described in medical literature since the middle of the 19th century (5). It is considered a complex systemic disease, which classically causes inflammation and swelling of the metacarpophalangeal- (MCP) and metatarsophalangeal (MTP) joints, as well as the proximal interphalangeal (PIP) joints of hands and feet (6). RA significantly impairs the life quality of patients and, in addition to joint inflammation, affects multiple organs such as lungs (7), skin (6), blood (8), and the cardiovascular system (9, 10), and is associated with psychiatric affective disorders such as depression (11). RA is a progressive and chronic disorder, but additional “flares” are common, that are characterized by a severe worsening of symptoms in a short time. Clinical data show that 67 % of RA patients sustain at least one flare during nine years of treatment, with a correlation between increased flare incidences and radiologically evident disease progression (12). The continuous destruction of articular tissue correlates with impaired life quality and suicidal ideations (13). Pain-related restrictions in everyday life, such as problems with dressing, walking stairs and grabbing objects may also cause socioeconomic problems as they often lead to work disability (14). Furthermore, more than 60 % of affected patients are at a pre-retirement age of under 65 years (15). Lastly, RA burdens the health care system by causing up to 80,000 USD of annual total costs per patient (16, 17).

1.1.2 Pathogenesis

1.1.2.1 Genetic Susceptibility

Unlike most autoimmune diseases, RA does not follow a clear hereditary pattern. However, there are some susceptibility factors, such as the human leukocyte antigen (HLA) variant DRB1*0401, DQA1*03 together with DQB1*03 or *04, among others that have been identified through desoxyribonucleic acid (DNA) analysis (18). These gene

variants lead to alterations in HLA type II sequences causing changes in immune pathways. In particular, modified interactions between $CD4^+$ T-cells and antigen-presenting cells (APC) are linked to RA (19). There is limited evidence for a slightly higher risk of developing RA when carrying specific combinations of these alleles (20). Recent micro-RNA (miRNA) studies also revealed associations between the mir-499 rs3746444 polymorphism and RA (21).

1.1.2.2 Environmental Risk Factors

Environmental risk factors associated with an increased risk for RA include smoking, female sex, air pollution, obesity and a red meat-based diet with high sodium (22). Heavy smoking directly increases citrullination of proteins in the lungs, leading to an increased risk of formation of antibodies against citrullinated protein antigens (ACPA) (23).
Microbiome alterations have been shown to play a relevant role in RA progression (24). According to current understanding, genetic risk factors and environmental conditions contribute to a misdirected immune system, finally causing systemic and joint inflammation.

1.1.2.3 Autoantibodies

Downstream to DNA variations, there are several autoantibodies, detectable in serum of patients, linked to the progression and severity of RA in humans. Here, predominantly rheumatoid factor (RF) and ACPA are associated with disease activity and severity. Both immunoglobulins are present in 50 - 80 % of RA patients (25) and are linked to a more severe course of RA (26). The assessment of ACPA and RF serum levels as well as the consecutive division into seropositive and seronegative patients has been an integral part of the EULAR RA classification criteria (27). Moreover, ACPA and RF appear in the blood long before clinical manifestations become evident. In particular, ACPA can already be found in up to 50 % of patients four years prior to first clinical symptoms (28). There is increasing evidence that, in this preclinical period, antibodies linked to RA might initiate systemic autoimmunity in extraarticular tissues such as the mucosa of the lung, gut and oral cavity (29). Beyond their diagnostic role in RA progression, autoantibodies may play a pathogenic role via activation osteoclasts, neutrophils and the complement system (30).

1.1.2.4 Synovium and Endothelium

During RA, an intraarticular hypertrophied fibrovascular joint layer called pannus promotes the progression of synovitis. Endothelial cells and synoviocytes are involved in the formation of pannus (31, 32).

First, intensified activation of angiogenesis via vascular endothelial growth factor (VEGF) and the corresponding signalling cascade causes a “flooding” of the extracellular space with immune cells (32). The VEGF sub form C is directly induced by tumour necrosis factor-alpha (TNF-α) and other cytokines *in vitro* (33). RA also causes a hypoxic environment in the joint which additionally induces VEGF via hypoxia-inducible factor 1-alpha (HIF-1α) (34). Therefore, endothelial cells are understood as signalling sources for inflammatory stimuli in RA.

Second, the central cellular part of the pannus is formed by fibroblast-like synoviocytes (FLS), also called type B synoviocytes. FLS, which physiologically appear in the synovium next to macrophages, are capable of synthesizing the synovial fluid component hyaluronan as well as degrading enzymes such as metalloproteinases (MMP) (35). FLS undergo a metabolic transformation during onset of RA. After being activated by increased intraarticular concentrations of pro-inflammatory cytokines such as TNF-α, interleukin (IL)-1 and IL-6, danger-associated molecular patterns (DAMP), direct stimulation of lymphocytes and crosstalk with synovial macrophages, FLS start to invade adjacent cartilage and bone tissue via integrin-mediated cell-cell contacts. A subsequent increase in nuclear factor kappa-B (NF-κB) signalling and upregulation of other pro-inflammatory pathways inside FLS stimulates the secretion of MMP and collagenases, inducing the degradation of the adjacent extracellular matrix (31, 36). Transformed FLS perpetuate a continuous inflamed status inside RA-affected joints.

1.1.2.5 Cytokines

During progression of RA, a vicious cycle of pro-inflammatory signalling promotes joint destruction, involving cytokines like TNF-α, IL-1 and IL-6 (30).

TNF-α was discovered in 1975 as a toxic peptide causing necrosis in sarcoma cells (37). In RA, macrophages, lymphocytes, neutrophils, osteoblasts and osteoclasts produce increased amounts of TNF-α (38). TNF-α stimulates osteoclasts, induces chemotaxis of monocytes and neutrophils into the joint, stimulates the production of

other inflammatory cytokines like IL-1 and IL-6 as well as chemokines like CXCL-8, and causes pathological neoangiogenesis via the VEGF pathway (38).

IL-1, which can be subdivided into IL-1α und IL-1β, is a pro-inflammatory cytokine that stimulates systemic inflammation and is involved in the development of fever and sepsis (39). During progression of RA, in particular, the upregulation of IL-1β signalling and simultaneous downregulation of its natural antagonist IL-1 receptor antagonist (IL-1ra) causes cartilage degradation via induction of matrix-degrading MMP and aggrecanases (40).

IL-6, a pro-inflammatory cytokine secreted by macrophages, acts as the key regulator of the inflammatory acute phase reaction during natural pathogen defense (41). Upregulation of IL-6 in RA is involved in the generation of autoantibodies, formation of pannus, stimulation of bone resorption and overexpression of MMP and aggrecanases (42).

IL-17, which is produced by $CD4^+$ T-cells and regulated by IL-1 and IL-6, was also reported to play a crucial role in progressive synovitis and joint destruction during RA by promoting osteoclastogenesis and inflammatory signalling (39, 42, 43).

Chemokine ligand 2 (CCL2) is a chemokine that induces migration of T-cells to the synovium and pathological angiogenesis during RA (44).

1.1.2.6 Cartilage

During RA, excessive activation of degrading enzymes leads to a breakdown of cartilage (40). The metabolism and regeneration potential of cartilage-building chondrocytes is controlled by the transcription factor SOX9 (45).

An *in vitro* study showed that SOX9 could be essential for the regeneration of cartilage in inflammatory arthritis. Overexpression of SOX9 in chondrocytes was able to prevent an IL-1β-induced breakdown of the cartilage matrix (46).

1.1.3 RA Therapeutics

Since RA is characterized by a rapid progression and destruction of joints in predominantly aged patients (15), high efforts are being put into developing new therapy strategies in order to decelerate the disease course. To date, there is no curative therapy for RA that may result in complete remission with permanent arrest of all symptoms. In the past, therapy options included a heterogeneous spectrum of

therapies, many of which were administrated without evidence and later turned out to be ineffective or even toxic (47).
The use of glucocorticoids in anti-inflammatory therapy of RA has been documented since the mid-20th century (48) and is still a fundamental base of anti-rheumatic treatment today.

1.1.3.1 Conventional DMARD

In the 1970s the term disease-modifying anti-rheumatic drugs (DMARD) emerged to define drugs, such as the early cyclophosphamide, that actually modified the course of the disease in contrast to glucocorticoids and nonsteroidal anti-inflammatory drugs (NSAID). Examples for NSAID are salicylic acid and diclofenac, which mainly help to reduce pain and swelling (49). Referring to the 2022 American College of Rheumatology recommendations, RA therapy today should be commenced with the monotherapeutic permanent usage of the conventional DMARD methotrexate (MTX) supplemented with short-term administration of glucocorticoids during the initiation phase or when changing the drug. The conventional DMARD leflunomide and sulfasalazine should be used when MTX is contraindicated (50).

1.1.3.2 Biological DMARD

A deeper understanding of the pro-inflammatory cytokines involved in the pathomechanism of RA revolutionized the treatment strategies in the subsequent years.
In 1999, a phase III trial investigated the efficacy of infliximab, a chimeric antibody against TNF-α, in treating active RA. Patients receiving infliximab in addition to standard MTX treatment showed significantly reduced disease activity after 30 weeks when compared to placebo (51). Infliximab was the first biological DMARD for RA that was approved by the Food and Drug Administration. Since then, the biological DMARD market has evolved and started to acquire an increasing amount of new targeted drugs from different categories of molecules, including monoclonal antibodies and small molecules. Today, the pool of therapeutics has grown impressively (52, 53). Inhibition of the signalling pathways of TNF-α, IL-1 and IL-6 are the main targets of modern RA treatment (53, 54).

According to the current treatment recommendations for RA, if the treatment target is not achieved under a conventional DMARD therapy, a biological DMARD should be added in the presence of poor prognostic factors (50). Following first-line therapy failure, tocilizumab, a monoclonal antibody against IL-6, can be used. Second-line therapy can also include the use of Rituximab (55).

Rituximab is a chimeric monoclonal antibody against CD20 that was approved for the treatment of non-Hodgkin's lymphoma in 1997. However, Rituximab also significantly alleviated the disease activity of RA in patients that were not responsive anti-TNF biologics (56).

CD20 is known to be an important surface protein for B-lymphocyte differentiation and development of antibody-producing plasma cells is believed to be involved in the production of autoantibodies during RA (56, 57).

1.1.3.3 Synthetic DMARD

Early in the 21th century, studies unraveled a pivotal role of the Janus kinase/signal transducer and activator of transcription proteins- (JAK/STAT) dependent pathway in RA. Dysfunction of the JAK/STAT pathway in RA causes hyperstimulation of apoptosis in chondrocytes, overexpression of MMP (58) and sensitization of synovial cells with a higher responsiveness to IL-6 (59). The synthetic small molecule JAK-antagonist tofacitinib achieved comparable results to biological DMARD in terms of efficacy and adverse effects (60). While most biological DMARD increase the risk of infectious diseases in treated patients especially in higher doses (54, 61), tofacitinib does not seem to cause this adverse effect (62).

1.1.4 RA Therapy Challenges

Current treatment concepts for RA allow significant symptom reduction and increased life quality (63). Yet, biologics are comparably expensive, costing up to 80,000 USD per patient per year in the United States of America (16, 64). Nevertheless, biologics can be cost-efficient in patients not responding to conventional DMARD (65).

One of the biggest problems with the application of DMARD is the risk of infection. All DMARD, especially biologic therapeutics, exert their disease-modifying effects by suppressing the immune system. Therapeutic immunosuppression is crucial in RA

treatment but can facilitate the invasion of microorganisms and may create critical vulnerability to opportunistic infections that would otherwise not manifest.

Meta-analyses identified anti-TNF-α biologics to notably elevate incidences of tuberculosis, displaying a serious and potentially lethal bacterial communicable disease (66, 67). Higher hospitalization rates in RA patients sustaining infections compared to non-RA patients indicate increased morbidity and risk for complications (68).

MTX is still considered the anchor drug of RA therapy. As a cytostatic drug, MTX causes various adverse effects, like most other conventional non-biological DMARD. Teratogenicity makes MTX an unsuitable drug for the use in fertile women, and evidence suggests male fertility being affected (69). MTX can also cause toxic pneumonitis and consecutive interstitial lung disease (70). Furthermore, MTX frequently induces nausea, vomiting and abdominal pain, leading to withdrawal of the medication in up to 9 % of treated RA patients (71). Concomitant folic acid substitution can reduce the incidence of adverse effects, but is unable to prevent them (72).

Administration of glucocorticoids creates another significant problem in RA treatment. Newer observational data estimate that 30 - 50 % of RA patients still receive long-term glucocorticoids in addition to their DMARD therapy, leading to a dose-dependent increase in risk for serious infections (73). Low-dose long-term treatment with glucocorticoids decreases swelling and tenderness of joints in RA patients (74). However, glucocorticoids additionally impair the already compromised bone quality, which is prevalent in RA (75).

A meta-analysis from 2015 showed that the expanded application of NSAID and glucocorticoids in RA patients also increases the risk for cardiovascular events (76). However, all side effects have to be put in perspective, as DMARD primarily decrease RA disease activity. Disease activity itself, on the other hand, is known to be linked directly to overall mortality in RA patients when compared to healthy individuals (77).

In brief, regardless of the desired therapeutic effects, medications for RA share side effects that may impair bone homeostasis and alter the patient’s immune competence. Therefore, the need for new more nuanced therapeutic strategies in handling RA is ever-present.

1.1.5 Prognosis

Depending on the source of data and defined criteria there are still 30 - 90 % of RA patients who fail to respond to therapy and do not show clinical remission, even when treated with intensified combination therapies of biological and conventional DMARD (78-80). Poor prognostic factors are high values in the disease activity score-28 (DAS-28) assessment, smoking, female gender, elevated erythrocyte sedimentation rate, old age and delayed RA diagnosis (79, 81).

Despite improved therapy strategies and reduced surgical interventions for RA-associated joint deformities of hands and feet, up to 20 % of RA patients need major surgical musculoskeletal interventions within 25 years after being diagnosed (82). According to the Spanish National Registry, rates of hip and other joint replacement surgeries in RA patients have not decreased by more than 7 % in the last 17 years. Furthermore, arthroplasty surgery in RA patients entails significantly higher rates of complications including dislocation and periprosthetic joint infections (83).

1.2 Rheumatoid Arthritis and Bone

While RA is characterized primarily by intermittent joint swelling and pain accentuated in the early morning hours, it also perturbs the bone metabolism leading to impaired bone quality. It has long been proven that RA disturbs the homeostasis between bone resorption and bone formation, resulting in a net bone loss (84). Moreover, impaired bone quality in patients with RA results in significantly increased fracture rates (85) and should be considered when therapeutic strategies are drafted.

1.2.1 Bone Resorption

In healthy bone tissue, bone is resorbed by osteoclasts, which are able to break down mineralized structures. Increased resorption in RA-altered bone is induced by an upregulation of several transcription factors and enzymes as well as a downregulation of resorption-inhibiting pathways.

The activity of osteoclasts can be visualized by measuring tartrate-resistant acid phosphatase (TRAP), an iron-containing enzyme, which is secreted by osteoclasts (86). RA induces osteoclast activity with increased expression of TRAP, leading to the formation of bone erosions during disease progression (87).

Cathepsin K (CTSK) is a cysteine protease that degrades collagen type I in bone tissue. Serum levels of CTSK seem to corelate with radiological destruction of joint-adjacent bone in RA patients (88).

Receptor activator of nuclear factor kappa-B ligand (RANKL), a member of the family of TNF proteins (89), plays a crucial role not only in the differentiation of osteoclasts but also in cellular crosstalk between osteocytes, osteoblasts, osteoclasts and professional cells from the adaptive immune system (90-92). In this pathway, the RANK receptor is a surface transmembrane protein which can be found on osteoclasts, and the ligand RANKL is secreted by premature osteoblasts (93, 94). RANKL promotes osteoclastogenesis and inhibits osteogenic differentiation by antagonizing the β-catenin pathway (90).

Furthermore, osteoprotegerin (OPG), a soluble receptor for the OPG ligand (OPGL = RANKL), which is also involved in multiple networks, is a key player in osteoclast maturation and bone turnover (95). Although the precise effect of OPG in pathologically altered bone diseases is still controversial (94), there is evidence for OPG as a natural inhibitor of RANK-associated osteoclastogenesis (96) to limit inflammatory bone loss. Administration of YCEIEFCYLIR (OP3-4), a peptide mimicking OPG, stabilized bone quality in arthritic mice (97). Moreover, a higher RANKL/OPG ratio in patients with RA correlated with a more rapid and erosive course of bone damage (98). Also, RANKL-deficient mice present significantly improved bone quality and the absence of typical bone loss when suffering from experimental arthritis (99).

Inflammation inside RA joints leads to an upregulation of osteoclasts. TNF, which is expressed in inflamed joints in huge concentrations, directly activates osteoclast maturation and enhances precursor cell proliferation through NF-κB, which is crucial for the progression of RA (100). Interestingly, reduced bone mineral density (BMD) is already present in patients in early disease stages, in which inflammation has not yet evolved (101). Reduced BMD in this early state could instead be explained by a direct induction of bone resorption processes via autoantibodies, namely RF and ACPA, which circulate before the onset of the disease, form immune complexes, and then directly stimulate osteoclasts (102). This later ascertained pathway highlights the pathogenic role of the serum markers RF and ACPA as important prognostic estimators not only for inflammation and joint destruction but also for bone affection.

In summary, bone resorption can be seen as a complex interplay of premature bone cells, mature bone cells and immune cells, interacting with multiple signalling peptides

and cytokines. Although many aspects of how RA affects osteoclast metabolism remain unexplored, it seems proven that an upregulated RANKL pathway leads to excessive osteoclast activity and increased bone resorption with consecutive decreased bone density and increased risk of fractures.

1.2.2 Bone Formation and Erosion Repair

The inflammatory setup, as it takes place in RA, not only boosts bone resorption but also unbalances bone turnover, thus disrupting osteoblast-delivered bone repair.
Bone gamma-carboxyglutamic acid-containing protein (BGLAP) and tissue non-specific alkaline phosphatase (ALPL) are two reliable markers of bone formation. BGLAP, also referred to as osteocalcin, is a calcium-binding protein that is secreted by osteoblasts. BGLAP controls the bone mineralization process and can be used as a serum bone formation marker (103). ALPL is also abundantly expressed by osteoblasts and can be used as a reliable marker of bone formation (104).
Bone formation is predominantly regulated by runt-related transcription factor 2 (Runx2). Runx2, as a master regulator of osteogenic differentiation, is an essential pleiotropic transcription factor that controls osteoblast-delivered bone formation (105). Peripheral blood samples from RA patients show decreased gene expression of Runx2 (106). *In vivo* experiments in mice further suggest that osteoblasts in inflammatory surroundings show an impaired ability to repair bone lesions and maintain bone formation due to downregulated Wnt signalling (107).

1.2.2.1 The Wnt Pathway

Proteins from the Wnt family are involved in various growth processes during ontogenesis and the evolution of different tissues. They facilitate osteoblast proliferation and differentiation. Wnt family proteins further stimulate osteoblasts via G-protein-coupled receptors (GPCR), interacting with the intracellular β-catenin pathway (108).
While being downregulated in osteoblasts, as RA proceeds, Wnt seems to be upregulated in FLS (109). Inflammation triggers FLS to release dickkopf-1 (DKK1), a Wnt antagonist that indirectly inhibits osteoblastic differentiation by binding to the LDL receptor–related protein 5/6 (LPR5/6) and the coreceptor Kremen-1/2 (110, 111). LPR5/6 are part of the normal Wnt cascade. DKK1 bridges both receptors to Kremen-

1/2, causing internalization of the ligand-receptor complex and inhibition of downstream Wnt signalling (112). This mechanism takes place especially in the early phase of arthritis, indicating that DKK1 could be another critically dysregulated master regulator in the development of bone erosions (113). Alterations in the rheumatic bone metabolism are visualized in Fig. 1. The Wnt pathway is dysregulated in opposite directions inside osteoblasts compared to FLS during RA, highlighting the complexity of physiological bone metabolism with crosstalk between osteoclasts, osteoblasts and FLS.

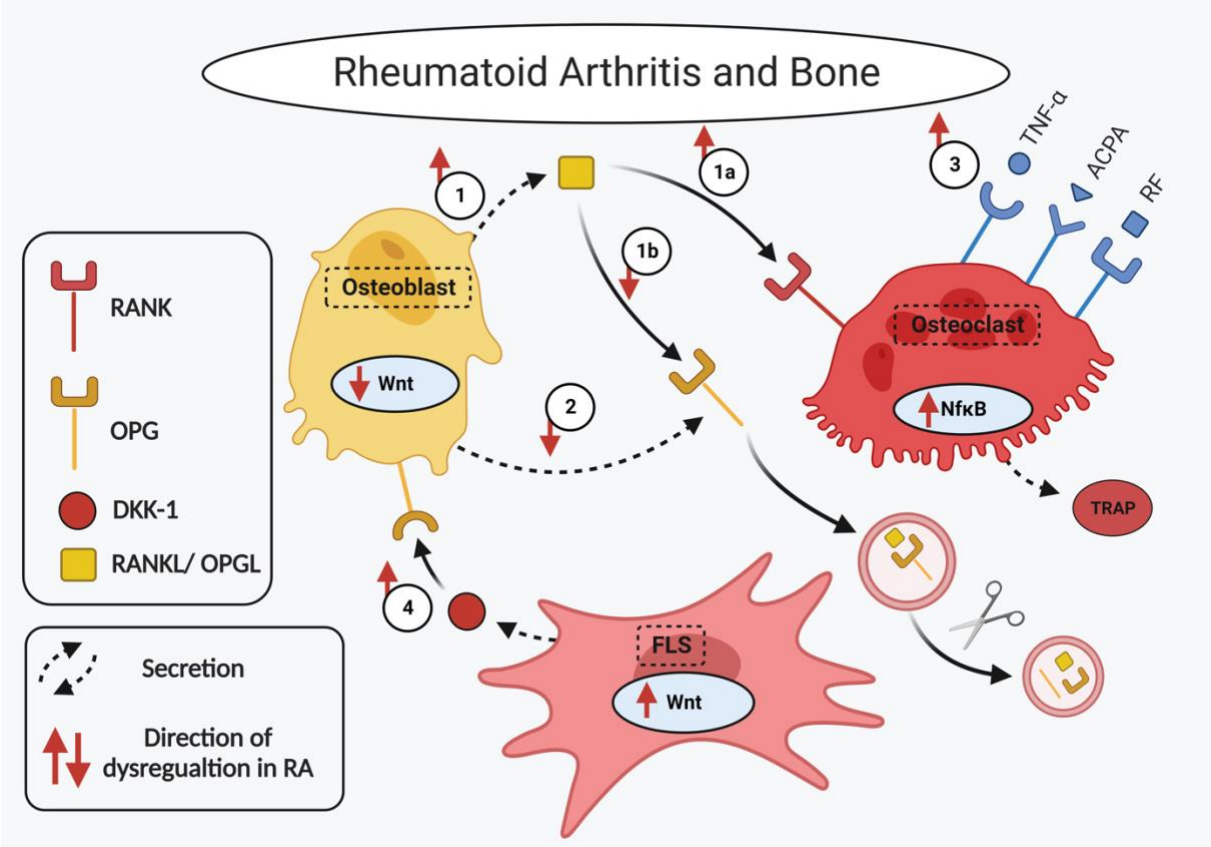

Fig. 1. RA-related dysfunctional pathways in bone metabolism (figure created with Biorender.com).

Fig. 1. RA-related dysfunctional pathways in bone metabolism. The figure shows alterations that lead to bone loss in RA patients. The Wnt pathway is downregulated in osteoblasts, while it is upregulated in FLS. TRAP is released by osteoclasts, indicating their activity.
(1) Osteoblasts secret RANKL (= OPGL) which either binds to (1a) RANK and stimulates the differentiation of osteoclasts, or to (1b) OPG, which leads to internalization and degradation, preventing RANKL/RANK-interaction. (2) The Wnt pathway stimulates osteoblasts to secrete OPG. In RA, the RANKL/OPG ratio is changed in favor of RANKL. (3) RF, ACPA and TNF-α, all increased in RA, bind to osteoclasts and upregulate the NF-κB pathway. (4) FLS release DKK-1, which interacts with osteoblasts and downregulates the Wnt pathway.

1.2.3 Cytokines and Bone

During the progression of RA, pro-inflammatory cytokines contribute to the development of bone erosions.
TNF-α has been proven to induce DKK1. Mice that lack DKK1 were spared from focal bone lesions when developing artificial arthritis (111).
There is evidence that increased IL-6 signalling could be a key factor for impaired bone erosion repair in RA. An observational study showed a significant reduction of erosion volumes in the metacarpal head and radius of RA patients when treated with tocilizumab (114). Furthermore, high circulating plasma levels of IL-6 seem to serve as an independent prognostic factor for accelerated radiological progression of bone erosions in untreated RA patients (115).

In summary, RA affects bone quality by enhancing bone resorption through elevated RANKL signalling but also by compromising osteoanabolic activities and repair mechanisms from osteoblasts via antagonising the Wnt cascade.
Osteoblastic Wnt signalling seems to upregulate the secretion of OPG from osteoblasts, suppressing the differentiation of osteoclasts (116) (Fig. 1). Despite bone quality being directly affected by RA-associated changes, the chronic disease is mostly treated by prolonged application of drugs. In the case of RA, therapy may add indirect disease-related bone damage. Glucocorticoids are administrated both for handling flares and supplementing low-dose permanent therapy (117). Long-term administration of glucocorticoids can lead to glucocorticoid-induced osteoporosis (GIOP), a dose-

dependent drug-induced derangement of bone homeostasis, leading to systemic bone loss and increased fracture risk (118). Since glucocorticoids are still widely prescribed in RA patients, GIOP constitutes a major concern in long-term RA treatment (73, 75).

1.3 The CAIA Model

RA is a complex, systemic inflammatory disease affecting multiple organ systems. To study RA outside the human body several *in vivo* RA models have been proposed (119-121). Synovial tissue explants, 2D monolayer cultures and 3D cartilage tissue models are *in vitro* approaches to investigate distinct aspects of the pathogenesis or response of RA to therapeutic agents. However, tissue explants often exhibit a short survival time and an altered metabolism from donor-associated diseases and medications. 2D cultures lack the complex 3D network of articular cartilage and recent 3D cartilage models are still in a process of evolution to fit human physiology (122). Different animal models with strengths and weaknesses have been invented over the years to reconstruct inflammatory arthritis in murine experiments. The collagen-induced arthritis (CIA) model is based on long-existing knowledge from the 1970s about autoreactive antibodies against collagen type II, which naturally occur during joint inflammation in RA. Collagen type II is the dominating collagen in hyaline cartilage (119, 123). CIA is a murine model which has been used widely since the 1980s to study arthritis in mice. Courtenay et al. managed to induce severe and fulminant arthritis in different mice strains approximately 30 days after intradermal injection of bovine collagen type II (124). Despite simplifying the induction of cartilage autoimmunity by just targeting collagen type II, CIA shows essential similarities to human RA, including cartilage degradation, synovial hyperplasia and mononuclear cell infiltration, as well as the presence of B- and T-lymphocytes reactive to collagen type II (125). The collagen antibody-induced arthritis (CAIA) model was developed as an improved CIA model, addressing some of its limitations and shortcomings, while still being based on autoantibodies targeting collagen type II. The CAIA model was therefore applicable in genetically modified mouse strains that were not susceptible to CIA, and the time to symptom development was considerably shorter in the CAIA model (121, 126).

The antibody-cocktail used for our experiments contains 4 antibodies against the C1, J1, D3 and U1 epitopes of collagen type II, which have all been shown to not only induce arthritis in mice, but further to be specifically involved in human RA (126, 127).

In general, antibodies show highly specific binding sites to distinct epitopes, and collagens display large matrix peptides with several of those epitopes (128). Therefore, trials with multiple combinations of potential epitopes were conducted to design antibody cocktails that reliably induce synovitis (129). While the onset of CIA takes several weeks via the activation of antibody-producing B-cells, CAIA is characterized by a rapid disease onset. Transgenic mice fail to respond to CIA, while they do respond to CAIA, because CAIA bridges the major histocompatibility complex type II (MHC) class-dependent activation of immune cells in collagen type II autoimmunity (130-132). The mosaic of involved epitopes that are suitable for inducing arthritis varies according to the strain and age of mice, hence it has to be matched to experimental conditions (132). Other approaches, based on autoantibodies, include the K/BxN-serum transfer arthritis model, which uses naturally occurring antibodies against glucose-6-phosphate isomerase (GPI) that lead to spontaneous development of severe arthritis in transgenic mice expressing the K/BxN T-cell receptor (*Krn* gene) and a specific MHC haplotype. Serum of K/BxN mice can be transferred to various mice strains to induce experimental arthritis (120).

1.4 CGRP

Calcitonin gene-related peptide (CGRP) must be divided into an α- and β- isoform. αCGRP is mainly expressed in sensory nerve fibres, while βCGRP is localized in the enteric nervous system (133). As most literature does not discriminate between the two isoforms, “CGRP” will be used if the authors did not report isoform specifics. Our experiments were, however, conducted using mice deficient for αCGRP, which is why we use the appropriate description when referring to our data.

1.4.1 Discovery

CGRP was originally discovered by Amara et al. in 1982 (134) when investigating the calcitonin gene and its functions. After serially transplanting calcitonin producing medullary thyroid carcinoma tumour pieces into rats, they surprisingly reported significantly deviating levels of tumour-derived calcitonin production in some samples. They inferred that distinct post-transcriptional modifications of messenger ribonucleic acid (mRNA) could lead to a quantitative variance in calcitonin biosynthesis (135, 136). They further experienced that this decrease in the calcitonin protein and calcitonin specific mRNA expression correlated with a compensatory increase in an alternative “pseudo calcitonin” or “calcitonin like” mRNA, which encoded for a new protein while originating from the exact same gene (137). Further investigations of the new *CGRP* mRNA revealed marked differences to mature calcitonin mRNA. While calcitonin mRNA expression accumulated in thyroid cells, *CGRP* mRNA seemed to be foremost present in hypothalamic cells, suggesting a potential humoral function of CGRP as a neuropeptide (134). More detailed research classified CGRP as a 37 amino acid containing neuropeptide, synthesized from the calcitonin gene through alternative splicing predominantly in the midbrain. CGRP can be detected not only in hypothalamic neurons but also seems to be widely distributed in the central and peripheral nervous system, as well as inside blood vessels (138). Later, a second protein with nearly similar amino acid structure but a different, non-calcitonin-related gene origin was observed, leading to the division of CGRP into αCGRP and βCGRP. αCGRP displays the original calcitonin-related peptide and βCGRP shows a slightly different mRNA code and a single difference in the amino acid structure. This difference seemed to explain the similar but still varying distribution patterns along the nervous system (139). The corresponding gene of βCGRP or “CGRP II“ was found to be located adjacent to the previously described *Calc1* gene on chromosome 11 and, therefore, was referred

to as the *Calc2* gene (140). Notably, αCGRP and βCGRP, due to their high homology, act similarly *in vivo* (141). Thus, they are often not differentiated in the literature and just referred to as CGRP. However, αCGRP is likely to be more present in sensory nerve fibres in the dorsal root ganglia (DRG), spinal cord and peripheral tissue, while βCGRP shows a dominating presence in the enteric nervous system, with up to seven-fold higher concentrations than αCGRP (133). CGRP follows a circadian release, with plasma concentration peaks at midday and in the afternoon depending on dietary factors (142). Today, the *Calc1* gene is mostly referred to as *Calca* and *Calc2* as *Calcb*, according to the Gene Nomenclature Committee of the Human Genome Organisation (HGNC). Interestingly, when removing the first N-terminal domain from human CGRP, the residual peptide fragment $hCGRP_{8-37}$ suddenly acts as a CGRP receptor antagonist but calcitonin receptor (CTR) agonist (143).

1.4.2 Release and Distribution

As described above, CGRP is predominantly found in nervous tissue with abundant binding site clustering in vascular proximity and a clear deviation from the distribution patterns of calcitonin (144). Perivascular nerve cells seem to be the most important source for active CGRP. It is important to highlight that the adherence of nerve tissue to blood vessels, which has been studied extensively in cerebral pain genesis, is fundamental for the acting mechanisms of CGRP. In brain perfusion, nerve fibres showing positive immunoreactivity for CGRP can be located abundantly in the close proximity of blood vessels, proving a direct, not only autonomous but also sensorial innervation of arterial vessels (145). In particular, non-myelinated C-fibres and thicker Aδ-fibres show expression of CGRP and its receptors (146).

The release of CGRP is controlled by the interaction with transient receptor potential cation channels (TRP), more precisely the subform vanilloid receptor 1 (TRPV1). TRPV1 is a non-selective cation channel that is located in terminal nerve endings from nociceptive fibres (Aδ and C-fibres), where it essentially mediates the signal transduction from pain-inducing stimuli such as heat, mechanical irritation or chemical activation via capsaicin into an electrical signal (147). Cell culture studies show that capsaicin and heat both increase CGRP expression in endothelial cells, suggesting a direct upregulation of α- and βCGRP, induced by recruitment of TRPV1 (148). Hypertensive models in rats further suggest rutaecarpine, a TRPV1 agonist, to perform antihypertensive effects via stimulation of CGRP release (149).

1.4.3 CGRP-Related Peptides

There are four peptides that show a structural or functional relation to CGRP. Amylin is a diabetes-associated 37 amino acid peptide with structural relation to CGRP which is released by Langerhans islets in the pancreas (150). Adrenomedullin (ADM), a 52 amino acid peptide from the renal medulla, originally extracted from pheochromocytoma samples, shares structural and functional homology with CGRP (151). ADM has successfully been linked to different functions such as vascular homeostasis in the pulmonary cycle, where it improves haemodynamics by inducing vasodilation in rats (152). Substance P (SP), a peptide discovered in 1931 after extracting tissue parts from horses (153), has been linked to CGRP not only in respect of an extensive overlay in distribution patterns inside the DRG, smooth muscle tissue and around blood vessels, but also in respect of similar and even mutual enhancing effects on vascular tone and vascular permeability (154, 155).
Procalcitonin is a large 111-amino acid precursor protein of calcitonin that was identified in 1984 (156). Being encoded by the *Calca* gene, procalcitonin represents a sensitive sepsis marker, specifically sensitive for bacterial infections (157).

1.4.4 Receptors

1.4.4.1 The CGRP Receptor

CLR and CRLR are used separately in the following, as the literature occasionally uses different abbreviations for these terms. In the following lines, CLR will stand for the CGRP receptor as a whole functional unit, consisting of 3 subunits: receptor activity modifying protein one (RAMP1); the calcitonin receptor-like receptor (CRLR), which displays one single subunit protein of the CLR; and the receptor component protein (RCP). CLR = CRLR + RAMP1 + RCP.

The underlying mechanisms of CGRP signalling have been discussed controversially for some years. Different receptors, originally described as CGRP1 and CGRP2 as well as others with coaffinity for CGRP, such as closely related amylin receptors, were identified in early investigations (158). Later, it turned out that the discovery of the calcitonin receptor-like receptor (159) replaced the initial terminology. Today, the CRLR plays the most significant role in effect mediation, and hence in pharmacological targeting. The nomenclature results from its extreme similarity with the longer known

calcitonin receptor amino acid sequence, reaching a 56 % homology rate in humans and 91 % in rats (160). After the first failed approaches in determining the receptor-ligand affinity of the CRLR in cell cultures, the family of RAMP was discovered and this finally led to a better understanding of CRLR physiology. RAMP are indispensable for CRLR function and activation. In particular, three types of RAMP can modify the final layout and function of the CRLR. While RAMP1 is necessary for the function, surface presentation and binding ability of the CRLR and therefore its function as the CLR, RAMP2 and RAMP3 build ADM receptors when being co-expressed with the CRLR protein at the cell surface (161). This fact explains the unsettled contradictions in early receptor classifications and shows how one receptor protein can exhibit different ligand binding behaviours depending on which supportive factor is co-expressed. Furthermore, RAMP seem to be able to modulate the binding activity of the CTR, including cross-binding to CGRP (162). The final CGRP receptor (CLR) belongs to the family of GPCR and can be seen as a composite receptor protein consisting of the CRLR, RAMP1 and RCP1, a second ancillary and longer known protein that plays an important part in CLR function and regulation (163, 164).

1.4.4.2 GPCR Signalling of CGRP

GPCR are a large receptor family. All GPCR share similar morphological characteristics and signalling attributes. The hallmarks have been found to be a structure consisting of 7 transmembrane domains with an extracellular N-terminus and intracellular C-terminus (165). GPCR are divided into several subfamilies characterized by parallels in pathway details. The CLR, just like the CTR, belongs to the type B secretin-like GPCR family, more precisely subfamily B1, from which all members seem to act predominantly through a $G\alpha_s$-subunit-mediated increase of cyclic adenosine monophosphate (cAMP) via coupling to the adenylate cyclase (AC) (166). The $G\alpha_s$-subunit has turned out to solely display one out of many subunits of possible G protein-receptor complexes, which can occur in many different layouts and activation pathways, leading to various functions all over the body, being a small part of a broad and complex GPCR network (167).

Although it has been implicated that CGRP also acts through additional types of known GPCR signalling ways like phospholipase C (PLC) activation (168) and several others, including gene expression effects (Fig. 2), cAMP signalling with consecutive protein

kinase A activation (PKA) is still the best investigated pathway and, therefore, the main subject of interest in pharmacological targeting (141, 169).

1.4.4.3 Desensitization, Degradation and Recycling of the CLR

Like other GPCR, the affinity and expression of the CLR is precisely regulated. GPCR signalling is an important way of cell-to-cell communication in human physiology and neurophysiology. The reward system (dopamine) and calcium homoeostasis (parathyroid hormone) are partially regulated by GPCR signalling. In general, all GCPR share a periodic cycle, in which the receptor is internalized into the cytosol. The internalization is directly induced by ligand binding-mediated activation of endocytosis, followed by receptor recycling and transfer to the surface membrane for the next activation (170). To guarantee a well-balanced homeostasis in cellular signalling, this cycle is controlled by long- and short-term regulatory processes that can desensitize the pathway. When being stimulated by the ligand excessively or continuously, the system reacts with a reduction of the number and excitability of available receptors on the membrane via phosphorylation and increased ubiquitination in lysosomes, whereas intermittent stimulation of the GPCR preserves adequate resensitization (171). Concerning the CLR and CGRP signalling, endothelin-converting enzyme-1 (ECE-1), a member of the metalloendopeptidases, is important. Metalloendopeptidases represent an enzyme family which has long been proved to play a pivotal role in the regulation of many signalling pathways by modifying peptides via cleaving. ECE-1 could serve as a highly specific regulator of CLR recycling. Another well-known example for metalloendopeptidases is angiotensin I converting enzyme (ACE), which regulates cardiovascular long-term adaption by converting angiotensin 1 (AT1) into the active AT2 and in addition inactivates bradykinin by cleaving off a terminal peptide part (172, 173). ECE-1, in particular, has been identified to impact the regulation of CLR recycling, hence mainly directing desensitization. As has been similarly observed in other GPCR, such as the β2-adrenoreceptor (174), the CLR is internalized via endosomal endocytosis under the participation of clathrin and β-arrestin2. More precisely, the CLR, RAMP1, CGRP and ECE-1 are co-internalized together. ECE-1 then specifically degrades CGRP inside the early endosome and enables recycling of the CLR to the surface, and thereby the restoration of the signalling sensitivity by releasing the CLR from β-arrestin2, which otherwise seems to trap the CLR inside the endosome (175). The enzymatic activity of ECE-1 could be

one exemplary attempt at an explanation for the mechanisms of re- and desensitization in CGRP signalling. Moreover, sustained stimulation of the CLR seems to increase the rate of lysosomal degradation of the CLR and thus the decimation of the available receptor, possibly displaying a natural mechanism of limitation of excessive GPCR activation (176). In summary, the current evidence points towards a complex physiology of the CLR in crosstalk with numerous regulatory mechanisms that maintain an adequate signal stream intensity by adapting the surface expression of active receptor proteins.

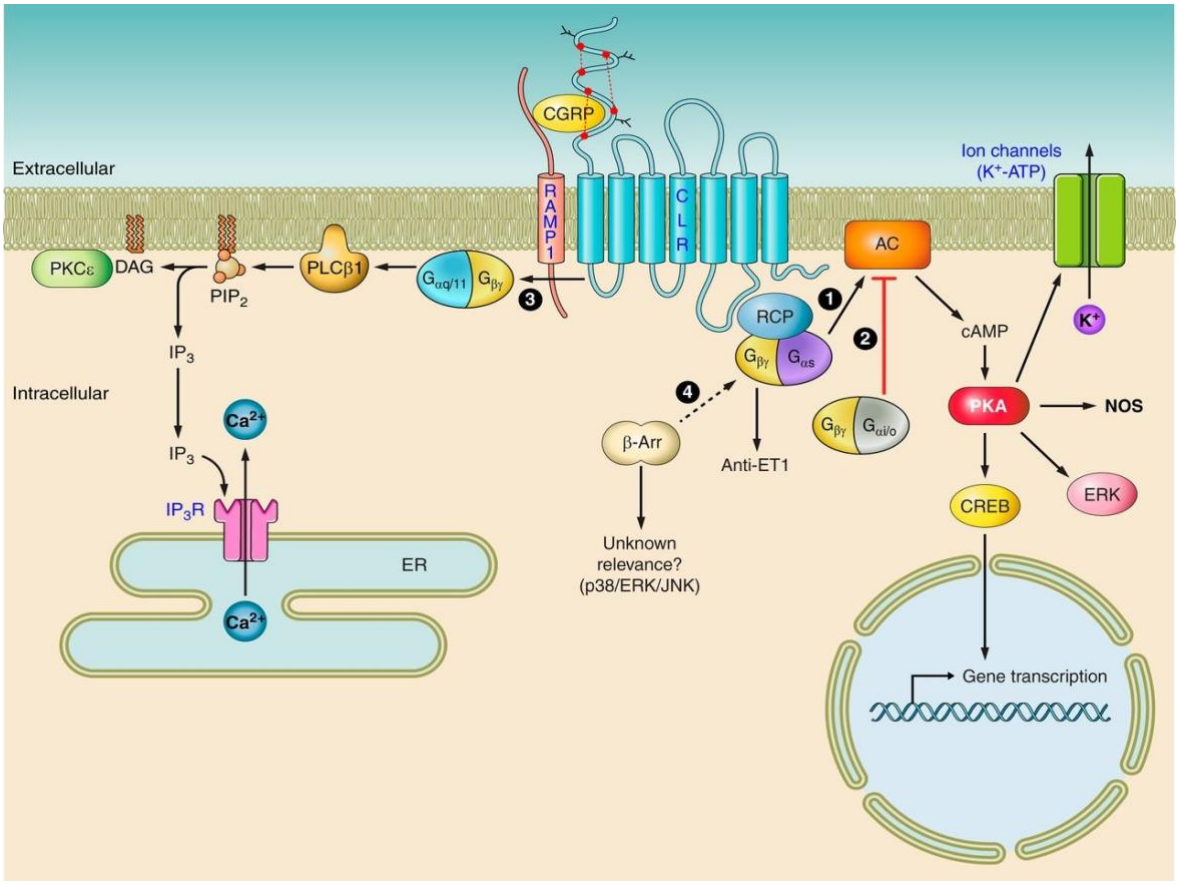

Fig. 2. Visualization of different mechanisms of acting for CGRP as a GPCR-representative (from Russell et al., 2014 (141), with written consent from the American Physiological Society).

Fig. 2. Visualization of different mechanisms of acting for CGRP as a GPCR-representative. CGRP binds to the CLR consisting of the CRLR, RAMP1 and RCP. The most investigated downstream pathway is marked with (1). (1) Binding to the $G_{\alpha s}$-subunit causes the cAMP concentration to rise via activation of AC with the consecutive activation of PKA and cAMP response element-binding protein (CREB) exerting effects on gene expression as well as activating nitric oxide synthase (NOS), potassium (K^+) channels and extracellular signal-related kinase (ERK). Effects which include β-arrestin (4) rest upon poorer evidence. (2) Alternative binding to the $G\alpha_i$-subunit resulting in inhibition of AC can be seen subordinately. (3) Binding to the $G_{\alpha q/11}$-subunit, causing activation of PLC and later phosphatidylinositol 4,5-bisphosphate (PIP2) with downstream activation of inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG) as second messengers with final activation of protein kinase C (PKC) and calcium ion (Ca^{2+}) release from the endoplasmic reticulum (ER), also known as the IP3/DAG pathway, has been reported but not linked intensively to clinical effects to date.

1.4.5 CGRP and Vasodilation – a Breakthrough in Migraine Therapy

Since the discovery of CGRP and its role in pain signal transduction made by Amara et al. in 1982 (134), αCGRP has been investigated intensively and a variety of different functions have been identified over the past few decades. Soon after unmasking the molecular structure of CGRP, its important function as a potent vasodilator was reported. CGRP was demonstrated early on to cause flushes after injecting femtomole doses of synthetic CGRP in human probands intradermally (177). These potent vasodilative effects can be explained by experiments which confirmed that CGRP relaxes smooth muscle tissue by opening adenosine triphosphate (ATP) -sensitive potassium channels (K_{ATP}) via the PKA/cAMP pathway (178), subsequently causing hyperpolarization and resistance to neuronal electrical stimulation (179). CGRP seems to open K_{ATP} channels, both directly via the cAMP/PKA (178) pathway and indirectly via an endothelium-delivered release of nitric oxide (NO) (180, 181). NO induces K_{ATP}-channel-dependent hyperpolarization of smooth muscle cells via activation of the guanylate cyclase and a subsequent increase of cyclic guanosine monophosphate (cGMP) (180). Furthermore, CGRP has been linked to angiogenesis and is thought to play an essential role in sufficient wound healing since αCGRP-deficient mice showed disturbed repairing processes in a wound healing model (182).

The presence of CGRP in cerebral arteries first led to a possible connection to migraine pathophysiology in 1985 (183). The dedicated search for novel targeted therapies in this field later enabled new innovative and selective drugs aiming for a blockade of CGRP signalling that made their way into clinical application. Today, numerous CGRP blocking drugs have been tested in clinical settings on migraine patients. The process of unveiling the role of CGRP as a part of neurogenic inflammation and pain induction has led to a deeper and more complete understanding of the pathophysiology of vascular headache (184).

1.4.6 CGRP-specific Pharmacotherapy

The first selective inhibitor of CGRP was BIBN4096BS (olcegepant), a small molecule that acts as a pure CGRP receptor antagonist and effectively inhibits neurogenic vasodilation *in vitro* and *in vivo* (185). Beside other CGRP receptor antagonists ("-gepants"), antibodies binding specifically to either CGRP itself or the CLR are available and under ongoing clinical investigation (186). The current focus of research has shifted slightly towards monoclonal antibodies (mAb), because serious concerns about

hepatotoxicity seem to be overshadowing the enthusiasm in pushing the implementation of “-gepants” into clinical use (187). Therefore, mAb are increasingly moving into the focus of research in CGRP-specific pharmacology. Here, erenumab, fremanezumab, galcanezumab and eptinezumab from the group of anti-CGRP mAb seem to display effective and safe medication opportunities that are already in clinical use for prevention therapy in chronic migraines (188, 189). Erenumab holds a special position here, as it directly binds to the CLR. Concerning safety in clinical application, mAb do not show a significantly increased risk of adverse effects like upper airway infection, sinusitis, constipation, arthralgia, fatigue and nausea when compared to placebo (189).

Cardiovascular side effects of blocking the CGRP signalling pathway have been a major concern since introducing anti-CGRP drugs to the market. CGRP was preventive in vascular wall remodelling after induced hypertension in mice (190) and protective for the myocardium by inducing coronary vasodilation in artificially induced heart ischemia in rats (191).

Even though no severe cardiovascular adverse effects have been detected in monotherapy with anti-CGRP mAb in safety analyses with smaller cohorts so far (192), there still remains doubt over the safety of anti-CGRP drugs.

1.5 CGRP and Inflammation

The presence of CGRP in DRG, the enteric nervous system and vascular environments has been investigated intensively. However, there is also evidence that CGRP signalling is connected to inflammation, especially joint inflammation. The phenomenon that the stimulation of afferent sensory nerve fibres can manipulate vascular tone, displaying the basis of neurogenic inflammation, was documented early in the 20th century (193). To date, a long journey of research has revealed that CGRP is an essential key player in neurogenic inflammation, especially in the skin. CGRP mediates vasodilation and increased vascular permeability together with SP and other neuropeptides (194). CGRP further seems to enhance the effects of IL-1 in skin edema formation (195), to increase IL-1β-mediated neutrophil accumulation in a mouse model (196), and to promote leukocyte adhesion to the endothelium in cultured cells as a key part of the initiation of general neutrophil inflammation (197). Taken together, these findings all point towards a not negligible participation of CGRP in neurogenic inflammation in the skin and potentially even beyond the skin.

However, there are also hints for anti-inflammatory effects of CGRP that complicate positioning it inside the immunological network. In murine peritoneal macrophages, CGRP showed attenuating effects on lipopolysaccharide (LPS) -induced TNF-α secretion (198) and murine lymphatic tissue showed depressed proliferative T-cell response after CGRP application (199). Furthermore, early results are indicating an inhibitory effect of CGRP on B-lymphocyte differentiation *in vitro* (200). Whether CGRP must be seen as a pro- or anti-inflammatory peptide thus remains a complicated question with substantial need for further investigation. The evidence about the role of CGRP within the immune system is still inconclusive.

1.5.1 CGRP and Synovial Inflammation

Regarding synovial inflammation and arthritis, there is little evidence for CGRP-related mechanisms that could be interesting for future research in RA. CGRP concentrations significantly increased in hypertrophied synovia of ankle joints of rats as well as in adjacent DRG when joints were challenged with inflammation, suggesting CGRP to be involved in arthritic settings (201). Also, increased CGRP levels in the DRG of arthritic mice were alleviated by anti-inflammatory corticosteroid therapy in another murine study using the CIA model (202). Synovial fluid analyses from a small patient cohort undergoing arthroscopy showed increased levels of CGRP in RA patients compared to patients suffering from other knee joint disorders (203). Furthermore, CGRP upregulated the production of IL-17 in isolated murine T_h-17 cells in a dose-dependent manner after stimulation with either αCGRP or βCGRP according to a study investigating experimental autoimmune encephalomyelitis (204). IL-17-producing T_h-17 cells have been found to play an important role in RA and other autoimmune diseases (43).

Owing to its origin and primary appearance inside terminal nerve endings, CGRP could also cause RA-related pain, which represents a massive burden for patients. CGRP-deficient mice were protected from hyperalgesia after arthritis induction in knee joints in a murine study (205).

1.6 CGRP and Bone

While calcitonin, as the oldest known representative of the *Calca* gene family, was found to be an indispensable player in calcium- and bone homeostasis long ago (206),

research has focused on the effects of CGRP on the cardiovascular and neurovascular system since soon after its discovery. Investigations focused on vasodilation, permeability of blood vessels, wound healing and pain induction, all of which are closely related.

Early investigations are, however, suggesting human CGRP (207), as well as rat CGRP (177), to influence bone metabolism by inhibiting osteoclast-delivered bone resorption *in vitro* when applied in extremely elevated dosages compared to calcitonin. Furthermore, immunohistochemical studies have shown enhanced CGRP expression originating from ingrowing nerve fibres inside the forming callus throughout bone healing in a rat fracture model (208). These CGRP-positive nerve fibres inside the bone matrix have been found to not only grow into the callus after the bone structure has been damaged, but also to be present in intact bone, especially inside the epiphysis near the joint, where the fibres directly reach close proximity to osteoclasts (209). Beyond the mere occurrence of CGRP in the bone environment, studies investigated the actual effect of CGRP on bone quality. For example, a murine study using transgenic mice artificially expressing CGRP in osteoblasts observed increased bone formation rates, bone volume and bone density when CGRP was expressed (210). We were previously able to show that mice specifically lacking αCGRP exhibit a significantly impaired bone quality (211). This osteopenic phenotype in $\alpha CGRP^{-/-}$ mice was observed earlier (212) and was recently verified to progressively intensify throughout the ageing process with significant manifestation at the age of 9 months in mice (213). In addition, mice lacking TRPV1, which is important for stimulating the release of CGRP, showed severe bone loss in a model of periodontitis (214). Additionally, transgenic $\alpha CGRP^{-/-}$ but not $\beta CGRP^{-/-}$ mice failed to respond to osteocyte-detected force adequately. The force in this study was created by applying ulnar loading, which usually represents a proliferation stimulus in intact bone (215).

Concerning subchondral sclerosis and cartilage loss, blocking CGRP via administration of olcegepant alleviated artificially induced murine knee osteoarthritis (OA) (216). This study indicates negative CGRP-mediated effects on bone that is affected by OA. However, the distinct impact of CGRP on cartilage in physiological and pathological conditions remains mostly unclear.

In summary, CGRP is required to preserve an intact equilibrium of bone formation and resorption. This equilibrium is essential, both for normal bone constitution and fracture healing throughout the ageing of the musculoskeletal system. However, the role of

CGRP in joint pathologies such as RA and OA that markedly affect bone is still mostly unkown.

1.7 Aim of this Study

RA is a progressive systemic disease leading to destructive joint inflammation and impaired bone quality. The role of αCGRP, a pleiotropic neuropeptide which acts on blood vessels, pain induction, bone metabolism and inflammation, in RA is however still unclear. The aim of this study is to characterize the function of αCGRP in the context of inflammatory arthritis and evaluate its potential as a therapeutic target for RA. Implementation of αCGRP as a potential target in RA therapy is principally uncomplicated since monoclonal antibodies inhibiting αCGRP signalling are already in clinical use for migraine therapy.

We combined the total knockout of endogenous αCGRP production in mice with an antibody-mediated arthritis model characterized by transient joint inflammation. The extent of joint inflammation and bone destruction was investigated clinically, histologically and on a molecular level.

2 Materials and Methods

2.1 Animals

All experiments were carried out in accordance with the Animal Welfare Act (Federal Law Gazette I, p.1094). We obtained ethical approval (G-0044/18) from the local animal rights protection authorities (LAGeSo). All animals used in the experiments were male C57BL/6J mice, aged 10 - 12 weeks and kept in standard cages in a 12 h light-/ 12 h dark cycle, having access to water *ad libitum* while receiving regular dry pellet food. All arthritic mice additionally received metamizole (1350 mg/ kg body weight/ day) *per os* via drinking water for analgesia. Animals were assigned randomly to their experimental group. Emergency procedures in case of critical conditions of the animals were determined in prior to the experiment. Humane endpoints included >30 % body weight loss compared to day 0 for more than 24 h, moribund appearance, limping and rigid movement behavior. To facilitate nesting and protect physical comfort, SAFE® compact crinklets (Safe Diets, Augy, France) and soft bedding were implemented in each cage.

2.1.1 αCGRP$^{-/-}$ Mice

To study *in vivo* mechanisms of CGRP in animals, a knockout model of mice specifically lacking αCGRP was introduced in 1999 (217). The specific creation of mice lacking αCGRP without impaired calcitonin synthesis was described by Lu et al. The knockout was created by insertion of a TGA stop codon via oligonucleotide-directed site mutagenesis right between the N-terminal (N-proCGRP) peptide and the CGRP-encoding region within exon 5 of the gene with additional duplication and reinsertion of the following carboxyl-terminal peptide sequence. This manipulation resulted in a gene which produces normal calcitonin but an altered αCGRP prohormone specifically lacking the αCGRP peptide sequence while coding intact N- and carboxyl-terminal peptides (217). All transgenic mice were backcrossed at least seven times to ensure a pure C57BL/6J genetic background.

2.2 Study Design

2.2.1 Experimental Groups

We compared 8 αCGRP$^{-/-}$ CAIA with 8 wild type (WT) CAIA animals, as well as 4 αCGRP$^{-/-}$ control (CTRL) and 4 WT CTRL animals on day 10. Six αCGRP$^{-/-}$ CAIA were compared to 5 WT CAIA, 4 αCGRP$^{-/-}$ CTRL and 4 WT CTRL mice on day 48 (Fig. 3). All animals were screened daily over the course of 10 or 48 days. The two observation periods were chosen to distinguish between the acute inflammation phase (day 10) and the resolution phase of the arthritis (day 48) (218).

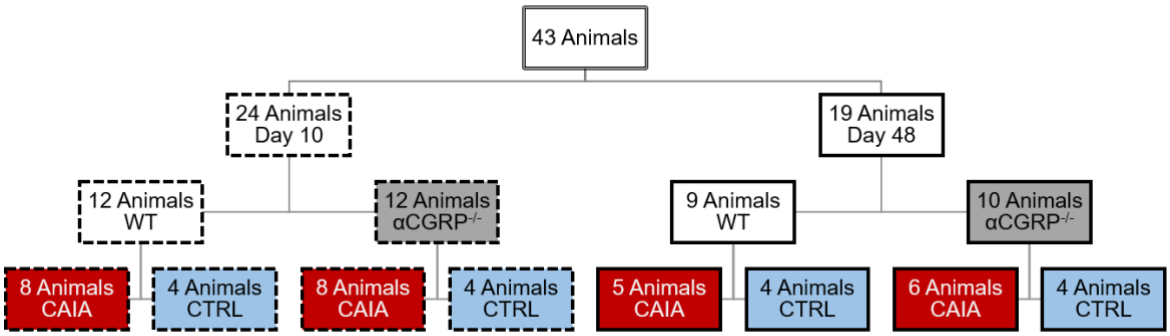

Fig. 3. Experimental groups.

Fig. 3. Experimental groups. Tree diagram shows allocation of animals to their experimental group.

2.2.2 Induction of Arthritis

Arthritis was induced by intraperitoneal injection of 8mg (400µl) of collagen type II antibody-induced arthritis cocktail (CAIA, ArthritoMab Antibody Cocktail, MD Bioproducts, Zurich, Switzerland) on day 0 and intraperitoneal injection of 100 µg (200µl) lipopolysaccharide (LPS) on day 3. Accordingly, the same volumes of PBS were administrated at the same time points in CTRL animals (Fig. 4).

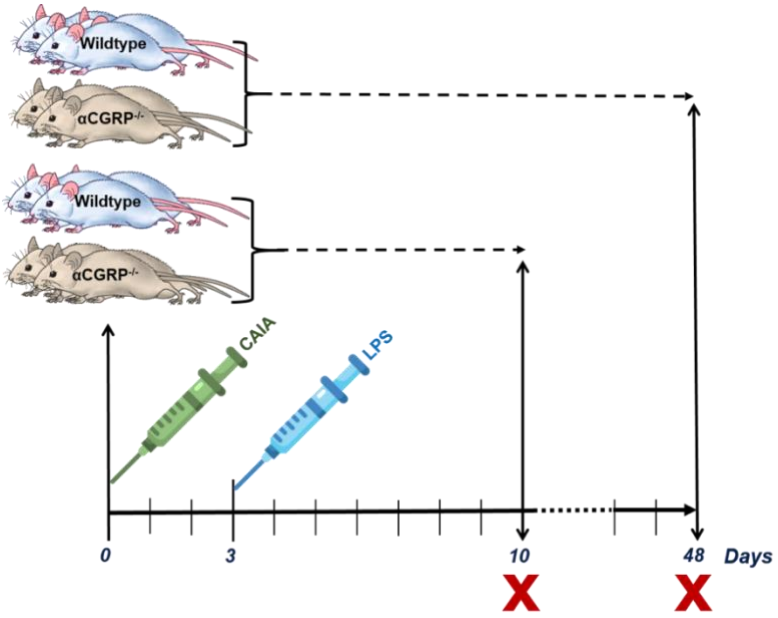

Fig. 4. Timeline for conducted experiments.

Fig. 4. Timeline for conducted experiments. The timeline of the experiments is illustrated as a diagram. Time points for specimen collection are highlighted (red cross).

2.3 Clinical Evaluation

2.3.1 Body Weight

From day 0 to day 10 and day 48, all of the parameters listed below were evaluated daily. Mice were weighed using a scale.

2.3.2 Grip Strength

The grip strength of the forepaws was measured using a grip strength device (BIO-GS3 grip strength test, Bioseb - *In Vivo* Research Instruments, USA/ Canada) (Fig. 5). To visualize grip strength results, data are presented as the mean of 5 consecutive measurements as well as the maximum force recorded in 5 measurements for each day. For grip strength, the restricted cubic spline model was utilized for analyzing values from day 5 to 20 (conducted with the help of D.Z. and R.R.).

Fig. 5. Grip strength testing.

Fig. 5. Grip strength testing. The picture shows the grip strength measuring setup. Mice were held by the base of the tail and gently pulled away horizontally from the grid of the device, which was connected to a sensor, detecting the applied traction. It was ensured that mice only used front paws to hold onto the grid.

2.3.3 Clinical Scoring

An established semi-quantitative score was used for clinical scoring (219). Each of the four paws was evaluated and the scores of each paw were summed up. The scores were applied as follows: 0 for no visible swelling or redness; 1 for slight swelling and

redness of the ankle or wrist; 2 for moderate swelling and redness of the ankle or wrist or a maximum of two swollen digits; 3 for severe swelling and redness of the entire ankle or wrist or swelling of all digits. A cumulative score of 12 was the maximum per mouse.

2.4 Tissue Collection and Preparation

All mice were euthanized at day 10 or 48 by cervical dislocation after anesthesia with an intraperitoneally injected ketamine 60 mg/kg bodyweight and medetomidine 0.3 mg/kg bodyweight mixture.

A midline incision was performed to expose the animal's abdominal organs. One cm long bowel samples from WT animals were obtained from different parts of the gastrointestinal tract (GIT), including the proximal jejunum (n = 3), distal ileum (n = 3) and distal colon (n = 3). These samples will later be referred to as the bowel control group.

For molecular analysis, the left wrist and left hind paw were obtained. The bones for molecular analyses were prepared thoroughly, wholly liberated from all soft tissue pieces and stored after shock freezing in liquid nitrogen at -80 °C until further usage. Serum was removed, and also shock frozen with liquid nitrogen and stored at -80 °C until further usage. The tail tips were taken for genotyping and stored at -20 °C until analysis.

For histological and radiological analysis, the right hind paw and right knee joint with adjacent proximal tibia and distal femur were obtained. The bones for histological and radiological analysis were prepared as cautiously as possible, and the skin and external muscles were carefully removed without injuring the joints. The obtained samples then were fixed at 4 °C in 4% paraformaldehyde (PFA) for 12 h and finally washed out and stored in PBS for analysis by micro-computed tomography (µCT). After µCT analysis and before cryo-embedding as described by Kawamoto (220, 221), all samples were dehydrated with ascending glucose solution according to the following plan: 24 h – 10 % / 24 h – 20 % / 24 - 48 h – 30 %. After embedding, all blocks were stored at -80 °C until 7 µm-thick sections were produced by cutting the embedded bones with microtome blades (Feather©, N35HR, pfm medical, United Kingdom). For histological scoring, the specimens were stained with hematoxylin and eosin (H&E) to assess inflammation, toluidine blue (TB) to assess cartilage destruction and TRAP to assess bone erosion.

2.5 Histological Scoring

Synovial inflammation can be visualized with a standard H&E overview staining to evaluate cell infiltration and hyperplasia. For cartilage degradation, TB, which is a cationic dye, is commonly employed. TB binds with high affinity to negatively charged groups of proteoglycans, which are incorporated in hyaline cartilage abundantly (222). TRAP staining is useful to uncover intraosseous fields of extensive matrix resorption mediated by osteoclasts (bone erosions).

For histological scoring, an established semi-quantitative score was used to assess the severity of inflammation, cartilage damage and bone erosion (223). Mean values of two independent and blinded observers (Tazio Maleitzke (T.M.) and Alexander Hildebrandt (A.H.)) regarding genotypes and experimental groups were used. The inflammatory activity was evaluated as follows: 0 for a normal joint; 1 for mild inflammation/infiltration without edema of connective tissue and without synovial hyperplasia; 2 for moderate inflammation or infiltration with surrounding connective tissue/capsular edema and discrete synovial hyperplasia; 3 for severe inflammation/infiltration with distinct surrounding connective tissue/capsular edema and synovial hyperplasia. Cartilage degradation was evaluated as follows: 0 for a normal articular surface; 1 for a slight loss of toluidine blue staining and chondrocyte loss; 2 for moderate loss of toluidine blue staining and chondrocyte loss; 3 for severe loss of toluidine blue staining and multifocal chondrocyte loss. Bone erosions were evaluated as follows: 0 for no erosions; 1 for mild erosions with few areas of bone resorption and few osteoclasts, visible only at higher magnification; 2 for moderate erosions with visible areas of bone resorption and osteoclasts; 3 for distinct erosions with large areas of bone resorption and some osteoclasts in all areas including the cortex.

2.6 Radiological Evaluation via Micro-Computed Tomography

2.6.1 Raw Data Setup

The setup details for radiological assessment including sample positioning, X-ray energy, intensity, voxel size, image resolution and key acquisition parameters were determined following established guidelines (224). PBS was chosen as the sample medium for scanning. All specimen were analysed and reconstructed post-mortem (each knee joint with adjacent tibia and femur, wrist with the distal forearm and ankle

with distal lower leg) by µCT (Skyscan 1172, 70 kV, 142 µA, slice thickness 5.1 µm/ 0.5 Al filter/ rotation step: 0.2/ averaging frames: 3/ random movement: 10). The reconstruction was performed with SkyScan NRecon software (beam hardening 30 %, ring artefact reduction 10 %, maximum output value 0.11, output format PNG) as well as Data Viewer (v1.5.2.4) and ImageJ (v1.52).
Threshold-based volumes of interest (VOI) with a lower threshold of 80 mg hydroxyapatite (HA) /ccm were placed around analysed bone structures.

2.6.2 Proximal Tibia

The VOI for proximal tibiae was set 0.5 mm below the growth plate of the proximal tibia reaching 1 mm in the caudal direction. Global bone parameters were obtained, including bone volume/ total volume (BV/TV in %), absolute bone density (BV mean in mg HA/ ccm) and trabecular parameters including trabecular thickness (Tb. Th.), trabecular number (Tb. Nb.), trabecular separation (Tb. Sp.) and bone surface (BS). For analysis of trabecular bone, the cortex was removed by shrinking the VOI, whereas global bone parameters were obtained with a complete VOI including the bone cortex (Fig. 6). To visualize the pattern of trabecular deterioration, a 3D heat map using BV values was obtained with ImageJ. The colours were converted to black and white.

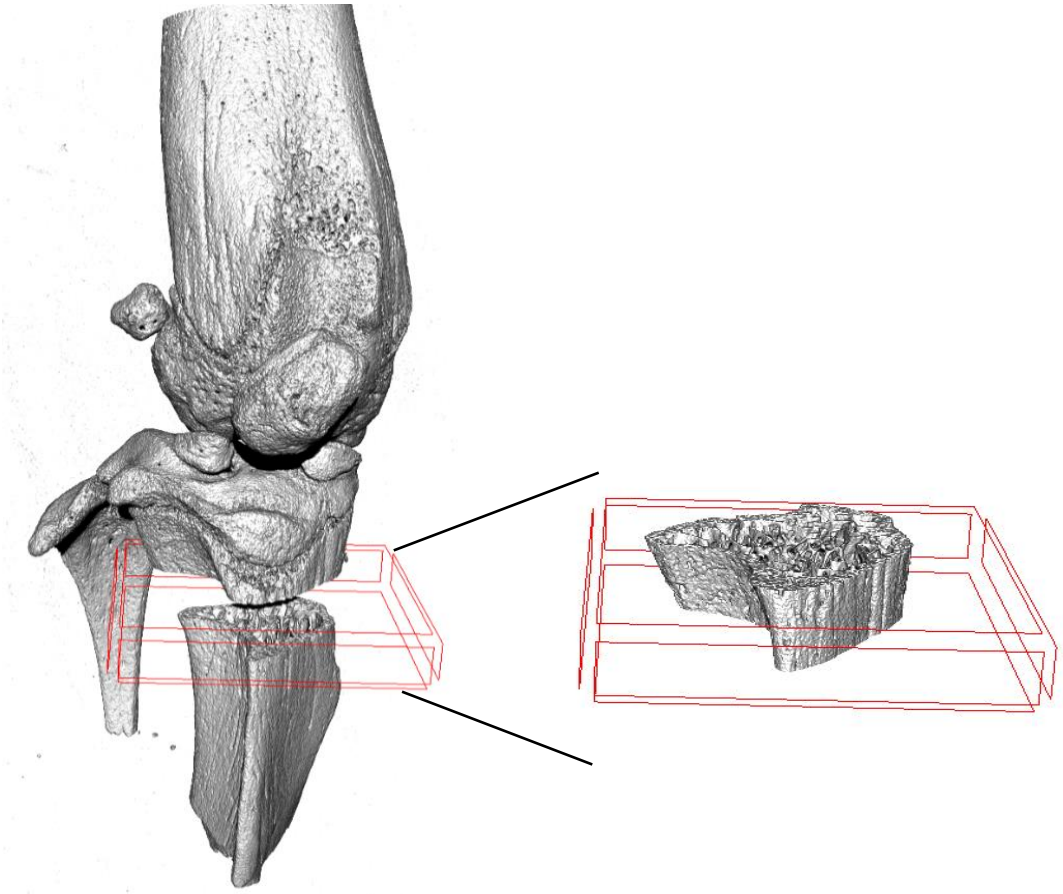

Fig. 6. 3D reconstruction of right knee joint on day 10 following CAIA (adapted from Maleitzke et al. (1), with written consent from the Oxford University Press).

Fig. 6. 3D reconstruction of right knee joint on day 10 following CAIA. Frontal view on femorotibial joint. Left side shows an overview of the knee joint. The VOI, located 1 mm distal to the tibial metaphyseal growth plate, is shown in detail on the right.

2.6.3 Joint Porosity

We assessed a radiological correlate for subchondral bone inside the talus, representing a potential spot for bone erosions.

To determine the porosity of local subchondral bone as a measure for bone erosions of affected rheumatic joints, a VOI was placed around the talar surface of the tibiotalar joint, reaching 80 µm toward the trabecular bone (Fig. 7). Porosity was quantified by obtaining cortical volume/ total volume (Ct.V/TV in %), bone and pore density (Bo&Po density in mg HA/ ccm) and average pore diameter (AvgPo.Dm in µm). To investigate the distribution of joint pores, colour-coded image stacks using AvgPo.Dm values were obtained with ImageJ. To investigate the distribution of bone density on the talar surface, coloured 3D heat maps using Bo&Po density were obtained with ImageJ.

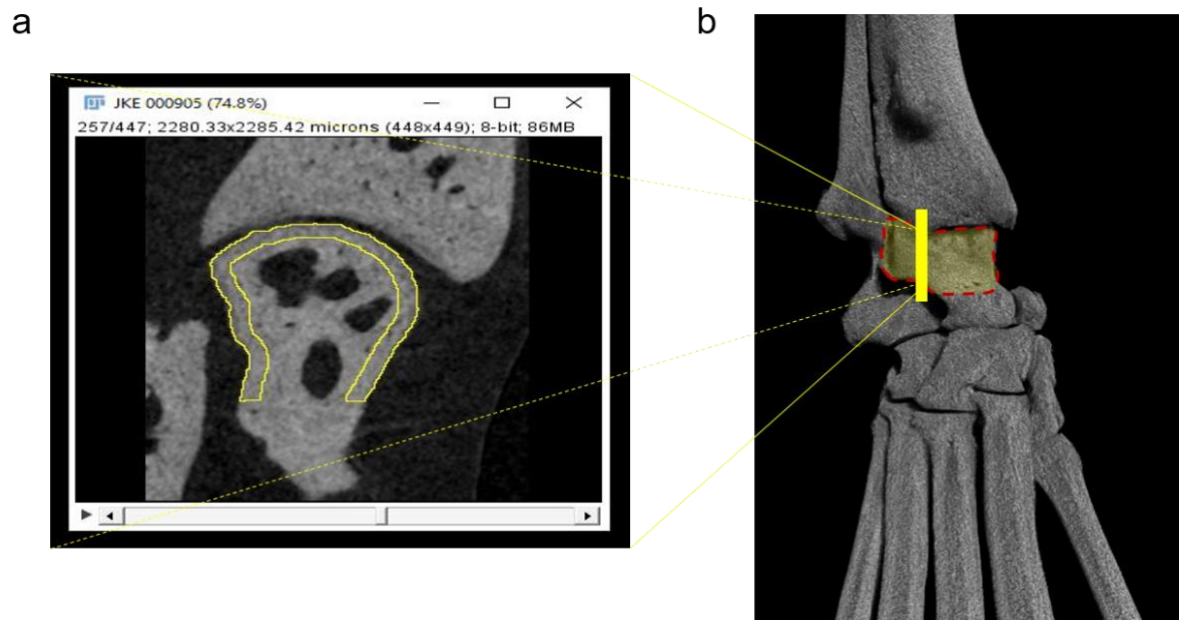

Fig. 7. Assessment of joint porosity.

Fig. 7. Assessment of joint porosity. (a) 2D sagittal-reconstruction of the tibiotalar joint. Yellow lines indicate the VOI which represents the subchondral bone layer of the articulating part of the talus. (b) 3D reconstruction of the tibiotalar joint. The yellow bar shows the section plane from Fig 7.a. The end of the VOI was set as the point where the talus starts to narrow as more distal parts were not assumed to be involved in the interaction of articulating joint parts. The dashed line (red) frames the VOI that measures the talus porosity.

2.7 Molecular Analysis

For molecular assessment, quantitative real-time polymerase chain reaction (qRT-PCR) was performed on inflamed tissues after mRNA extraction. Snap-frozen ankle and wrist joints were first homogenized with an Ultra Turrax disperser (Sigma Aldrich) before being incubated in 1 ml Trizol. RNA was then isolated with the RNeasy Mini Kit (Qiagen) and DNase I (Thermo Fisher). RNA Concentrations were determined and quality was tested with an A260/A280 ratio of 1.9 - 2.1 using a NanoPhotometer P360 (Implen GmbH). 0.25 - 1 µg RNA was analysed by re-transcribing RNA into complementary DNA (cDNA) using the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher). 7 µl Power SYBR Green PCR Master Mix (Sigma Aldrich) was mixed with 5 µl cDNA volume per well. qRT-PCR was then performed on a 384-well plate reader in a 7900HT Fast Real-Time PCR System at an annealing temperature of 60 °C with 2 ng cDNA per well. The raw data were then evaluated with SDS v2.4 software

(Applied Biosystems). For each run, SDS v2.4 calculated the threshold cycle (CT), as well as the melting curve, which confirmed PCR product specificity. The primers for analysis were individually designed with the GRCm38.p6 C57BL/6J reference genome and employing Primer3 software (http://bioinfo.ut.ee/primer3-0.4.0/), including at least two exons with a large intron in between in order to avoid genomic DNA amplifications. The primers were provided by Eurofins Genomics GmbH and pipetted at a final concentration of 0.2 µM. Primer sequences of assessed genes can be found in the Supplementary Methods section. The final relative gene expression was calculated using the virtual copy number (vCN) method (([vCN sample]/ [vCN GAPDH])*10000] which issues a fictive gene transcript count compared to the housekeeping gene. For this purpose, primary CT values were transformed into vCN values ($vCN = 10^{([CT]-35)/-3.3219}$). For the chosen housekeeping gene glycerinaldehyde-3-phosphate-dehydrogenase (*Gapdh*), the mean value of two pipetted runs was used.

All results should be interpreted as the expression of the transcriptional activity of distinct protein-coding genes. This likely (but not necessarily) correlates with the relative up- or downregulation of the corresponding protein inside the cell type that was dominant inside the dissected tissue. Regarding our ankle joint samples, the dominating cell types are assumed to be chondrocytes, synovial cells and periarticular bone cells.

2.8 Immunohistology

To verify intraarticular existence of αCGRP in inflamed joints, immunohistochemistry was performed for localization of αCGRP, Endomucin (Endm) and CD31 within the tibiotalar joints. Here, unstained 7 µm-thin cryo-sections were permeabilized with 0.25 % Triton/ PBS for 10 min followed by ample washing with PBS and then blocking with 3 % bovine serum albumin (BSA)/ 5 % Donkey serum/ PBS. Primary antibody incubation with anti-CGRP (1:100, cat# ab47027, RRID:AB_1141573) was then achieved by adding anti-CD31 (1:100, cat# AF 3628, RRID:AB_2161028) and anti-Endm (1:100, cat# sc-65495, RRID:AB_2100037) overnight. On the next day, sections were again washed amply in PBS and then incubated with the secondary antibody (1:400, anti-rabbit 647, Cat# A-32728, RRID:AB_2633277; 1:400, anti-goat 546, Cat# A-11058, RRID:AB_2534105; 1:400 anti-rat 488, Cat# A-21208, RRID:AB_2535794) before being mounted in Fluoromount-G with 4’,6-diamidino-2-phenylindole (DAPI) (Thermo Fisher). Final images were rendered using a Leica DM RB microscope (Leica

Microsystems) and AxioVision Rel. 4.8 software (Zeiss) and a Leica TCS SP5 confocal microscope (Leica Microsystems) with a Mai Tai HP multiphoton laser (Spectra Physics).

2.9 Statistical Analysis

Linear mixed models were employed for outcomes that were measured repeatedly, including clinical score, body weight and grip strength. For this purpose, time was parameterized with a restricted cubic spline and 4 knots (225). The process of transformation was necessary to consider the non-linear and repetitive structure of the clinical data. Group and time*type interaction were defined as independent variables. The utilized statistical test for interaction then showed significances for general interactions concerning the whole data set rather than testing single time points separately. This complex model provides adequate approximations for repetitive data collection and can be applied in customized time frames. Interactions between all of the groups (WT CAIA, WT CTRL, αCGRP$^{-/-}$ CAIA, αCGRP$^{-/-}$ CTRL) were analysed. The calculations of all mixed models were kindly conducted by Dario Zocholl (D.Z.) and Robert Röhle (R.R.).

For other simple endpoint comparisons between two groups, a non-parametric Wilcoxon-Mann-Whitney test (MWU) was used since Gaussian distribution, which would be required for the parametric Student's t-test, is hard to prove in small data sets. All MWU tests were calculated using GraphPad PRISM© 5. When day 10 data were compared to day 48 data longitudinally, a 2-way analysis of variance (ANOVA) with multiple comparisons was used. Unless stated otherwise, data are presented as means ± standard error of the mean (SEM). Results were deemed significant where $p < 0.05$. Data visualized in boxplots is presented as median ± minimum and maximum with additional plotting of every data point. To warrant a qualitative research structure, ARRIVE guidelines were followed for data storage (226). The relatively small sample size used in our experiment, including punctual dropouts caused by unforeseen events, was calculated in the run-up to the experiment. Expecting an effect size of 1.5 with α of 0.05 and β of 0.2, while considering that CAIA is a safe and validated method with nearly 100 % of arthritis incidence, we calculated that 8 animals for the intervention group and 4 animals for control groups, the latter of which were anticipated to exhibit lower variances naturally, would be sufficient to produce significant results.

3 Results

3.1 Clinical outcomes

3.1.1 Body Weight

The induction of CAIA resulted in significant body weight loss both in αCGRP$^{-/-}$ and WT animals when compared to their respective CTRL animals ($p<0.01$). However, while both genotypes significantly differed from their CTRL group, no significant difference between WT CAIA and αCGRP$^{-/-}$ CAIA mice could be seen in the acute inflammation phase of arthritis ($p=0.69$). Over 48 days, the restricted cubic spline model revealed a significant influence of CAIA treatment ($p=0.003$), while the knockout of αCGRP had no significant influence ($p=0.81$) (Fig. 8).

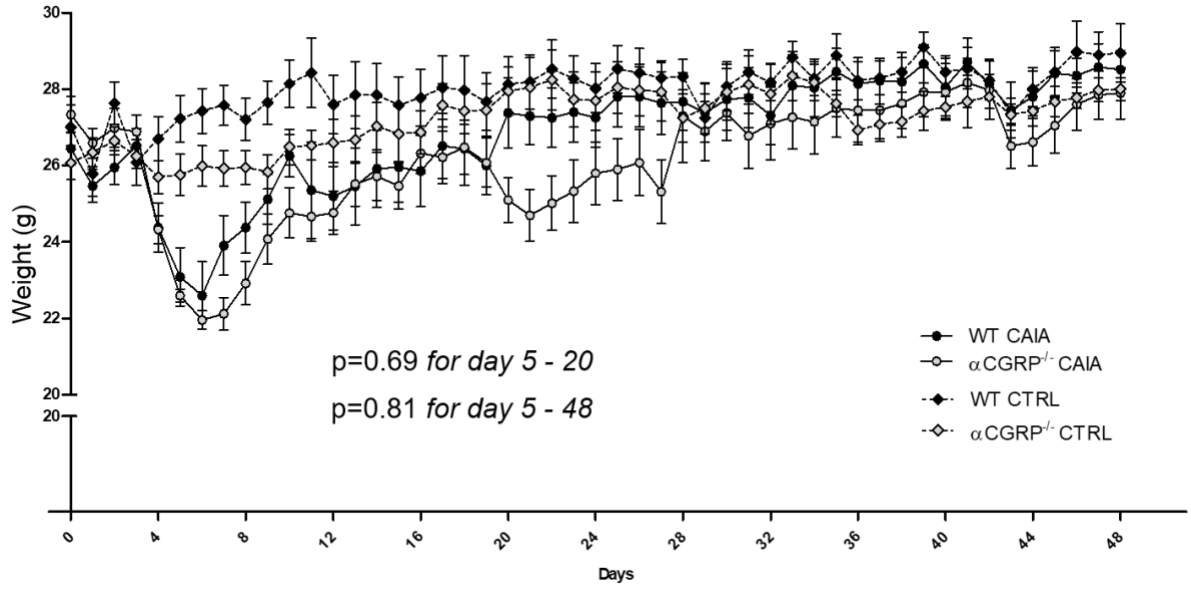

Fig. 8. Body weight over 48 days (adapted from Maleitzke et al. (1), with written consent from the Oxford University Press).

Fig. 8. Body weight over 48 days. Graph shows body weight development of each experimental group during the experiment. Given values are means ± SEM.

3.1.2 Grip Strength

A loss of grip strength was observed in all animals (Fig. 9) during the first four days, which may be due to habituation resulting in reduced interest towards the grid.

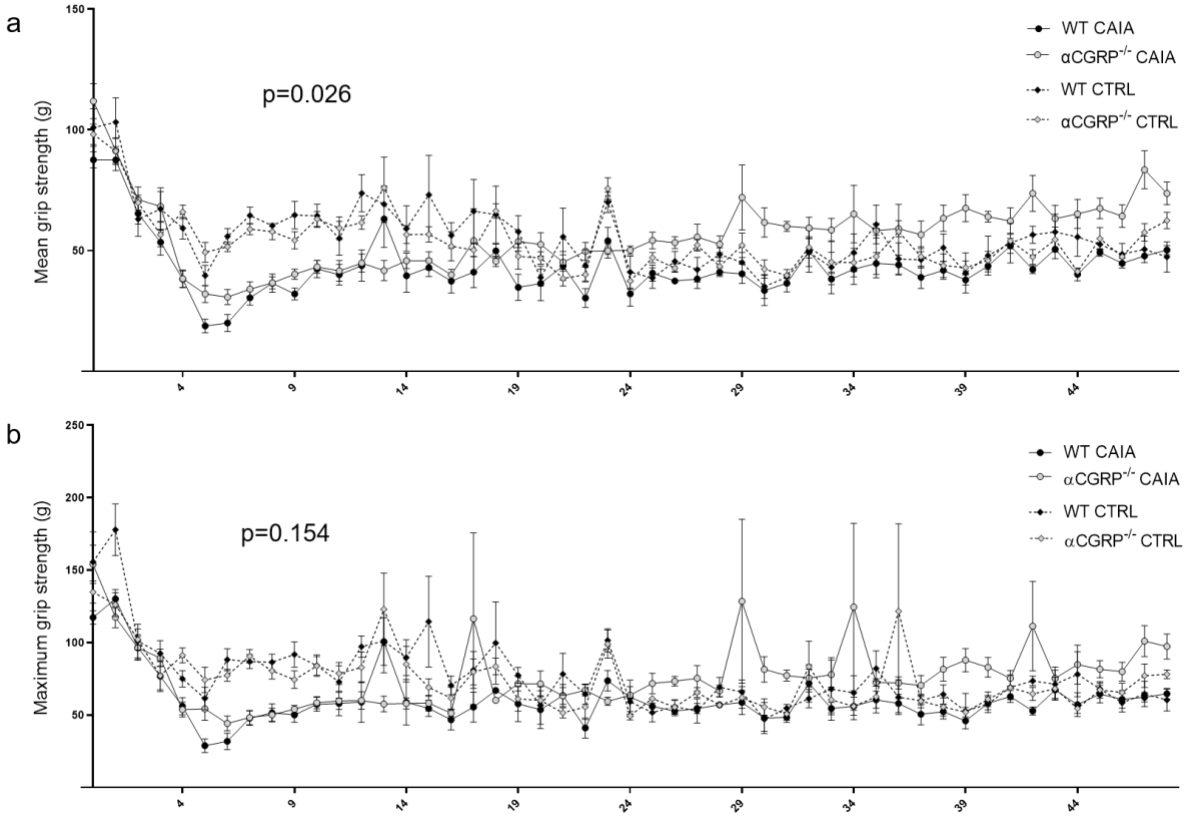

Fig. 9. Assessment of grip strength (adapted from Maleitzke et al. (1), with written consent from the Oxford University Press).

Fig. 9. Assessment of grip strength. Grip strength development during the experiment presented as the (a) mean and (b) maximum of 5 consecutive measurements. Given values are means ± SEM.

However, despite the fact that all CAIA animals suffered from significantly greater loss of strength than CTRL animals both for mean and max. grip strength ($p<0.001$ and $p=0.007$, respectively), a comparison between αCGRP$^{-/-}$ CAIA and WT CAIA animals during the acute inflammation phase (day 5 - 20) also revealed significantly lower pulling force in WT CAIA animals for mean grip strength ($p=0.026$) (Fig. 9a). The results suggest that WT animals were more severely affected by the arthritis. As expected from clinical observations, all animals recovered after approximately 20 days from when values converged with CTRL animals.

3.1.3 Clinical Score

All mice injected with the antibody cocktail developed an arthritic phenotype with significant paw swellings within 7 days (Fig. 10). All four paws were scored daily, employing the semi-quantitative scoring system (219).

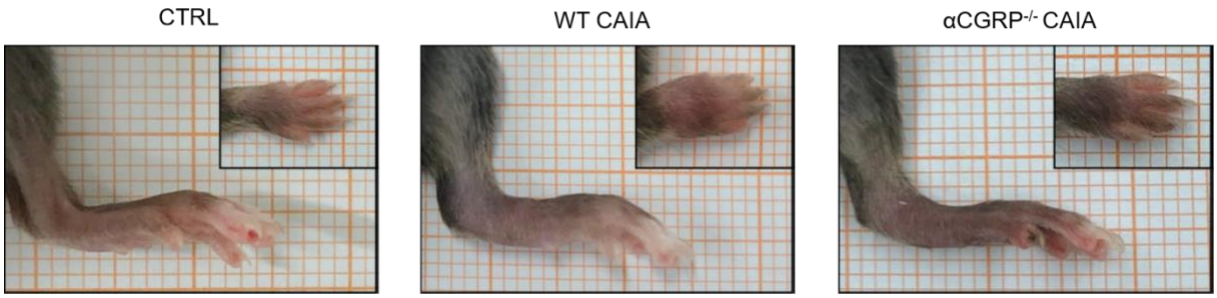

Fig. 10. Images of paw swellings (adapted from Maleitzke et al. (1), with written consent from the Oxford University Press).

Fig. 10. Images of paw swellings. Representative images of sagittal ankles and dorsal wrists 8 days after CAIA induction. In CTRL animals, rosy skin colour and lean extremity shape with visible bone and tendon parts can be observed (corresponding to score = 0 for wrist and ankle). In WT CAIA animals, remarkable swelling and erythema can be seen in wrists and paws. The swollen soft tissue conceals visible landmarks of bone architecture affecting phalanges as well as the carpal and tarsal region (score = 3 for wrist and ankle). In αCGRP$^{-/-}$ CAIA mice, less severe redness and swelling is seen, often only affecting single digits or with mild swelling of ankle joint (corresponding to score = 1 for ankle and wrist).

The cubic spline model was applied to clinical data from day 5 - 20. Both CAIA groups showed significantly higher scores than CTRL animals ($p=0.0016$), indicating the marked influence of CAIA treatment. In addition, WT CAIA mice showed significantly higher clinical scores ($p=0.0018$) than αCGRP$^{-/-}$ CAIA mice, while no joint inflammation was present in CTRL animals. WT CAIA animals were clinically more affected by CAIA than αCGRP$^{-/-}$ CAIA mice (Fig. 11).

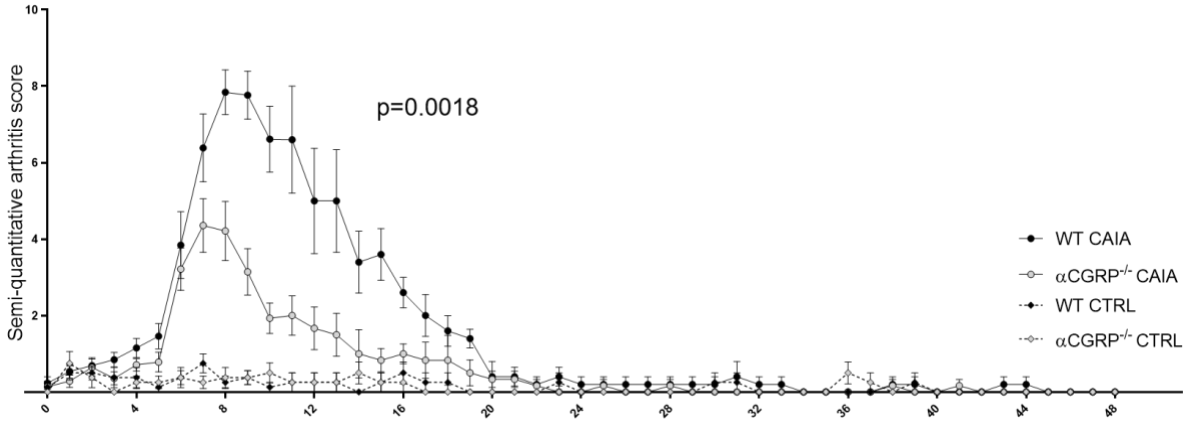

Fig. 11. Arthritis score over 48 days (adapted from Maleitzke et al. (1), with written consent from the Oxford University Press).

Fig. 11. Arthritis score over 48 days. Development of the semi-quantitative arthritis score during the experiments. Given values are means ± SEM.

3.2 Histological Findings

3.2.1 Inflammation

Confirming clinical findings, the histological inflammation was significantly higher in WT CAIA animals after 10 days compared to CTRL animals (p=0.006), while αCGRP$^{-/-}$ animals showed no significant difference to their CTRL animals (Fig. 12).

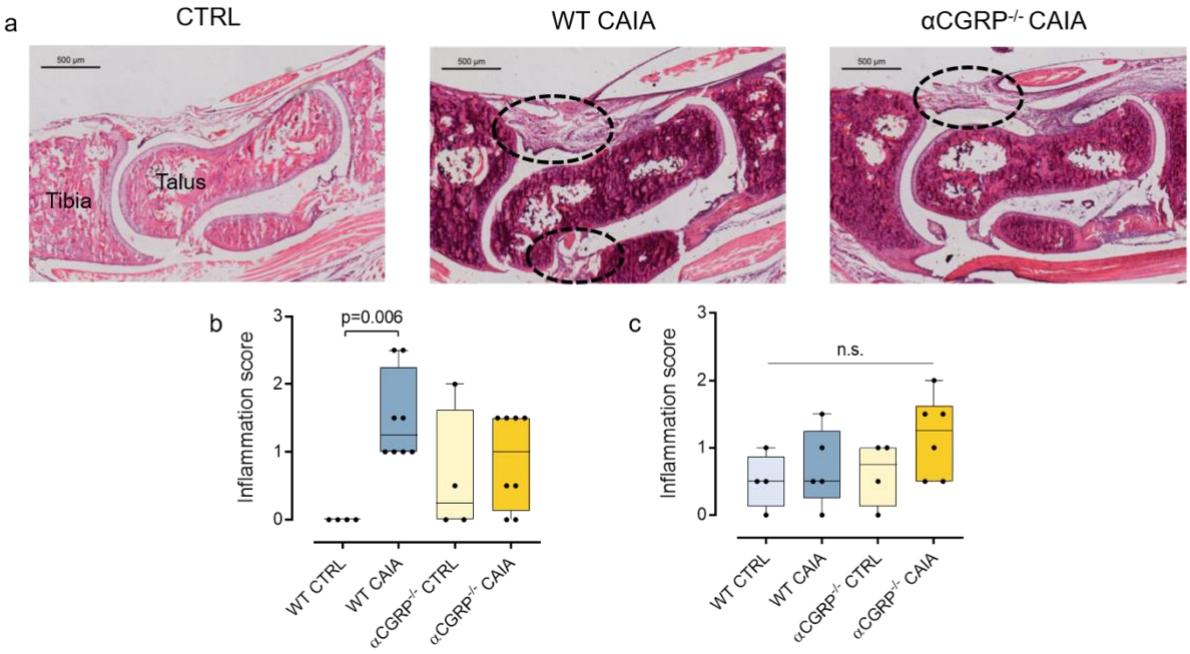

Fig. 12. Histological joint inflammation (adapted from Maleitzke et al. (1), with written consent from the Oxford University Press).

Fig. 12. Histological joint inflammation. (a) Representative sagittal images of the tibiotalar joints from mice 10 days after CAIA induction show histological inflammation in H&E-stained sections with severe synovial cell lining hyperplasia and lymphocyte infiltration (dotted frames (black) show inflammatory regions) in WT CAIA mice (corresponding to score = 3), while joints of αCGRP$^{-/-}$ mice exhibit milder expression of capsular reactivity (score = 2). CTRL mice show minimal abnormalities in soft tissue composition, with a mostly slim capsular strap and only one or two layers of flat FLS (score = 0). (b) Semi-quantitative scores obtained at day 10 (inflammation phase). (c) Semi-quantitative scores obtained at day 48 (resolution phase). Given values are median ± minimum and maximum.

At day 48, no significant difference could be seen between any of the experimental groups, although a few abnormalities caused scores above 0. This goes in accordance with clinical observations that indicated remission with regression of symptoms and visible swellings after 20 days.

3.2.2 Cartilage Degradation

Cartilage degradation is a major criterion for severity of RA, and it appeared in WT CAIA mice in the acute inflammation phase of arthritis. 10 days after CAIA induction, WT mice showed significantly more pronounced losses of dye-storing cartilage mass than αCGRP$^{-/-}$ mice (p=0.03), while CTRL mice exhibited undamaged joint surface matrices (Fig. 13).

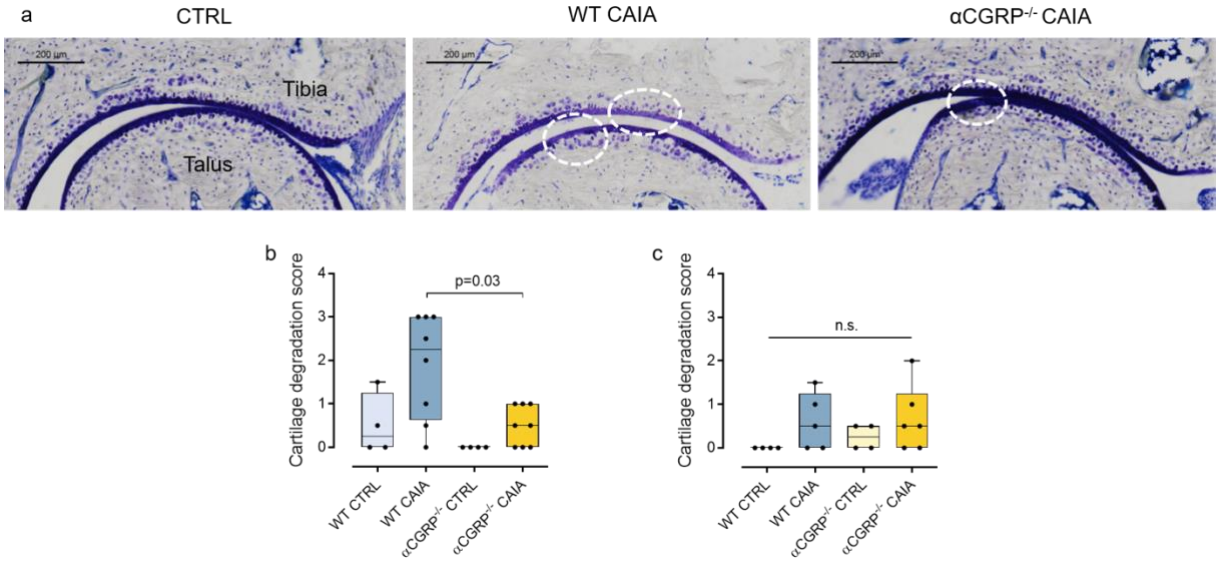

Fig. 13. Histological cartilage degradation (adapted from Maleitzke et al. (1), with written consent from the Oxford University Press).

Fig. 13. Histological cartilage degradation. (a) Representative images from the ankle joint in sagittal view (TB staining) reveal severe colour loss (dotted frames (white) mark affected spots) in WT CAIA mice after 10 days, with multifocal lesions affecting cellular chondrocyte lining as well as hyaluronic cartilage matrix (corresponding to score = 3). Control mice show a homogenous cartilage surface with continuous chondrocyte lining (score = 0). αCGRP$^{-/-}$ animals show minimal unifocal paleness with discreet cell lining disruption (score = 1). The collapsed joint space is owed to fixation and has no pathological value. (b) Boxplots show semi-quantitative scores of animals at day 10. (c) Boxplots show semi-quantitative scores of animals at day 48. Given values are median ± minimum and maximum.

Interestingly, there was no significant cartilage damage in the animals after 48 days in any of the groups except for occasional small lesions.

3.2.3 Bone Erosions

The TRAP staining finally unravelled marked proliferation fields of intraosseous osteoclasts near to local inflammation spots in CAIA mice after 10 days (Fig. 14a). Matching clinical findings, WT CAIA mice presented a significantly (p=0.03) higher number of focal erosions in the tibiotalar region than their CTRL littermates. However, in respect of bone erosions, αCGRP$^{-/-}$ mice suffering from CAIA also developed significantly (p=0.01) higher bone erosion scores than their CTRL animals (Fig. 14b). Although no statistical difference could be seen between WT and αCGRP$^{-/-}$ mice, TRAP staining of osteoclast distribution suggested that arthritis not only affected cartilage but moreover spread into adjacent bone areas. In line with our observations for cartilage loss and inflammation, no bone erosions were visible after 48 days (Fig. 14c).

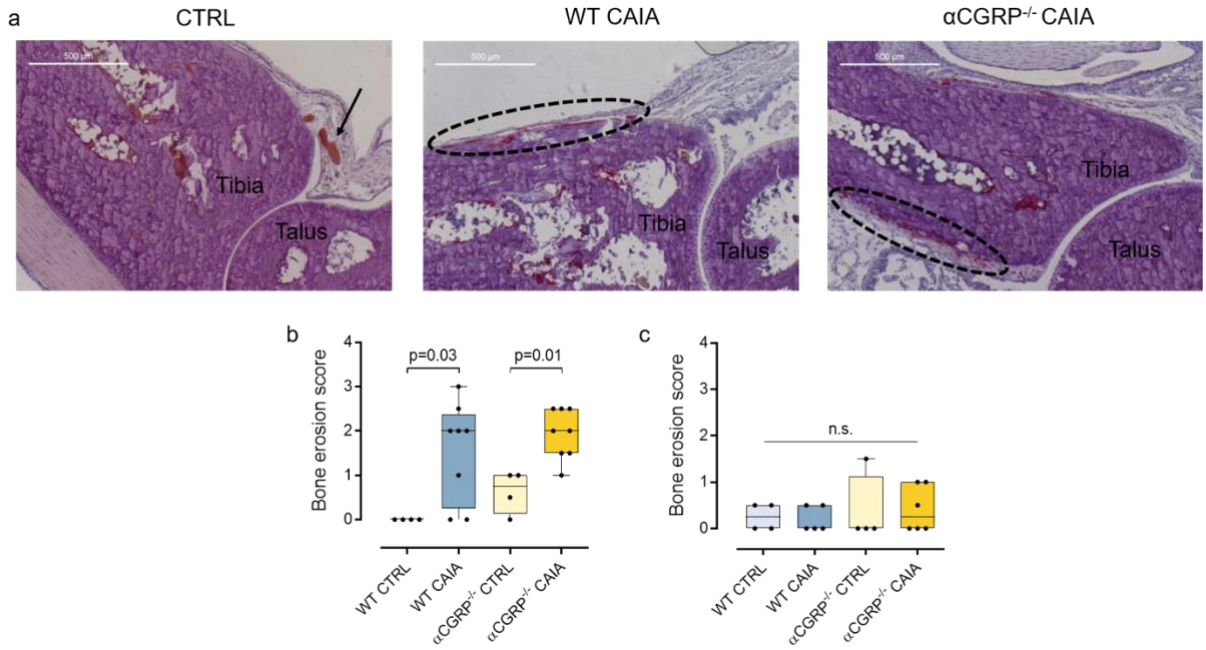

Fig. 14. Histological bone erosions (adapted from Maleitzke et al. (1), with written consent from the Oxford University Press).

Fig. 14. Histological bone erosions. (a) Representative images of TRAP-stained sagittal ankle joints show erosion sites (dotted frames (black) show red/brown-coloured clusters of osteoclasts) with abundant subchondral osteoclast accumulation as a sign of osteocatabolic processes both in WT CAIA mice and αCGRP$^{-/-}$ mice after 10 days (score = 3), while CTRL mice only show physiologically smaller fields of trabecular endosteal osteoclasts (score = 0). Dye accumulation inside vessels has to be seen as an artefact (arrow (black)). (b) Semi-quantitative bone erosion scores after 10 days. (c) Semi-quantitative bone erosion scores after 48 days. Given values are median ± minimum and maximum.

3.2.4 Immunohistochemistry

We also wanted to evaluate if the αCGRP peptide was secreted to the inflamed areas as a correlate for pro-inflammatory signalling within the biochemical milieu inside the joint. We therefore searched for αCGRP signals in ankle joints by means of immunohistochemical staining of ankle slides. Sections of WT CAIA mice revealed lucid signals of αCGRP together with CD31. CD31 is known as an immunoactive protein localized on the surface of many immune cells, taking a crucial role in the interaction between neutrophils and the endothelium during inflammation (227). Both markers could be visualized on the synovial surface (Fig. 15). This non-quantitative observation was essential to clarify the definite involvement of αCGRP during CAIA inside the joint.

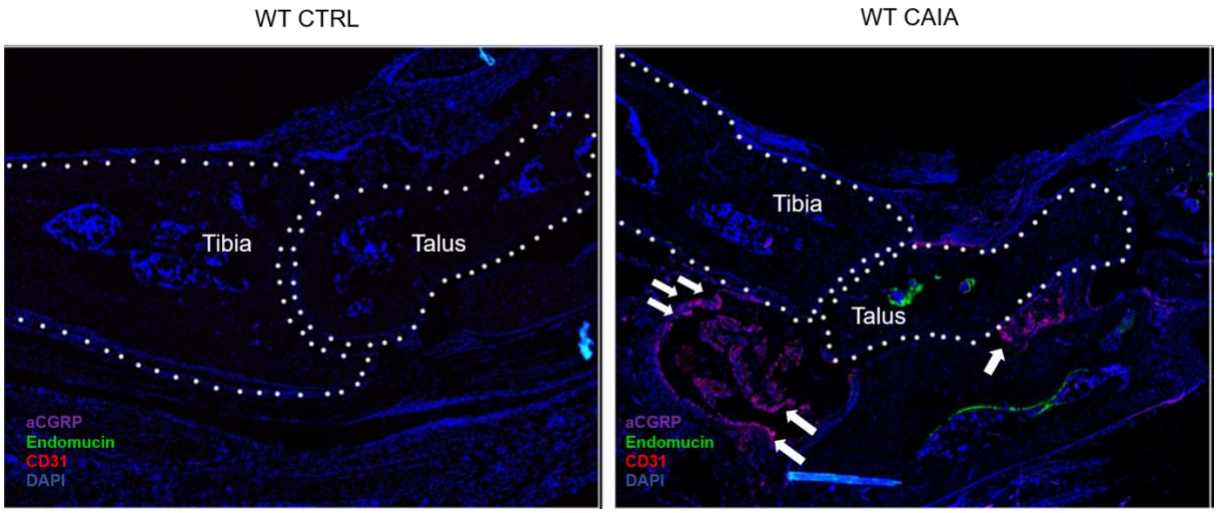

Fig. 15. Presence of αCGRP visualized with fluorescent dye (adapted from Maleitzke et al. (1), with written consent from the Oxford University Press).

Fig. 15. Presence of αCGRP visualized with fluorescent dye. Exemplary images from fluorescence microscopy of sagittal ankle sections of WT mice at day 10 after CAIA induction stained for 4 typical markers. Endomucin is used to identify vessels, and DAPI is used as a background staining to get anatomical orientation. Arrows (white) show intensive synovial signals for αCGRP (violet) together with CD31 (red) in WT CAIA mice.

3.3 Radiological Evaluation

3.3.1 Systemic Bone Changes/ Proximal Tibiae

No significant alterations in cortical and trabecular bone quality in respect of systemic changes occurred after 10 days of arthritis (Fig. 16).

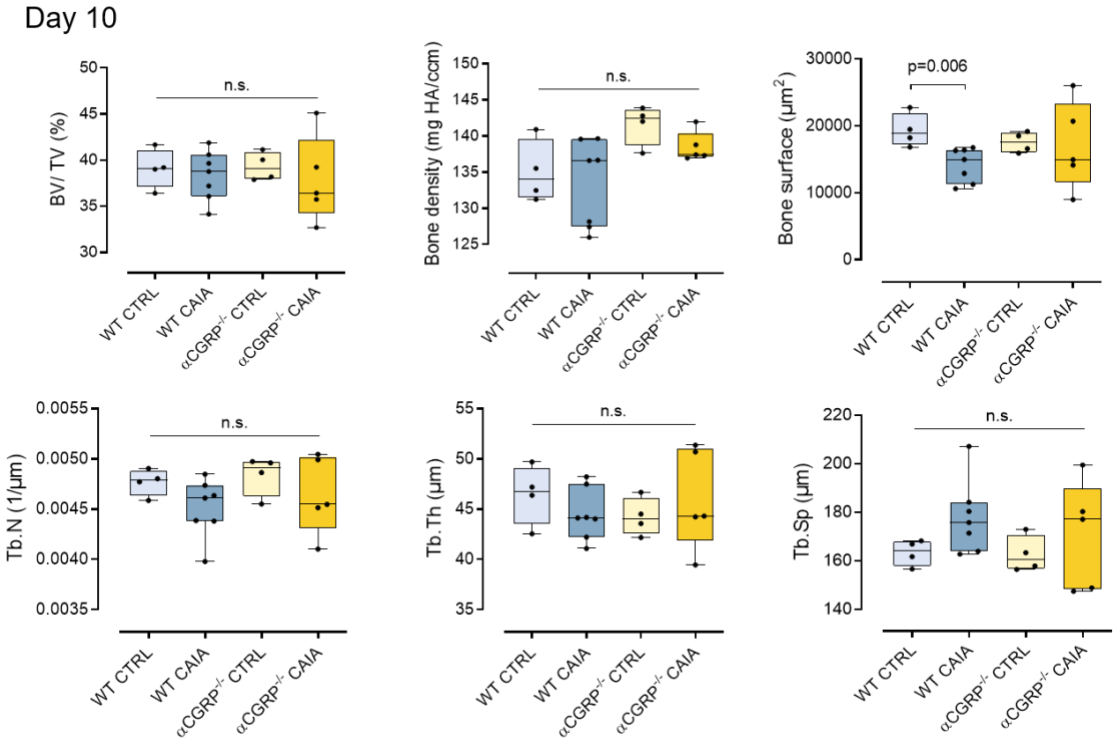

Fig. 16. Early systemic bone changes (adapted from Maleitzke et al. (1), with written consent from the Oxford University Press).

Fig. 16. Early systemic bone changes. Boxplots show parameters of bone quality of the proximal tibiae 10 days after the induction of CAIA. Given values are median ± minimum and maximum.

The bone surface was significantly decreased in WT CAIA mice when compared to CTRL animals, indicating signs of trabecular loosening compared to WT control mice (p=0.006). However, there was no difference in all other parameters for radiologically measurable deficits in bone architecture between CAIA and CTRL animals in the acute inflammation phase. After clinical initiation just a few days before sample preparation, arthritis did not seem to impact bone quality in the proximal tibiae. Of note, the area of interest for this observation was consciously set to the proximal tibia at a noteworthy distance to the inflammatory action site. Therefore, changes in bone composition in this region could not be explained by local cytokine interactions or paracrine effects of inflammatory mediators towards osteoclasts and osteoblasts. After short-time

evaluation of proximal tibiae revealed no clear tendency for effects in terms of bone affection, long-time results after 48 days raised stronger suspicions about how arthritis could influence bone metabolism.

Forty-eight days after induction of CAIA, we recognized a clear tendency for αCGRP$^{-/-}$ mice to suffer from deteriorated bone quality after sustaining arthritis in comparison with WT animals. The obtained parameters only illustrate a small part of the skeletal system. The proximal tibia serves as a typical hollow bone, with both trabecular and cortical parts.

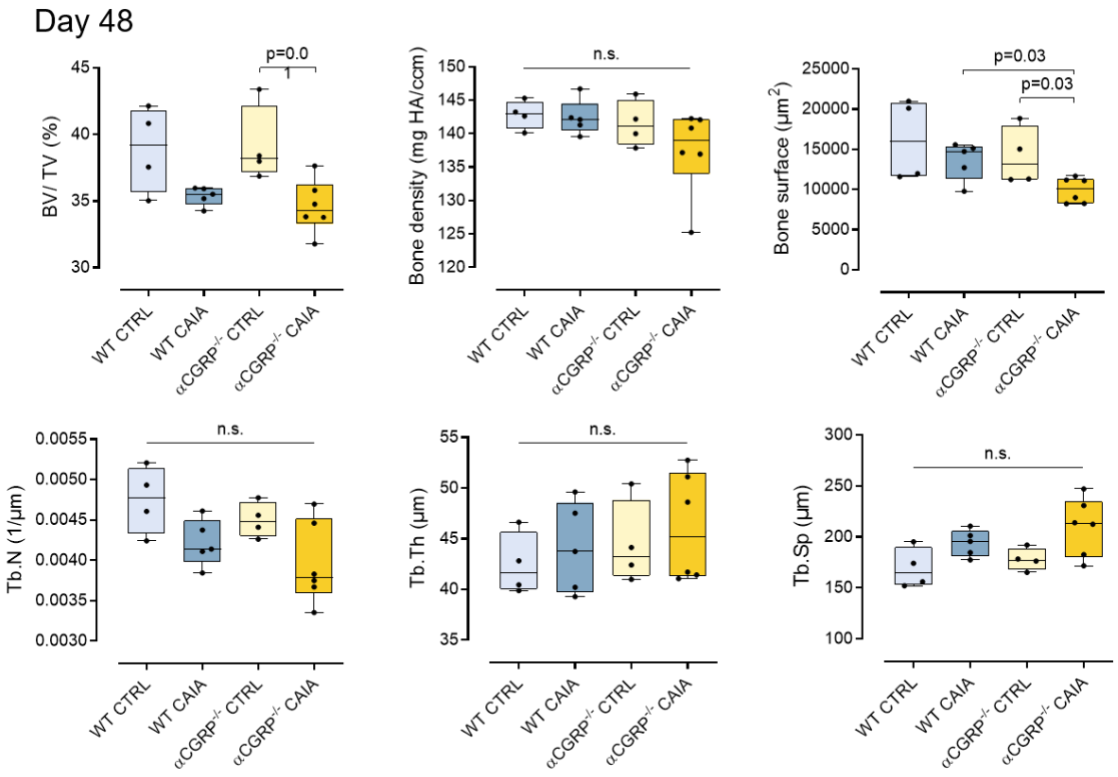

Fig. 17. Late systemic bone changes (adapted from Maleitzke et al. (1), with written consent from the Oxford University Press).

Fig. 17. Late systemic bone changes. Boxplots show parameters of bone quality of the proximal tibiae 48 days after the induction of CAIA. Given values are median ± minimum and maximum.

In comparison with their CTRL group, αCGRP$^{-/-}$ animals showed lower values for BS (p=0.03) as well as for BV/TV (p=0.01), with ancillary but not significant tendencies for Tb. Sp. and Tb. Nb., indicating a worsening in both trabecular and cortical structure after more extended exposure to CAIA (Fig. 17).

Finally, it was of great interest for us to investigate bone parameters longitudinally. Intra-group comparisons between long and short observation periods should provide more precise and accurate information about the dynamic structural changes throughout the artificial course of induced arthritis. Longitudinal data confirmed our prior observations as a 2-way ANOVA showed significantly decreased BS ($p<0.05$) and Tb. Nb. ($p<0.01$) as well as increased Tb. Sp. ($p<0.01$) in αCGRP$^{-/-}$ CAIA mice after 48 days compared to αCGRP$^{-/-}$ CAIA animals 10 days after induction of CAIA (Fig. 18). Taken together, all parameters point towards a clearly suppressed bone phenotype in arthritic αCGRP$^{-/-}$ mice after being exposed to arthritis, although exhibiting milder clinical symptoms than their WT littermates.

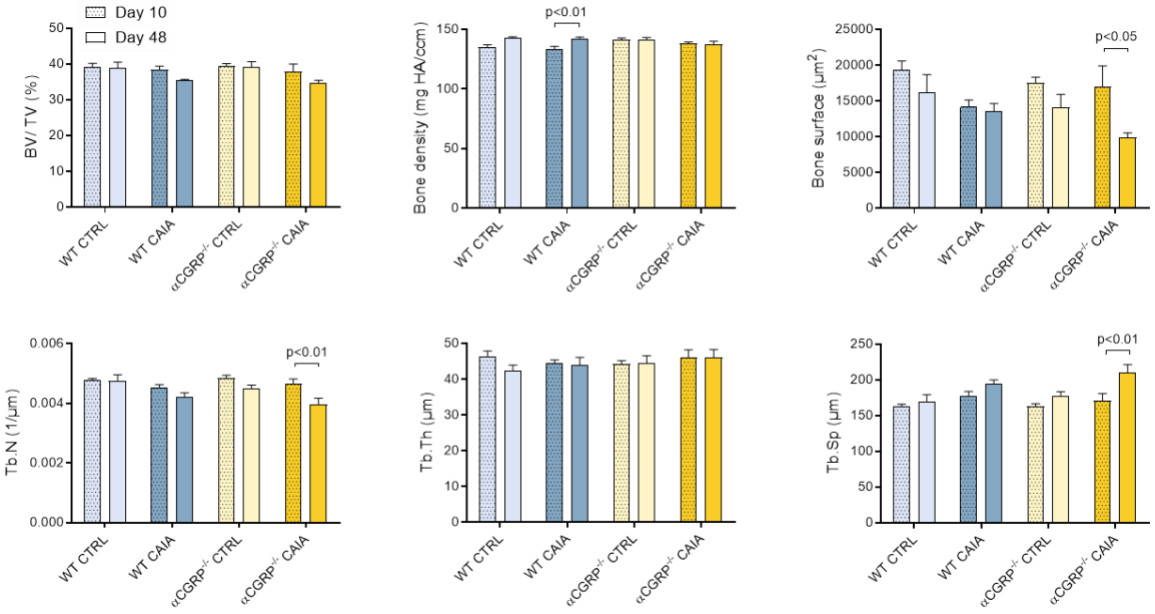

Fig. 18. Longitudinal bone changes (adapted from Maleitzke et al. (1), with written consent from the Oxford University Press).

Fig. 18. Longitudinal bone changes. Radiological data presented as longitudinal intra-group comparisons show differences in bone quality of the proximal tibiae between the acute inflammation phase and the resolution phase. Given values are means ± SEM.

3.3.2 Local Bone Changes/ Talus Porosity

Next, local bone formation was investigated as human RA patients often suffer from severe bone erosions, which are always localized within the subchondral bone in immediate vicinity to the inflammatory action site.

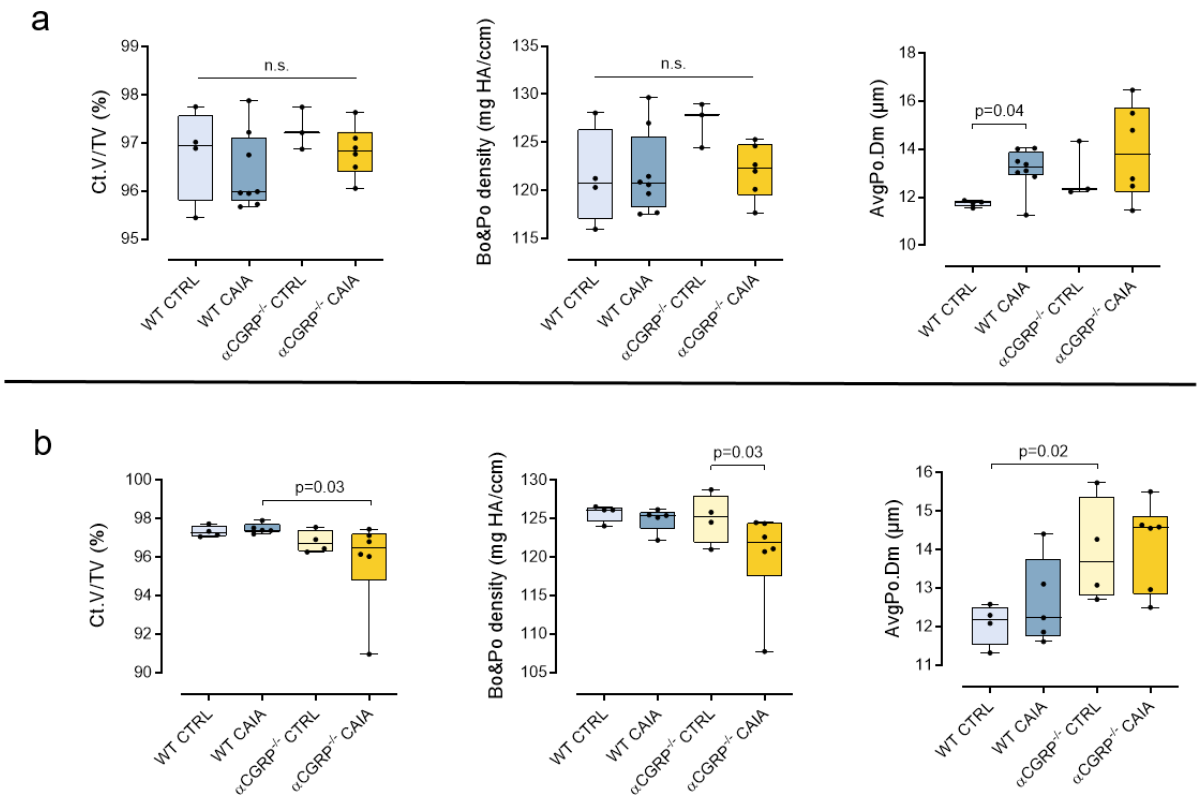

Fig. 19. Local bone porosity (adapted from Maleitzke et al. (1), with written consent from the Oxford University Press).

Fig. 19. Local bone porosity. (a) Parameters of porosity of talar subchondral bone at (a) day 10 and (b) day 48 after induction of CAIA. Given values are median ± minimum and maximum.

WT CAIA mice showed increased AvgPo.Dm (p=0.04) in comparison with their CTRL animals after 10 days, indicating bigger pores inside the subchondral layer in normal animals with severe acute arthritis (Fig. 19). In contrast, αCGRP$^{-/-}$ CAIA animals seemed to be spared from bone erosions, corresponding to a mitigated clinical course of the arthritis. After 48 days, WT CAIA mice, in accordance with results from our global bone data, showed no visible erosions. αCGRP$^{-/-}$ CAIA animals, on the other hand, had significantly decreased Ct. V/TV (p=0.03) after 48 days when compared to WT CAIA animals as well as Bo&Po density (p=0.03) compared to WT CAIA animals.

3.3.3 Visible Bone Changes over Time

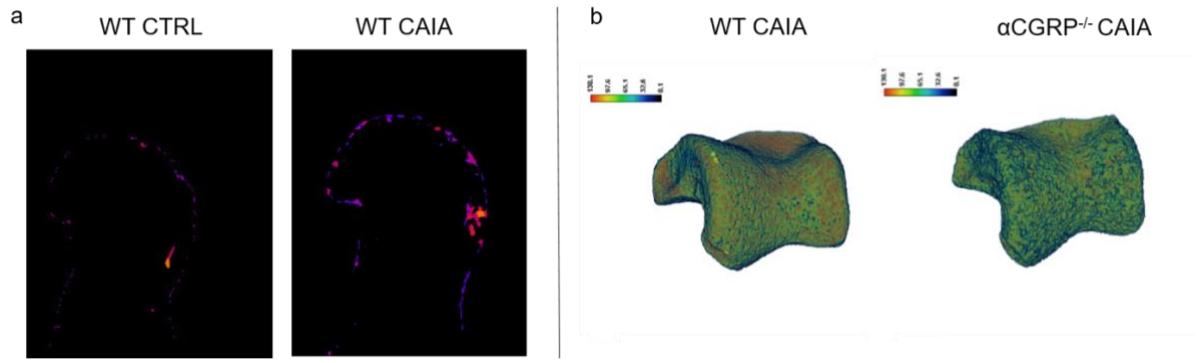

Fig. 20. Visualization of the surface of the talus (adapted from Maleitzke et al. (1), with written consent from the Oxford University Press).

Fig. 20. Visualization of the surface of the talus. (a) Colour-coded sagittal view of the bony talar joint surface 10 days after the induction of CAIA shows distribution patterns of erosions as a visual illustration of the AvgPo.Dm. WT CAIA mice show marked erosions (dark violet for small pores, lighter red and yellow for bigger pores), while joint surfaces of WT CTRL animals look smooth. (b) Coloured heat map of talar surfaces 48 days after the induction of CAIA from a frontal/anterior point of view illustrates Bo&Po density values on a scale (red for high density, blue for low density). Although Bo&Po density did not reach significant differences, severe multifocal penetrations of the joint surface could be witnessed in αCGRP$^{-/-}$ mice.

We visualized our radiological results for local bone porosity to assess the exact distribution of bone lesions along the joint surface. WT CAIA mice showed evenly penetrated joint surfaces after 10 days, while CTRL mice had smooth joint lines. In animals in the resolution phase (day 48), visualization of the Bo&Po density showed evenly distributed erosions along the talar surface (Fig. 20).

We also visualized the trabecular architecture of the proximal tibia. αCGRP$^{-/-}$ mice exhibited a visible deterioration of bone structure after longer exposition to experimental arthritis, also reaching regions which are not directly affected by the inflammation. The deterioration pattern involved a decline in thickness and the number of trabeculae (Fig. 21).

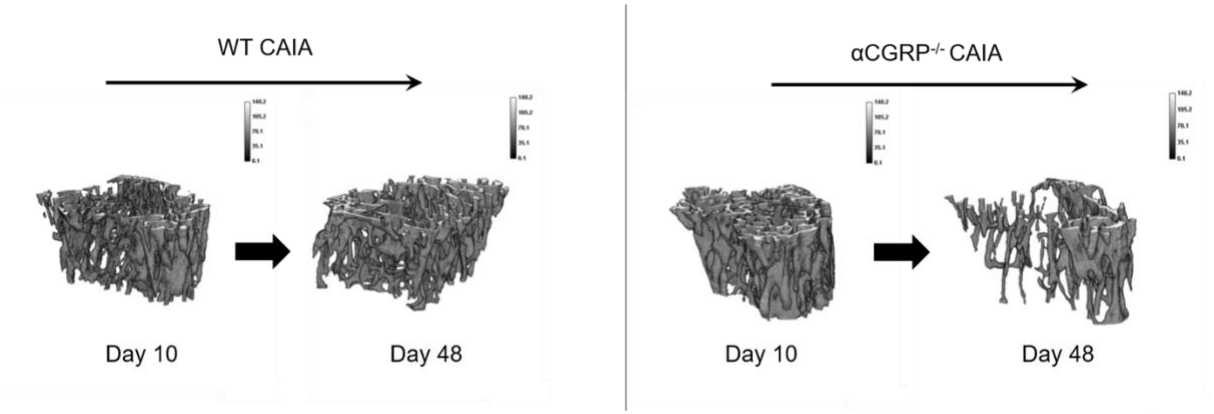

Fig. 21. Visualization of the tibial trabecular network (adapted from Maleitzke et al. (1), with written consent from the Oxford University Press).

Fig. 21. Visualization of the tibial trabecular network. 3D visualization of the tibial trabecular architecture shows deterioration of the bone structure in αCGRP$^{-/-}$ CAIA mice, while WT animals did not exhibit decreased trabecular richness.

3.4 PCR - Molecular Results

3.4.1 βCGRP and the CGRP-Receptor

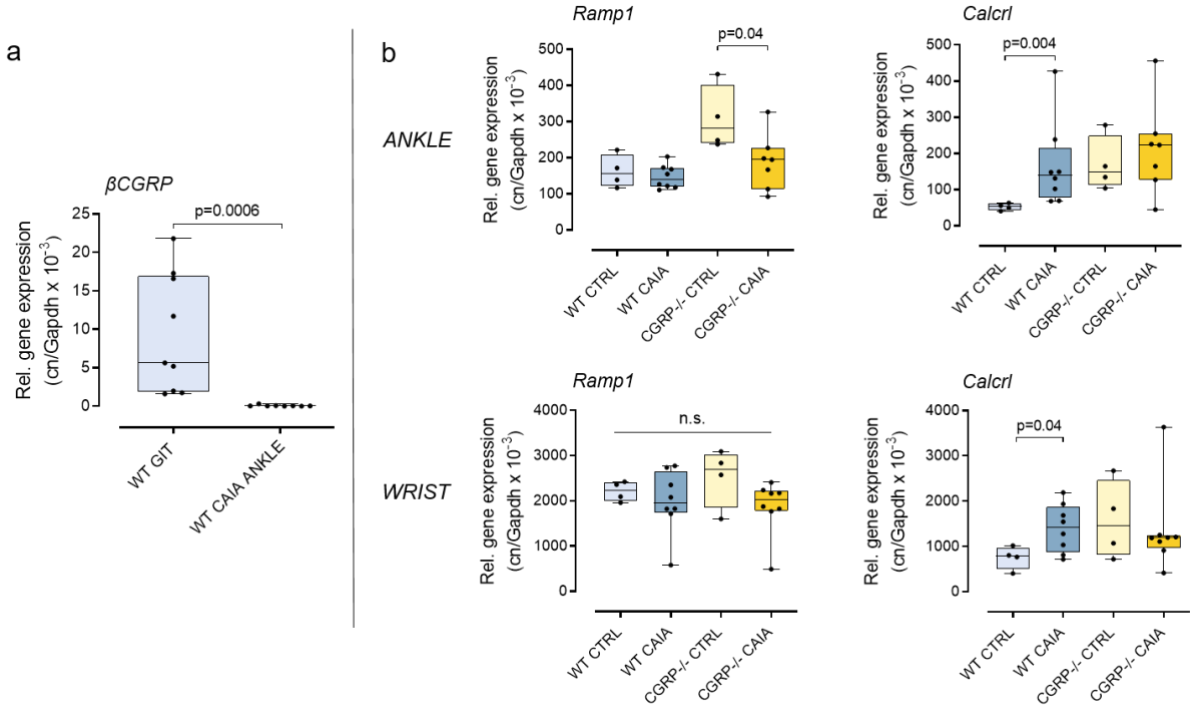

Fig. 22. CGRP signalling inside the arthritic joint (adapted from Maleitzke et al. (1), with written consent from the Oxford University Press).

Fig. 22. CGRP signalling inside the arthritic joint. (a) Direct comparison of expression levels of β*CGRP* in GIT and WT CAIA ankle joints. (b) Boxplots indicate gene expression of *Ramp1* and *Calcrl*. Given values are median ± minimum and maximum.

To assess the distinct distribution pattern of CGRP and its receptor, *αCGRP* mRNA and *βCGRP* mRNA were analysed in ankle joints. As predicted, *βCGRP* could not be detected in inflamed ankle joints, while bowel tissue of WT animals, serving as a positive control group, contained higher levels of *βCGRP* mRNA (p= 0.0006) (Fig. 22a). In contrast, α*CGRP*, *Ramp1* and *Calcrl* were detected in joint tissue (Fig. 22b). Interestingly, *Ramp1,* as a part of the CLR, is downregulated in αCGRP$^{-/-}$ CAIA animals when compared to their controls. WT CAIA animals did not show significant changes in *Ramp1* expression. mRNA levels of *αCGRP* are presented in the following chapter dealing with inflammation (Chapter 3.4.2), as αCGRP was assumed to be a pro-inflammatory peptide.

3.4.2 Inflammation

To assess molecular mechanisms of joint inflammation, several pro-inflammatory markers in homogenized tissue from swollen joints were analysed. Our aim was to detect elevations or reductions in the transcription of relevant mRNA which could possibly indicate inflammatory processes inside the joint. It was important to detect the behaviour of *αCGRP* expression, which was higher in WT CAIA mice compared to CTRL mice (p=0.03), as expected from clinical results and previous investigations. The presence of αCGRP inside the inflamed joints represents a fundamental pillar of our hypothesis, which suspects αCGRP to play a crucial role during joint inflammation.

Tnfa (p=0.04) and *Cd80* (p=0.04), the latter of which is a well-known monocyte surface marker involved in inflammatory cytokine signalling (228), were seen to be upregulated in WT CAIA mice in comparison to their CTRL group. αCGRP$^{-/-}$ CAIA mice did not show any significant differences when compared to their control group (Fig. 23). However, *Il1a*, *Il1b*, *Ccl2*, *Cd14*, *Mmp13* and *Il6*, measured in tissue originating from the lower limb, did not show any significant changes.

We next analysed the front paws, which showed similar results. In front paws, *Tnfa* (p= 0.04) and *Cd80* (p=0.004) expression levels in ankle joints were significantly higher in WT CAIA mice when compared to their CTRL group (Fig. 24), while αCGRP$^{-/-}$ CAIA mice stayed unaffected. In addition, *Il1b* (p= 0.008), *Ccl2* (p=0.04) and *Mmp13* (p=0.01) were significantly higher in WT CAIA animals compared to CTRL animals. Concerning wrists, our results provide evidence for ongoing intraarticular inflammation in WT CAIA mice in contrast to αCGRP$^{-/-}$ CAIA mice, which revealed less mRNA expressions of pro-inflammatory cytokines.

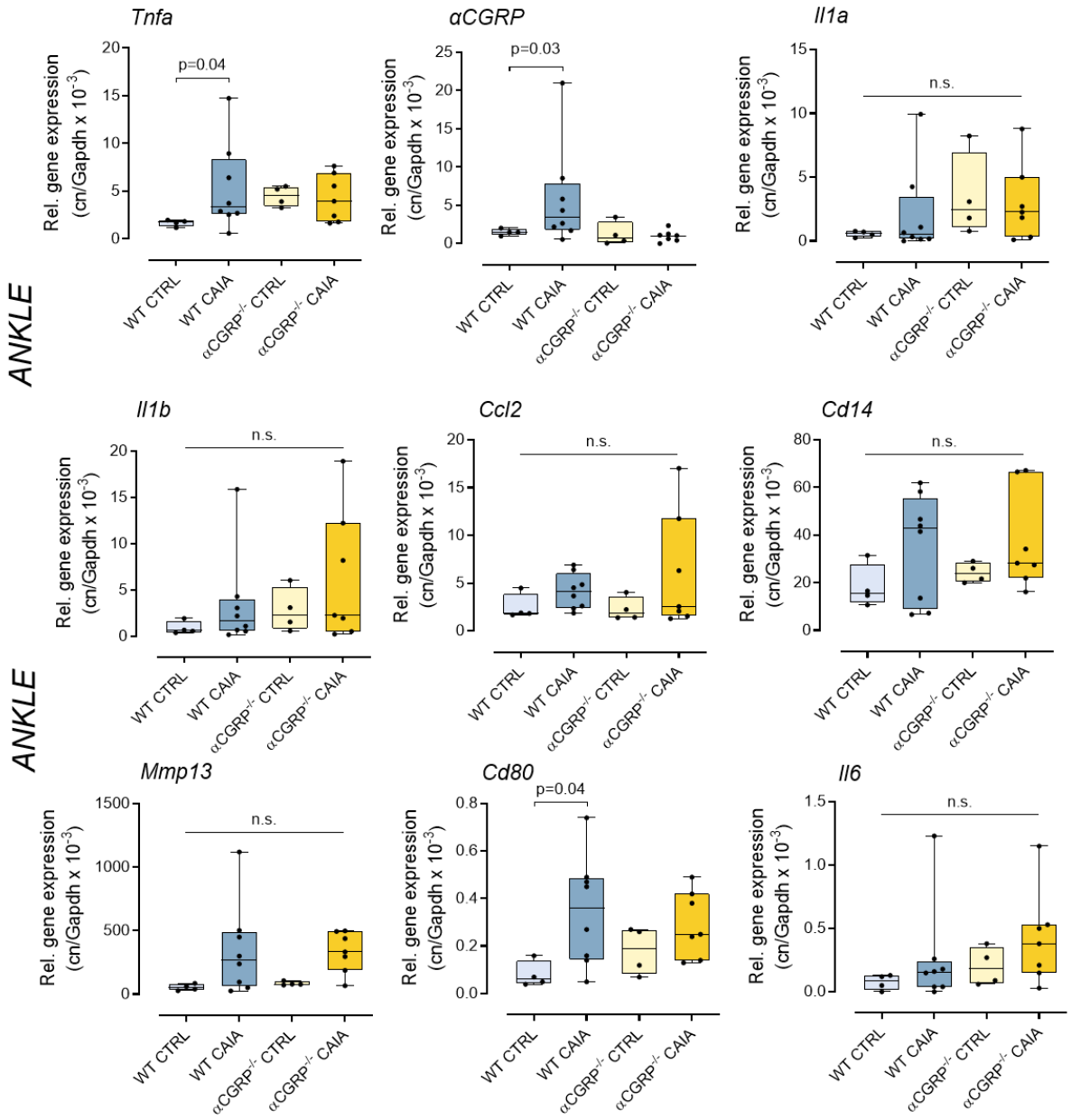

Fig. 23. PCR data of inflammation in ankle joint tissue (adapted from Maleitzke et al. (1), with written consent from the Oxford University Press).

Fig. 23. PCR data of inflammation in ankle joint tissue. Boxplots show PCR-delivered results of gene expression of pro-inflammatory markers in ankle joint tissue 10 days after the induction of CAIA. Given values are median ± minimum and maximum.

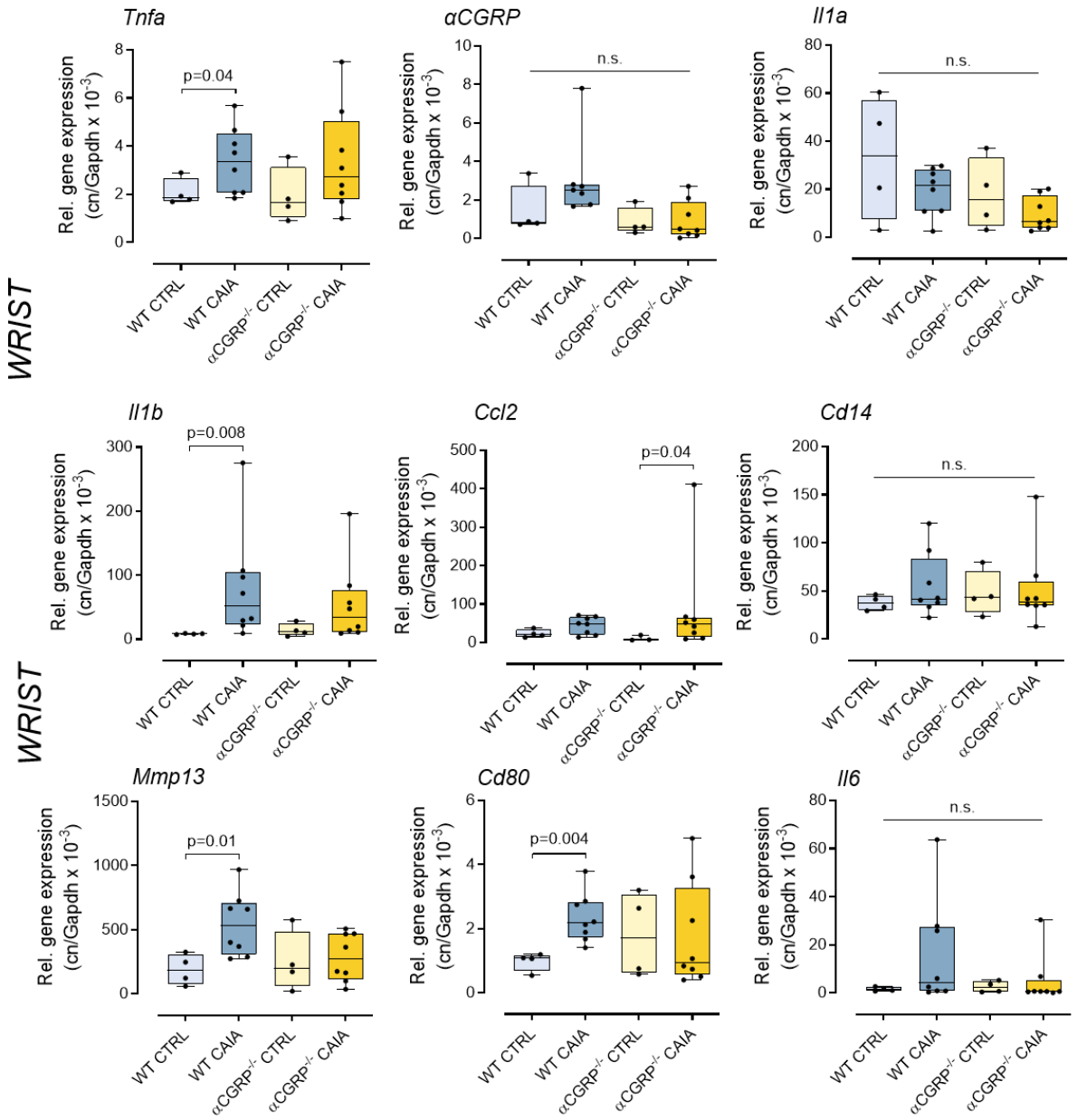

Fig. 24. PCR data of inflammation in wrist joint tissue (adapted from Maleitzke et al. (1), with written consent from the Oxford University Press).

Fig. 24. PCR data of inflammation in wrist joint tissue. Boxplots show PCR-delivered results of gene expression of pro-inflammatory markers in wrist joint tissue 10 days after the induction of CAIA. Given values are median ± minimum and maximum.

Taken together, our results from ankle and wrist joint tissue seem to be in line with clinical and histological findings pointing towards a significantly mitigated acute inflammation phase in CAIA-treated mice when naturally lacking intact αCGRP signalling.

3.4.3 Bone

We next investigated changes in the gene expression of osteoanabolic and osteocatabolic bone markers. Samples from mice at day 10 after the induction of CAIA were utilized as PCR material. The acute inflammation phase of the arthritis caused an upregulation in the *Sp7* (Osterix) transcription factor in wrist samples of WT CAIA mice when compared to their CTRL group. *Sp7* plays a role in osteoblast differentiation. Beyond *Sp7*, the two other analysed markers for osteoblast activity, *Bglap* and *Alpl*, showed no significant changes either in wrist or in ankle samples (Fig. 25a). This is in line with radiological results from animals at day 10 that did not show major alterations in bone structure when compared to their CTRL group.

Wrist samples showed a significant increase in *Runx2* transcription in WT CAIA mice compared to WT CTRL mice (p=0.004) (Fig. 25b).

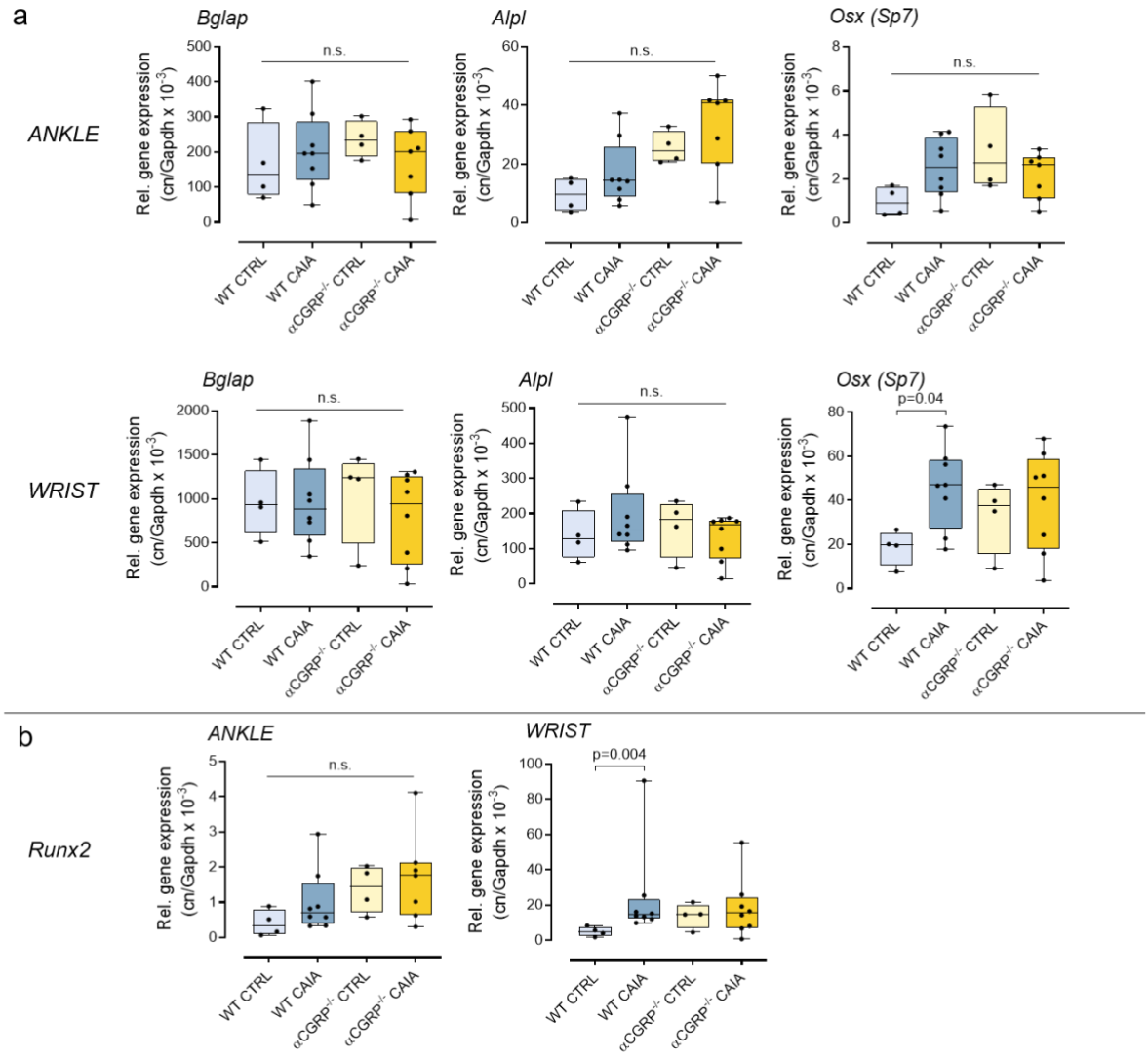

Fig. 25. PCR data of gene expression of bone formation markers (adapted from Maleitzke et al. (1), with written consent from the Oxford University Press).

Fig. 25. PCR data of gene expression of bone formation markers. (a) Boxplots show results from the molecular analysis of ankle and wrist joint tissue 10 days after the induction of CAIA. (b) PCR results of *Runx2* as a master regulator of osteoblast differentiation. Given values are median ± minimum and maximum.

To detect osteoclast reactivity, *Acp5* and *Ctsk*, two peptides abundantly enriched in actively secreting osteoclasts, were screened for expression.

Unlike the mostly unaffected bone formation, bone resorption in joint tissue at day 10 was upregulated when animals were exposed to CAIA. Wrist samples of WT CAIA mice exhibited significantly higher levels of *Acp5/ Trap* (p=0.04) and *Ctsk* (p=0.04) transcripts when compared to their CTRL group. Ankle tissue showed contrary results, with a significant increase only for *Acp5/ Trap* (p=0.04) in αCGRP$^{-/-}$ CAIA mice compared to their CTRL group (Fig. 26a). In summary, the molecular evaluation of bone turnover provided coherent results with radiological data from mice during the acute inflammation phase. A direct disruption in joint-adjacent areas was slightly more pronounced in WT animals, while an indirect, late disruption in systemic bone constitution was seen only in αCGRP$^{-/-}$ mice.

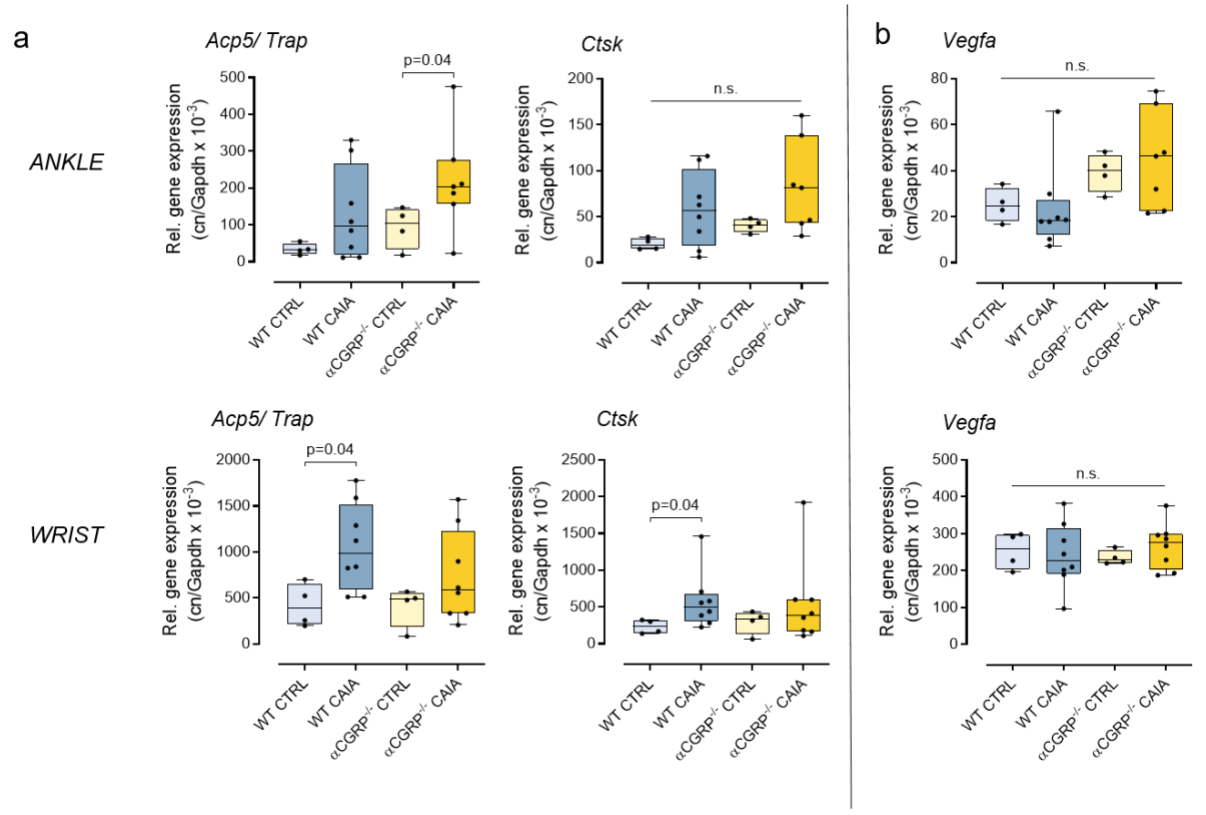

Fig. 26. PCR data of gene expression of bone resorption and angiogenesis markers (adapted from Maleitzke et al. (1), with written consent from the Oxford University Press).

Fig. 26. PCR data of gene expression of bone resorption and angiogenesis markers. (a) Boxplots show results from the molecular analysis of ankle and wrist joint tissue 10 days after the induction of CAIA. (b) PCR results of *Vegfa* as an angiogenesis marker. Given values are median ± minimum and maximum.

3.4.4 Angiogenesis

Vegfa expression surprisingly did not show any significant changes in CAIA affected joints either for ankles or wrists in the acute inflammation phase. Molecular results could not support the hypothesis that CAIA induces angiogenesis and neovascularization in acutely inflamed joints (Fig. 26b).

3.4.5 Cartilage

Cartilage is the essential element in the induction, progression and regeneration of arthritis, and thus had to be investigated intensively.

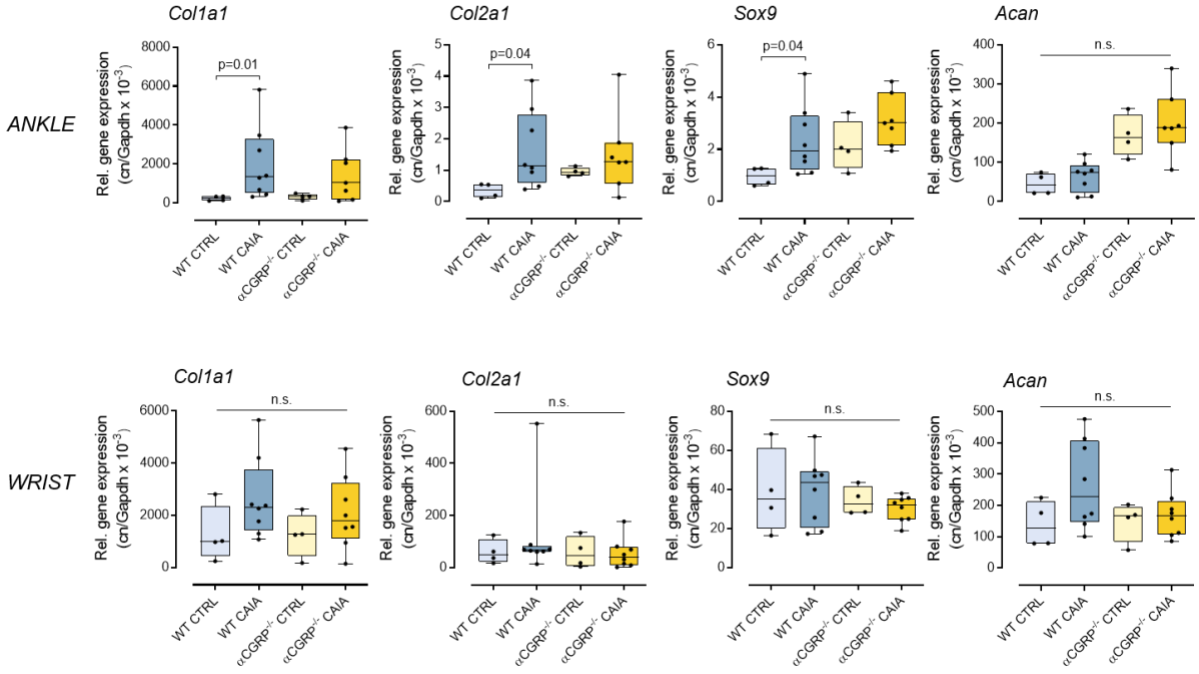

Fig. 27. PCR data of gene expression of cartilage markers (adapted from Maleitzke et al. (1), with written consent from the Oxford University Press).

Fig. 27. PCR data of gene expression of cartilage markers. Boxplots show results from the molecular analysis of ankle and wrist joint tissue from mice 10 days after the induction of CAIA. Given values are median ± minimum and maximum.

PCR screening for cartilage-specific collagen (*Col2a1* gene), ubiquitous collagen (*col1a1* gene), SOX9 (*Sox9* gene) and the peptide aggrecan (*Acan* gene) was

performed. Aggrecan serves as an integral part of the extracellular matrix of cartilage. A significant increase in the transcription of *Col1a1* (p=0.01), *Col2a1* (p=0.04) and *Sox9* (p=0.04) was seen in ankle tissue samples from WT CAIA mice compared to their CTRL group (Fig. 27). In contrast to WT CAIA mice, αCGRP$^{-/-}$ CAIA animals did not show significant changes in the expression of cartilage markers when compared to their CTRL group (Fig. 27).

No significant changes in the expression of cartilage markers were seen in the wrist tissue of CAIA animals when compared to their CTRL group.

4 Discussion and Limitations of this Study

4.1 Discussion of Results

4.1.1 General Aspects

In this study, we were able to show for the first time that endogenous disruption of αCGRP signalling has anti-inflammatory effects during experimental arthritis. Intact αCGRP signalling seemed to be important for healthy bone quality and repairing capacity for bone erosions.

All CAIA animals developed significant signs of arthritis around day 7 with swelling and redness, that subsided around day 21. This transient course of arthritis matches original observations made by Nandakumar et al. (2007) when describing CAIA in different mouse strains (121). The observed body weight loss matched that of previous studies using the same dosage of CAIA antibody cocktail (229, 230). In addition, histological results for WT CAIA animals were comparable to results reported by Buttgereit et al. (231) and Tu et al. (223), who both experienced similar scores for inflammation and cartilage degradation in WT mice with a CD1 background receiving CAIA or K/BxN serum.

αCGRP$^{-/-}$ CTRL mice did not exhibit phenotypical differences compared to WT CTRL animals. This goes in accordance with previous findings from Lu et al., who reported normal cardiovascular function and neuromuscular development for αCGRP$^{-/-}$ mice (217). However, other findings suggest some alterations in αCGRP$^{-/-}$ mice, such as a slightly altered vestibular organ innervation (232) and a markedly upregulated renin-angiotensin system (RAS) (233). In addition, it has been documented that αCGRP$^{-/-}$ mice display higher baseline mean arterial blood pressure (MAP) and heart rates than their WT littermates (234). Although the role of αCGRP in blood pressure homeostasis has not yet been clarified, its role as a potent vasodilative and blood pressure lowering peptide *in vivo* has been verified multiple times (177, 235).

Though αCGRP$^{-/-}$ mice lack αCGRP, they exhibit normal βCGRP signalling. As βCGRP might exhibit similar effects to αCGRP, it could possibly compensate for the absence of αCGRP (217). To overcome this problem in murine αCGRP studies, transgenic mice, lacking both αCGRP and βCGRP, would be necessary. Since *Calca* and *Calcb* are located on the same chromosome, the creation of such a transgenic line is complicated and cannot be achieved by simple breeding, and thus has still not implemented in research successfully to date (211). To partially overcome this

problem, we performed an additional PCR screening for *βCGRP* which did not appear in inflamed joints in relevant concentrations. In line with studies investigating distribution patterns of αCGRP and βCGRP, our observations indicate βCGRP to act predominantly inside the GIT, while αCGRP plays a predominant role in the musculoskeletal system (133, 141).
In our study, *Ramp1* was downregulated in αCGRP$^{-/-}$ CAIA animals when compared to CTRL animals, while WT animals did not show significant changes in *Ramp1* expression.

4.1.2 Discussion of Results for Inflammation and Cartilage Degradation

Concerning cartilage degradation, our histological results showed pronounced lesions and loss of hyaluronic matrix in WT mice suffering from CAIA, while αCGRP$^{-/-}$ CAIA mice did not exhibit significant changes in comparison to their CTRL group. The CAIA-induced processes in the joint matched the molecular results, with increased expression of cartilage markers for collagen types I and II as well as SOX9 proteins. This significant upregulation in WT CAIA mice might indicate early repair mechanisms of chondrocytes reacting to inflammatory changes. As this was not observed in αCGRP$^{-/-}$ CAIA mice, our data indicate an inhibitory effect of αCGRP on CAIA-induced cartilage turnover. This is in line with Nakasa et al., who observed chondroprotective effects when blocking CGRP via administration of olcegepant in experimental OA (216).

Ahmed et al. showed a marked presence of CGRP in inflamed rat joints and DRG (201). This is in line with the increased immunohistochemical reactivity we detected in ankle joints of WT CAIA mice. In our study, we induced arthritis via the administration of autoantibodies in combination with LPS and observed a marked loss in grip strength and body weight. However, administration of LPS itself may cause loss of body weight and grip strength in mice (236, 237). In line with histological findings from ankle joints and gene expression patterns from joint tissue, we observed less pronounced ankle swellings in αCGRP$^{-/-}$ CAIA mice compared to WT CAIA mice.
Zhang et al. observed similar alleviating effects on joint swelling when inducing arthritis in transgenic αCGRP-deficient mice (205). We found elevated levels of *Il1b* expression in inflamed wrists of WT CAIA mice. Ahluwalia et al. showed that IL-1β is a mediator in inflammatory activation of neutrophil accumulation that can be enhanced by CGRP

(196). Since αCGRP$^{-/-}$ mice did not show elevated *Il1b* expressions, IL-1β could be an essential part of how CGRP contributes to developing arthritis.

Furthermore, the rise in *Tnfa* both in wrists and ankle joints of WT CAIA animals can be seen as a surrogate marker for inflammatory activity. TNF-α is a key player in many inflammatory processes and TNF-α inhibition is effective in RA symptom control and, therefore, implemented in most modern RA therapy regimes (117). In addition, *Cd80* was elevated significantly in WT CAIA joints compared to CTRL animals. This was not the case for αCGRP$^{-/-}$ CAIA mice. CD80, which interacts with TNF-α, has been previously shown to activate T-cells in RA, thus its blockade has been integrated in recent therapy strategies for RA (228). Together with CD86, CD80 drives effector T-cell responses and IL-17 production in the development of experimental arthritis, which has displayed important parallels to human RA (238). The fact that WT CAIA but not αCGRP$^{-/-}$ CAIA mice exhibited increased levels of these markers indicates a disrupted inflammation cascade in αCGRP$^{-/-}$ animals concerning CD80-, CD86-, TNF-α- and IL-1β-mediated responses.

4.1.3 Discussion of Results for Bone

RA severely impacts skeletal integrity, and bone erosions represent one primary manifestation of the disease. The multitudinous ways in which RA unbalances the bone homeostasis and alters cellular interactions and activity levels of cells regulating bone plasticity have been reviewed in detail (100, 239). In brief, alterations in RA promote osteoclastogenesis by upregulating RANKL signalling and increasing stimulation of the NF-κB pathway, while osteoblast function is impaired due to the downregulation of Wnt signalling and suppression of OPG secretion.

Histological bone analysis showed increased bone resorption in WT CAIA mice at day 10, while WT CAIA mice at day 48 showed normalized bone morphometry. After 48 days, both systemic and local bone quality was severely impaired in αCGRP$^{-/-}$ mice suffering from CAIA, even though they presented less serious clinical arthritis symptoms in the acute inflammation phase. Our results indicate bone loss in the absence of αCGRP, being most pronounced in trabecular bone where active turnover is localized. In line with our results, Ballica et al. reported that estrogen-deficient mice are protected from trabecular bone loss when being exposed to elevated CGRP levels

(210). In summary, there is marked evidence for a bone protective role of CGRP *in vitro* (207, 240) and *in vivo* (210-213).

Except for AvgPo.Dm as a local bone porosity parameter after 48 days, we could not detect a significant difference between WT CTRL and $CGRP^{-/-}$ CTRL mice in respect to radiological indices of bone quality. This could possibly be owing to the fact that our mice at the age of 10 - 12 weeks were younger than the age at which Nidermair et al. (213) predicted the clinically observable manifestation of osteopenia in αCGRP-deficient mice. The osteopenic phenotype in our αCGRP-deficient mice treated with CAIA was more likely to be the result of a preexisting osteopenic genotype in combination with provoked external erosive damage from the arthritis. Transient arthritis in αCGRP-deficient mice might trigger a preliminary onset of global osteopenia. Although we clinically investigated animals continuously, our post-mortem analysis misses the whole detailed process of recovery from transient arthritis. Our data can only provide information about the acute inflammation phase at day 10 and the final bone quality during the resolution phase at day 48, but it fails to detect everything that takes part in the meantime. WT CAIA mice showed increased bone resorption and bone formation markers at day 10, but seemed to be reconstituted entirely at day 48, with even slightly increased bone density compared to day 10. The intact bone architecture in WT CAIA mice after 48 days could indicate that transient CAIA, which is characterized by rapid onset and easing after approximately 20 days (121), induced transient bone damage, from which WT mice seemed to recover entirely within 48 days. However, the short time of exposure in healthy WT mice could also be too short to cause bone damage at all. $\alpha CGRP^{-/-}$ mice clearly showed signs of compromised bone integrity at day 48 after the induction of CAIA.

The increase in bone density in WT CAIA animals over time could be caused by a healthy regeneration process with physiological stimulation of bone formation after weathering an inflammation.

In line with Matzelle et al. (218), we found that transient arthritis temporarily increases bone turnover in WT mice. However, it lastingly disrupted bone metabolism in αCGRP-deficient mice. The potential adverse effects of anti-CGRP drugs on bone homeostasis could be relevant for their long-term use. So far, there are no studies examining the long-term effects of anti-CGRP agents on bone quality in humans.

4.2 Discussion of the CAIA Model

An experimental model for medical research purposes should always try to imitate events and interactions inside a vital organism to the best of its ability. To generate a transferability of conclusions from experiments with CAIA-injected mice to human patients with RA, the advantages as well as deficits of the CAIA model mimicking human RA should be first discussed.

The rapid and simple onset of CAIA fosters advances for its experimental use in several research questions concerning the mouse strain and time-dependent aspects (130).

However, since CAIA does not require any presence of either active B- or T-cells (241), it negates an essential cellular driven part of the complex signal chain contributing to autoimmunity in human RA. Human RA, beside many other involved mechanisms, actually includes the activation of T- (19) and especially B-cells. B-cells, inter alia, produce ACPA-IgG, displaying a stable serum-detectable marker in all phases of RA as well as a key antibody correlate for disease-causing autoimmune responses from B-cells (30, 242).

Despite these deficits in mimicking pathogenic sequences in RA, CAIA is still an effective approach for experimental arthritis. Despite bringing some helpful features when compared to CAIA, other animal models also contain their own problems. The K/BxN model, unlike human RA, entirely depends on the activation of neutrophils (243). The CIA model develops slowly because it involves the activation of lymphocytes (244).

The different RA animal models furthermore cause different patterns of joint inflammation over the body. In accordance with previous findings (223), our study confirmed that CAIA manifests more in the front paw joints including the digits, while K/BxN apparently shifts the site of active inflammation more towards the ankles (231). CAIA therefore demonstrates some deviations from human RA, which predominantly affects the smaller joints of the hands, but as part of a progressive inflammatory march, may also occur in bigger joints including knee and hip and even shoulder at a later stage (245). Hence, animal models such as CAIA, though possibly providing essential information for diseases such as RA, always have to be treated with caution before translating any results to human application.

4.3 Discussion of Sex in Rheumatoid Arthritis

Differences between male and female patients represent a critical factor not only in prevalence (4), but more importantly in disease onset, course and severity. Accelerated disease progression and higher disease activity can be seen in females (246). To explain this impressive gap in RA manifestation between both sexes, genetically as well as hormonally and other biologically driven factors have to be considered (247). Gonadal hormones seem to crucially influence the course of RA, because disease activity diminishes in women during pregnancy, followed by a postpartum rebound with increased symptoms (248). These clinical observations are in line with molecular studies where both androgens and oestradiol exert multiple effects on T- and B-cells and other parts of immune activity (247), and therefore display immunomodulatory players that should not be neglected when performing research in RA. However, it is still hard to identify a clear pro- or anti-inflammatory role of sex hormones in RA. Sex hormones seem to modulate immune pathways in a complex manner, including various target sites within the humoral and cellular networks. This may sometimes even contribute to contrary effects in different autoimmune diseases (249).

For experimental purposes, male mice seem to be more susceptible both for CIA and CAIA. Young animals are especially suitable because they show the most continuous and stable clinical reaction to this method of arthritis induction (132). The increased reactivity to arthritis models could be due to the fact that oestradiol suppresses T-cell-delivered inflammatory reactions in murine arthritis (250). Nevertheless, it is not possible to directly translate results from male mice receiving CAIA to human RA.

4.4 Discussion of Materials and Methods

4.4.1 Discussion of Subjectivity in Outcome Data Collection

Subjectivity and the lack of objective measuring tools for the evaluation of RA are still a major problem in every clinical trial and cannot be eliminated in experimental studies. The DAS-28 is still the most commonly and most validated tool for endpoint comparisons in clinical RA trials (251). Aside from serum levels of C-reactive protein (CRP) and the sedimentation rate as objective markers, the DAS-28 is based on clinical observations of joint swelling. Other applied tools such as the Clinical Disease Activity Index (CDAI) and many more all share the same problem of subjectivity in

evaluation, although they could be verified as valid predictive markers in some cases (252). This fundamental problem occurs in numerous other diseases. Caution should be applied when referring to research results that were generated with measurements of semi-quantitative scores, even if obtained by blinded investigators. Any results generated in this way hold a considerable risk for biased outcome data. To extract reliable objective biomarkers that represent the status of patients suffering from RA is still a big challenge. RA affects life quality in many multiaxial aspects rather than just causing rapid death. This should be borne in mind when appraising RA research results. In our study, primary endpoints regarding the histological and clinical evaluation of arthritis severity were conducted via visual assessment from human observers, which, even if blinded for experimental group and time point, is always biased and unstable. To overcome this potential imprecision, the results were amended using objective molecular expression data and µCT parameters.

Additionally, our PCR data, which were obtained by *in vivo* analysis of homogenized tissue, have some restrictions, owing to several aspects of the extraction process. Tissue from ankles and wrists was acquired via careful removal of undesired soft tissue such as muscle, tendons and skin. This precise sample processing was performed to minimize contamination with unwanted tissue parts. Yet, the resulting tissue compound might contain ancillary cell types that potentially distort gene expression levels in addition to the already heterogeneous tissue-mix that included synovia, cartilage and bone. Therefore, results of *in vivo* PCR analysis should be interpreted with caution unlike gene expression data from cell cultures.

4.5 Conclusion and Outlook

In this study, we showed for the first time that αCGRP has a dual role in experimental arthritis *in vivo*. Our experimental design was based on the genetic deficiency of αCGRP. However, a shorter exogenous blockade of the protein itself or the receptor via administration of a monoclonal antibody could lead to divergent results and should be addressed separately. If exogenous blockade of CGRP could exert similar anti-inflammatory effects in transient arthritis in mice, anti-CGRP drugs, such as erenumab, could be discussed as a supplement for the pool of RA medications in the future. The markedly impaired bone quality in young αCGRP-deficient mice suffering from CAIA should also sensitize researchers and clinicians for possible adverse effects in long-term usage of CGRP antagonists in migraine therapy (188, 189). Although CGRP, in

the long run, seems to be essential for efficient bone remodelling, its suppression in acute inflammatory arthritis could potentially alleviate the symptoms of RA patients. Our results shed light on a new area in the field of RA research and further evidence is required before our preclinical findings can be confirmed in a clinical setting.

5 Supplementary Methods

5.1 Primer Sequences

Primer sequences:

Gapdh forward ACTGAGCAAGAGAGGCCCTA,

Gapdh reverse TATGGGGGTCTGGGATGGAA;

αCGRP forward CAGGCCTGAACAGATAACAGC,

αCGRP reverse TGTGTCTTTCATCAGCCTTTCTT,

Tnfa forward ACGCTGATTTGGTGACCAGG,

Tnfa reverse GACCCGTAGGGCGATTACAG,

Il1a forward TCGCAGCAGGGTTTTCTAGG,

Il1a reverse *TGCAGGAATGTACGGAGAGC,*

Il1b forward ACCTAGCTGTCAACGTGTGG,

Il1b reverse TCAAAGCAATGTGCTGGTGC,

Cd14 forward CCCAGTCAGCTAAACTCGCT,

Cd14 reverse AGGGTTCCTATCCAGCCTGT,

Cd80 forward CAAGTTTCCATGTCCAAGGC,

Cd80 reverse GGCAAGGCAGCAATACCTTA,

Ccl2 forward AGCTGTAGTTTTTGTCACCAAGC,

Ccl2 reverse GACCTTAGGGCAGAT,

Mmp13 forward GATGGCACTGCTGACATCAT,

Mmp13 reverse TTGGTCCAGGAGGAAAAGC,

Il6 forward CCCCAATTTCCAATGCTCTCC,

Il6 reverse CGCACTAGGTTTGCCGAGTA,

Col1a1 forward TGTTCAGCTTTGTGGACCTC,

Col1a1 reverse TCAAGCATACCTCGGGTTTC,

Col2a1 forward GGTCCCCCTGGCCTTAGT,

Col2a1 reverse CCTTGCATGACTCCCATCTG,

Sox9 forward AAGACTCTGGGCAAGCTCTG,

Sox9 reverse GGGCTGGTACTTGTAATCGGG,

Acan forward CAATTACCAGCTGCCCTTCA,

Acan reverse CAGGGAGCTGATCTCGTAGC,

Sp7 forward GCCCCCTGGTGTTCTTCATT,

Sp7 reverse CCCATTGGACTTCCCCCTTC,

Runx2 forward GTGGCCACTTACCACAGAGC,

Runx2 reverse TGAGGCGATCAGAGAACAAA,

Bglap forward CCTGGCTGCGCTCTGTCT,

Bglap reverse TGCTTGGACATGAAGGCTTTG,

CtskK forward GTCGTGGAGGCGGCTATATG,

CtskK reverse AGAGTCAATGCCTCCGTTCTG,

Acp5 forward GGTATGTGCTGGCTGGAAAC,

Acp5 reverse ATTTTGAAGCGCAAACGGTA,

Ramp1 forward GGGACCCTGACTATGGGA,

Ramp1 reverse CACCATAGCGTCTTCCCAAT,

Calcrl forward GGGGACAGTTACATGAGTCCA,

Calcrl reverse GGGCTGAGTCACTCCTCTCA,

Calcb forward CAGGCCTGAGTCACTAGCAG,

Calcb reverse TCCTTGAGGCCTTCACATCG,

Vegfa forward TCTCCCAGATCGGTGACAGT,

Vegfa reverse AAGGAATGTGTGGTGGGGAC,

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7 Appendix

7.1 Eidesstattliche Versicherung

Eidesstattliche Versicherung

Ich, Alexander Hildebrandt, versichere an Eides statt durch meine eigenhändige Unterschrift, dass ich die vorgelegte Dissertation mit dem Thema: „The dual role of αCGRP in experimental arthritis/ „Die duale Rolle von αCGRP in experimenteller Arthritis“ selbstständig und ohne nicht offengelegte Hilfe Dritter verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel genutzt habe.
Alle Stellen, die wörtlich oder dem Sinne nach auf Publikationen oder Vorträgen anderer Autoren/innen beruhen, sind als solche in korrekter Zitierung kenntlich gemacht. Die Abschnitte zu Methodik (insbesondere praktische Arbeiten, Laborbestimmungen, statistische Aufarbeitung) und Resultaten (insbesondere Abbildungen, Graphiken und Tabellen) werden von mir verantwortet.

Ich versichere ferner, dass ich die in Zusammenarbeit mit anderen Personen generierten Daten, Datenauswertungen und Schlussfolgerungen korrekt gekennzeichnet und meinen eigenen Beitrag sowie die Beiträge anderer Personen korrekt kenntlich gemacht habe (siehe Anteilserklärung). Texte oder Textteile, die gemeinsam mit anderen erstellt oder verwendet wurden, habe ich korrekt kenntlich gemacht.

Meine Anteile an etwaigen Publikationen zu dieser Dissertation entsprechen denen, die in der untenstehenden gemeinsamen Erklärung mit dem/der Erstbetreuer/in, angegeben sind. Für sämtliche im Rahmen der Dissertation entstandenen Publikationen wurden die Richtlinien des ICMJE (International Committee of Medical Journal Editors; www.icmje.og) zur Autorenschaft eingehalten. Ich erkläre ferner, dass ich mich zur Einhaltung der Satzung der Charité – Universitätsmedizin Berlin zur Sicherung Guter Wissenschaftlicher Praxis verpflichte.

Weiterhin versichere ich, dass ich diese Dissertation weder in gleicher noch in ähnlicher Form bereits an einer anderen Fakultät eingereicht habe.

Die Bedeutung dieser eidesstattlichen Versicherung und die strafrechtlichen Folgen einer unwahren eidesstattlichen Versicherung (§§156, 161 des Strafgesetzbuches) sind mir bekannt und bewusst.“

Datum Unterschrift

7.2 Anteilserklärung

Anteilserklärung an etwaigen erfolgten Publikationen

Alexander Hildebrandt hatte folgenden Anteil an den folgenden Publikationen:

Publikation 1: [Autoren: Maleitzke T, Hildebrandt A, Weber J, Dietrich T, Appelt J, Jahn D, Zocholl D, Baranowsky A, Duda GN, Tsitsilonis S, Keller J], [Pro-inflammatory and bone protective role of calcitonin gene-related peptide alpha in collagen antibody-induced arthritis], [Zeitschrift: Rheumatology (Oxford)], [Erscheinungsjahr: 2021 Apr]
Beitrag von Alexander Hildebrandt im Einzelnen:

- Durchführung der Experimente, Erhebung der Primärdaten (Anfertigung und Aufbereitung der Proben sowie Durchführung der Analysen für Histologie, MicroCT, qPCR, Immunhistochemie), Zusammenfassung und statistische Auswertung der Primärdaten, Erstellen einzelner Graphiken (die Graphiken 6 und 9-27 enthalten ganz oder teilweise von mir modifizierte bzw. ergänzte oder erweiterte Elemente aus der o.g. Publikation. Die Originalgraphiken aus der Publikation sind in Zusammenarbeit mit Tazio Maleitzke, Jerome Weber und Tamara Dietrich entstanden, alle anderen nicht bereits im Vorwort erwähnten Graphiken habe ich selbst erstellt), Bearbeitung des Manuskriptes

_____
Unterschrift, Datum und Stempel des/der erstbetreuenden Hochschullehrers/in

_____
Unterschrift des Doktoranden/der Doktorandin

7.3 Lebenslauf

Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht.

7.4 Publikationsliste

- Maleitzke T, **Hildebrandt A**, Weber J, Dietrich T, Appelt J, Jahn D, Zocholl D, Baranowsky A, Duda GN, Tsitsilonis S, Keller J. Pro-inflammatory and bone protective role of calcitonin gene-related peptide alpha in collagen antibody-induced arthritis. Rheumatology (Oxford). 2021 Apr 6;60(4):1996-2009. doi: 10.1093/rheumatology/keaa711. PMID: 33221885.
- Maleitzke T, **Hildebrandt A**, Dietrich T, Appelt J, Jahn D, Otto E, Zocholl D, Baranowsky A, Duda GN, Tsitsilonis S, Keller J. The calcitonin receptor protects against bone loss and excessive inflammation in collagen antibody-induced arthritis. iScience. 2021 Dec 24;25(1):103689. doi: 10.1016/j.isci.2021.103689. PMID: 35036874; PMCID: PMC8753130.
- Zhou S, Maleitzke T, Geissler S, **Hildebrandt A**, Fleckenstein FN, Niemann M, Fischer H, Perka C, Duda GN, Winkler T. Source and hub of inflammation: The infrapatellar fat pad and its interactions with articular tissues during knee osteoarthritis. J Orthop Res. 2022 Apr 21. doi: 10.1002/jor.25347. Epub ahead of print. PMID: 35451170.
- Maleitzke T, Weber J, **Hildebrandt A**, Dietrich T, Zhou S, Tsitsilonis S, Keller J. Standardized protocol and outcome measurements for the collagen antibody-induced arthritis mouse model. STAR Protoc. 2022 Dec 16;3(4):101718. doi: 10.1016/j.xpro.2022.101718. Epub 2022 Sep 22. PMID: 36152302; PMCID: PMC9519592.
- Maleitzke T, Dietrich T, **Hildebrandt A**, Weber J, Appelt J, Jahn D, Otto E, Zocholl D, Jiang S, Baranowsky A, Duda GN, Tsitsilonis S, Keller J. Inactivation of the gene encoding procalcitonin prevents antibody-mediated arthritis. Inflamm Res. 2023 Apr 11. doi: 10.1007/s00011-023-01719-x. Epub ahead of print. PMID: 37039837.

7.5 Danksagung

The following section is dedicated to all people who helped me out throughout the hard and long way to finish a dissertation project. I would have never been able to finish my work without the continuous teaching and support from different directions.

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7.6 Bescheinigung Statistik

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Bescheinigung

Hiermit bescheinige ich, dass Herr Alex Hildebrandt innerhalb der Service Unit Biometrie des Instituts für Biometrie und Klinische Epidemiologie (iBikE) bei mir eine statistische Beratung zu einem Promotionsvorhaben wahrgenommen hat. Folgende Beratungstermine wurden wahrgenommen:

- Termin 1: *17.08.2020*
- Termin 2: 25.*11.2020*

Folgende wesentliche Ratschläge hinsichtlich einer sinnvollen Auswertung und Interpretation der Daten wurden während der Beratung erteilt:

- Komplexe Interpretation von p-Werten in linear mixed models.
- Erklärung der Verwendung von restricted cubic splines.

Diese Bescheinigung garantiert nicht die richtige Umsetzung der in der Beratung gemachten Vorschläge, die korrekte Durchführung der empfohlenen statistischen Verfahren und die richtige Darstellung und Interpretation der Ergebnisse. Die Verantwortung hierfür obliegt allein dem Promovierenden. Das Institut für Biometrie und Klinische Epidemiologie übernimmt hierfür keine Haftung.

[redacted]