Thesis

Knee osteoarthritis in the context of the metabolic syndrome

submitted by

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Statutory Declaration

I hereby declare that this thesis is my own original work and that I have fully acknowledged by name all of those individuals and organizations that have contributed to the research for this thesis. Due acknowledgement has been made in the text to all other material used. Throughout this thesis and in all related publications I followed the “Standards of Good Scientific Practice and Ombuds Committee” at the Medical University of Graz.

Paul Schadler, eh Graz, 02.02.2022

Disclosures

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Contributions

The following people/organizations have contributed to the work of this thesis:

- This project was a collaboration of the Medical University of Graz, Austria, and the Ludwig Boltzmann Institute for Cluster Arthritis and Rehabilitation, Department for Rehabilitation, Saalfelden/Gröbming, Austria, and the Department for Degenerative Joint Diseases, Vienna, Austria

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- Mag. Dr. Bettina Thauerer conducted ELISA and Luminex measurements and quality controls; helped in data analysis of ELISA and Luminex data; revised the manuscript;
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- PD Mag. Dr. Birgit Lohberger planned and designed the experiments, including approval by the ethics committee and funding; assisted in data analysis and interpretation; revised the manuscript;
- Mag. Nicole Stündl assisted in planning and design; instructed me on cell culture standards, PCR, PCR quality control; supported me during cultivation of chondrocytes; helped with PCR experiments and PCR quality control; and gave advise on PCR data analysis;
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List of Abbreviations

Abbreviation	Description
ADAMTS4	a disintegrin and metalloproteinase with thrombospondin motifs 4
ADAMTS5	a disintegrin and metalloproteinase with thrombospondin motifs 5
ADAMTS	a disintegrin and metalloproteinase with thrombospondin motifs
AGEs	advanced glycation endproducts
AGE	advanced glycation endproduct
AMPK	AMP-activated protein kinase
AMPKα	AMP-activated protein kinase α
BCAA	branched-chain amino acids
BIPEDS	Burden of disease (B), Investigative (I), Prognostic (P), Efficacy of intervention (E) and Diagnostic (D), Safety (S)
BMI	Body mass index
BMP	bone morphogenic protein
COMP	cartilage oligomeric matrix protein
COX2 inhibitor	cyclooxygenase-2 inhibitor
CRP	C-reactive protein
CV	cardiovascular
CXCL-10	C-X-C motif chemokine ligand 10
CXCL-9	C-X-C motif ligand 9
ECM	extracellular matrix
ELISA	enzyme-linked immunosorbent assay
FABP4	fatty acid-binding protein 4
FDA	U.S. Food and Drug Administration
FGF-23	fibroblast growth-factor 23
FGF	fibroblast growth-factor
GDF-5	growth differentiation factor 5
H1-NMR spectroscopy	Proton nuclear magnetic resonance spectroscopy
HDL	high density lipoprotein
IGF-1	insulin-like growth factor 1
IL#	interleukin #
IL	interleukin
KL score	Kellgren-Lawrence scoring
KOA	Knee Osteoarthritis
KOOS	Knee injury and Osteoarthritis Outcome Score
MCP1	monocyte chemotactic protein 1
MMP#	matrix metalloproteinase#
MRI	Magnetic resonance imaging
MetS	Metabolic Syndrome
NSAID	Non-steroidal anti-inflammatory drug
OARSI	Osteoarthritis Research Society International
OA	Osteoarthritis
PLA2G2A	phospholipase A2
RA	rheumatoid arthritis
ROS	reactive oxygen species

Abbreviation	Description
SF	synovial fluid
T2DM	Diabetes Mellitus, type 2
T2DM	type 2 diabetes mellitus
TGFα	tumor necrosis factor alpha
TGFβ	transforming growth factor beta
TIMPs	tissue inhibitors of metalloproteinases
WOMAC	Western Ontario and McMaster Universities Osteoarthritis Index
anti-CCP	Anti-citrullinated protein antibody
mRAGE	membrane-bound receptor for advanced glycation endproducts
oxLDL	oxidized low-density lipoprotein
sRAGE	soluble receptor for advanced glycation endproducts
uCTXII	urinary C-terminal cross-linked telopeptide type II collagen

1 Zusammenfassung

Adipositas und das metabolische Syndrom sind bekannte Risikofaktoren für die Entstehung von Knieosteoarthrose (KOA). Diabetes mellitus, eine Komponente des metabolischen Syndroms, ist ein besonders wichtiger Faktor in der Progression von Arthrose. Das orale Antidiabetikum Metformin ist die Standardtherapie bei Typ 2 Diabetes mellitus. Ein möglicher Zusammenhang zwischen Adipositas und KOA kann einerseits durch eine mechanische Überbelastung erklärt werden, andererseits auch durch eine niederschwellige, systemische Entzündungsreaktion. Die ursächlichen Zusammenhänge sind allerdings noch weitgehend ungeklärt. Bisher konnte kein Effekt von body mass index (BMI) oder einer Metformin-Stimulation auf die Expression von Matrixgenen, wie Metalloproteinasen oder Aggrecanasen, in menschlichen Chondrocyten nachgewiesen werden. Ebenso konnte für die hoch prävalente Erkrankung Arhtrose trotz massiver Anstrengungen bisher kein effizienter diagnostischer Blut-Biomarker identifiziertt werden. Weiters ist auch eine Beziehung zwischen BMI und Knorpeldicke bislang ungeklärt.

Das Ziel dieses Dissertationsprojekts war es, BMI-assoziierte Veränderungen im Knorpel, gemessen mittels Expression von Matrixgenen sowie der sonographischen Knorpeldicke, zu verstehen. Weiters sollte die Anwendbarkeit verschiedener metabolischer Biomarker als diagnostische Werkzeuge (in Korrelation mit dem Schweregrad der Erkrankung, gemessen mittels Lequense Index und sonographischer Knorpeldicke), sowie deren Verbindung mit Adipositas, untersucht werden.

Um diese Fragen zu beantworten, wurden 3 Querschnittsstudien von erwachsenen Patienten, die sich einer Implantation einer Kniegelenksendoprothese unterzogen, durchgeführt. Dabei wurde 1), die Expression von Matrixgenen in menschlichen Chondrozyten, mittels PCR und multivariabler Regressionsanalyse untersucht. 2) Im Rahmen der Studien wurden die Serumspiegel verschiedener, metabolischer Biomarker mittels ELISA und Luminex bestimmt, das Metabolom mittels nuclear magnetic resonance (NMR) Spectroskopie detektiert, und der Zusammenhang der Biomarker mit dem klinischen Schweregrad der KOA, sowie der Adipositas überprüft. 3) Abschließend wurden Serumspiegel verschiedener Biomarker bestimmt und die Beziehung mit der sonographischen Knorpeldicke im Kontext von Adipositas untersucht.

Wir konnten zeigen, dass adipöse Patienten eine höhere Expression des Knorpel abbauenden Enzyms a disintegrin and metalloproteinase with thrombospondin motifs 5 (ADAMTS5) hatten, und dass Metformin diese Expression verringern konnte. Weiters war es möglich, einen

Zusammenhang zwischen der Knorpeldicke und dem BMI, sowie dem Biomarker fatty acid-binding protein 4 (FABP4) herzustellen. Zwischen dem klinischen Schweregrad der KOA (Lequesne) und den untersuchten Biomarkern konnte kein Zusammenhang nachgewiesen werden.

Über dieses Dissertationsprojekt konnte eine neue Perspektive bezüglich des Zusammenhanges Adipositas und KOA hergestellt, FABP4 als vielversprechenden, diagnostischen Biomarker identifiziert, und die Rolle von Metformin als mögliche, neue Behandlungsoption exploriert werden.

2 Abstract

Obesity and the metabolic syndrome are risk factors for and contribute to the pathogenesis of knee osteoarthritis (KOA). Diabetes in particular, a component of the metabolic syndrome, has been associated with disease progression. Metformin is the first-line treatment in type 2 diabetes mellitus. The connection of obesity and KOA remains unclear, but might be attributed to both mechanical loading and systemic low-grade inflammation. The relationship of BMI with and the effect of metformin stimulation on chondrocyte matrix gene expression is unknown. Furthermore, despite massive efforts over the past decades, no single diagnostic marker has been discovered that can reliably and accurately diagnose KOA. Finally, the relationship of BMI with cartilage thickness is controversial.

The purpose of this thesis project was, first, to understand BMI-associated and metformin-induced changes in cartilage, as measured by the expression of extracellular matrix genes and the sonographic cartilage thickness. Second, to assess several metabolic biomarkers as diagnostic markers for the assessment of burden of disease (measured using the Lequesne index or sonographic cartilage thickness) and to evaluate their connection with obesity / the metabolic syndrome.

To answer these questions we conducted 3 cross-sectional studies of adult patients undergoing knee arthroplasty. In three separate investigations, we assessed 1), the effect of metformin and BMI on matrix gene expression in primary, human chondrocytes, using polymerase chain reaction and multivariable regression analysis. 2) We measured serum levels of several metabolic biomarkers using the enzyme linked immunosorbent assay (ELISA) and the Luminex® technology. Furthermore we assessed the metabolic state using NMR spectroscopy, and examined their relationship with clinical KOA severity and obesity. 3) We measured serum biomarkers levels and examined their connection with sonographic cartilage thickness in the context of obesity.

We found that obese patients had a higher expression rate of the degrading enzyme ADAMTS5, and this could be mitigated by metformin stimulation. We detected an association of cartilage thickness and BMI as well as the biomarker fatty acid-binding protein 4 (FABP4). We could not find an association of any biomarker with clinical KOA severity.

This thesis project provided new insights to the relationship of obesity and KOA, identified FABP4 as promising new diagnostic blood biomarker, and explored the role of metformin as a possible therapeutic agent.

3 Introduction

3.1 Knee osteoarthritis

3.1.1 Definition and Epidemiology

According to the Osteoarthritis Research Society International (OARSI), osteoarthritis (OA) is defined as “a disorder involving movable joints characterized by cell stress and extracellular matrix degradation initiated by micro- and macro-injury” that involves " maladaptive repair responses including pro-inflammatory pathways of innate immunity" and manifests “first as a molecular derangement (abnormal joint tissue metabolism) followed by anatomic, and/or physiologic derangements (characterized by cartilage degradation, bone remodeling, osteophyte formation, joint inflammation and loss of normal joint function), that can culminate in illness.” (2) Diagnosis is currently based on clinical symptoms alone.

Incidence and prevalence vary considerably among studies due to definitions used, including symptomatic, radiographic, self-reported and doctor-diagnosed.

Due to the low predictive value of radiographic findings for pain, prevalence of symptomatic knee osteoarthritis tends to be lower compared to radiographic OA. (3)

Hip and knee OA affected more than 300 million people worldwide in 2021 (4). In the US, the prevalence of OA has increased from 21 million in 1990 to 32 million in 2021 (4). Global prevalences are increasing due to an ageing population and obesity (5). Prevalence of OA is also age and sex-specific, with higher prevalence in the 50 to 70 years-old and women (5). Similar numbers are observed in knee OA: In the US 14 million people suffered from symptomatic KOA, mostly at the age below 65 years (6). In the Framingham OA study, the prevalence of symptomatic KOA was 7% (7). (3) Among the joints affected by OA, knee OA was the 2nd most common type, with more than 40% of people older than 80 years suffering from it in 2013 (8).

Based on data from the 2017 Global Burden of Disease study, the incidence of hip and knee OA was estimated as 195 cases per 100.000 worldwide, with incidences as high as 385 cases per 100.000 compared to 75 per 100.000 in World Bank high-income countries. Incidence rates were higher in white compared to Black people in the US and also increased rapidly with ages of around 50 years, with falling rates after the age of 70 (4,9,10). OA is more common in women compared to men (11). The lifetime risk to develop symptomatic knee OA has been estimated 40% in men and 47% in women (3,12).

3.1.2 Socio-Economics

3.1.2.1 OA treatment cost is rising rapidly

Treatment currently is substantial for an individual: In Italy, the direct cost per person per year was estimated at €934 and the indirect at €1236 in 2004 (13). In a Medicare dataset in the US, there was an average total expense of $5,364 per patient attributable to knee OA in 2014 (14). The estimated lifetime medical cost attributable to knee osteoarthritis ranged between $12.000 and $16.000 in the US in 2015 (15). For a society, this leads to higher expenses: In Germany, health care expenditure for knee arthroplasty was estimated at around €1 billion per year between 2003 to 2009 (16). In the US, numbers are even higher: The estimated annual cost of knee OA treatment ranged between $5.7 and $15 billion in the US in 2017 (17). These high costs, in combination with ever increasing incidence and prevalence, put an enormous pressure on health-care systems and societies worldwide. At the same time, the majority of KOA patients are of working age (15-64 years) (18).

3.1.2.2 Disability in OA

In 2010 globally hip and knee OA ranked as the 11th highest contributor to global disability, and the 38th highest in disability-adjusted life years (DALYs), out of 291 conditions (19). In 2015, OA was ranked 15th among the 30 leading causes of years lived with disability (YLDs) in both sexes in a 2015 report from Nordic countries. In the same report, globally it ranked as the 35th/66th) leading cause of total DALYs in women/men (20).

3.1.3 Morbidity and Mortality

3.1.3.1 OA is associated with increased all-cause mortality

Even more troublesome, OA in hip and knee has been associated with an increased risk for cardiovascular (CV) death, including ischemic heart disease and congestive heart failure, as well ass all-cause mortality (21,22). Possible explanations include lower physical activity levels due to pain, increased non-steroidal anti-inflammatory drug (NSAID) consumption and its adverse affect, comorbid conditions and complications associated with joint surgery and joint replacement. (3)

3.1.4 Diagnosis of Osteoarthritis

The diagnosis of osteoarthritis is currently based on clinical signs and symptoms in at-risk groups without the further need to use imaging or laboratory studies (23,24). OA can be diagnosed when the following symptoms are present:

- Age ≥ 45 years
- Activity-related pain in one or few joints
- No morning stiffness / stiffness for less than 30 minutes

Other typical findings that can aid in the diagnosis are listed in the section “clinical manifestation”.

When any of the following signs are present, further studies are warranted:

- young patients
- atypical symptoms
- systemic findings, such as weight loss
- obvious anatomic derangements, such as instability

Additional studies may include laboratory investigations to exclude inflammatory arthritis, such as erythrocyte sedimentation rate, C-reactive protein, anti-citrulinated peptide antibodies (anti-CCP) to exclude rheumatoid arthritis.

It is important to remember that no further imaging is required in the high-risk patients mentioned above. In particular, radiography cannot be considered part of the routine examination, as many patients with KOA do not show changes on radiography and vice versa. (23,25)

3.1.4.1 Differential diagnosis

Several differential diagnoses can usually be distinguished easily from KOA. These include rheumatoid arthritis (stiffness at rest, periarticular erosions on X-ray, presence of rheumatoid factor and anti-CCP), crystalline arthritis (gout; urate / CPP crystals in synovial fluid, presence of tophi), and infectious arthritis (severe pain associated with sever joint inflammation, resembled by >2000 cells/mm³ in synovial fluid (SF), pathogens detected by synovial fluid cultivation). (23)

3.1.4.2 Clinical manifestation

OA in general is associated with activity-related pain that is relieved by rest. This is usually reflected by worse pain in the afternoon and evening, sometimes leading to poor sleep quality. Pain usually progresses through three stages (26,27):

1. Pain caused by high-impact mechanical stress without severe limitation of function
2. Constant pain that starts to affect daily activities
3. Severe limitation in function due to aching pain.

Furthermore, in OA there is joint tenderness, limitations of motion (due to osteophytes, capsular swelling and effusion), joint deformity (caused by sub-/total tears in ligaments, tendons, or mensical tears leading to subluxation) and instability (leading to an increased risk of falls and injury).

In KOA usually both knees are involved with one knee more severely affected than the other. In medial-compartment tibio-femoral joint OA, pain is localized anterio-medially (28), while in patello-femoral joint OA, pain is usually centered around the anterior aspect of the knee. Pain associated with sitting, standing up and climbing stairs is typical of patello-femoral joint KOA (29,30). Posterior knee pain can be caused by popliteal joint cyst (Baker's cyst). Persistent pain that disturbs sleep quality is a finding in advanced KOA (11). (23)

3.1.4.3 Imaging

As mentioned before, KOA can be diagnosed solely based on clinical symptoms and signs. Imaging can, however, aid in diagnosis, particularly when symptoms are unclear and it can also help in assessing severity (31). Conventional X-ray imaging is the most widely used modality as it is cheap and widely available. It can detect typical changes in KOA, such as subchondral sclerosis, joint space narrowing, osteophytes and cysts (32). However, X-ray images correlate poorly with symptoms and are insensitive to early signs of KOA. On the other hand, many patients with characteristic OA findings on X-ray do not suffer from pain or functional limitations (25,33). Magnetic resonance imaging (MRI) in OA is more important for ruling out OA than ruling in, based on its lower sensitivity than clinical or radiographic diagnosis (34). MRI can detect early changes and assess surrounding soft-tissue pathology (35). It is not necessary in routine work-up, but can be used in patients to rule out other pathology, such as ligament tears. (23)

Ultrasound

Ultrasound is becoming a popular tool in the diagnosis of KOA. Ultrasound can detect and assess minimal structural abnormalities of soft tissue, including cartilage, muscles, tendons and ligaments. Furthermore, pathological findings, such as osteophytes, bony defects, joint effusion and cysts can be easily detected (32,36). Several studies have validated ultrasound imaging as a diagnostic tool: There is a strong agreement of ultrasound findings with MRI measurements and ultrasound findings are reportedly associated with pain and function (37). In cadaver studies, there was a strong

agreement of anatomic and sonographic cartilage thickness on the medial femoral condyle, while it was weak on the lateral condyle and intercondylar notch (38). These studies show that ultrasound is a valid and accurate diagnostic tool in KOA.

3.1.4.4 Biomarkers

As indicated before, currently the diagnosis of KOA is based on clinical symptoms and insensitive radiography. MRI and ultrasound are limited due to high cost (MRI) and acoustic shadowing (ultrasound). Detection of biochemical markers in biologic fluids not only provides an easy, less expensive, and highly efficient method for diagnosis of (K)OA, but can also provide a quantification, is reliable and can help in early detection. (26)

In addition, biomarkers are of critical importance for disease knowledge and treatment innovation: One of the many purported reasons for the historical slow pace in gain of knowledge and treatment innovation of diseases might be the lack of valid and responsive biomarkers to ascertain efficacy (18). Considering the time and cost to develop new compounds (estimated to $802 million in 2003 (39), the low probability of success (in all clinical trail phases and approval, around 8% (40)), the discovery of a valid, sensitive and reliable biomarker that can aid in assessment of drug efficacy is of critical importance. According to Hunter et. al., in (K)OA there are additional barriers to the development of new therapeutic compounds: First, the current reference gold standard of disease severity and diagnosis, the joint space width on radiography, is insensitive, has a low responsiveness and bad correlation with clinical symptoms. Second, there is no consensus as to what constitutes a meaningful clinical endpoint. And third, OA is a complex disease with a marked heterogeneity of disease onset and clinical presentation (18). In an effort to overcome these barriers, the BIPEDS (acronym see below) classification system for biomarkers has been introduced (41): This scheme classifies biomarkers into six categories: burden of disease, investigational, prognostic, efficacy of intervention, diagnostic and safety biomarkers. As of 2015 there were 12 molecular biomarkers of bone or cartilage turnover that could be quantified with commercially available kits. These represent highly-qualified OA-related biomarkers but none of them have a role in daily clinical practice yet. (18)

Many other biomarkers have been evaluated in different settings and for different purposes according to the BIPEDS scheme (9,42–45). However, so far no biomarker has proven to be of clinical relevance when assessing (K)OA burden of disease. Below, several potential candidate biomarkers for the assessment of burden of diseases, according to the BIPEDS scheme, will be reviewed:

Oxidized low-density lipoprotein (oxLDL) is the oxidized form of LDL-cholesterol. It is involved in the formation of atherosclerotic plaque and plays an important role in cartilage degeneration via induction of chondrocyte death (46). Studies have shown that oxLDL levels in KOA patients are higher compared to control and there seems to be a correlation with severity (47).

Fatty acid-binding protein 4 (FAB4) is a member of the FABP superfamily of lipid chaperons. It is mostly expressed by adipocytes and regulates intracellular fatty acid transport by increasing fatty acid solubility and facilitating transport to specific cellular compartments or enzymes. In addition, FABP4 is a regulator of energy homeostasis. FABP4 expression is higher in women. Elevated levels of circulating FABP4 are associated with obesity, increased cardiovascular risk and cancer. (48) FABP4 was also found to be associated with KOA severity: Levels were higher in KOA compared to healthy control, and also correlated with severity on radiographic examinations (49). FABP4 knock-out or pharmacological inhibition caused a slowing of cartilage degeneration in mouse models of induced obesity (50).

Phospholipase A2 (PLA2G2A), a member of the phospholipase A2 (PLA2) family, is the membrane bound form of PLA2. It acts as a lipolytic enzyme in the hydrolysis of membrane phospholipids. Intra-articular injection of PLA2 is known to start an acute inflammation reaction and expression of multiple isoforms in OA chondrocytes can be increased by pro-inflammatory stimulation. (51,52)

Advanced glycation endproducts (AGE) are formed during episodes of hyperglycemia. These proteins, modified by non-enzymatic glycosylation, play an important role in inflammation regulation. The interaction of AGE with the membrane bound receptor (mRAGE) induces local inflammation. This can be minimized by soluble AGE receptors (sRAGE) that block the interaction with mRAGE and are associated with anti-inflammatory responses. However, so far no study has found a positive effect of reduced AGE levels in OA. (53)

Leptin is a hormone mostly produced in adipocytes and plays an important role in energy homeostasis, among many other effects. Studies have found a positive association with obesity and female gender (54,55). In OA leptin levels were found to be higher in synovial fluid than serum and there seems to be an enhanced expression of the leptin receptor Ob-Rb in osteoarthritic cartilage compared to normal cartilage (56). Leptin alone or in combination with pro-inflammatory cytokines, such as IL-1, was reported to enhance collagen degradation via the increased expression of MMP-1 and MMP-13 (57). This is supported by a study that showed that leptin levels were positively associated with MMP-1 and MMP-3 levels in synovial fluid in OA patients (58). These results seem to be largely independent of obesity: In leptin receptor- or leptin-deficient mice with

extreme obesity, there was no difference in cartilage degeneration compared to wildtype mice (59). (60,61)

Several clinical studies have tried to explore the role of leptin specifically in KOA. However, the findings on leptin levels and KOA severity in clinical studies remain inconclusive: While some have found an association with KOA severity, others have not (62–64).

Similar to leptin, resistin is a peptide hormone with an important role in energy homeostasis and insulin resistance (65). In KOA, resistin has been associated with disease severity in some studies, while others have not found an association (63,64,66–68).

Ghrelin is a hormone secreted by entereo-endocrine cells of the gastrointestinal tract and among others is involved in the regulation of food intake. In KOA, ghrelin was associated with disease severity, measured on the Western Ontario and McMaster Universities Arthritis Index (WOMAC) scale (69).

Fibroblast growth factor 23 (FGF-23), an important chondrocyte differentiation factor, is upregulated in OA cartilage in comparison to healthy controls. Accordingly, serum levels were higher in KOA patients and there was a correlation with KOA severity. (70,71)

In addition to the above mentioned biochemical markers, previous studies have also found interesting changes in the metabolites in KOA: Using both targeted and untargeted metabolomics approaches, studies have shown changes particularly in branched-chain amino acids (BCAA) and arginine pathways in KOA patients compared to healthy controls. This is supported by large epidemiologic studies that found a link of BCAA concentrations with diabetes mellitus, cardiovascular disease and metabolic syndrome. (72,73)

3.1.5 Pathogenesis of Osteoarthritis

3.1.5.1 Inflammation is a key driver of destruction in OA

The pathogenesis of OA is complex. A multitude of genetic, environmental, social, biomechanical and cellular factors lead to destruction of and changes in ligaments, cartilage, joint capsules and muscles that accumulate in end-stage pathology. In the past, osteoarthritis was thought to be caused by a simple, degenerative “wear and tear” process that progresses with age. However, research indicates that OA is a complex disease caused by a multitude of factors that converge in an inflammatory process, as indicated by the “-itis” in osteoarthritis. These factors are discussed below: (74)

- **Inflammation**: In contrast to rheumatoid arthritis where there is a synovial infiltrate with a high abundance of leukocytes and lymphocytes and fibroblast proliferation, in OA there is only weak cellular inflammation, mostly involving macrophages. At the same time, local and systemic cytokines and adipokines fuel the production of degrading enzymes, proteases that cause destruction in all tissues of the joint, including bone, cartilage and soft tissues. Cytokines that might play an important role in OA pathogenesis include interleukin-6 (IL-6), Monocyte Chemotactic Protein 1 (MCP-1), C-X-C motif chemokine ligand 10 (CXCL-10) and C-X-C motif ligand 9 (CXCL9) (75). Several other cytokines have also been detected in the synovial fluid (75,76). These pro-inflammatory factors lead to the chemotaxis of macrophages and induce the production of proteases. Recently, the innate immune system has gained more attention as a possible driving factor: Activation of the innate immune response by fragments of matrix proteins, such as cartilage oligomeric matrix protein (COMP), fibronectin and collagen promotes pro-inflammatory processes and thereby might cause a vicious cycle (76).

- **Proteases**: Activation of above mentioned immune processes leads to the production of matrix degrading enzymes. OA research has focused on proteases that degrade cartilage extracellular matrix proteins. Important examples include members of the aggrecanase ‘disintegrin and metalloproteinase with thrombospondin motifs’ family, such as ADAMTS-4 and ADAMTS-5. Both have been linked to OA and degrade aggrecan, a large proteoglycan in the cartilage. Similarly, other important enzymes are matrix-metalloproteinase (MMP) 1, 3 and 13 that are responsible for collagen II degradation. These MMPs are inhibited by tissue inhibitors of metalloproteinases (TIMPs) in healthy cartilage. This balance is

disturbed in OA, however. (77)

- **Other pathways**: Other pathways include fibroblast growth factor (FGF) signaling, bone morphogenic protein (BMP) and Wnt pathways. (74)

3.1.5.2 Pathologic findings in OA involve the entire joint

Above mentioned pathologic processes lead to pathologic changes that affect the entire joint and are listed below: (74)

- **Articular cartilage**: The earliest changes seen in OA involve the cartilage surface where fibrillation occurs in areas of maximal load. In a healthy state, chondrocytes, the only cell type present in cartilage, are quiescent cells that maintain the cartilage through anabolic and catabolic activities. In OA, chondrocytes proliferate to form clusters and undergo changes in the phenotype towards hypertrophic chondrocytes, similar to the hypertrophic zone of the growth plate, to produce collagen type X and MMP-13. This occurs most likely due to loss in extracellular matrix. With disease progression there is more extracellular loss due to local inflammatory mediators leading to degradation and protein fragments that stimulate chondrocytes to produce even more cytokines and degrading enzymes. Once collagen is lost, it cannot be replaced in the cartilage. In end-stage disease, cell death occurs, leading to areas without chondrocytes.

- **Bone**: In OA subchondral bone thickening (bone sclerosis) is commonly seen due to increased secretion of collagen that does not mineralize properly. Osteophytes form at the insertion site of ligaments and tendons. Typically, subchondral bone cysts can be seen on X-ray. Bone marrow lesions, detected on MRI, can be seen at regions with maximal load and can be associated with local fibrosis and necrosis.

- **Synovia**: While in OA commonly there is some degree of synovitis, this is not thought to be the initiating factor, in contrast to rheumatoid arthritis (RA) and involves other cell types infiltrating the synovia. But synovitis contributes to pain and disease progression via the production of inflammatory mediators.

- **Soft tissue**: In addition to the above mentioned changes, there is extracellular matrix destruction and cell death in surrounding tissues, including ligaments, tendons, capsules, menisci and other structures. This can lead to atraumatic tears and ruptures that are commonly observed in end-stage OA and that can contribute to inflammation again. In addition, OA also affects periarticular muscles and nerves, leading to weakness and pain.

3.1.5.3 There are several OA phenotypes based on risk factors

These pathologic changes are common to all forms of OA and are independent of the inciting event. Thus, some experts have suggested to distinguish several OA phenotypes based on risk factors and inciting events. These are discussed in more detail below: (74)

- **Age**: As mentioned before, age is associated with both incidence and prevalence of OA. However, development of OA and ageing are two separate processes. Changes seen in joint ageing include cellular ageing and ageing of the extracellular matrix (ECM). Changes in the ECM include thinning of the cartilage, reduced hydration and altered biomechanical properties (increased "brittleness"). This is due to the accumulation of advanced glycation endproducts (AGEs) leading to cross-linking of collagen. Cellular changes include mitochondrial dysfunction, reduced responsiveness to growth factors, such as insulin-like growth factor 1 (IGF-1) and transforming growth factor beta (TGFβ), cell senescence-related secretory phenotype and reduction of autophagy. These changes seen in ageing make joints more susceptible to the development of OA. (74,78)

- **Trauma**: Acute injury of ligaments, meniscal tears, or intra-articular fractures induce an acute inflammation process, involving inflammatory mediators, such as IL-6 and tumor necrosis factor alpha (TNFα), that is sustained over time. Pathologic changes of posttraumatic OA can usually be seen within 10 years after the traumatic injury. (79,80)

- **Obesity**: Obesity is a risk factor for the development of OA in weight-bearing joints such as hip and knee, but also non-weight bearing joints, such as the hands (81). Obesity and the metabolic syndrome lead to a systemic low-grade inflammation that, together with high adipokine levels and altered glucose and lipid metabolism lead to local joint destruction and metabolic OA. This will be discussed in more detail below. (82)

- **Genetics**: Rare hereditary forms of OA include mutations in collagen type II, IX, XI - the structural collagens in articular cartilage. In genome-wide association studies, several loci have been discovered that predispose to OA, such as polymorphisms in the gene growth differentiation factor 5 (GDF-5). It is a member of the bone morphogenetic protein family and important for the joint development. However, these mutations confer a slight increase in risk only and other factors, such as environment or epigenetics might play an important role. (83)

- **Anatomic factors**: Joint shape plays an important role in the development of OA: In the

knee, varus- and valgus alignment are associated with increased risk of medial and lateral compartment knee OA, respectively. This can be explained by abnormal and altered loads that result in an increase of inflammatory mediators. (84)

- **Gender**: The distribution of OA depends on gender: In hand and knee OA there is a female predominance, while hip OA is equally common in men and women. The decline in estrogen with age in women is a major contributor to cartilage degeneration. (26)

3.2 Metabolic Syndrome

Obesity, particularly abdominal obesity, is associated with insulin resistance and disturbances in both glucose and lipid metabolism, often leading to diabetes type 2. Hyperinsulinemia, hyperglycemia and hyperlipidemia lead to endothelial and vascular dysfunction, arterial hypertension and vascular inflammation that end in the development of atherosclerotic vascular disease. The co-occurrence of these vascular risk factors suggested the existence of a “metabolic syndrome”. It is debated, however, if this is a separate disease entity, and if it confers more risk beyond its separate components. (85)

3.2.1 Definition & Diagnosis

There are several definitions for metabolic syndrome (MetS) leading to difficulties when comparing studies. The definition of The National Cholesterol Educational Programme NCEP Adult Treatment Panel III (ATP) 2001 (and updated by the American Heart Association, AHA, in 2005) is the most widely used definition. MetS is defined as the presence of any three of the following (86):

- Abdominal obesity (≥102cm in men and ≥88cm in women)
- serum triglycerides ≥150mg/dl or drug therapy of hypertriglyceridemia
- serum HDL < 40mg/dl in men, < 50mg/dl in women, or drug therapy for low HDL
- blood pressure ≥130/85mmHg or drug therapy for hypertension
- fasting blood glucose ≥110mg/dl or drug therapy for hyperglycemia

3.2.2 Epidemiology and risk factors

The prevalence of MetS has increased over the past decades. While data from the 1994 National Health and Nutrition Exam Survey (NHANES III) showed a prevalence of 22%, this has increased to 34.7% in 2016 (87). Among several risk factors, weight was the most important one: in the Framingham Study, an increase of 2.25kg was associated with an increase in the risk for developing MetS of up to 45% (88). In the NHANES III study only 5% of normal weight, but 60% of obese patients were diagnosed with MetS. Age plays an important role as well: While the prevalence was 6.7% in the 20-29 year old, this increased to 42% in the over 70 year old. Other risk factors include race, postmenopausal status, smoking, low income, high carbohydrate diet, no alcohol consumption, physical activity, soft drink consumption and use of atypical antipsychotics (clozapine). (85,89)

3.2.3 Complications of Metabolic Syndrome

Patients with MetS are at considerable risk of developing type 2 diabetes mellitus (T2DM). In a meta-analysis of 16 multi ethnic cohort studies, the relative risk ranged from 3.53 to 5.17. Several studies showed an increased risk with increasing number of MetS components found. (90)

Similarly, several meta-analyses showed that MetS increased the risk for cardiovascular disease (CVD; 1.53-2.18) and all-cause mortality (1.27-1.6). However, it remains unclear if MetS adds more risk than each individual component. Other relevant associations include fatty liver disease leading to cirrhosis, hepatocellular carcinoma, chronic kidney disease, polycystic ovary-syndrome, gout and obstructive sleep apnea. Recently, several studies have also linked OA and metabolic syndrome. This and the pathogenesis of metabolic OA will be reviewed in more detail below. (85,91,92)

3.2.4 Treatment of Metabolic Syndrome

The AHA and other societies recommend setting two major therapeutic goals for patients with MetS: 1) Treatment of the underlying cause (obesity) by dietary and physical actions. 2) Treatment of CV risk factors, if they persist despite lifestyle modifications. The cornerstone of treatment is lifestyle modification: Adhering to the Mediterranean diet and regular exercise are very important. This can help prevent progression to overt T2DM and of other CV risk factors. (86)

In the prevention of T2DM, metformin might play a role, in the treatment of T2DM it is considered the optimal initial treatment. The role of metformin in OA will be reviewed below. (93)

3.2.5 Metabolic Osteoarthritis

The prevalence of MetS in patients with OA is 59%, while it is lower (23%) in the population without OA (94). Several studies have found that patients with MetS are at higher risk of development and progression of OA: In the NHANES III study, the prevalence of MetS was increased in patients with OA independent of age and BMI (94). In the Japanese Research on Osteoarthritis / Osteoporosis Against Disability (ROAD) study, there was a gradual increase of risk for KOA development or progression with the accumulation of individual MetS components (95). Others have found that in women the presence of MetS was associated with a higher risk of KOA and hand OA compared to obesity alone (96). (82,97)

Each component of MetS is associated with an increased risk for (K)OA by itself. Several studies have linked diabetes and OA: In the NHANES III study, a 1-point increase in the Homeostatic Model Assessment Insulin Resistance (HOMA-IR) correlated with an increase of KOA risk of 18% in non-obese and 34% in obese males independent of other risk factors (98). T2DM was also linked to severe OA, indicated by the need for total need arthroplasty, independent of age or gender (99). In vitro studies found that OA chondrocytes had a disturbed glucose homeostasis: Accumulation of glucose led to the accumulation of ROS, directly and indirectly via pro-inflammatory cytokines (100). Glucose can induce the expression of catabolic enzymes (MMP-1 and 13) in OA chondrocytes (101). In addition, hyperglycemia leads to the accumulation of AGE. These can not only induce a pro-inflammatory response and production of proteases, such as MMP1 (102), but also alter the biomechanical property of the cartilage matrix, leaving it more vulnerable to mechanical stress (103).

The link of hypertension and OA is supported by the findings from the ROAD, showing an association of hypertension with KOA (95). Hypertension might lead to endothelial dysfunction and decreased blood flow in the subchondral microvessels, ultimately leading to ischemia (82,104).

Dyslipidemia was also associated with KOA independent of BMI in several studies (97). oxLDL might also play an important role in KOA, by its involvement in progression of atherosclerosis (105).

The association of obesity and OA has been shown by several studies. While the detailed mechanism is unclear, mechanical and metabolic factors appear to be the main culprits. Increased mechanical load leads to the expression of pro-inflammatory factors and degenerative enzymes (106). However, mechanical stress cannot explain the association of obesity and OA in non-weight

bearing joints, such as hands. Thus, the metabolic and endocrine effects of adipose tissue have been recognized as an important driver in OA: Several adipokines, most importantly leptin, have been linked to OA (see above).

Low-grad inflammation is recognized both in OA, metabolic syndrome and diabetes mellitus. This condition is associated with higher expression of pro-inflammatory factory, such as IL-1, -6, -10 and TNFα and leads to accelerated ageing and cell senescence via oxidative stress conferred by ROS. It remains difficult to assess whether chronic low-grade inflammation is the cause or consequence of OA and/or MetS in these patients. (82,97)

3.3 Treatment of Osteoarthritis

3.3.1 State of the art

Treatment of KOA involves a combination of several non-pharmacological, pharmacological and surgical interventions. All patients with KOA should undergo personalized counseling and education about KOA. Misconceptions, e.g. that KOA will inevitably get worse, need to be addressed. It is important to create realistic and positive expectations for treatment efficacy and adherence to lifestyle changes. Treatment decisions should be based on disease severity (107):

- Mild KOA: Patients with mild KOA have intermittent pain with preserved function and quality of life. Treatment should focus on non-pharmacological intervention, exercise, weight management and topical analgesics.
- Moderate/severe KOA: Patients with impaired functionality on quality of life need a more aggressive, multimodal therapeutic approach with non-pharmacoligcal intervention as first-line treatment. Aquatic exercises are better tolerated in patients with severe pain. Extraarticular factors, such as sleep problems, mood disorders and chronic pain need to be addressed. In addition to oral and topical non-steroidal anti-inflammatory drugs (NSAIDS), braces and walking aids may be necessary to relieve pain. In patients with refractory symptoms, surgery is indicated. Surgical options include total joint replacement / arthroplasty, the gold standard, as the only definitive treatment. Studies have shown improved function and greater pain relief compared to nonsurgical treatment alone (108). However, also nonsurgical patients improved in this study, while surgical patients had a higher complication rate. Alternative options include unicompartmental knee arthroplasty in unicompartmental disease and osteotomy in younger, more active patients.

3.3.2 Treatments of no or uncertain benefit

Treatments that are not recommended included platelet-rich plasma injection, insoles, nutritional supplements and knee arthroscopy.

3.3.3 Experimental treatment and the role of metformin

While currently there is no disease modifying OA drug (DMOAD), new compounds targeting metabolic pathways and growth-factors, such as inhibitors of metalloproteinases, are being investigated. (109)

One substance of recent focus is metformin. As mentioned before, metformin is the gold standard in diabetes management. Several studies in mouse models have shown protective effects: Metformin biguanide derivative, could mitigate cytotoxic effects of IL-1b and was able to reduce reactive oxygen species (ROS) (110). It was also able to alleviate IL-1b-induced cytokine production by regulation of AMPK and it reduced oxidative stress (111,112). It was also able to reduce cartilage degeneration (113).

Findings from clinical studies are equivocal, however: While one prospective study found reduced cartilage loss in patients taking metformin, and another study showed increased improvements in functionality and pain compared to NSAIDs alone, a study on a large epidemiologic data set could not find a role of metformin in OA treatment [(110);(114);mohammed2014;barnett2017].

3.4 Hypothesis and Aim

The purpose of this thesis project was to evaluate the role of obesity and the metabolic syndrome in the context of osteoarthritis. We wanted to understand, if we can find a metabolic biomarker that is associated with KOA and can be used as a marker for burden of disease, as measured on a functional / pain level (Lequesne index), or using ultrasound imaging (cartilage thickness). Furthermore, we wanted to understand the association of obesity/BMI and KOA on a biochemical / cellular level by measuring gene expression using RT-qPCR. Specifically, the following questions were addressed in this project:

1. What is the relationship of BMI, the metabolic syndrome and metabolic biomarkers (measured using ELISA, Luminex, or NMR spectroscopy) in KOA?
2. Can we find an association of biomarkers and KOA severity (Lequesne index) or radiography (hip-knee-ankle-angle)?
3. Is there an association of obesity/BMI, serum biomarkers and sonographic cartilage thickness in KOA?
4. What is the relationship of obesity/BMI and the expression of certain matrix genes, with a major role in the pathogenesis of OA, in KOA?
5. Can treatment with metformin, an important drug in T2DM management, affect chondrocyte gene expression (measured by qPCR on human primary chondrocytes)?
6. How do other clinical parameters relate to gene expression in chondrocytes?

To answer these questions, we conducted 3 prospective studies that have been published before and are reprinted, with the permission from the respective journal, below.

4 Publications

4.1 The Association of Blood Biomarkers and Body Mass Index in Knee Osteoarthritis: a Cross-Sectional Study

Original Article

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The Association of Blood Biomarkers and Body Mass Index in Knee Osteoarthritis: A Cross-Sectional Study

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Abstract

Objective. Despite massive efforts, there are no diagnostic blood biomarkers for knee osteoarthritis (KOA). This study investigated several candidate diagnostic biomarkers and the metabolic phenotype in end-stage KOA in the context of obesity.
Design. In this cross-sectional study, adult patients undergoing knee arthroplasty were enrolled and KOA severity was assessed using the Lequesne index. Blood biomarkers with an important role in obesity, the metabolic syndrome, or KOA (oxidized form of low-density lipoprotein [oxLDL], advanced glycation end product [AGE], soluble AGE receptor [sRAGE], fatty acid binding protein 4 [FABP4], phospholipase A2 group IIA [PLA2G2A], fibroblast growth factor 23 [FGF-23], ghrelin, leptin, and resistin) were measured using enzyme-linked immunosorbent assay (ELISA; $n = 70$) or Luminex technique (subgroup of $n = 35$). H1-NMR spectroscopy was used for the quantification of metabolite levels (subgroup of $n = 31$). The hip-knee-ankle angle was assessed. Multivariable and multivariate regression analysis was used to examine the relationship of biomarkers with body mass index (BMI) and KOA severity in complete case and multiple imputation analysis.
Results. While most of the investigated biomarkers were not associated with KOA severity, FABP4 and leptin were found to correlate with BMI and gender. Resistin was associated with Lequesne index in complete case analysis. Using a targeted metabolomics approach, BMI-dependent changes in the metabolome were hardly visible.
Conclusions. Our findings confirm studies on FABP4, leptin, and resistin with regard to obesity and the metabolic syndrome. There was no association of the investigated biomarkers with KOA severity, most likely due to the patient selection (end-stage KOA patients). Based on this absence of BMI-dependent changes in the metabolome, we might assume that BMI is not correlated with KOA severity in this specific patient group.

Keywords
FABP4, biomarkers, obesity, knee osteoarthritis

Introduction

Background

Knee osteoarthritis (KOA) is a major cause of disability and pain worldwide.[1] Despite massive efforts over the past decades, no blood serum marker has emerged as diagnostic marker so far.[2]

Based on recent research results, inflammation, obesity, and the metabolic syndrome have been described as risk factors for KOA manifestation and progression of OA.

Obesity, defined as a body mass index (BMI) of greater than 30, is a silent epidemic associated with several chronic conditions and affecting more than 2.1 billion individuals worldwide.[3,4] It is also a major contributing factor to the pathogenesis and progression of KOA due to adipocyte-derived

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systemic inflammation. Obesity-related osteoarthritis has been described as a complex, multifactorial condition that can cause significant impact on quality of life of affected patients.[5] There is a close correlation of BMI and clinical and functional consequences of KOA.[6] This observation is relevant when considering KOA treatment strategies based on obesity. Investigating candidate biomarkers to assess a possible correlation to BMI and/or KOA severity seems to be an important step in finding diagnostic blood biomarkers.

In addition, obesity represents a key finding for the metabolic syndrome (metS). The metS is defined as a combination of several disorders associated with cardiovascular risk. It causes local and systemic changes that induce a proinflammatory state leading to oxidative stress and eventually cartilage degradation.[7]

Although adipocytokines and the metabolome are considered to be important pathogenic factors in obesity-related OA, there has been little research of these factors as diagnostic biomarkers.

Leptin, resistin, and grehlin play a major role in obesity and metS. These markers contribute to the imbalance of joint homeostasis and are involved in the pathogenesis of osteoarthritis.[8,9]

The oxidized form of low-density lipoprotein (oxLDL) is not only a marker of metabolic disease, but plays a major role in cartilage degeneration.[10]

Fatty acid binding protein 4 (FABP4) is a adipokine that is closely associated with obesity and metabolic disease. In knock-out models and after pharmaceutical inhibition of FABP4, OA induced by high-fat diet in mice was alleviated.[11]

The receptor/ligand system of advanced glycation end products (AGEs) and their cell receptor (RAGE) are similarly involved in the pathophysiology of metabolic diseases. The AGE-RAGE interaction results in increased generation of oxygen radicals and pro-inflammatory cytokines. Circulating soluble AGE receptors (sRAGE) interact with AGEs to counterbalance the negative effects of AGEs-RAGE interaction.[12]

The finding that PLA2G2A is highly abundant in biological fluids of patients suffering from inflammatory diseases, such as arthritis, sepsis, and myocardial infarctions, emphasizes the important role of PLA2G2A in inflammation. Elevated levels of PLA2G2A have been described as biomarker for cardiovascular disease and the metS.[13]

Fibroblast growth factor 23 (FGF-23) is a regulator of cartilage differentiation and might also play a role in the pathogenesis of osteoarthritis.[14]

Previous metabolomics studies have identified changes in branched-amino acid (BCAA) and arginine levels in serum and synovial fluid of KOA patients.[15] Another study, using an untargeted metabolomics approach, found several differences in obese KOA patients compared with nonobese KOA patients.[16]

To the best of our knowledge, these peptides and proteins with important roles in cartilage matrix turnover (PLA2G2A), glucose metabolism (AGE, soluble RAGE), and chondrocyte differentiation (FGF-23) have not been studied extensively in the context of obesity and end-stage KOA before.

In this article, we examined abovementioned biomarkers as markers of burden of disease in end-stage KOA, measured using the Lequesne index. This study was conducted to investigate whether any of these biomarkers are associated with obesity, the metS, and KOA and to test their ability in characterizing KOA severity.

Methods

This study was conducted as a cross-sectional study at the Department of Orthopedics and Trauma of the Medical University of Graz, Austria, from January 2019 to April 2020. The procedures described were in accordance with the ethical standards of the ethics committee of the Medical University of Vienna (IRB#2029/2016) and the Medical University of Graz (IRB#31-133ex18/19) and with the Helsinki Declaration.

Study Population

Adult patients undergoing knee arthroplasty were enrolled in the "Better Life in Osteoarthritis Registry" (BLOAR) after informed consent. The BLOAR is an Austrian multicenter registry for OA patients with the goal to further research in the prevention and treatment of osteoarthritis. A total of 70 patients were grouped based on BMI (group 1 = "underweight": BMI $<$ 20 [n = 19], group 2 = "normal weight": 20 to 30 [n = 32], group 3 = "obese": BMI $>$ 30 [n = 19]) and chosen randomly (enrollment based on date of surgery and available resources on the day of enrollment) from the registry for analysis in this study.

Blood samples were taken preoperatively and centrifuged within 1 hour. Serum was stored at −20°C until further processing. To achieve a more distinct separation, and due to limited resources, only patients with the lowest and highest BMI in the underweight and obese group, respectively, were further analyzed with metabolic phenotyping and Luminex assays.

Patient Characteristics

Height, weight, and abdominal circumference and blood pressure measurements were taken. For the purpose of this study, obesity was defined as a BMI of greater than 30, normal weight as a BMI of 20 to 30, and underweight as a BMI less than 20. Previous nonoperative and operative treatment (including physiotherapy, physical therapy, weight-reduction program, intra-articular injections, topical and systemic analgesics) was recorded. In women, hormonal status was recorded (previous gynecologic surgeries, oral contraceptive pills, hormone-replacement treatment). Comorbidities

and medication were recorded based on history taking and medication was grouped based on mechanism of action. Based on the National Cholesterol Education Program (NCEP) definition of the metabolic syndrome, patients were diagnosed with metabolic syndrome when 3 or more of the following conditions were met: (1) on antihypertensive medication, known arterial hypertension, or systolic office measurement greater than 130 mmHg; (2) known dyslipidemia or on lipid-lowering drugs; (3) known diabetes mellitus or on antidiabetic drugs; and (4) abdominal obesity (waist circumference $>$ 102 cm for men or $>$ 88 cm for women).[17] The Lequesne index, a patient-reported outcome index, is a questionnaire with 3 sections on pain and discomfort, walking distance, and activities of daily living. A higher score is associated with a greater disability. This index was used to assess KOA severity.[18] The ASA physical status classification system was used to assess the overall patient health status. The ASA physical status classification is a system that can be used to assess the health status and evaluate perioperative risks.

X-Ray Measurements

Preoperative full-length limb standing x-ray images were taken and analyzed for the hip-knee-ankle angle.[19] Measurements were performed using imageJ (version 1.52j) by P.S. and by M.F. in a subset of 16 patients.[20] Interrater agreement was assessed using intraclass correlation coefficient 3,A (ICC3,A). Interrater agreement was excellent (ICC3,A = 0.99, $P = 0$).

Enzyme-Linked Immunosorbent Assay (ELISA)

The following ready-to-use sandwich ELISA kits were used according to the manufacturer's instruction: oxidized low-density lipoprotein cholesterol (oxLDL; Mercodia, Uppsala, Sweden), advanced glycation end products (AGE; Cell Biolabs, San Diego, CA), soluble receptor for advanced glycation end products (sRAGE; BioVendor, Brno, Czech Republic), fatty acid binding protein 4 (FABP4; BioVendor), and membrane-bound phospholipase A2 group IIA (PLA2G2A; RayBiotech Life, Peachtree Corners, GA). The AGE-sRAGE ratio was calculated based on these measurements. All measurements were performed in duplicates at 450 nm with a microplate reader (Infinite F50 from Tecan, Austria).

XMAP Human Bone Metabolism Magnetic Bead Panel

Using the Luminex xMAP platform in a magnetic bead format, we simultaneously analyzed the following targets from human serum samples in a subgroup of 35 patients who were selected based on BMI (see above; BMI group 1: $n = 16$, BMI group 3: $n = 19$): fibroblast growth factor 23 (FGF-23), ghrelin, leptin, and resistin. For detection, we used the commercially available ProcartaPlex (Thermo Fisher, Waltham, MA) on a Bio-Plex 200 system (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's instructions. There was no cross-reactivity between the antibodies for an analyte and any of the other analytes in this panel. Measurement of median fluorescence intensity (MFI) was performed using the Bio-Plex Manager software, version 4.1 (Bio-Rad Laboratories) and used for further analysis.[21]

Metabolic Phenotyping

In another, partly overlapping subgroup of 31 patients (BMI group 1: $n = 15$, BMI group 3: $n = 16$), changes in metabolic phenotypes were explored using nuclear magnetic resonance (NMR) spectroscopy (Bruker BioSpin GmbH, Rheinstetten, Germany). Human serum samples from KOA patients were lyophilized and 500 µl of NMR buffer (5.56 g Na_2HPO_4, 0.4 g trimethylsilylpropanoic acid [TSP], 0.2 g NaN_3, in 400 ml of D_2O; pH 7.4) were added. All NMR experiments were performed at a temperature of 310 K on a Bruker AVANCE Neo Ultrashield 600 MHz spectrometer equipped with a triple resonance probe head and processed as described previously.[22] Bruker TopSpin version 3.1 (Bruker BioSpin GmbH) was used for NMR data acquisition and automatic processing (exponential line broadening of 0.3 Hz, phasing, and referencing to TSP at 0.0 ppm). Regions around water, TSP, and remaining MeOH signals were excluded. The spectra for all samples were further analyzed, deconvoluted, and quantified automatically, using the R package *ASICS*.[23] Probabilistic quotient normalization was performed to correct for sample metabolite dilution. Concentrations shown are normalized concentrations.

Statistical Analysis

All analyses were performed using R version 4.0.3 (2020-10-10) on Manjaro Linux 5.6.15-1.[24] The following R packages were used: *mice*, *lmerTest*, *tidyverse*, *visreg*, *irr*, *ASICS*, *ClustOfVars*, *car*.[23,25-31] For Luminex data analysis, a collection of R scripts provided by Breen *et al*.[32] was used.

In the ELISA data set, hierarchical cluster analysis, based on principal component analysis (PCA) for a mixture of continuous and categorical variables (PCAMIX), was performed on variables to gain a better understanding of the relationship among variables collected.30 For the purpose of this study, we cut the cluster dendogram based on aggregation levels and the Rand index into a total of 11 clusters.

Missing data and sensitivity analysis. There were missing data in the ELISA biomarkers and the Lequesne index (all less than 16%, see Suppl. Tables S1 and S2). It was assumed that

Table 1. Study Population by BMI Group.

	Underweight (n = 19)	Normal (n = 32)	Obese (n = 19)	Total (N = 70)
Age	75.16 (6.38)	70.28 (9.41)	64.68 (7.33)	70.09 (8.92)
BMI	22.88 (1.63)	30.18 (2.62)	38.35 (3.07)	30.42 (6.27)
Female	15 (78.9%)	20 (62.5%)	15 (78.9%)	50 (71.4%)
HKAA	7.40 (3.94)	7.63 (4.36)	8.52 (4.02)	7.81 (4.12)
ASA				
1	1 (5.3%)	3 (9.4%)	0 (0.0%)	4 (5.7%)
2	9 (47.4%)	17 (53.1%)	8 (42.1%)	34 (48.6%)
3	9 (47.4%)	10 (31.2%)	11 (57.9%)	30 (42.9%)
4	0 (0.0%)	2 (6.2%)	0 (0.0%)	2 (2.9%)
Lequesne index	14.30 (3.73)	12.79 (3.82)	14.83 (2.92)	13.80 (3.61)
TUGT	14.07 (3.84)	12.89 (6.38)	14.71 (5.92)	13.71 (5.67)
Diabetes mellitus	4 (21.1%)	5 (15.6%)	3 (15.8%)	12 (17.1%)
Hyperlipidemia	6 (37.5%)	10 (38.5%)	4 (21.1%)	20 (32.8%)
Abdominal circumference	89.08 (7.30)	106.96 (11.22)	122.76 (9.90)	107.63 (15.95)
CAD	4 (21.1%)	5 (15.6%)	3 (15.8%)	12 (17.1%)
Hysterectomy	0 (0.0%)	5 (17.2%)	7 (36.8%)	12 (19.0%)

BMI = body mass index; HKAA = hip-knee-ankle angle; ASA = ASA physical status classification system; CAD = coronary artery disease; TUGT = timed up and go test.

data were missing completely at random (MCAR). Details on assumed mechanisms of missingness are outlined in the Supplement. Based on the MCAR assumption, missing values were imputed using multiple imputation chained equation (MICE) with the R package *mice*.[28] All analyses were performed on complete cases and the multiply imputed data sets. To explore violations of the MCAR assumption, sensitivity analysis was performed. The δ adjustment technique was used for this purpose:[28,33] A δ of ±15% and ±30% of the complete case mean was added to the imputed values to understand how deviations from the MCAR assumptions influence analysis.

Inferential analysis. Normality was assessed using Shapiro-Wilk tests, Q-Q, and density plots. Means were compared using a Student t test or Wilcoxon rank sum test where appropriate. Correlations were assessed using Spearman rank correlation.

To answer our research questions, the following regression analyses were performed: (1) Multivariable linear regression was performed on each ELISA biomarker as dependent variable. All models were adjusted to the known risk factors such as age, gender, BMI, and hip-knee-ankle angle. To understand the relationship with the Lequesne index and the metabolic syndrome, these variables were also added to all models. To detect multicollinearity in fixed-effects models, the variance inflation factor (VIF) was calculated. All VIF values were smaller than 2. (2) Logarithmic transformation was applied to Luminex fluorescence values and these were used in linear mixed models as outcome variable.[21,32] Patient identity was used as cluster variable in a model with random intercepts. Models were adjusted for age, gender, BMI, and HKAA. To understand the association with the Lequesne index and the metabolic syndrome, these variables were also added to the model. (3) For metabolic phenotyping, data were analyzed using PCA. The number of principal components was determined using the cumulative variance (exceeding 50% of cumulative variance). Partial least squares regression and discriminant Analysis (PLSR, PLS-DA) using the R package *ASICS* was used to test the relationship of metabolite concentrations and BMI group and test scores. PLS and PLS-DA models were assessed using R^2 to estimate goodness of fit and Q^2 to estimate predictive performance. The number of components added to the model was determined based on R^2 and Q^2 scores using 7-fold cross-validation. The predictive performance of the full model was assessed based on the cumulative Q^2 score using permutation testing (1000 permutations). All models were assessed using diagnostic plots.

All P values were adjusted using the Benjamini-Hochberg correction as implemented in R and rounded to 3 digits, with a value of $<.05$ indicating significance.

Results

Study Population

A total of 70 patients were enrolled. Patient characteristics are shown in **Table 1**. The mean age was 70.09 ± 8.92 and

mean BMI was 30.42 ± 6.27. A total of 50 women (71.43%) were included. Supplementary Tables S3-A and S3-B show the subgroup patient characteristics.

Hierarchical Cluster Analysis

Data on 66 variables were collected. Hierarchical cluster analysis groups highly correlated variables in clusters to give a better understanding of the meaning of each variable. **Figure 1** shows the cluster dendogram. The clusters and variables are listed in Supplementary Table S4.

Hierarchical cluster analysis showed that FABP4 level was closely related to gender and height, whereas sRAGE and oxLDL were closely connected among each other and with hysterectomy. Furthermore, AGE and PLA2G2A revealed a close correlation with each other as well as with osteoporosis. The Lequesne index was in the same cluster as the HKAA. Conservative treatment options (physiotherapy and physical and occupational therapy) formed an additional cluster. These findings were as expected and showed the close connection of variables.

Association of ELISA/Luminex Measurements With Clinical Parameters

FABP4 level was associated with BMI and gender, but not Lequesne index, in KOA.

In univariable analysis, we found a significant positive correlation of BMI and FABP4 levels ($\rho = 0.4851$, $P = 0$). When adjusting for age, gender, HKAA, and metS in complete cases, FABP4 was still associated with BMI and gender (see Suppl. Table S5). The same result could be observed in the imputed data sets, where BMI and gender were associated with FABP4 after adjustment (see **Table 2**, **Fig. 2**). Thus, higher FABP4 levels were found in women and obese patients. No association with the Lequesne index was observed.

In the sensitivity analysis, results were stable (see Suppl. Tables S6-A and S6-B). Thus, even if the imputed values deviated by up to 30% from the mean of the observed values, we would still observe a similar effect.

All other biomarkers explored by using the ELISA technique (oxLDL, AGE, sRAGE, PLA2G2A) did not show a significant association with BMI, Lequesne index, or metS (Suppl. Tables S7-A to S7-E).

Leptin was associated with gender and BMI group. In complete cases, leptin was associated with BMI group and gender (Suppl. Table S8). The same results were observed after multiple imputation (**Table 3**). This association was further examined using sensitivity analysis and remained stable (Suppl. Tables S9-A and S9-B). Leptin levels were higher in obesity compared with underweight and in women compared with men. **Figure 3** shows the relationship of BMI and gender as predicted by the linear mixed model.

Resistin was associated with Lequesne index in complete case analysis. In complete cases, resistin showed an association with Lequesne index (Suppl. Table S8). After applying multiple imputation, this association was not observed, however (**Table 3**). This was confirmed in the sensitivity analysis (Suppl. Tables S9-A and S9-B) demonstrating that only under the most were able to explain the data. extreme imputation scenario (+30%), resistin was associated with Lequesne index. Thus, this indicated that resistin levels were probably not correlated with Lequesne index.

FGF-23 and ghrelin were not associated with BMI or Lequesne index. Neither FGF-23 nor ghrelin was associated with BMI, metS, or Lequesne index in complete case analysis and multiple imputation (Suppl. Table S8, **Table 3**, Suppl. Tables S9-A and S9-B).

Metabolic Phenotyping

Serum probes from the 2 BMI groups were analyzed for their metabolite composition and summarized in a heat map (**Fig. 4**). While, on the heatmap, there is clearly a pattern, we were not able to detect an association with BMI or metS.

To understand the relationship of metabolites with clinical parameters, PCA, group comparison with *t* tests, and PLS or PLS-DA were performed.

PCA results did not indicate a possible difference in metabolites of BMI groups 1 (underweight) and 3 (obese; see **Fig. 5**). In univariate analysis, we did not detect any differences. Accordingly, in PLS-DA, we were not able to predict BMI group membership (R2Y = 0.96, Q2Y = 0.27, $P_{R2Y} = .27$, $P_{Q2} = .043$).

Using PLS and PLS-DA, we were also not able to predict scores of the Lequesne index or metabolic syndrome (Suppl. Tables S10-S12).

Discussion

In this study, we investigated several candidate biomarkers, as well as the metabolome, in the context of obesity, in end-stage KOA. A cross-sectional clinical/lab study was conducted in end-stage KOA patients using serum biomarkers as measured by ELISA/Luminex technique or NMR spectroscopy to conduct a multivariable and a multivariate analysis for possible associations with clinical parameters such as BMI and the Lequesne index. Our hypothesis was that biomarker levels change with respect to BMI and that biomarkers were associated with KOA severity.

We found that higher FABP4 and leptin were significantly associated with obesity and female gender. In agreement with

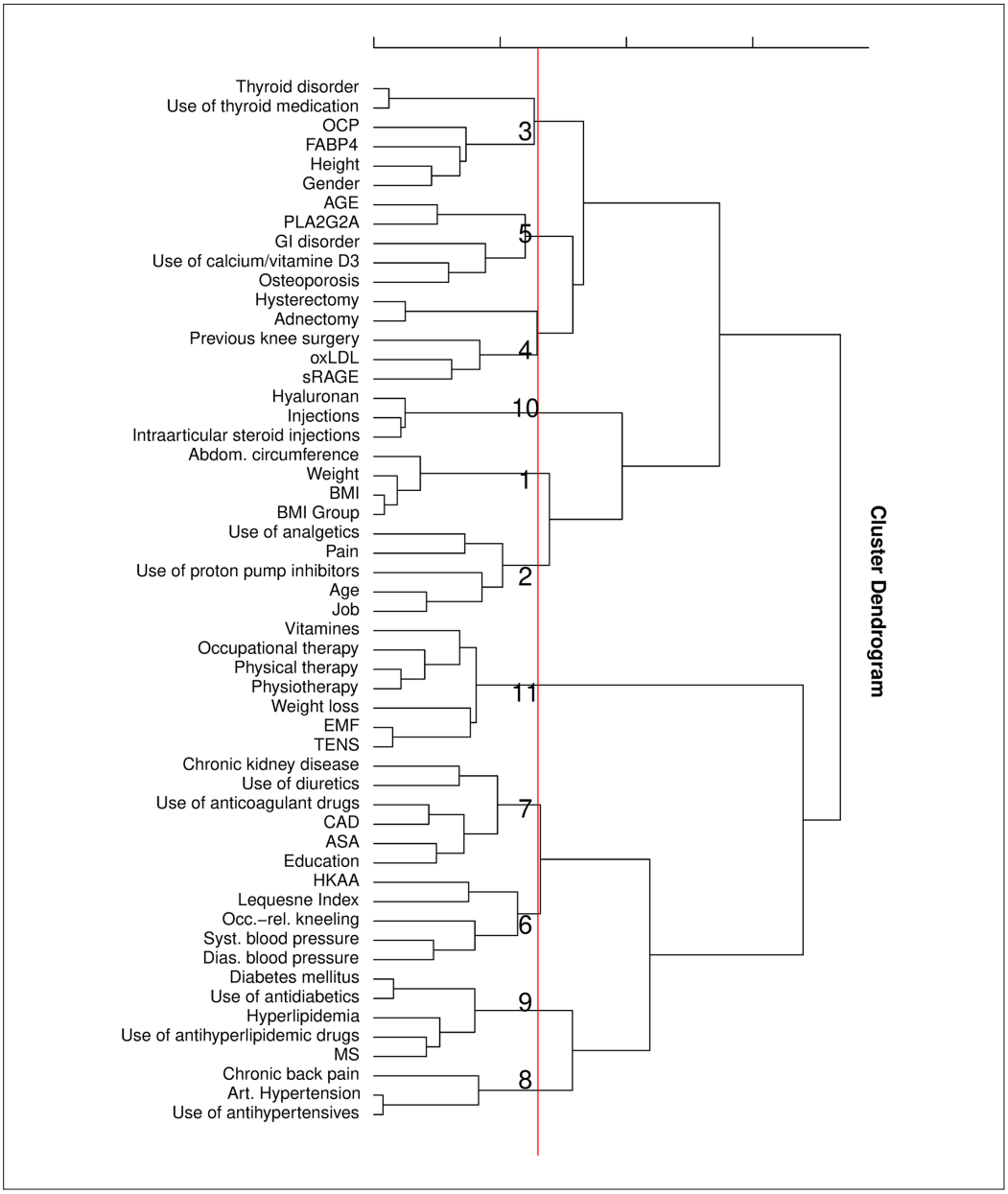

Figure 1. Dendogram of hierarchical cluster analysis of variables. A synthetic variable is computed on the basis of principal component analysis for a mixture of continuous and categorical variables (PCAMIX). Variables are grouped based on the correlation with this variable. The cluster dendogram was cut (red line) into 11 clusters based on the agglomeration level and the Rand index. The dendogram height indicates the agglomeration level and thus lower height indicates more similarity. OCP = oral contraceptive pill; GI disorder = gastrointestinal disorder; EMF = dielectric heating; TENS = transcutaneous electrical nerve stimulation; CAD = coronary artery disease; ASA = ASA physical status classification system; HKAA = hip-knee-ankle angle; MS = metabolic syndrome; numbers indicate cluster number.

Table 2. Results of Linear Regression With FABP4 as the Dependent Variable in Multiple Imputation Models.

Variable	Crude Est.	Adj. Est.	2.5%	97.5%	Adj. *P* Value
Age	−0.35	0.169	−0.355	0.693	.521
BMI	1.64	1.610	0.851	2.370	.001***
Gender (male)	−19.34	−17.201	−26.052	−8.349	.001***
HKAA	0.26	−0.372	−1.411	0.666	.521
Lequesne index	1.25	0.858	−0.416	2.131	.425
No metabolic syndrome	−8.49	−3.871	−12.659	4.916	.521

The model was adjusted for the age, gender, BMI, HKAA, and metabolic syndrome. *P* values are adjusted. There is a significant positive association of FABP4 levels and BMI and female gender. Crude est. = crude estimate; Adj. est. = adjusted estimate; 2.5% and 97.5% = 2.5% and 97.5% confidence intervals; no metabolic syndrome = patient did not meet metS criteria as outlined in the text; FABP4 = fatty acid binding protein 4; BMI = body mass index; HKAA = hip-knee-ankle angle.

previous studies, we did not detect an association of leptin and the Lequesne index, but BMI group and gender. Resistin did not show an association with Lequesne index in end-stage KOA patients. Finally, we were not able to detect BMI-dependent changes in the metabolome of KOA patients.

FABP4 is one of several members of a family of lipid chaperons. It is mainly expressed in adipocytes and is involved in intracellular fatty acid transportation. There is a strong correlation between FABP4 and obesity and FABP4 expression is higher in females than males.[34] In mice models of induced obesity, FABP4 knock-out and pharmaceutical inhibition alleviated cartilage degeneration.[11] In KOA patients, FABP4 concentrations are higher compared with non-KOA controls and are associated with obesity and KOA severity on the Kellgren-Lawrence scale.[35] In agreement with all these findings, our results confirm higher FABP4 levels in obese females in KOA, independent of age, HKAA, and metabolic syndrome. We did not detect an association with the Lequesne index, however. Only a homogeneous group of end-stage KOA patients on the day before knee arthroplasty was included in our study and this might explain why no such association could be observed.

PLA2G2A has been implicated in the pathogenesis of OA as a cartilage degrading enzyme.[36] To our knowledge, no study has previously assessed PLA2G2A as a blood biomarker for KOA. Our study did not demonstrate an association of PLA2G2A with BMI and/or KOA severity.

Hyperglycemia leads to an accumulation of advanced glycation end products (*AGE*), proteins modified by nonenzymatic glycosylation that cause local inflammation by binding to membrane-bound receptors (mRAGE). The interaction of AGEs with soluble receptors (*sRAGE*) is associated with anti-inflammatory responses by blocking interaction with mRAGE.[12] Our results did not indicate an association with BMI or KOA severity. A study on fasting in KOA also found no association of changes in pain and function scores and AGE/sRAGE.[37]

oxLDL, the oxidized form of LDL-cholesterol, is a significant regulator of cartilage degeneration[10]: In KOA, oxLDL levels are higher compared with controls. Furthermore, in another study, oxLDL was found to be positively correlated with KOA severity. This study did not

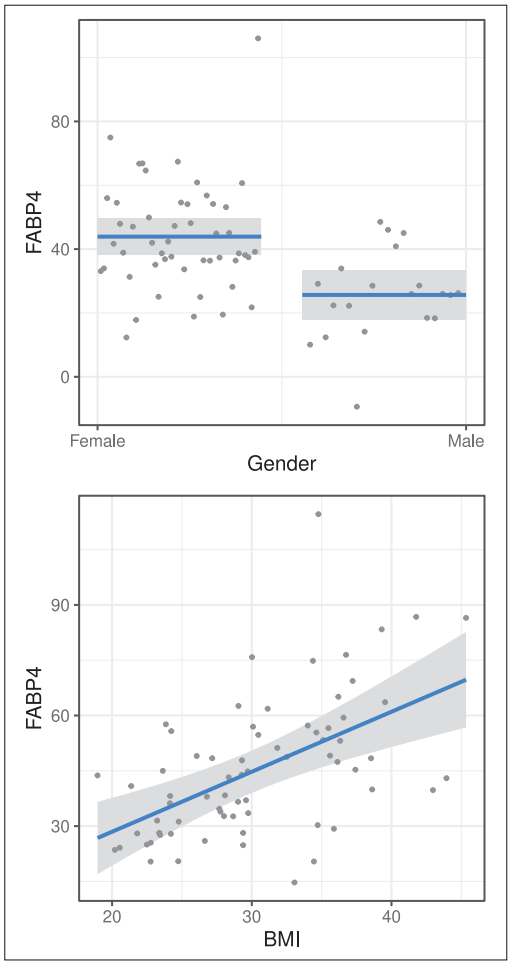

Figure 2. Top: Association of gender and FABP4 as predicted by linear regression. FABP4 is higher in women. Bottom: Correlation of BMI and FABP4 as predicted by regression. FABP4 increases with BMI. FABP4 = fatty acid binding protein 4; BMI = body mass index.

Table 3. Results of Linear Mixed Models on Imputed Data Showing Interactions Only.

Coefficient	Unadj. Est.	Adj. Est.	2.5%	97.5%	Adj. *P* Value
Ghrelin: Age	0.002	–0.004	–0.058	0.049	.972
Ghrelin: BMI group 3	–0.135	–0.222	–1.173	0.729	.972
Ghrelin: Gender (male)	0.052	0.175	–0.723	1.073	.972
Ghrelin: HKAA	–0.013	–0.008	–0.114	0.098	.972
Ghrelin: Lequesne Index	–0.012	–0.019	–0.141	0.102	.972
Ghrelin: No metabolic syndrome	–0.125	–0.251	–1.083	0.581	.972
Leptin: Age	–0.031	0.028	–0.026	0.081	.787
Leptin: BMI group 3	1.014	1.642	0.688	2.596	.011*
Leptin: Gender (male)	–1.039	–1.418	–2.323	–0.513	.021*
Leptin: HKAA	0.033	0.040	–0.069	0.149	.972
Leptin: Lequesne Index	0.005	–0.031	–0.182	0.121	.972
Leptin: No metabolic syndrome	–0.565	–0.535	–1.383	0.313	.669
Resistin: Age	–0.003	–0.028	–0.082	0.026	.787
Resistin: BMI group 3	–0.321	–0.785	–1.752	0.183	.391
Resistin: Gender (male)	–0.279	–0.234	–1.137	0.668	.972
Resistin: HKAA	0.030	0.127	0.020	0.234	.113
Resistin: Lequesne Index	–0.097	–0.166	–0.301	–0.030	.113
Resistin: No metabolic syndrome	0.169	–0.028	–0.884	0.827	.972

The model was adjusted for age, gender, BMI group, HKAA, and metabolic syndrome. There is a significant positive association of leptin levels and obesity (BMI group 3) and female gender. Unadj. est. = unadjusted estimate; Adj. est. = adjusted estimate; 2.5% and 97.5% = 2.5% and 97.5% confidence intervals; ':' = interaction term; BMI = body mass index; HKAA = hip-knee-ankle angle.
*$P < .05$.

adjust for confounders, however.[38] Our study did not demonstrate such a relationship, neither with BMI nor with KOA severity, after adjusting for several important confounders. Furthermore, in contrast to the aforementioned study, only end-stage KOA patients were included for this investigation and differences in KOA severity might be minimal.

Leptin is mostly produced by adipocytes and leptin levels are positively associated with obesity and female gender.[39,40] Leptin levels are increased in KOA compared with controls and there is an association with BMI and gender.[41-43] Our results confirm that obese female patients have higher serum leptin levels. In agreement with a previous study, we could not detect an association with KOA severity.[42,43] This contrasts with another study including patients at different stages of KOA and might be explained by a more rigorous adjustment for confounding covariates in our study and the fact that we included end-stage KOA patients only.[41]

In KOA patients with joint effusion, *resistin* is associated with severity even after adjusting for several confounders in serum and synovial fluid.[43,44] Another case-control study in obese, female patients found higher resistin levels in KOA patients and resistin levels were positively correlated with WOMAC scores.[45] Another study in different KOA stages did not find an association of pain and resistin levels in serum, but synovial fluid only.[46] In contrast to aforementioned study in female patients with joint effusion, in our study resistin levels were weakly, inversely associated with the Lequesne index in complete case analysis.[44] This negative correlation was observed in complete case analysis and, in the most extreme scenario, in multiple imputation only. Our results are supported by a study showing that serum resistin levels are not associated with preoperative visual-analog scale (VAS) pain levels. Although not significant, in that study the association was also a negative one.[47] Similarly, a previous report found that serum resistin levels were not associated with cartilage damage in end-stage KOA.[42] It is unclear why there was a negative association in complete case analysis, if any, when compared with studies of KOA at different stages showing a positive association. It is possible that missing data introduced a bias in our data that was mitigated through multiple imputation. To summarize, our results show that resistin is not associated with Lequesne index.

In KOA, *ghrelin* was found to be associated with Western Ontario and McMaster Universities Osteoarthritic Index (WOMAC), even after adjusting for BMI, age, and gender.[48] We could not confirm this finding, possibly due to more rigorous adjustment applied in our study and due to the inclusion of end-stage KOA patients only.

FGF-23 plays an important role in chondrocyte differentiation. FGF-23 is upregulated in osteoarthritic cartilage compared with normal controls.[14] FGF-23 levels were found to be higher in *FGF-23* KOA patients compared with normal patients in serum and there was a correlation of

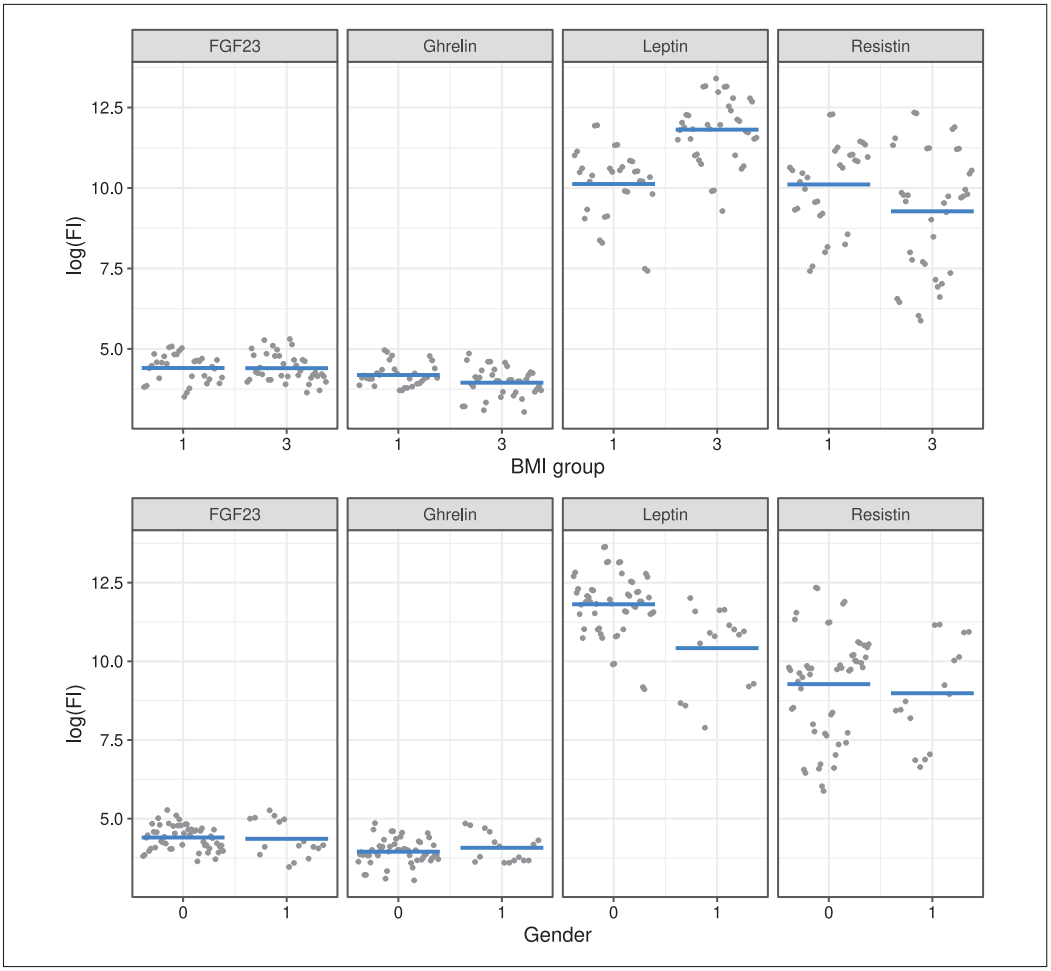

Figure 3. The relationship of BMI group (top) and gender (bottom) with fluorescence intensity per cytokine is shown as predicted by linear mixed modeling. Leptin levels are higher in women in obese patients. BMI = body mass index; BMI group 1 = underweight; BMI group 3 = obesity; 0 = female gender.

KOA severity and FGF-23 level.[49] In contrast, in our study we could not detect an association of serum FGF-23 level with KOA severity nor BMI. The aforementioned study did not control for confounding factors and this might explain the different findings.

Previous studies, using a targeted or untargeted *metabolomics* approaches, showed significant changes, particularly in branched-chain amino acids (BCAA) and arginine pathways, in KOA compared with control in agreement with epidemiologic studies indicating that changes in BCAA serum concentrations are associated with diabetes, cardiovascular disease, and metabolic syndrome.[15,50] In another study, obese KOA patients showed higher oxidative stress levels compared with nonobese KOA patients.[16] In contrast, in our study we did not detect any BMI-dependent changes in end-stage KOA patients. Again, the reason might be that only end-stage KOA patients were included, there was no control group, and differences in metabolites may be minimal within this group. Furthermore, while the above-mentioned study showed BMI-dependent changes in KOA

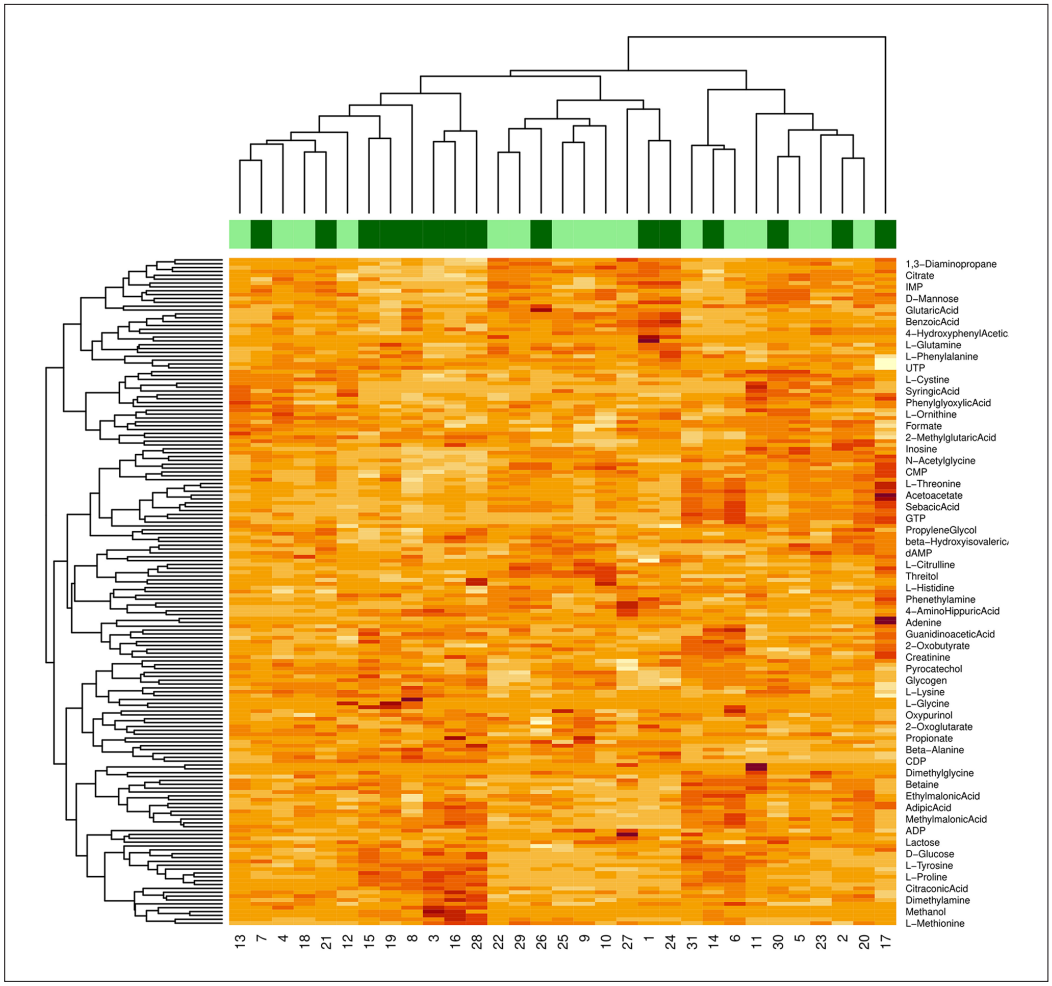

Figure 4. Heat map of metabolite concentrations based on BMI group (dark green = underweight, light green = obese). While there is obviously a pattern in metabolite concentrations, we could not detect an association with BMI group or metabolic syndrome in our study. Numbers at the bottom = sample number; BMI = body mass index; IMG = inositol monophosphate; GMP = guanosine monophosphate; GTP = guanosine-5-triphosphate; NADP = nicotinamide adenine dinucleotide phosphate.

patients, that study used an untargeted approach with mass spectroscopy and these differences in the methodology might explain the discrepancies compared with our findings.[16]

Limitations

This study has the following limitations: Only a small sample size was enrolled in this study. Due to resource limitations, there were missing data. This could be mitigated by multiple imputation and sensitivity analysis, as demonstrated, however. Furthermore, blood samples were taken at random and patient fasting state was unknown. This might influence ELISA, Luminex, and NMR spectroscopy results. However, based on the agreement with previous studies, we are confident that our results are valid. Finally, our data are applicable to patients undergoing knee arthroplasty for end-stage KOA only.

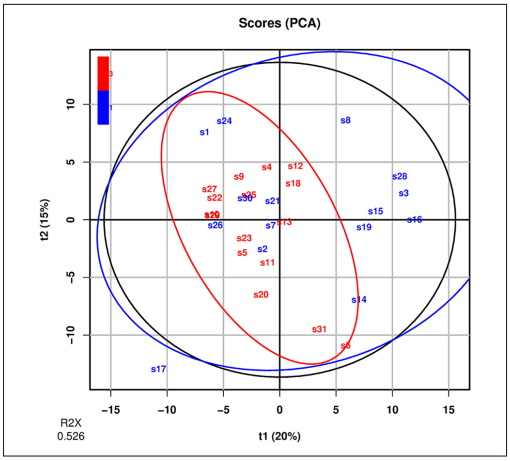

Figure 5. PCA score plot of the first 2 principal components. Samples were colored according to their BMI group. There is an overlap of BMI groups 1 and 3. "t1, t2" = first 2 principal components (explained variance) and deviation from the respective component (axis); s1-s31 = patient samples; PCA = principal component analysis; BMI = body mass index.

Conclusion

This study investigated the relationship of several previously not extensively examined biomarkers with BMI and KOA severity. We could confirm the association of BMI and gender with FABP4 and leptin. None of the explored biomarkers was associated with KOA severity and no BMI-dependent changes in the metabolome of end-stage KOA patients were observed.

Author Contributions

B.L. and B.S.F. contributed to conception and design. B.L., B.S.F., and P.S. contributed to analysis, interpretation, and drafting of the article. A.L., B.L., B.T., B.S.F., M.F., M.O., M.S., P.S., V.R., and W.K. contributed to collection and assembly of data. All authors critically revised and finally approved the article.

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Declaration of Conflicting Interests

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Compliance With Ethical Standards

The procedures followed were in accordance with the ethical standards of the responsible committee (Ethics committee of the Medical University of Graz, Austria, IRB#31-133) on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000.

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Data Availability Statement

All data and R code used in this study are available upon reasonable request.

Supplemental Material

Supplementary material for this article is available on the *Cartilage* website at http://cart.sagepub.com/supplemental.

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4.2 Fatty Acid–Binding Protein 4 (FABP4) Is Associated with Cartilage Thickness in End-Stage Knee Osteoarthritis

Original Article

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Fatty Acid–Binding Protein 4 (FABP4) Is Associated with Cartilage Thickness in End-Stage Knee Osteoarthritis

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Abstract

Background. There is no single blood biomarker for the staging of knee osteoarthritis (KOA). The purpose of this study was to assess the relationship of obesity, serum biomarkers, the hip-knee-ankle angle (HKAA) with sonographic cartilage thickness. *Methods*. We conducted a cross-sectional study of $n = 33$ patients undergoing knee arthroplasty. Body mass index (BMI) was recorded, and patients were grouped based on BMI. Serum blood samples were collected, and the following biomarkers were measured using the ELISA technique (subgroup of $n = 23$): oxidized low-density lipoprotein (oxLDL), soluble receptor for advanced glycation end-products (sRAGE), fatty acid–binding protein 4 (FABP4), membrane-bound phospholipase A2 (PLA2G2A). The HKAA was analyzed on full-length limb standing x-ray images. Cartilage thickness was assessed on ultrasound images. Multivariable regression analysis was performed to account for confounding. *Results*. After adjusting for age, gender, and HKAA, obese patients had thicker medial femoral cartilage ($\beta = 0.165$, $P = 0.041$). Furthermore, lateral cartilage thickness was negatively correlated with FABP4 level after adjusting for of age, gender, BMI, and HKAA ($\beta = -0.006$, $P = 0.001$). Confirming previous studies, after adjustment, FABP4 level was associated with a higher BMI group ($\beta = 42.99$, $P < 0.001$). None of the other markers (oxLDL, PLA2G2A, and sRAGE) was associated with BMI or cartilage thickness. *Discussion*. Our results indicate that BMI has a weak, positive association with cartilage thickness in end-stage KOA patients. FABP4 levels were negatively associated with cartilage thickness. While our study is limited by a small sample size, these results further highlight the role of FABP4 as promising biomarkers of burden of disease in KOA.

Keywords

ultrasound, obesity, biomarkers, FABP4, knee

Introduction

Knee osteoarthritis (KOA) is a leading cause of disability worldwide.[1] For the prevention and treatment, early diagnosis, disease staging, and assessment of treatment response are key factors. While currently diagnosis is based on symptoms and signs, several imaging modalities can aid in diagnosis. Several studies have explored and validated the role of ultrasound in KOA. Ultrasound findings have a moderate to strong agreement with measurements in magnetic resonance imaging (MRI) studies and are also associated with pain and function.[2] This was also shown in a cadaveric study, where agreement of sonographic and anatomic cartilage thickness was strong on the medial femoral condyle and weak on the lateral condyle and intercondylar notch.[3]

While there are several established risk factors, such as gender, age, and trauma, obesity also plays an important role in the pathogenesis of KOA.[4] It not only causes an increased load on weightbearing joints but is also associated with systemic and local joint inflammation and is a key component of metabolic syndrome. In metabolic syndrome, a combination of arterial hypertension, dyslipidemia, insulin resistance, and obesity cause a pro-inflammatory state that is associated with cartilage destruction. Both KOA and metabolic syndrome have been associated with systemic mediators of inflammation, such as C-reactive protein and interleukin-1. Oxidative stress, commonly observed in metabolic syndrome, is a known contributor to accelerated cellular aging and apoptosis in cartilage. Atherosclerosis and endothelial dysfunction have been associated with knee and hand osteoarthritis severity.[5]

Despite a huge ongoing effort in the detection of blood-based diagnostic markers, no single blood biomarker has emerged so far, however.[6] Previous studies have suggested a potential role of fatty acid–binding protein 4 (FABP4), oxidized low-density lipoprotein (oxLDL), membrane-bound

phospholipase A2 (PLA2G2A) and soluble receptor for advanced glycation end-products (sRAGE) in the pathogenesis of osteoarthritis (OA).[7-10] Cytoplasmic FABP4, a member of the FABP superfamily and mostly expressed in adipocytes, regulates intracellular fatty acid transportation by increasing fatty acid solubility and facilitating transport to cellular compartments and specific enzymes.[11]FABP4 also acts as a regulator of energy homeostasis and high circulating levels have been associated with obesity, increased cardiovascular risk, cancer and also KOA severity.[8,12] FABP4 levels were found to be higher in KOA patients compared to non-KOA controls and increased with KOA stage based on the radiographice Kellgren-Lawrence scale in a previous study.[8] OxLDL is involved in the formation of atherosclerotic plaques, and a known regulator of cartilage degeneration via induction of chondrocyte cell death.[13] PLA2G2A, member of the phospholipase A2 family (PLA2), is the membrane-bound form of phospholipase A2, a lipolytic enzyme that catalyses the hydrolysis of membrane phospholipids.[9] Several phospholipase A2 isoforms have been implicated in the pathogenesis of OA via regulation of inflammation. Intra-articular injection of human phospholipase A2 in rats was found to induce acute inflammation and cause chronic degenerative changes. Furthermore, the expression of multiple PLA2 isoforms could be induced in response to stimulation with pro-inflammatory cytokines in OA chondrocytes in another study.[14,15] Finally, the interaction of soluble receptors for advanced-glycation endproducts (sRAGE) and advanced glycation endproducts (AGE), commonly seen in hyperglycemic states, inhibits pro-inflammatory responses.[16] Advanced glycation endproducts might be helpful in the early detection of OA.[17]To the best of our knowledge, no other study has examined the relationship of obesity and aforementioned serum biomarkers with sonographic cartilage thickness in KOA.

In this study, we investigated the relationship of obesity and aforementioned serum biomarkers with sonographic cartilage thickness in end-stage KOA. The aim of the study was to identify a possible relationship between BMI, serum biomarkers, and sonographic cartilage thickness in end-stage KOA.

In order to answer these questions, we conducted a cross-sectional study in end-stage KOA patients, using knee ultrasound examination, aforementioned blood-based markers measured using the ELISA (enzyme-linked immunosorbent assay) technique, and anthropometric variables in multivariable analysis.

Methods

Study Population

To answer our research questions, we conducted a cross-sectional clinical/laboratory study at the Department of Orthopedics and Trauma of the Medical University of Graz, Austria, from January 2019 to April 2020. This study was conducted in accordance with the ethical standards of the Ethics Committee of the Medical University of Graz (IRB#31-133 ex18/19) and with the Declaration of Helsinki.

This study population ($n = 33$) was enrolled at random from patients undergoing total knee arthroplasty. Adult patients older than 18 years undergoing knee arthroplasty were eligible. All patients were enrolled after informed consent. BMI was recorded and patients were grouped based on BMI (group 1: BMI < 25 kg/m^2 [$n = 6$], group 2: BMI 25-35 kg/m^2 [$n = 21$], group 3: BMI > 35 kg/m^2 [$n = 6$]). The preoperative ASA (American Society of Anesthesiologists) physical status classification score was recorded for every patient.

Enzyme-Linked Immunosorbent Assay

Serum blood samples were collected on the day before surgery, centrifuged within an hour, and stored at −20 °C. We measured the following biomarkers in a subgroup of 23 patients (group 1, $n = 6$; group 2, $n = 11$; group 3, $n = 6$): oxLDL (Mercodia, Uppsala, Sweden), soluble receptor for advanced glycation end-products (sRAGE; BioVendor,

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Supplementary material for this article is available on the *Cartilage* website at https://journals.sagepub.com/home/car.

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Brno, Czech Republic), fatty acid–binding protein 4 (FABP4; BioVendor, Brno, Czech Republic), membrane phospholipase A2 (PLA2G2A; RayBiotech Life, Peachtree Corners, GA, USA) using ready-to-use kits according to the manufacturer's recommendation. Measurements were performed at 450 nm on a microplate reader (Infinite F50, Tecan, Austria) in duplicates. For quality reasons, the controls (high and low) included in the kits (oxLDL, sRAGE, FABP4) were also used. An intra-assay coefficient of variance (CV) <10% and an inter-assay CV <15% were used as run approval criteria. All CV values were within aforementioned limits. All samples measured were within detection limits of the respective ELISA kit. For detailed description on the performance of assays used in this study, please see Supplementary Appendix A.

X-Ray Measurments

Full-length limb standing x-ray images were taken preoperatively. The hip-knee-ankle-angle (HKAA) was assessed using imageJ (National Institutes of Health, version 1.52j) by PS and by MF in a subset of 16 patients.[18] Interrater agreement on HKAA was assessed using intraclass correlation coefficient 3,A was found to be excellent (ICC3,A = 0.99, $P < 0.001$).

Ultrasound Measurements

Ultrasound measurements were performed on a Siemens Sonoline G50 (Siemens, Germany) using a linear 8 MHz transducer. B-Mode examination of the knee were performed by PS under supervision of RH. The grayscale settings of the machine were kept the same for every patient. Medial, intercondylar notch and lateral femoral cartilage thickness was measured on a suprapatellar transverse view in millimeter.[3,19,20] (**Fig. 1**). Images were taken in supine position on the evening before surgery. Images were measured twice with a break of 2 weeks using imageJ (see above). Intrarater agreement was assessed using ICC3A,1. Intrarater reliability of cartilage thickness was moderate to good (medial: ICC = 0.609, $P = 0.001$; lateral: ICC = 0.411, $P = 0.006$; intercondylar notch: ICC = 0.447, $P = 0.004$).

Statistical Analysis

All statistical analyses were performed using the statistical programming language R version 4.0.3 (2020-10-10) on Manjaro Linux 5.6.15-1.[21]The following R packages were used: *tidyverse, visreg, irr*.[22-24] Continuous variables are expressed as mean (standard deviation), categorical data as counts (percentage). Normal distribution was assessed using Shapiro-Wilk test and Q-Q plots. If data were normally distributed, groups were compared using Student *t* test or univariate analysis of variance (ANOVA), otherwise Wilcoxon rank sum test or Kruskal-Wallis test. Categorical variables were tested using the chi-square test. Correlations were assessed using the Spearman's rank correlation coefficient. To adjust for age, gender, BMI, and HKAA as confounders, multivariable regression analysis was performed. Sonographic cartilage thickness was used as the outcome variable for multivariable analysis. *P* values were adjusted using the Benjamini-Hochberg adjustment and considered significant if <0.05. There were no missing data.

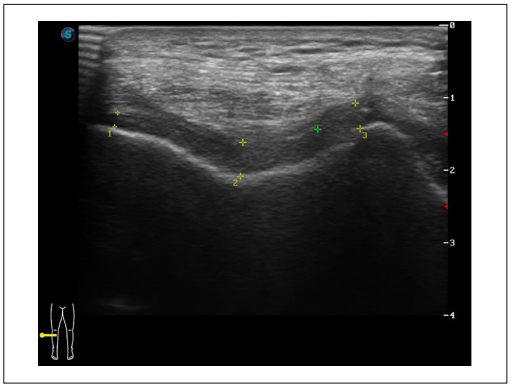

Figure 1. Measurement of cartilage thickness using ultrasound on suprapatellar transverse image on the medial (right), intercondylar notch, and lateral femoral condyle. This figure shows a standard transverse suprapatellar view of the knee. The hyperechoic (white) band at the bottom shows the femoral bone. Sitting on it, the cartilage is depicted by a hypoechoic (dark) band with a small hyperechoic (white) line right on top of it. The yellow markers 1-3 indicate the distances measured medially, laterally, and on the intercondylar notch (center), respectively.

Results

A total of 33 patients (23 women, 69.7%), were enrolled (**Table 1**). The mean age was 70.06 ± 7.91, mean BMI was 29.93 ± 4.94 kg/m^2. Of the enrolled patients, only 1 patient had an ASA score of 1 and 1 patient had an ASA score of 4.

Correlation of BMI and Cartilage Thickness

Multivariable linear regression was used to test the association of BMI and cartilage thickness, adjusting for age, gender, and HKAA.

In univariate analysis, medial cartilage thickness was higher in BMI group 3 (BMI > 35 kg/m^2) compared with BMI groups 1 (BMI < 25 kg/m^2) and 2 (BMI 25-35 kg/m^2) ($P = 0.033$ and $P = 0.013$). After adjusting for age, gender, and HKAA, BMI group 3 (BMI > 35 kg/m^2) compared with BMI group 1 (BMI < 25 kg/m^2) was still positively associated with medial cartilage thickness (**Table 2**, **Fig. 2**).

Table 1. Study Population by Body Mass Index (BMI) Groups.[a]

	BMI < 25 kg/m^2 (n = 6)	BMI 25-35 kg/m^2 (n = 21)	BMI >35 kg/m^2 (n = 6)	Total (n = 33)
BMI, kg/m^2, mean (SD)	22.81 (2.05)	29.95 (2.54)	36.99 (2.38)	29.93 (4.94)
Gender, *n* (%)				
Male	0 (0.0)	10 (47.6)	0 (0.0)	10 (30.3)
Female	6 (100.0)	11 (52.4)	6 (100.0)	23 (69.7)
Age, years, median (range)	76.00 (60.00-79.00)	73.00 (50.00-82.00)	61.50 (55.00-69.00)	72.00 (50.00-82.00)
HKAA, deg, mean (SD)	5.61 (5.85)	6.90 (4.21)	7.57 (3.69)	6.79 (4.35)
ASA, *n* (%)				
1	0 (0.0)	1 (4.8)	0 (0.0)	1 (3.0)
2	4 (66.7)	8 (38.1)	5 (83.3)	17 (51.5)
3	2 (33.3)	11 (52.4)	1 (16.7)	14 (42.4)
4	0 (0.0)	1 (4.8)	0 (0.0)	1 (3.0)
CT, mm, mean (SD)				
Intercondylar notch	0.31 (0.05)	0.33 (0.06)	0.45 (0.15)	0.35 (0.09)
Lateral	0.25 (0.04)	0.25 (0.06)	0.34 (0.16)	0.26 (0.09)
Medial	0.24 (0.08)	0.26 (0.07)	0.38 (0.14)	0.28 (0.10)

CT = cartilage thickness; HKAA = hip-knee-ankle angle. ASA = American Society of Anesthesiologists physical status classification system.
[a]Continuous variables are expressed as mean (SD) and categorical data as counts (percentage).

Table 2. Results of Linear Regression with Medial Cartilage Thickness as the Dependent Variable.[a]

Variable	Crude Estimate	Adjusted Estimate	2.5%	97.5%	Adjusted *P* Value
Age	0.00	0.003	−0.002	0.007	0.437
BMI group 2	0.01	−0.018	−0.108	0.073	0.843
BMI group 3	0.13	0.165	0.050	0.281	0.041*
Gender (male)	0.02	0.066	−0.012	0.145	0.285
HKAA	0.00	0.001	−0.007	0.008	0.843

BMI = body mass index; HKAA = hip-knee-ankle angle.
[a]The model was adjusted for age, gender, and HKAA; 2.5% and 97.5% indicate 2.5% and 97.5% confidence interval.

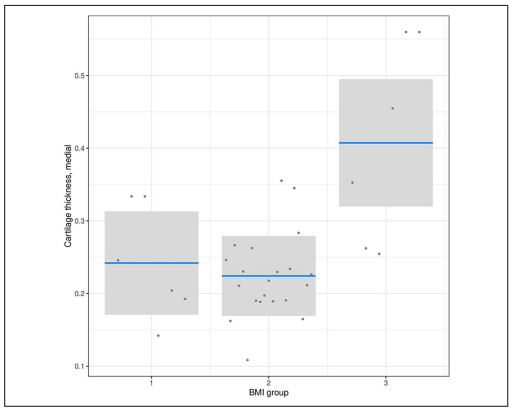

Figure 2. The relationship of body mass index (BMI) group and medial cartilage thickness as predicted by linear regression analysis, adjusted for age and gender. Patients in BMI group 3 had a significantly higher cartilage thickness on the medial femoral condyle.

Intercondylar notch and lateral cartilage thickness showed a positive association and trend toward a positive association with BMI group 3 ($P = 0.013$ and $P = 0.068$), respectively, but not after adjusting ($P = 0.692$ and $P = 0.692$, respectively). Thus, these results indicate that patients with a higher BMI had thicker medial cartilage.

FABP4 Level Is Associated with Cartilage Thickness Independent of BMI

Multivariable linear regression was used to test the association of ELISA biomarkers with cartilage thickness. We were interested to see if any of the blood biomarkers was associated with cartilage thickness. Several of the biomarkers measured in this study, are associated with BMI. In order to understand, if biomarkers are related to cartilage thickness independently, the model was adjusted for BMI. In addition, to account for the effects of gender, age and HKAA, the model was also adjusted for these covariates.

We found that the FABP4 level was associated with lateral cartilage thickness independent of age, gender, BMI,

Table 3. Results of Linear Regression with Lateral Cartilage Thickness as the Dependent Variable.

Variable	Crude Estimate	Adjusted Estimate[a]	2.5%	97.5%	Adjusted *P* Value[a]
Age	0.00	0.002	−0.003	0.007	0.477
BMI group 2	−0.01	0.069	−0.023	0.162	0.308
BMI group 3	0.13	0.280	0.150	0.409	0.001**
FABP4	0.00	−0.006	−0.008	−0.003	0.001**
Gender (male)	−0.01	−0.022	−0.121	0.077	0.649
HKAA	0.00	0.003	−0.003	0.010	0.438

BMI = body mass index; FABP4 = fatty acid–binding protein 4; HKAA = hip-knee-ankle angle; ** = *P*-value < 0.01.
[a]The model was adjusted for age, gender, and HKAA; 2.5% and 97.5% indicate 2.5% and 97.5% confidence interval.

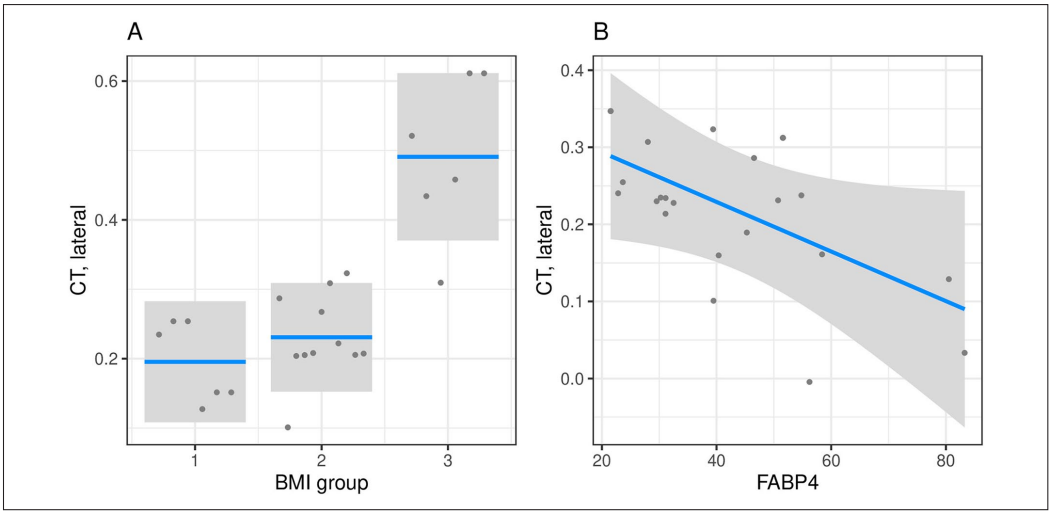

Figure 3. The relationship of body mass index (BMI) group, fatty acid–binding protein 4 (FABP4) level, and lateral cartilage thickness (CT, lateral) as predicted by linear regression analysis, adjusted for age, gender, hip-knee-ankle angle (HKAA). Figure **A** shows that a higher BMI group is associated with higher lateral cartilage thickness. Figure **B** shows the linear regression line of lateral cartilage thickness, predicted by FABP4, holding all other model covariates constant. There is a negative correlation of lateral cartilage thickness and FABP4.

and HKAA (**Table 3**, **Fig. 3**). This relationship was not significant in the medial or intercondylar area (not shown). In our model, there was no association of cartilage thickness and HKAA (**Table 3**). In this model, there was also a significant association of BMI group and lateral cartilage thickness (**Table 3**).

As expected from previous studies, in univariate analysis and after adjusting for age, gender and HKAA, FABP4 was associated with BMI group 3 (ANOVA: $P = 0.002$, Regression: $\beta = 42.99$, $P < 0.001$, respectively).

These results indicate that higher BMI was associated with greater cartilage thickness on the lateral and medial (see above) condyle. We also found that higher FABP4 levels were independently associated with lower cartilage thickness on the lateral condyle.

Other Markers Were Not Associated with BMI or Ultrasound Findings

None of the other markers (oxLDL, PLA2G2A, and sRAGE) was associated with cartilage thickness using multivariable regression (data not shown). Thus, these markers did not show a relationship with cartilage thickness and cannot be considered candidate biomarkers of burden of disease.

Discussion

The purpose of this study was to assess the relationship of obesity, serum biomarkers and sonographic cartilage thickness. We conducted a cross-sectional study and assessed associations using multivariable regression analysis. Our results

indicate that patients with a higher BMI had a weak, positive association with cartilage thickness on the medial and lateral femoral condyle. In addition, we found that FABP4 was negatively associated with lateral cartilage thickness even after adjustment for BMI, gender, age, and HKAA.

The relationship of obesity and cartilage thickness or volume is complex and there are contradictory findings. Several MRI studies have detected no association of BMI and cartilage thickness or cartilage loss. However, in these studies there was a positive trend in multivariable analysis or a positive association in univariate analysis.[25-30] In children, there seems to be no obesity-dependent difference in cartilage volume.[31] Similarly, one study found a higher cartilage loss in KOA patients but not obese patients.[32] There is also evidence suggesting fat-free body mass and muscle mass or muscle strength, but not BMI, is positiviely associated with cartilage thickness, while the opposite might be true for fat mass.[29,30,33,34]

Contrary to this, in one study, there was a positive association of BMI and cartilage thickness in the femoral groove.[35] On the other hand, there are also studies that have found a BMI-dependent reduction of cartilage volume.[36,37]

Other important factors that influence cartilage thickness are sex (males have higher cartilage volume), physical activity, and age.[33,38,39] Obesity and age also seem to impact gait and alter the positive relationship of cartilage thickness and ambulatory loads.[40] Systemic inflammation also affects cartilage: High-sensitivity C-reactive protein levels (hsCRP) were higher in patients with lower cartilage thickness.[26] Metabolic syndrome is a complex systemic disease that is associated with systemic low-grade inflammation, and other metabolic derangements, such as hyperglycemia, hyperlipidemia, and arterial hypertension.[5] Thus, not surprisingly, metabolic syndrome also affects cartilage volume significantly: In an MRI study, metabolic syndrome was associated with medial compartment cartilage loss even after adjustment for BMI and central obesity.[41]

While there was no significant association of BMI and femoral cartilage thickness in a sonographic study comparing obese with nonobese patients, obese patients tended to have higher cartilage thickness values.[42] We found a significant, weakly positive association of obesity with cartilage thickness. The variations in literature above and our findings can be explained by differences in study portocol, site (femoral vs. tibial), technique of cartilage measurement, and imaging modality.

FABP4 is mainly expressed in adipocytes and acts as a lipid chaperon in intracellular fatty acid transportation. As a member of the FABP superfamily, it facilitates the transport of fatty acids to specific enzymes and cellular compartments.[43] Several studies have shown that FABP4 plays an important role in the development of atherosclerosis, insulin resistance and other findings of metabolic syndrome. FABP4 deficiency is known to protect against atherosclerosis in apolipoprotein E–deficient mice. In this study, we could confirm that FABP4 is correlated with obesity, and expression is higher in females.[43-45] Pharmaceutical inhibition of FABP4 has been shown to improve insulin resistance, diabetes mellitus, and atherosclerosis in mice.[46] Similarly, FABP4 knock-out or pharmaceutical inhibition mitigated cartilage degeneration by induced obesity in mouse models.[47] A previous study suggested FABP4 as a promising biomarker as FABP4 levels are higher in KOA patients and are associated with obesity.[8] In this study, we not only found an association of FABP4 with obesity but also with cartilage thickness measured on ultrasound images. This further supports the role of FABP4 as a biomarker candidate for KOA burden of disease.

Limitations

This study has several limitations. Only a small sample size was enrolled in this study and because of the exploratory nature, no sample size calculation could be performed. Blood samples were taken only in a subpopulation due to resource limitations. Blood samples were taken at random times with unknown fasting status and this might influence ELISA results. Intake of sugar or high-fat meal can significantly reduce FABP4 levels consistently by 20% from baseline over a 4- to 6-hour period.[48] It is also known that FABP4 levels are higher in obese patients and women.[43-45] While we cannot exclude changes of FABP4 levels due to meal intake, we believe this effect is negligible due to a consistent decrease over 4 to 6 hours and given the strong agreement (higher FABP4 levels in obesity and women) with previous studies. oxLDL levels are not altered by meal intake in overweight and obese patients.[49] We are not aware of an effect of food intake on isoform PLA2G2A. sRAGE levels seem to be dynamically regulated postprandially, and we therefore might have missed a possible association.[50]

Furthermore, we also adjusted for age, gender, BMI, and HKAA, in multivariable analysis. However, we cannot exclude any confounding effects of covariates not observed in this study. Further research in a larger study population is necessary to confirm our findings. Our results are only applicable to end-stage KOA patients.

Conclusion

Our results indicate that BMI has a weak, positive association with cartilage thickness in end-stage KOA patients. FABP4 levels were negatively associated with cartilage thickness. While our study is limited by a small sample size, these results further highlight the role of FABP4 as a promising biomarker of KOA burden of disease.

Authors' Note

All data and R code used in this study are available on reasonable request.

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Declaration of Conflicting Interests

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Ethical Approval

The procedures followed were in accordance with the ethical standards of the responsible committee (Ethics committee of the Medical University of Graz, Austria, IRB#31-133) on human experimentation (institutional and national) and with the Declaration of Helsinki of 1975, as revised in 2000.

Informed Consent

All patients were enrolled after informed consent.

Trial Registration

Not applicable.

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4.3 The Effect of Body Mass Index and Metformin on Matrix Gene Expression in Arthritic Primary Human Chondrocytes

Original Article

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The Effect of Body Mass Index and Metformin on Matrix Gene Expression in Arthritic Primary Human Chondrocytes

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Abstract

Objective. Obesity is a known risk factor for knee osteoarthritis (OA). Diabetes has been associated with progression of OA and metformin is the first-line treatment in type 2 diabetes. The effect of the body mass index (BMI) and metformin on the expression of certain matrix genes in human chondrocytes is unclear. The purpose of this study was to investigate the effect of BMI and metformin on the expression of matrix genes in primary human chondrocytes. *Design*. Adult female patients undergoing knee arthroplasty for end-stage OA were enrolled. Primary chondrocytes were cultivated and stimulated with metformin. Matrix gene expression was analyzed using polymerase chain reaction. Clinical data were used in multivariable regression models to assess the influence of BMI and metformin stimulation on gene expression. *Results*. A total of 14 patients were analyzed. BMI was a predictor of increased expression in ADAMTS5 ($\beta = -0.11$, $P = 0.03$). Metformin slightly reduced expression in ADAMTS5 ($\beta = 0.34$, $P = 0.04$), HIF-1a ($\beta = 0.39$, $P = 0.04$), IL4 ($\beta = 0.30$, $P = 0.02$), MMP1 ($\beta = 0.47$, $P < 0.01$), and SOX9 ($\beta = 0.37$, $P = 0.03$). The hip-knee-ankle angle and proton pump inhibitors (PPIs) intake were associated with reduced SOX9 expression ($\beta = 0.23$, $P < 0.01$; $\beta = 2.39$, $P < 0.01$). Higher C-reactive protein (CRP) levels were associated with increased MMP1 expression ($\beta = -0.16$, $P = 0.02$). *Conclusion*. We found that BMI exerts a destructive effect via induction of ADAMTS5. Metformin reduced the expression of catabolic genes ADAMTS5 and MMP1 and might play a role in disease prevention. Limb malalignment and PPI intake was associated with a reduced expression of SOX9, and higher CRP levels correlated with increased MMP1 expression, indicating a destructive process.

Keywords

metformin, osteoarthritis of the knee, obesity, gene expression, primary chondrocytes

Introduction

Osteoarthritis (OA) of the knee is among the most debilitating diseases.[1] In the United States, an estimated 14 million people are suffering from symptomatic knee OA,[2] with numbers increasing as the population becomes ever older. This leads to a massive economic burden on the health care system. The lifetime cost for patients diagnosed with knee OA was estimated at around $140,300.[3]

The pathophysiologic mechanisms underlying knee OA are complex as knee OA is a multifactorial disease.[4,5] There are many known risk factors, such as age, gender, body mass index (BMI), trauma, and genetic background.[6] BMI has been found to alter expression of certain matrix genes such as MMP13 (matrix metallopeptidase 13) or aggrecan.[7] The relationship with other major players in the pathophysiologic process of OA remains unclear, however.

In addition to damage caused by overweight, recent scientific findings indicate that systemic inflammation plays an important role in onset and progression of the disease.[4] This is also evidenced by the fact that in addition to OA of the weightbearing joints, obese patients often suffer from OA of the hand as well.[8] Low-grade systemic

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inflammation is common in metabolic syndrome.[9] Diabetes, in particular, has been associated with OA and is a risk factor for disease progression.[10,11] The first-line treatment in diabetes type 2 is metformin. Clinical studies have found that this biguanide derivative might have a protective effect against knee OA.[12-14] In animal models, metformin was found to reduce the expression of the catabolic genes.[15,16] So far the effects of metformin on the expression of cartilage matrix genes in human chondrocytes have not been investigated.

The purpose of this study was therefore to address the following questions: (1) What is the effect of BMI on the expression of certain matrix genes playing a major role in the pathogenesis of knee OA? (2) How can metformin influence this gene expression? (3) How do other clinical parameters and medication relate to gene expression? Our hypothesis was that BMI and metformin have significant effects on these matrix genes. To answer these questions, primary human chondrocytes were cultivated and stimulated with metformin. Interrelations of quantitative polymerase chain reaction (qPCR) data and clinical data were analyzed by a multivariable regression analysis.

Methods and Materials

This study was designed as a cross-sectional clinical/experimental laboratory study and conducted at the Department of Orthopaedics of the Medical University of Graz, Austria, starting in February 2019 until December 2019. Patients were asked for written informed consent. The procedures followed were in accordance with the ethical standards of the responsible committee (Ethics Committee, Medical University of Graz, IRB #31-133) and with the Helsinki Declaration.

Study Population

Female patients undergoing knee arthroplasty for end-stage knee OA at the age of 52 to 82 years were enrolled.

Cartilage Samples and Cell Culture

Femoral cartilage was collected intraoperatively and stored in growth medium at 4 °C for 2 to 4 hours. Cartilage was dissected using a scalpel, washed using phosphate buffered saline (PBS) 1× (Gibco Invitrogen, Carlsbad, CA, USA) and digested using 2 mg/mL collagenase B (Gibco) in growth medium at 36 °C for 24 hours. Cells were then filtered using a 70 µm filter, centrifuged, and seeded in cell culture flasks. For cultivation DMEM/F12 (Dulbecco's modified Eagle medium F-12) growth medium, containing 10% fetal bovine serum (FBS), 1% penicillin-streptomycin (5.000 U/mL), 1% L-glutamine, 1% insulin-transferrin-selen, 0.01% transforming growth factorβ (TGF-β; 10 ng/µL), and 0.01% fibroblast growth factor (FGF; 10 ng/µL; all Gibco), were used. Cells were incubated at 37 °C in a humidified atmosphere of 5% CO_2 under normoxic conditions. Cell passages 1 and 2 were stored in liquid nitrogen using a 5% dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO, USA) solution. Passage 2 was used for experiments. For transfer and splitting, PBS 1× containing 10% trypsin 2.5% (Gibco) was used.

Cell Viability Assay

In order to find a nontoxic drug concentration for experiments, cell viability assays were performed. Chondrocytes (1×10^4 and 5×10^3 cells for measurement after 24 and 48 hours respectively) were seeded on 96-well plates and either used as control or treated with metformin in various concentrations (0.5 µM, 0.1 mM, 1 mM, 2 mM). Cell viability was evaluated in 2 patient samples using the CellTiter-Glo Luminescence Cell Viability Assay (Promega, Madison, WI, USA) after 24 and 48 hours according to the manufacturer's instructions. Background reference values were derived from the culture media. Absorbance values were measured with the Lumistar microplate luminometer (BMC Labtech, Ortenberg, Germany).

Metformin Treatment

Metformin (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in DMSO (Sigma-Aldrich) and stored at a concentration of 100 mM. Cells were stimulated with 1 mM metformin at a confluence of 30% to 50% and incubated for 48 hours. For each patient, a control and a metformin sample (1 mM) was obtained for further analysis.

Gene Expression Analysis by Reverse-Transcription Quantitative Polymerase Chain Reaction (RT-qPCR)

RNA was extracted using the RNeasy Mini kit using spin technology (Qiagen, Hilden, Germany). DNA was digested using RNase-free DNase Set (Qiagen). RNA concentration was determined using spectrophotometry (Nanodrop, ThermoFisher, Waltham, MA, USA). RNA was then analyzed by RNA gel electrophoresis (Bioanalyzer, Agilent, CA, USA). The RNA integrity number (RIN) for all samples was greater than or equal to 9. cDNA was synthesized using 2 µg of extracted RNA using the iScript kit (Bio-Rad Laboratories Inc., Hercules, CA, USA) according to the manufacturer's recommendations. The following amplification settings were used on a thermocycler (MyCycler, Biorad): Priming at 25 °C for 5 minutes, reverse transcription at 46 °C for 20 min and reverse transcription inactivation at 95 °C for 1 minute. cDNA was stored at −20 °C.

Table 1. Polymerase Chain Reaction (PCR) Primers Used in Quantitative PCR Experiments.[a]

Symbol	Name	Catalog Number/Primer Sequence (Forward/Reverse)[b]	Source
ADAMTS4	ADAM metallopeptidase with thrombospondin type 1 motif, 4	QT00032949	Qiagen
ADAMTS5	ADAM metallopeptidase with thrombospondin type 1 motif, 5	QT00011088	Qiagen
COL2A1	Collagen, type II, alpha 1	QT00049518	Qiagen
HIF-1a	Hypoxia inducible factor 1, alpha subunit	QT00083664	Qiagen
EPAS1 (HIF2)	Endothelial PAS domain protein 1	QT00069587	Qiagen
IL4	Interleukin 4	5′-ATCTTTGCTGCCTCCAAGAACAC-3′ 5′-GTAGAACTGCCGGAGCACAG-3′	self-designed
IL6	Interleukin 6	QT00083720	Qiagen
CXCL8 (IL8)	Chemokine (C-X-C motif) ligand 8	QT00000322	Qiagen
MMP1	Matrix metallopeptidase 1	5′-CTGTTCAGGGACAGAATGTGCT-3′ 5′-TCGATATGCTTCACAGTTCTAGGG-3′	Self-designed
MMP3	Matrix metallopeptidase 3	5′-TTTTGGCCATCTCTTCCTTCA-3′ 5′-TGTGGATGCCTCTTGGGTATC-3′	Self-designed
MMP13	Matrix metallopeptidase 13	5′-TCCTCTTCTTGAGCTGGACTCATT-3′ 5′-CGCTCTGCAAACTGGAGGTC-3′	Self-designed
SOX9	SRY-box transcription factor 9	5′-CGCCATCTTCAAGGCGCTGC-3′ 5′-CCTGGGATTGCCCCGAGTGC-3′	Self-designed
SREBP2	Sterol regulatory element binding protein	5′-AAGTCTGGCGTTCTGAGGAA-3′ 5′-AGGTCCACCTCATTGTCCAC-3′	Self-designed
TIMP1	TIMP metallopeptidase inhibitor 1	5′-CTTCTGGCATCCTGTTGTTG-3′	Self-designed

[a]Self-designed primers were generated using the online tool Primer BLAST (U.S. National Library of Medicine) and purchased from Sigma/Aldrich.
[b]Qiagen primer sequences are not disclosed by the manufacturer.

RT-qPCR was performed using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad Laboratories Inc.) according to the manufacturer's recommendations using technical triplicates. A reaction volume of 10 µL containing 12.5 ng target DNA was used. The following PCR amplification specifications were set on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad): incubation at 95 °C for 30 seconds for polymerase activation and DNA denaturation, followed by 40 amplification cycles of 10 seconds of denaturation at 95 °C and 20 seconds of annealing-elongation at 60 °C. Table 1 shows the primers used for PCR analysis. Results were analyzed using the CFX Maestro Software (Bio-Rad Laboratories Inc., version 1.1). Quantification cycle values (Ct) were exported for statistical analysis. Results with Ct values greater than 32 were excluded from analysis. The arithmetic mean of expression levels of the reference genes RPL and TBP was used for normalization (ΔCt).

Limb Alignment and Joint Space Width

Preoperative full limb standing x-ray images were obtained for every patient. Images were stored and analyzed using the hospital-wide PACS (picture archiving and communications system) (pacsview, version 3.00.123, Institute for Medical Informatics, Statistics and Documentation, Medical University of Graz, Austria). Analysis was performed by 2 investigators (MS, PS), images were read for medial and later joint space width (JSW) and the hip-knee-ankle angle (HKAA) as outlined previously (**Fig. 1**).[17] Intraclass correlation coefficient (ICC) was found to be excellent (ICC for HKAA 0.964 [0.891-0.988], ICC for medial JSW 0.923 [0.778-0.975], ICC for lateral JSW 0.881 [0.671-0.960]). For analysis the arithmetic mean of the aforementioned values was used.

Clinical Data

Clinical data collected included: demographic data (age), BMI, inflammation markers (leukocyte count, C-reactive protein [CRP] levels), x-ray analysis (JSW, HKAA), and medication. Medication was grouped based on mode of action.

Statistical Analysis

All statistical analyses were performed using the statistical software R (R Foundation for Statistical Computing, Vienna, Austria, version 3.5.2) on GNU/Linux Debian 10 and SPSS 25 (IBM, Armonk, NY, USA) on Windows 10

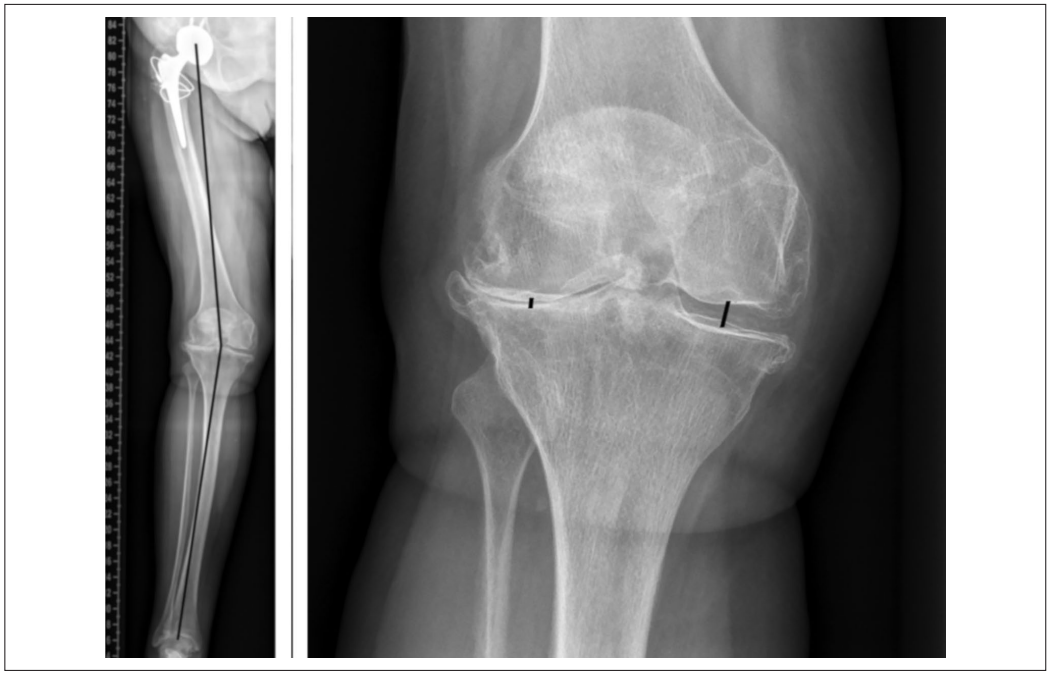

Figure 1. The measurement of the hip-knee-ankle angle (HKAA) (left) and joint space width (JSW) (right). The HKAA is defined as the angle between the mechanical axis of the femur and the mechanical axis of the tibia. The mechanical axis of the femur is a line from the center of the femoral head to the femoral reference point at the knee joint. The mechanical axis of the tibia is formed by a lined from the proximal tibial reference point to the mid ankle reference point.[17] The mechanical axes of the femur and tibia are shown on the left image by the black lines. The JSW was measured on the medial and lateral side as indicated by the black lines on the right image.

(Microsoft, Redmond, WA, USA).[18] The following R packages were used: lme4, lmerTest, tidyverse, car.[19-22] Groups were compared using paired Student *t* test,Wilcoxon rank or Kruskal-Wallis test, where appropriate, after testing for normality using the Kolmogorov-Smirnov test and Q-Q plots. Correlations were assessed using Spearman's correlation coefficient. Variables showing an association with ΔCt values with a *P* value <0.1 were used for multivariable analysis. To assess the effect of BMI on gene expression, for each gene of interest a linear regression model was calculated for the control group, using the expression level as outcome and the dependent variables as described above. Similarly, to assess the effect of metformin stimulation and BMI on gene expression, linear mixed models were calculated. A similar approach using regression models for analysis of qPCR data has been described and validated previously and has the advantage of increased flexibility, as it can account for multiple sources of variation compared with the traditional ΔΔCt method.[23] The following modifications were made to the aforementioned protocol: Ct values were averaged, normalized and ΔCt values were then used as the response variable. In mixed models, patient identity was entered as random effect to account for repeated measures. Known risk factors (age, BMI, HKAA) were entered into the models to adjust for them. The final model was selected based on model Akaike information criterion (AIC). Using the β-coefficient of regression models as the ΔΔCt value, the fold increase was calculated as $2^{-\Delta\Delta Ct}$.[24] Model assumptions were assessed using residual versus fitted, normal Q-Q, scale-location and residuals versus leverage plots. A *P* value <0.05 was assumed to be statistically significant. Due to this study's exploratory nature, sample size calculation was not performed. A total enrollment of 15 patients was the aim of this study. To asses interrater reliability on x-ray analysis, ICC (ICC 3,1, absolute agreement) was determined. Numerical variables are expressed as mean ±

Table 2. Baseline Characteristics of Patients Enrolled in the Study.[a]

	Overall	BMI <25 kg/m^2	BMI 25-35 kg/m^2	BMI >35 kg/m^2
n	14	4	6	4
BMI, kg/m^2	30.54 (6.28)	23.49 (1.19)	29.96 (2.67)	38.46 (2.70)
Age, years	70.05 (10.21)	73.16 (12.58)	72.40 (9.55)	63.41 (7.70)
ASA score of 3	7 (50.0)	3 (75.0)	2 (33.3)	2 (50.0)
JSW lateral	4.81 (2.36)	6.27 (1.37)	4.27 (2.90)	4.16 (2.06)
JSW medial	4.25 (1.93)	2.29 (1.65)	5.37 (1.62)	4.52 (1.12)
HKAA	6.68 (4.28)	7.38 (4.35)	6.33 (4.84)	6.50 (4.53)
Leukocyte count, $\times 10^3$/L	8.75 (3.17)	7.73 (2.35)	8.95 (4.18)	9.46 (2.58)
CRP, mg/dL	5.07 (3.80)	1.38 (0.26)	6.08 (3.83)	7.25 (3.38)
PPI intake	6 (42.9)	1 (25.0)	3 (50.0)	2 (50.0)
Diabetes mellitus	3 (21.4)	1 (25.0)	1 (16.7)	1 (25.0)

BMI = body mass index; ASA = American Society of Anesthesiologists physical status classification system (all patients were either classified as 2 or 3); JSW = joint space width on the medial or lateral side; HKAA = hip-knee-ankle angle; CRP = C-reactive protein; PPI = proton pump inhibitor.
[a]Continuous variables are displayed as mean (standard deviation), categorical data as count (percentage).

standard deviation. Categorical variables are expressed as count (percentage).

Results

A total of 15 patients were enrolled. Due to technical problems in the PCR experiments, 1 patient had to be excluded from the analysis, so that a total of 14 patients were analyzed. All 14 patients were female, with a mean age of 70.1 ± 10.2 years and a mean BMI of 30.5 ± 6.3 kg/m^2. Patients were either classified with an American Society of Anesthesiologists (ASA) score of 2 or 3. Three patients were diagnosed with type 2 diabetes, and no patient was insulin dependent or taking metformin. One patient was managed with lifestyle intervention only and not taking any antidiabetic medication, 1 patient was on linagliptin, the other patient was on gliclazide. **Table 2** shows the baseline characteristics.

Cell Viability Assay

A metformin concentration of 1 mM (129.16 µg/mL) was found to have no cytotoxic effect in the CellTiter-Glo assay ($\chi^2 = 9$, $P = 0.342$ for values after 24 and 48 hours) (**Fig. 2**). Steady-state metformin plasma levels in human beings range between 1 and 5 µg/mL (7.7-38.7 µmol/L).[25] A concentration of 1 mM was found to mitigate loss of cell viability by a decreased production of reactive oxygen species (ROS).[14] We are not aware of any study investigating the metformin concentration in synovial fluid. Thus, based on the positive results of the aforementioned study, and supported by the cell viability assay findings, a final metformin concentration of 1 mM was used for all experiments in order to detect changes in expression, if any at all.

Association of BMI and Gene Expression

BMI correlated only with ADAMTS5 ($r = -0.69$, $P < 0.01$) and MMP1 ($r = -0.59$, $P = 0.03$) (**Fig. 3**, **Table 3**) in univariable analysis. Multivariable linear regression was performed for each gene. The following variables were included in the regression models for ADAMTS5 and MMP1: age, BMI, HKAA, CRP level. Linear regression model coefficients are shown in **Table 4**. BMI was found to be a significant predictor of gene expression in ADAMTS5 ($\beta = -0.11$, $P = 0.034$, $n = 14$, 1.079-fold increase for a BMI increase of 1 point) after adjusting for age, BMI, and HKAA. This indicates that patients with a BMI of 30 kg/m^2 had a 2.14-fold increase in ADAMTS5 expression compared to patients with a BMI of 20 kg/m^2 in the control group. Considering the exponential nature of PCR replication, this also indicates an exponential relationship of BMI and ADAMTS5. In our study, this exponential relationship was observed in a BMI range of 22 to 40 kg/m^2. For an increase of 1 point of BMI, there is a 1.08-fold increase, while for 10 points of BMI there is a 2.14-fold increase of ADAMTS5 gene expression relative to reference genes (**Fig. 4**). BMI was not a significant predictor of MMP1 expression in multivariable regression ($\beta = -0.08$, $P = 0.148$). Similar results were obtained in mixed models, were patients with a BMI of 30 kg/m^2 had a 1.87-fold increase in ADAMTS5 expression compared to patients with a BMI of 20 kg/m^2 (**Table 5**).

Effect of Metformin Treatment of Chondrocytes on Gene Expression

Metformin had a significant treatment effect on ΔCt values in the following genes compared with the control group:

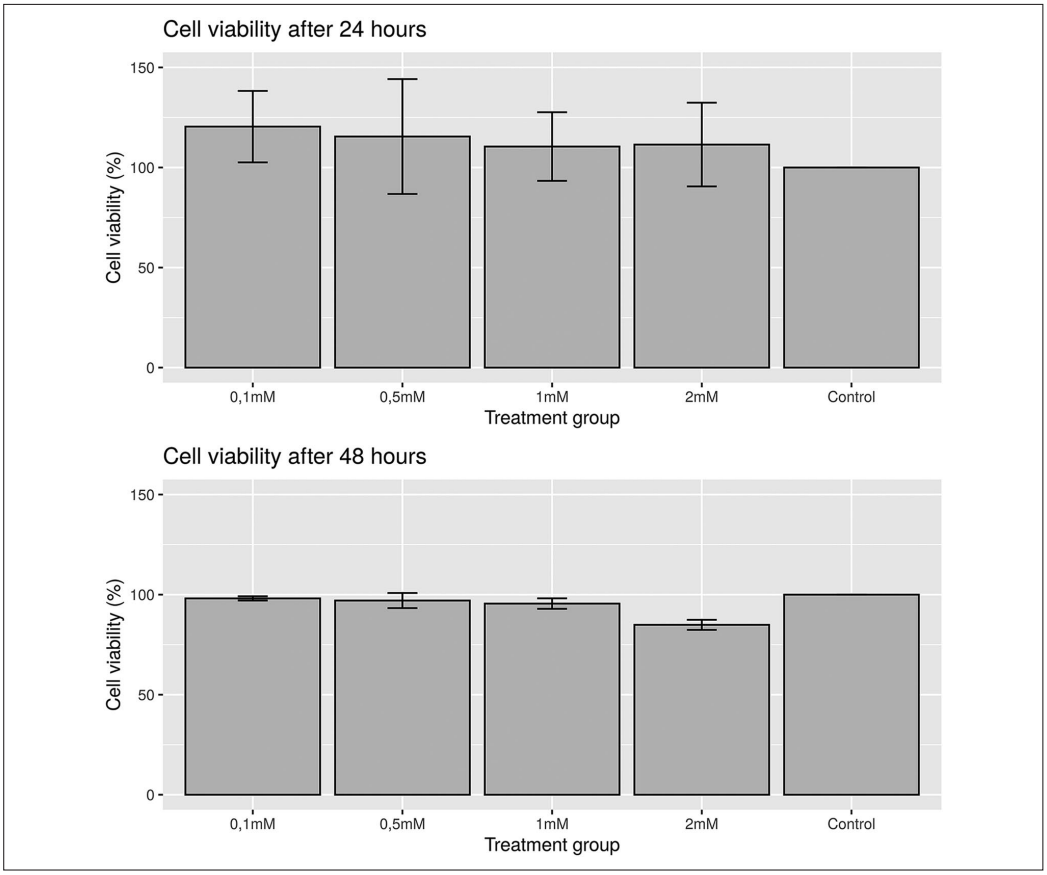

Figure 2. Results from CellTiter-Glo cell viability assay after 24 hours (top) and 48 hours (bottom). Bars indicate mean cell viability in percentage compared with control. Error bars show the standard deviation. No difference between treatment groups after 24 hours and 48 hours could be observed using a Kruskal-Wallis test.

IL4 (11.6 ± 0.6 vs. 11.3 ± 0.6, $P = 0.03$), MMP1 (7.2 ± 1.2 vs. 6.8 ± 1.1, $P < 0.01$), and SOX9 (5.1 ± 1.3 vs. 4.7±1.7, $P = 0.04$) (**Fig. 5**). After controlling for confounding variables, this effect was significant in the following genes: ADAMTS5 ($\beta = 0.34$, $P = 0.043$, $n = 14$ per treatment group, 0.79-fold decrease) (controlled for BMI, HKAA, age), HIF-1a ($\beta = 0.39$, $P = 0.04$, $n = 14$ per treatment group, 0.76-fold decrease) (adjusted for BMI, HKAA, age), IL4 ($\beta = 0.30$, $P = 0.02$, $n = 8$ per treatment group, 0.81-fold decrease) (accounting for BMI, HKAA, age), MMP1 ($\beta = 0.47$, $P < 0.01$, $n = 14$ per treatment group, 0.72-fold decrease) (adjusted for BMI, HKAA, age, CRP level), SOX9 ($\beta = 0.37$, $P = 0.03$, $n = 14$ per treatment group, 0.77-fold decrease) (controlling for BMI, HKAA, age, PPI intake). **Table 5** shows linear mixed models and coefficients. This indicates that stimulation with metformin causes a 21% decrease in ADAMTS5, a 24% decrease in HIF-1a, a 19% decrease in IL4, a 23% decrease in SOX9, and a 28% decrease in MMP1 gene expression. Other catabolic markers were not found to be affected by metformin stimulation. This relationship is graphically depicted in **Fig. 4**, which shows predicted regression lines for MMP1 by HKAA, and for ADAMTS5 by BMI, holding all other variables constant. **Fig. 6** and **Table 6** show the fold change in gene expression based on regression results.

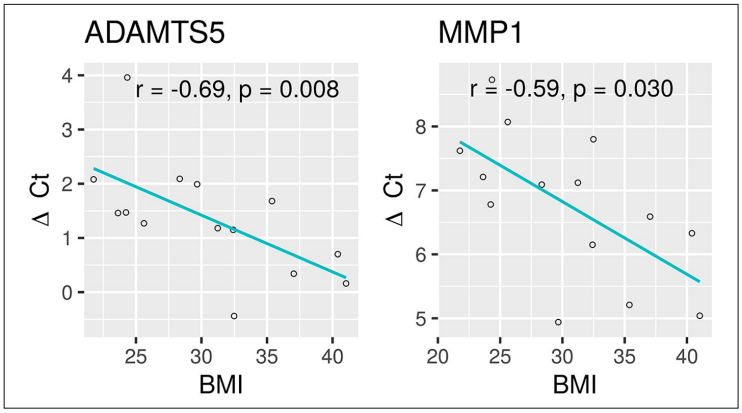

Figure 3. A scatterplot of body mass index (BMI) and ΔCt values of ADAMTS5 (left) and MMP1 (right) with a regression line showing a significant negative correlation. In patients with higher BMI, there is an increased expression of these two genes (a small ΔCt value indicates higher expression levels). *r*, Spearman's rank correlation coefficient; *P*, *P* value.

Table 3. Correlation Coefficients of BMI with the ΔCt Values of Genes of Interest Using Spearman's Rho in the Control Group.[a]

	Age		BMI		HKAA		JSW M		CRP	
	r	*P*	*r*	*P*	*r*	*P*	*r*	*P*	*r*	*P*
ADAMTS4	−0.35	0.266	0.56	0.063†	0.02	0.957	0.71	0.012*	0.66	0.022*
ADAMTS5	0.41	0.150	−0.69	0.008*	0.25	0.385	−0.3	0.302	−0.57	0.035*
COL2A1	0.31	0.564	−0.09	0.919	0.12	0.827	0.66	0.175	0.03	1.000
HIF1	−0.36	0.203	0.39	0.170	0.02	0.934	0.27	0.357	0.42	0.137
HIF2	−0.1	0.727	0.04	0.892	−0.21	0.471	0.12	0.682	−0.14	0.627
IL4	0.29	0.501	−0.33	0.428	0.48	0.243	0.12	0.793	0.14	0.752
IL6	−0.42	0.132	0.19	0.512	−0.05	0.863	0.19	0.512	0.04	0.892
IL8	0.15	0.604	−0.51	0.063†	−0.19	0.505	−0.54	0.047*	−0.55	0.040*
MMP1	0.09	0.762	−0.59	0.030*	−0.1	0.724	−0.33	0.246	−0.49	0.075†
MMP13	−0.33	0.253	0.15	0.610	−0.37	0.188	0.24	0.404	0.15	0.610
MMP3	−0.02	0.940	−0.06	0.832	0.07	0.822	0.09	0.750	0.01	0.976
SOX9	−0.04	0.904	−0.03	0.928	0.15	0.613	0.11	0.704	0.24	0.400
SREBP	−0.03	0.928	0.06	0.844	0.28	0.331	0.01	0.988	−0.05	0.880
TIMP1	−0.25	0.382	0.02	0.964	0.11	0.707	0.38	0.175	−0.05	0.856

BMI = body mass index; CRP = C-reactive protein; HKAA = hip-knee-ankle angle; *r* = Spearman's rank correlation coefficient; JSW M = medial joint space width; JSW L = lateral joint space width.
[a]There is a moderate, significant, negative association of ΔCt values of the 2 extracellular matrix degrading enzymes ADAMTS5 and MMP1 and the BMI indicating that higher BMI correlates with increased ADAMTS5 or MMP1 expression (**Fig. 3**).
†$P < 0.1$. *$P < 0.05$.

Association of HKAA and JSW and Gene Expression

Medial JSW was positively correlated with ADAMTS4 ($r = 0.71$, $P = 0.012$), while there was a negative correlation with IL8 ($r = -0.54$, $P = 0.047$) in the control group (**Table 3**). This could not be confirmed by mixed model multivariable regression, however (the variable was dropped in the final model). Instead we found that HKAA was a significant predictor of SOX9 expression ($\beta = 0.23$, $P < 0.01$, $n = 14$ per treatment group, 0.85-fold decrease for 1° of malalignment) (controlled for age, BMI, and PPI intake) in multivariable mixed model regression. Patients with a deviation of 10° HKAA showed a substantially reduced expression of SOX9 (0.2-fold) (**Tables 5** and **6**, **Fig. 6**).

Table 4. Fixed-Effects Linear Regression Model for ADAMTS5 and MMP1.[a]

	β Coefficient (Univariable)	95% CI	*P*	β Coefficient (Final)	95% CI	*P*	AIC (Final)
ADAMTS5							
BMI	−0.10	−0.19, −0.02	0.018*	−0.11	−0.22, −0.01	0.034*	43.2
HKAA	0.02	−0.14, 0,17	0.826	0.01	−0.16, 0.16	0.952	
Age	0.02	−0.04, 0.08	0.519	−0.01	−0.09, 0.06	0.684	
CRP	−0.14	−0.29, 0.01	0.063†	—	—	—	
MMP1							
BMI	−0.11	−0.20, −0.02	0.018*	−0.08	−0.20, 0.03	0.148	39.0
HKAA	−0.06	−0.22, 0.11	0.451	−0.08	−0.22, 0.06	0.215	
Age	−0.01	−0.07, 0.07	0.933	−0.03	−0.09, 0.04	0.340	
CRP	−0.19	−0.34, −0.04	0.016*	−0.15	−0.34, 0.03	0.094†	

BMI = body mass index; HKAA = hip-knee-ankle angle; CRO = C-reactive protein; AIC = Akaike information criterion; "—" = variable dropped in the final model.

[a]The results of the linear regression model for ADAMTS5 accounting for known risk factors are shown. BMI decreases ΔCt values and thus increases gene expression. The left column shows the univariable coefficients of the variables included in the final model using backward stepwise selection (accounting for known risk factors for osteoarthritis and variables with an association with a *P* value <0.1).

†$P < 0.1$. *$P < 0.05$.

Association of Inflammation Markers and Gene Expression

CRP showed a significant correlation with ADAMTS4 ($r = 0.7$, $P = 0.02$), ADAMTS5 ($r = -0.6$, $P = 0.04$), and IL8 ($r = -0.6$, $P = 0.04$) in the control group (**Table 3**). In the multivariable mixed model regression, CRP was a significant predictor of MMP1 gene expression ($\beta = -0.16$, $P = 0.02$, $n = 14$ per treatment group, 1.12-fold increase for 1-point increase in CRP level) (adjusted for BMI, HKAA, and age) (**Tables 5** and **6**, **Fig. 6**). Patients with a CRP level of 10 mg/dL had a 3-fold increase in MMP1 expression compared to patients with a level of 0 mg/dl. CRP was not a significant predictor of ADAMTS4, ADAMTS5, and IL8, however (the variable was dropped in the respective final model).

Association of Comedication

Oral use of PPIs was a significant predictor of reduced SOX9 expression in regression models ($\beta = 2.39$, $P < 0.01$, $n = 14$ per group, 0.19-fold decrease in PPI intake) (controlled for age, BMI, and HKAA) (**Tables 5** and **6**, **Fig. 6**).

Discussion

The purpose of this study was to assess the effect of BMI and metformin on the expression of important matrix genes in human chondrocytes using RT-qPCR. The hypothesis was that BMI and metformin have significant effects on these matrix genes and thus on the pathogenesis of OA of the knee joint. We found that an increase in BMI influences the expression of matrix degrading gene ADAMTS5, while metformin treatment at the cellular level causes a reduction of the catabolic genes ADAMTS5 and MMP1. Furthermore, we found a reduction of anabolic SOX9 gene expression in chondrocytes in patients with increased HKAA, and patients on PPIs. Finally, higher CRP levels were associated with increased MMP1 expression.

BMI is a known risk factor for knee OA. In addition, BMI is also associated with OA of the hand which indicates that other effects such as systemic inflammation might play a role in pathogenesis.[8,9] In our study, BMI was a significant predictor of the expression of ADAMTS5: Higher BMI values led to a small, exponential increase in gene expression. ADAMTS5, a member of aggrecanases, causes cartilage destruction. In contrast to our findings, a study applying *ex vivo* physiologic mechanical compression on human cartilage found a change of collagen type II and aggrecan, but no effect on ADAMTS4.[26] Another study found a positive association of BMI and TGFβ, aggrecan, MMP-13, IGF1, and TIMP2. As in our study, no effect on type 2 collagen could be found.[7] Our results show that baseline expression of matrix genes is affected by BMI.

Metformin is the first-line treatment in type 2 diabetes mellitus, a risk factor for OA. The effects of metformin on human cartilage gene expression have not been investigated extensively. The mechanism of action of metformin is complex and not fully understood. It acts via both AMPK-dependent and AMPK-independent mechanisms.[27] Findings from clinical studies on metformin treatment in OA are equivocal. In one study using prospective data, metformin was found to reduce cartilage loss.[12] Another study showed that patients with knee OA treated with a combination of

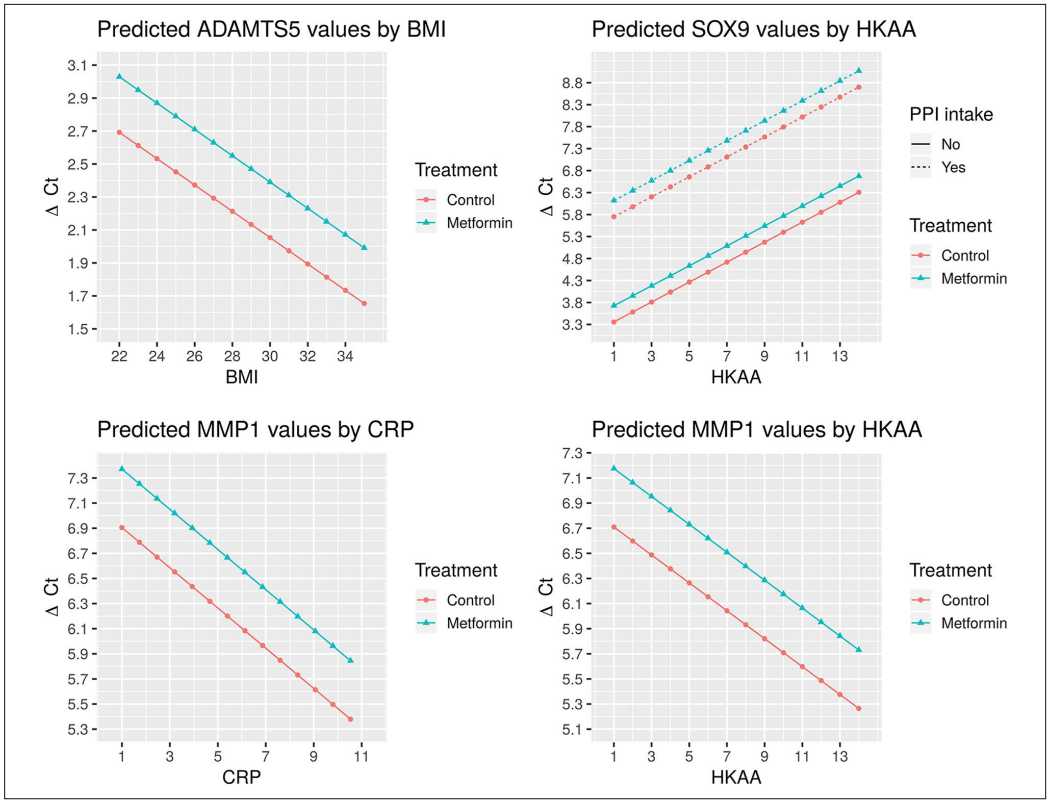

Figure 4. The predicted ΔCt values based on regression models for ADAMTS5 (top left), SOX9 (top right), and MMP1 (bottom left and right), holding all variables except metformin treatment, as well as body mass index (BMI) (top left), hip-knee-ankle angle (HKAA), and proton pump inhibitor (PPI) use (top right) and C-reactive protein (CRP) (bottom left) and HKAA (bottom right), constant. With higher BMI, CRP, or HKAA values, there is a decrease in the respective ΔCt values of ADAMTS5 and MMP1 (top left, bottom right and left) and thus increase in gene expression of the respective gene. Metformin leads to a small increase in ΔCt and thus a decrease in gene expression as depicted in all graphs. PPI intake is associated with a strong increase in ΔCt and thus decrease in SOX9 gene expression (top right).

meloxicam and metformin had a higher improvement in several clinical scores.[28] Another study on patients prescribed metformin, however, could not find an association with the risk of OA.[29]

In mouse chondrocytes, metformin was able to mitigate cytotoxic effects of IL-1β. Furthermore, metformin also reduced production of reactive oxygen species in that study.[14] In another study, AMPK activation by metformin improved mitochondrial function and reduced oxidative stress.[30] In OA mouse model metformin reduced cartilage degradation.[31]

In agreement with these studies, our results showed that expression of catabolic genes can be suppressed after 48 hours of exposure to metformin. We found that metformin slightly decreased expression of catabolic genes ADAMTS5, MMP1. On the other hand, expression of anabolic genes HIF-1a, IL4, and SOX9 was slightly reduced as well.

Our findings are supported by another study that found a reduction of MMP3 and MMP13 in OA mouse knee model after metformin treatment.[15] Also, metformin reduced MMP3 and ADAMTS5 expression in rat nucleus pulposus cell culture.[16] Our results indicate that metformin might reduce expression of these genes and exert a protective effect on cartilage.

SOX9 is a transcription factor and plays an important role as regulator of cartilage formation and chondrocyte

Table 5. Linear Mixed Regression Models of Selected Genes Accounting for Confounding Variables and Repeated Measures.[a]

	β Coefficient (Univariable)	95% CI	*P*	β Coefficient (Final)	95% CI	*P*	AIC (Final)
ADAMTS5							
Metformin	0.34	0.02, 0.66	0.043*	0.34	0.02, 0.61	0.043*	76.0
BMI	−0.08	−0.14, –0.012	0.015*	−0.09	−0.16, –0.02	0.020*	
HKAA	0.01	−0.11, 0.12	0.919	−0.01	−0.11, 0.10	0.857	
Age	0.02	−0.03, 0.06	0.457	−0.01	−0.05, 0.04	0.810	
CRP	−0.12	−0.23, –0.02	0.028*	—	—	—	
HIF-1a							
Metformin	0.39	0.02, 0.75	0.044*	0.39	0.02, 0.75	0.044*	82.7
BMI	−0.01	−0.11, 0.10	0.966	−0.04	−0.15, 0.07	0.455	
HKAA	0.01	−0.14, 0.17	0.852	0.08	−0.08, 0.25	0.311	
Age	−0.03	−0.1, 0.03	0.276	−0.06	−0.14, 0.01	0.108	
IL4							
Metformin	0.30	0.06, 0.53	0.022*	0.30	0.06, 0.53	0.022*	30.0
BMI	−0.04	−0.12, 0.04	0.284	−0.04	−0.14, 0.07	0.444	
HKAA	0.03	−0.09, 0.15	0.584	0.01	−0.10, 0,13	0.795	
Age	0.01	−0.03, 0.06	0.472	<–0.01	−0.06, 0.05	0.997	
MMP1							
Metformin	0.47	0.17, 0.76	0.005*	0.47	0.17, 0.76	<0.001*	62.9
BMI	−0.11	−0.19, –0.03	0.010*	−0.07	−0.15, 0.01	0.088	
HKAA	−0.07	−0.21, 0.07	0.297	−0.11	−0.21, –0.01	0.031*	
Age	<0.01	−0.06, 0.06	0.978	−0.02	−0.06, 0.03	0.447	
CRP	−0.19	−0.32, –0.06	0.008*	−0.16	−0.29, –0.03	0.019*	
SOX9							
Metformin	0.37	0.06, 0.68	0.026*	0.37	0.06, 0.68	0.026*	74.3
BMI	−0.02	−0.15, 0.11	0.798	−0.08	−0.18, 0.01	0.070	
HKAA	0.06	−0.13, 0.25	0.006*	0.23	0.08, 0.37	0.006*	
Age	−0.02	−0.10, 0.06	0.640	−0.05	−0.12, 0.01	0.098	
PPI intake	1.60	0.27, 2.93	0.024*	2.39	1.29, 3.49	<0.001*	

BMI = body mass index; CRP = C-reactive protein; HKAA = hip-knee-ankle angle; PPI = proton pump inhibitor; AIC = Akaike information criterion; "—" = variable dropped in the final model.

[a]The left column shows the univariable β coefficients of the variables included in the full model (accounting for known risk factors for osteoarthritis and variables with an association with a *P* value <0.1). The right column shows the coefficients included in the final model after model selection based on AIC. Variables, where no β coefficients for the final model are given. Metformin was found to cause a significant reduction in the expression of ADAMTS5, MMP1, HIF-1a, IL4, and SOX9. For example, in SOX9, there was an increase in the ΔCt value of 0.37 after metformin treatment, indicating a reduction in gene expression.

*$P < 0.05$.

differentiation.[32] In late-stage OA, SOX9 expression is reduced and its upregulation inhibits IL-1β induced inflammatory response.[33,34] Supporting our findings, metformin reduced SOX9 expression in esophageal cancer cells.[35] Contrary to this, metformin was found to increase levels of SOX9 expression in mouse cartilage.[15] How this affects cartilage metabolism and clinical results in humans remains unclear.

HIF-1a is expressed at higher levels in degenerating cartilage than in intact cartilage. Under normal conditions, HIF-1a protein undergoes rapid degradation in proteasomes.[36] In hypoxemic conditions, HIF-1a expression is increased and acts cartilage protective.[37] We found that metformin caused a decrease in HIF-1a expression under normoxic conditions. This might be an indirect sign of lower inflammation and ROS levels and less oxygen consumption. However, it remains unclear how metformin affects HIF-1a expression under hypoxia when HIF-1a does not undergo rapid degradation. This is in agreement with studies that found reduced HIF-1a levels in various cancer types after treatment with metformin.[38-40]

Finally, IL4 exerts anti-inflammatory effects via reduction of inflammatory cytokines and cytokines.[41] In our study, metformin reduced IL4 expression. This confirms studies showing that metformin can reduce IL4 expression in cancer cells.[42] Likewise, in nondiabetic heart failure

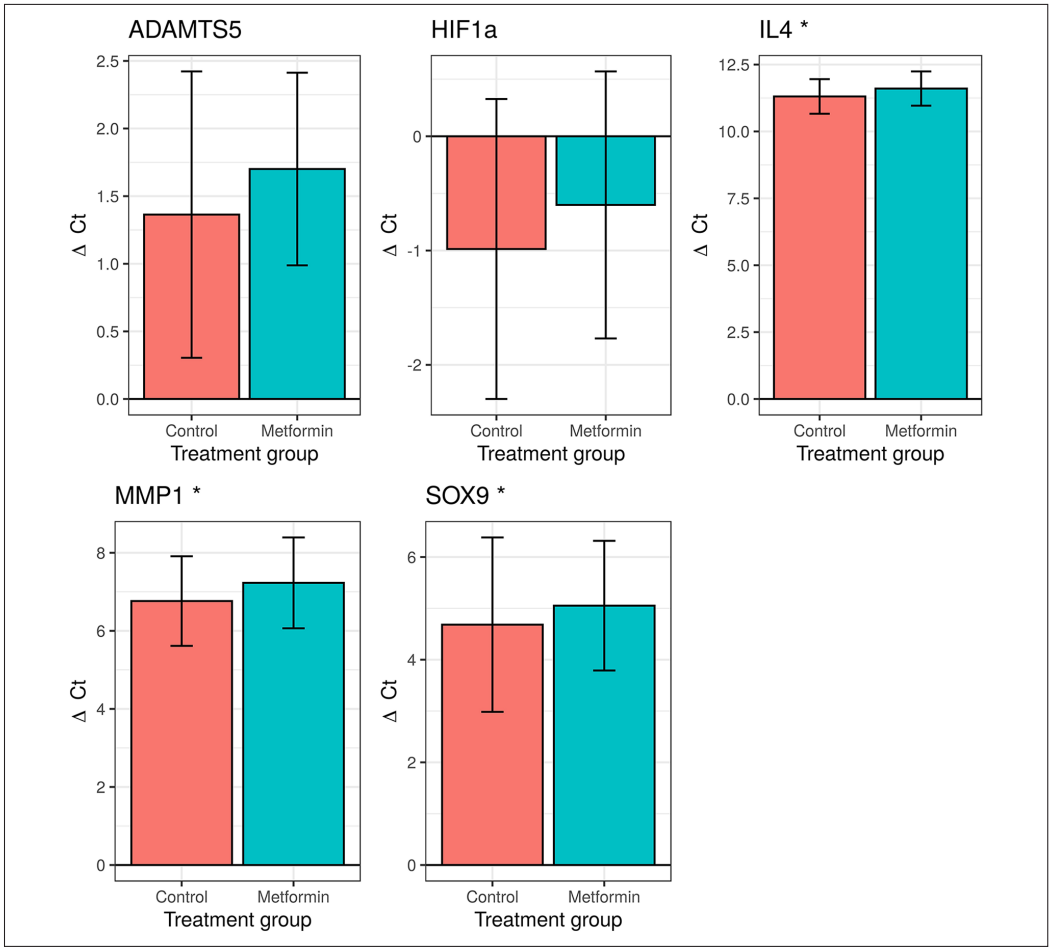

Figure 5. The metformin treatment effect of selected genes of interest. This figure shows the mean ΔCt values in the control versus treatment group. The error bars indicate the standard deviation. Metformin significantly decreased expression of IL4, MMP1, and SOX9 in univariable analysis. "." $P < 0.1$. *$P < 0.05$.

patients treated with metformin, IL4 plasma levels were significantly reduced.[43] This might be another indirect sign of lower inflammation level and thus lower IL4 expression caused by metformin.

Our results showed a significant negative correlation of CRP levels and ΔCt values of MMP1 in multivariable regression analysis, indicating increased MMP1 expression in patients with higher CRP values. This is supported by a study showing increased MMP1 expression in endothelial cells after incubation with CRP.[44] At the same time, a systematic review found significantly increased CRP levels in patients with OA.[45] This again highlights the role of inflammation in OA.

Looking at the association of limb alignment on matrix gene expression, we found that patients with a varus or valgus malalignment had significantly reduced SOX9 gene expression. In animal models of induced OA, there was an increase in subchondral bone thickness, decrease in aggrecan content and increase in MMP3 expression.[46-48] SOX9 plays an important role in cartilage formation and chondrocyte hypertrophy and a study showed a reduction of SOX9 expression in arthritic chondrocytes.[32,49] The

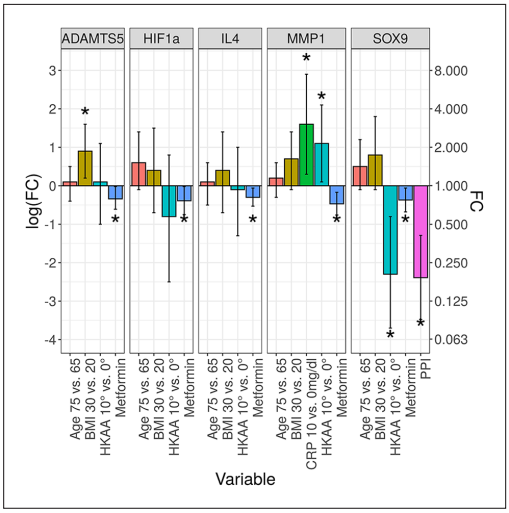

Figure 6. The fold change in gene expression in select genes. In this bar chart, we see the deviation from the baseline gene expression in select genes and various treatment groups or variables of interest (0 in the logarithmic scale on the left, 1 in the fold change ratio scale on the right). A bar above baseline indicates an increase in gene expression. Error bars indicate the 95% confidence intervals on the log scale. Fold change was calculated based on regression models (**Tables 5** and **6**). For example in ADAMTS5 we see a 1.87-fold increase of gene expression in patients with a body mass index (BMI) of 30 kg/m^2 compared with those with BMI of 20 kg/m^2. FC, fold change; log(FC) log2 of fold change. *$P < 0.05$.

authors of that study concluded that the reason for this reduction can only be speculated upon and might be caused by an imbalance in SOX9 signaling pathways. As SOX9 is a potent inhibitor of hypertrophic differentiation, this might eventually lead to increased subchondral bone. We found that patients with a strong varus or valgus malalignment had a reduced SOX9 expression compared to patients with no or little malalignment in this end-stage OA population. It is possible that this observed association is at least partly due to a more severely damaged cartilage in patients with a high HKAA.

We also found that patients taking proton pump inhibitors had a reduced expression of SOX9. A similar effect was found in esophageal cells where PPIs downregulated SOX9 expression and thereby slowed progression of Barrett's esophagus.[50] It is known that proton pump inhibitors change bone metabolism, especially in the elderly.[51] The effect of proton pump inhibitors on cartilage, however, remains unclear. Our results are suggestive of a potentially negative effect. However, we cannot exclude confounding effects.

This study has several limitations: First, it was conducted using an exploratory study design, no sample size calculation was performed and only a small sample size was analyzed. This is at least partly counterbalanced by the significant findings. Significant results in underpowered studies overestimate the effect size, however.[52] While further research is necessary to confirm our findings, we hope that this study can serve to generate new hypotheses and facilitate future research, considering the potential role of metformin in disease prevention. Similarly, the results of the cell viability assay have to be interpreted with caution due to the small sample size. Second, only female patients were included and results might be different in male patients. Third, the concentration of metformin used in our experiments was 20 to 30 times higher than physiologic steady-state plasma concentrations after oral intake. Thus, the findings in this study cannot be applied readily in clinical practice. Fourth, cells were stimulated for 48 hours only and thus long-term effects were not assessed. Fifth, cells were harvested for RNA extraction only after 2 cell passages. While prolonged cell culture of chondrocytes certainly induces cell dedifferentiation, this is a cumulative and linear process from hyaline cartilage toward fibrocartilage.[53,54] In clinical studies, passage 2 chondrocytes haven been used successfully to treat osteochondritis dissecans.[54] In order to stimulate cells with metformin and obtain enough cell material for further analysis, it was necessary to use chondrocytes of passage 2. Sixth, 3 patients included in this study suffered from diabetes. High glucose levels are known to alter gene expression.[55] These 3 patients were managed optimally with regard to diabetes, however, and thus any altered effects of diabetes on gene expression seem minimal or very unlikely. Finally, cartilage was collected from random sites intraoperatively, and there might be a site specific gene expression pattern in cartilage.

To summarize, this study is the first to investigate the effects of BMI and metformin on certain important cartilage matrix genes in primary human chondrocytes. We found that BMI is associated with a destructive effect linked to an increased expression of ADAMTS5. On the other hand, metformin stimulation can reduce expression of catabolic genes ADAMTS5 and MMP1. Thus, it might play a role in prevention of OA in obese patients. Limb malalignment was also linked to a reduction in the expression of SOX9. Likewise, intake of proton pump inhibitors was associated with a reduced SOX9 expression. This might be suggestive of destructive process. Higher CRP levels were associated with increased MMP1 expression, highlighting the role of systemic inflammation.

Table 6. Fold Change in Gene Expression in Select Genes (Based on Mixed Model Regression Analysis).[a]

Target	Variable	log2(FC)	95% CI	FC	*P*
ADAMTS5	Metformin	−0.34	−0.02, –0.61	0.79	0.043*
ADAMTS5	BMI 30 vs. 20 kg/m^2	0,9	0.2, −1.6	1.87	0.020*
ADAMTS5	HKAA 10° vs. 0°	0.10	1.1, –1	1.07	0.857
ADAMTS5	Age 75 vs. 65 years	0.10	0.5, –0.4	1.07	0.810
HIF1a	Metformin	−0.39	−0.02, –0.75	0.76	0.044*
HIF1a	BMI 30 vs. 20 kg/m^2	0.40	1.5, –0.7	1.32	0.455
HIF1a	HKAA 10° vs. 0°	−0.80	0.8, –2.5	0.57	0.311
HIF1a	Age 75 vs. 65 years	0.60	1.4, –0.1	1.52	0.108
IL4	BMI 30 vs. 20 kg/m^2	0.40	1.4, –0.7	1.32	0.444
IL4	Metformin	−0.30	−0.06, –0.53	0.81	0.022*
IL4	HKAA 10° vs. 0°	−0.10	1, –1.3	0.93	0.795
IL4	Age 75 vs. 65 years	0.10	0.6, –0.5	1.07	0.997
MMP1	Metformin	−0.47	−0.17, –0.76	0.72	<0.001*
MMP1	HKAA 10° vs. 0°	1.10	2.1, 0.1	2.14	0.031*
MMP1	BMI 30 vs. 20 kg/m^2	0.70	1.4, –0.1	1.62	0.088
MMP1	Age 75 vs. 65 years	0.20	0.6, –0.3	1.15	0.447
MMP1	CRP 10 vs. 0 mg/dL	1.60	2.9, 0.3	3.03	0.019*
SOX9	PPI	−2.39	−1.29, –3.48	0.19	<0.001*
SOX9	Age 75 vs. 65 years	0.50	1.2, –0.1	1.41	0.098
SOX9	BMI 30 vs. 20 kg/m^2	0.80	1.8, –0.1	1.74	0.070
SOX9	HKAA 10° vs. 0°	−2.30	−0.8, –3.7	0.20	0.006*
SOX9	Metformin	−0.37	−0.68, –0.06	0.77	0.026*

BMI = body mass index; CRP = C-reactive protein; HKAA = hip-knee-ankle angle; PPI = proton pump inhibitor; FC = fold change.
[a]The first 2 columns show the target gene and treatment group or variable of interest. The next 2 columns show the logarithm of the FC with the 95% confidence interval. Finally, on the last 2 columns we see the FC as well as the *P*. FC was calculated as FC = $2^{-\Delta\Delta Ct}$, $\Delta\Delta C_t$ values were obtained from regression models (**Table 5**).
*$P < 0.05$.

Author Contributions

Conception and design: BL, BSF, PSc, NS, MS, PSa. Analysis and interpretation of the data: PSc, NS, BL, MS, BSF, DG. Drafting of the article: PSc. Critical review of the article: BL, NS, BSF, AL, PSa. Provision of study materials or patients: AL, PSa. Statistical expertise: PSc. Collection and assembly of data: PSc, BL, NS, MS, DG, BSF. Administrative, technical, or logistic support: BL, NS, DG, BSF.

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Ethical Approval

The procedures followed were in accordance with the ethical standards of the responsible committee (Ethics Committee of the Medical University of Graz, Austria, IRB #31-133) on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000.

Informed Consent

Written informed consent was obtained from all patients before the study.

Trial Registration

Not applicable.

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5 Discussion

5.1 Summary and general discussion of the findings in this project

The purpose of this study was threefold: First, we wanted to understand the relationship of BMI / obesity and the pathogenesis of KOA. Second, we were hoping to find a biomarker that could help in the diagnosis and staging of KOA and might also show the close interrelation of KOA and the metabolic syndrome. Third, we wanted to see, how obesity is associated with altered gene expression in primary arthritic human chondrocytes, and if metformin can be used to mitigate degenerative effects of the metabolic syndrome on chondrocytes.

To answer these questions, we conducted 3 prospective clinical and laboratory studies from 2019 to 2020 in patients undergoing knee arthroplasty at the Department for Orthopaedics and Trauma, Medical University of Graz.

5.1.1 The Association of Blood Biomarkers and Body Mass Index in Knee Osteoarthritis: a Cross-Sectional Study

To answer our first question, we enrolled patients on the day before knee arthroplasty, took clinical data, including data on BMI and other typical MetS components and collected blood specimens. These were further analyzed using the ELISA and Luminex® technology, as well as NMR spectroscopy to quantify several biomarkers that play a role in metabolic disease and / or osteoarthritis and to quantify metabolites. To assess disease severity, we used the Lequesne index that measures pain and functioning. We found that FABP4 is highly correlated with BMI and gender. The same was true for leptin. We could not detect an association with severity of KOA (as measured on the Lequesne index). In contrast to others, we did not find a pattern in the metabolome that could show an association of MetS with KOA.

5.1.2 Fatty Acid–Binding Protein 4 (FABP4) Is Associated with Cartilage Thickness in End-Stage Knee Osteoarthritis

In another study we tried to quantify the association of BMI / obesity and several biomarkers with cartilage directly: For this study we enrolled patients and measured blood biomarkers using ELISA. We also assess cartilage thickness using ultrasound imaging. Paradoxically, we could show that in this cohort, BMI / obesity was associated with a slightly greater cartilage thickness compared to normal or underweight patients. Interestingly, in addition to the already known link of FABP4 and BMI and gender, we were able for the first time to detect a negative correlation of FABP4 levels and cartilage thickness: Patients with higher FABP4 levels had smaller cartilage thickness, even when adjusting for age and gender.

5.1.3 The Effect of Body Mass Index and Metformin on Matrix Gene Expression in Arthritic Primary Human Chondrocytes

Zooming in even more on a cellular level, in our third study, we wanted to understand gene expression patterns in arthritic human chondrocytes. To that end, we collected chondrocytes intraoperatively during knee arthroplasty. We cultivated and stimulated cells with metformin and measured matrix gene expression using RT-qPCR. We could show the important role of obesity in KOA: Chondrocytes harvested from patients with higher BMI had a higher expression level of pro-inflammatory and / or destructive genes, such as ADAMTS5. We were also able for the first time to show that metformin stimulation of human chondrocytes led to a decrease in the expression of several, mostly pro-inflammatory, genes such as ADAMTS5 and MMP1.

Our data support the hypothesis that obesity and the metabolic syndrome contribute to cartilage degeneration via systemic action and low-grade inflammation, in addition to direct mechanical impact and load. Furthermore, we could show that FABP4 is a promising candidate as biomarker of burden of disease in KOA. And finally, these results hint a potential role of metformin in the prevention and treatment of KOA for the future.

5.2 The relationship of MetS, selected serum biomarkers and KOA severity

There are numerous scores and scales for the assessment of KOA severity. Among these, the Lequesne index and the Western Ontario and McMaster Universities Osteoarthritis Index (WOMAC) score are widely used. The Lequesne index is a self-reported questionnaire that addresses the areas of pain and comfort, walking distance as well as activities of daily living. It is a validated and accurate diagnostic tool to assess KOA severity clinically. So far, however, only few studies have attempted to find an association of KOA severity, measured using the Lequesne index, or the WOMAC score, and many of the above mentioned serum biomarkers. Most studies still rely on assessing patients using the radiographic Kellgren-Lawrence scale. However, it is known that this scale is insensitive to early signs, change in pathology, i.e. progression, and does not correlate well with clinical symptoms.

In a recent study on 600 patients from the osteoarthritis initiative, a longitudinal cohort study sponsored by the NIH, of 19 biomarkers only 4 were consistently associated with radiographic and clinical features in a multivariate model. These 4 markers were serum Coll2-1 NO2, CS846, COMP and uCTX II, of which, according to the authors, uCTX II had the strongest and most consistent association with OA. (115). Apart from these well known and commercially available markers, several other candidates have received attention recently:

5.2.1 The promising role of FABP4

FABP4 has been associated with obesity, metabolic syndrome, insulin resistance, T2DM, hypertension, dyslipidemia, atherosclerosis and heart disease (48). In OA it was found to be associated with severity measured on radiographs and levels were higher in KOA patients compared to healthy controls (50). Knock out and pharmaceutical inhibition could alleviated OA in a mouse model (50). These findings support our results that show a negative correlation of cartilage thickness and FABP4 levels. Like others, we found a correlation of FABP4 and BMI.

5.2.2 The contradictory findings on adipokines

The three adipokines assessed in this project, leptin, ghrelin and resistin, have also increasingly received attention in OA research. Several papers could show higher levels in KOA patients compared to normal controls and some studies even detected a significant association with KOA severity: One study found a significant association of leptin and the pain component on the

WOMAC score (64). However, this connection was lost after adjustment for obesity and MetS. On the other hand, in the same study, resistin was associated with WOMAC-function and total score, even after adjustment. A study from the same group could also find a significant connection of ghrelin synovial fluid levels and KL-score, as well as the Lequesne index (66). A study in 96 patients at various KOA stages could detect a positive correlation of leptin and KOA severity. This study also found a decreased leptin synovial fluid (SF) to plasma ratio with advanced disease (62). This association of SF leptin and pain was confirmed by others (116). Another study on symptomatic KOA patients detected an association of ghrelin levels with the WOMAC score, pain and dysfunction even after adjustment for age, sex and obesity. Interestingly, there was also a positive association of ghrelin and serum levels of MMP3 and 13, but not COMP or CTXI (69).

Patients enrolled in these studies were mostly graded 2-3 on the K-L scale, whereas in our project, only end-stage patients were enrolled. Unfortunately, due to the large homogeneity of KOA patients included in these studies (such as pooling of various phenotypes) and different scoring systems applied, more research is necessary for a definite conclusion.

5.2.3 Metabolomic insights

Metabolic analysis of OA biologic fluids has advanced knowledge of OA. In animal models, H1-NMR spectroscopy detected increased levels of pyruvate, lactate, fatty acids, glycerol and glucose, furthermore, elevated levels of N-acetyl-glycoproteins, acetate, creatine/creatinine and glutamine/glutamate levels, in comparison to normal controls. Interestingly, there was also an increase in alanine and isoleucine amino acid concentration (117,118). (72)

Similar findings were observed in human urine samples. NMR spectroscopy was able to easily discriminate healthy from OA in urine samples and the metabolite profile showed a high correlation with KL score (119). Integrating NMR spectroscopy and GC-MS 11 important metabolites could be identified that are involved mostly energy metabolism. (120) A combined MS-based targeted metabolic approach and liquid chromatographic tandem MS approach was further able to classify two distinct OA subgroups based on high acylcarnitine, but low carnitine levels. (121) This suggested an altered energy metabolism in one of the subgroups. (72)

Several studies have found an association of BCAA (including valine, leucine and isoleucine) to histidine ration on KOA (122). Elevated BCAA are also linked to metabolic syndrome and CV disease (123): Increased levels have been linked to insulin resistance and T2DM. These changes might represent a common pathway in metabolic disease and OA. In addition to BCAA metabolism,

several studies have detected derangements in arginine metabolism (124). Arginine levels were lower and this was highly discriminative compared to normal controls. This was also linked to poor NO availability. Despite this compelling evidence, we were not able to detect a metabolic pattern associated with obesity or the metabolic syndrome. However, we included end-stage KOA patients only, and there might have been significant heterogeneity with regard to OA phenotypes in our study. (72)

5.3 Obesity and cartilage degeneration

Obesity is a well known risk factor for the incidence, progression of and the disability caused by KOA. The exact mechanism that drives KOA in the context of obesity remains unclear and seems to be difficult to elucidate. The impact of mechanical loading is of significant importance in OA (125). Obesity causes increased dynamic mechanical loading during walking and BMI is correlated with increased absolute mechanical stress during gait (126) and external knee adduction moment (KAM) (127), a surrogate measure of loading of the medial compartment. KAM was found to be a risk factor in KOA progression (128). Furthermore, obesity is associated with changes in muscle function and can lead to muscle dysfunction and weakness (129) which is again a known risk factor for KOA progression. (125)

At the same time, countless studies have suggested a link between metabolic disorder and KOA. It is known, for example, that high glucose concentration can induce the expression of catabolic enzymes, such as MMP 1 and 13 in chondrocytes (82). Both metabolic disease and OA share findings of and are associated with markers of systemic low-grade inflammation (82,130). Systemic inflammation can lead to pro-inflammatory responses, including the release of cartilage degrading enzymes, such as MMPs and aggrecanase (130). One enzyme with a major role in cartilage degeneration is ADAMTS5: While it is known that expression of this enzyme is increased in OA compared to normal controls (131), for the first time we were able to show that obesity, measured as BMI, was associated with increased ADAMTS5 expression in OA chondrocytes.

This finding is indirectly supported by several studies that have found a clear association of cartilage defects and obesity: A systematic review of cartilage imaging studies concluded that there was consistent data showing a detrimental effect of BMI with regards to cartilage defects. However, only two of seven studies found a significant, negative association of BMI and cartilage volume (132). Another study indicated a significant, positive association with body weight (132). A study not included in this above mentioned review found a positive association of BMI and femoral groove cartilage thickness (133). We also detected a positive association of BMI and cartilage in our sonographic study. These variations might be explained by the fact that BMI does not describe body composition, as shown by the phenomenon of sarcopenic obesity (134). Adding to this, another study found that while there was a significant association of BMI and cartilage volume in univariate analysis, only fat-free mass remained positively associated with cartilage volume after adjustment for other factors (135). In our study, obese patients tended to be younger and thus it is possible that these patients also had more fat-free mass compared to their older, non-obese counterparts. This

might in turn explain a higher cartilage volume found in our study. More research is necessary to understand the effect of body composition on cartilage volume.

5.4 Metformin as a potential KOA treatment candidate

For the first time, we were able to demonstrate the effect of metformin on gene expression in primary human osteoarthritic chondrocytes: Metformin in supratherapeutic dose of 1mM (physiologic serum levels range in concentrations of 1µM, serum fluid concentrations are unknown), reduced the expression of the degenerative genes MMP1 and ADAMTS5. At the same time, the expression of SOX9 and HIF1α were also reduced. It is unclear how this would affect chondrocytes in vivo, but might be a sign of reduced oxygen consumption, and therefor less HIF1α expression.

These results are in agreement with and supported by the findings of many other laboratory and clinical studies: Metformin, derived from biguanide, acts via AMPK-dependent and -independent mechanisms. In an IL1β induced cell culture OA model, metformin was able to alleviate the effects of IL1β-induced pro-inflammatory cytokine expression, increased the proliferation of cells and caused an anti-apoptotic affect. It blocked the NF-κB pathway by activating AMPK (111). Quite similar findings were reported by a study on a surgical OA mouse model: After surgical induced OA and 8 weeks of intra-articular injection of metformin or placebo, metformin was able to restore levels of MMP13 and downregulated collagen 2 expression. In IL1β induced cultured OA model chondrocytes, metformin increased AMPKα activation and upregulated expression of SIRT1, thereby mitigating pro-apoptotic effects of IL1β and catabolism (136). This was found to be true for SIRT3 as well which was upregulated by metformin (112). In agreement, in a similar study (this time metformin was given orally), metformin caused a significant reduction in cartilage damage in surgically induced OA murine model. These protective effects were not observed in AMPKa-knock-out mice, indicating again, that this protective effect was mediated via this pathway (113). Another study concluded similarly that both intragastric, as well as intra-articular metformin application reduced pain levels, as well as MMP13 levels, while increasing collagen II levels in mice after surgically induced OA in comparison to saline application (137). Another similar study also supported above mentioned results, but also showed that metformin delayed ageing of cartilage, indicated by lower cell senescence markers. Furthermore, it was able to inhibit the mTOR pathway which plays a critical role in cell apoptosis (138). The change in MMP3 and 13 levels were mediated by the PINK1 kinase which also mediated autophagic elimination of damaged mitochondria (112). In co-culture of IL1β stimulated murine chondrocytes and metformin stimulated human adipose tissue derived mesenchymal stem cells, the latter inhibited expression of degenerative genes (including MMP1, MMP3 and MMP13) in the former, and increased expression

of TIMP 1 and -3 (139).

Data from clinical studies are also promising: A study analysing data from the large, prospective Osteoarthritis Initiative cohort showed that metformin users had lower cartilage volume loss (0.7% vs 1.6% per year) over a 4 year period, even after adjustment for several known risk factors. There was also a trend towards a reduction of total knee replacement surgery, this was not significant however (110). In a nationwide Taiwanese study on patients using metformin in combination treatment with COX2-inhibitors, compared to metformin naive patients, the combination treatment was associated with lower joint replacement surgery rates (114). A double-blinded, randomized controlled trial on 98 patients, comparing the COX2-inhibitor meloxicam alone with its combination with either metformin or pioglitazone, found that both forms of combination therapy significantly improved pain and functioning (on KOOS questionnaire) over a period of 12 weeks (140). Contrary to these findings, a study on a diabetic cohort from a primary care clinical database in the UK did not find that patients on metformin were protected from onset of OA. However, this study did not adjust for BMI, did not consider dosing and duration of metformin intake, and suffered from variability in the definition of OA in the involved practices (141).

While these results are promising, more randomized controlled trials are necessary to draw definite conclusions.

5.5 Limitations

Looking at the results of this thesis project, there are several limitations that need to be considered, in addition to those mentioned in the respective study: First, all three studies were designed as laboratory or pilot studies, no sample size calculations were conducted (due to the lack of previous evidence) and only a small sample size was included. Second, patients were not grouped based on OA phenotype and comorbidities were not considered with enough rigor which might have introduced heterogeneity in our population. Also the definition of obesity was based on the BMI which, as outlined before, cannot describe body composition in detail. Third, patients were not fasted when blood samples were taken which might have impacted the results of ELISA, Luminex® and NMR spectroscopy.

5.6 Future research

Recently, the focus in biomarker research has shifted to the identification of OA phenotypes (142) which seems to be of importance considering the large heterogeneity of patient samples across studies. Future studies need to account for the differences in KOA phenotypes. In order to get a better understanding of the effects of metformin, it would be interesting to conduct a large randomized controlled trial to evaluate the potentially protective effect of metformin in a prospective, clinical setting.

5.7 Conclusion

In this project, we evaluated the connection of obesity and KOA in terms of gene expression, biomarkers and cartilage thickness. We also assessed the therapeutic effect of metformin and the connection of serum biomarkers with cartilage thickness. We found that obesity was associated with increased expression of degrading enzymes, while, paradoxically, obese patients had higher cartilage thickness. We also showed that metformin was able to reduce the expression of degrading enzymes and we found a link of serum biomarkers and cartilage thickness. While these results are largely supported by other studies, our experiments need to be followed up with larger investigations to confirm our findings.

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7 Appendix

7.1 Timeline and Milestones