Inflammation:
a link between metabolic syndrome and osteoarthritis?

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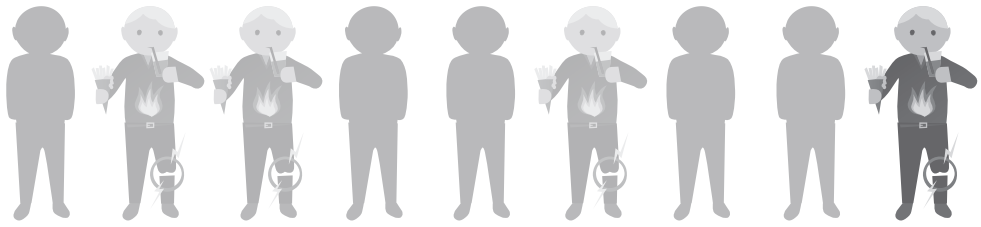
General introduction to osteoarthritis



1.1 Introduction

In 1743 William Hunter stated; From Hippocrates to the present age it is universally allowed that ulcerated cartilage is a troublesome thing and that once destroyed, is not repaired (1). The fact that destroyed cartilage is the main feature of the disease osteoarthritis (OA), makes it that researchers since decades are aiming to find the holy grail how to interfere in the pathogenesis of OA. In general, OA is described as a heterogeneous joint disease characterized by a progressive loss of cartilage.

1.1.1 Facts and figures

Worldwide it is estimated that 9.6% of men and 18% of women aged >60 years have symptomatic OA (2). However, these numbers should be interpreted with caution as the definition of OA is unclear and its onset is difficult to determine. Based on radiographs, OA in the hand joints is most frequently followed by knee and hip (3, 4). In general, OA is more prevalent in Europe and the USA than in other parts of the world (figure 1). OA is a major cause of impaired mobility and it belongs to the top ten of leading causes of burden of disease in high-income countries (5). In the Netherlands, most recent data from General Practices indicate that the prevalence of OA is 29/1000 for males and 50/1000 for females in the adult population (6).

1.1.2 Symptoms

Osteoarthritis is a heterogeneous disorder which is diagnosed based on symptoms, joint pathology or a combination of these two. Symptoms attributable to OA include pain, cracking (crepitus) and stiffness in the affected joints. The presence of osteophytes, joint space narrowing, sclerosis and altered shape of the bone end can be assessed, e.g. by using radiograph, and are in general classified on behalf of the Kellgren-Lawrence grading system (7). The most used diagnostic criteria were developed by the American College of Rheumatology (ACR) (8-10). These criteria include a combination of symptoms (pain) with radiographs.

1.1.3 Risk factors

Several risk factors for the development and progression of OA have been determined. The increase of age is one of the major contributors (figure 1) and is seen in all joints. After the age of 50, the prevalence and incidence of OA in the female gender is significantly greater than in men (11). Other risk factors which are frequently shown to increase OA occurrence are e.g. obesity, sex hormones, ethnicity and race, genetic predisposition and joint trauma (12). The mechanisms by which these risk factors contribute to the development and progression of OA are far from understood.

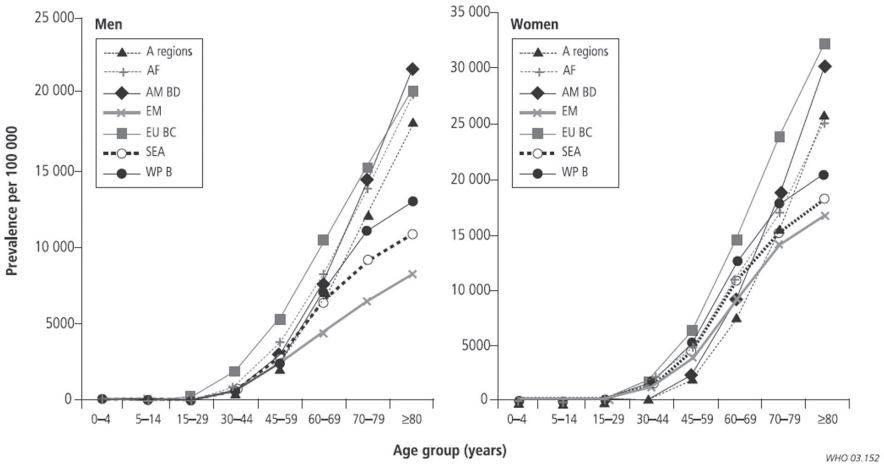


Figure 1. Prevalence of osteoarthritis of the knee, by age group, sex and region in 2000 (World Health Organization). A regions=developed countries in North America, Western Europe, Japan, Australia and New Zealand. AF=countries in sub-Saharan Africa. AM BD=developing countries in the Americas. EM=countries in the Eastern Mediterranean and North African regions. EU BC=developing countries in Europe. SEA=countries in Southeast Asia. WP B=countries in the Western Pacific region (2).

1.1.4 Problems and challenges

Existing therapies are primarily aimed to reduce pain and no cure is available which can interfere in the pathology of OA. Based on different risk factors (and mechanisms involved) and frequently observed differences in disease progression (between patients but also between joints) it becomes increasingly clear that OA is a disease which involves multiple tissues (13).

It is hypothesized that a variety of OA forms may exist that are similar with respect to outcome, however have a different underlying pathophysiological process. This may explain the variable outcomes of clinical trials, biomarker studies and genetic association studies, and therefore the difficulties observed when analyzing the efficacy of novel drugs. Without patient stratification, clinical trials may be 'contaminated' with patients that respond differently to interventions, which results in very large and costly clinical trials and prohibits the process of the development of new disease interfering therapies. To improve prediction of disease outcome, to optimize clinical trial efficiency and to analyze the efficacy of novel drugs, it is crucial to better understand the various mechanisms leading to the clinical outcome of OA.

Currently, OA management is directed toward patients in the latter phase of the disease. Although it is difficult to detect OA in an earlier stage, as radiographs are not sensitive enough, emphasis on early diagnosis and prevention could have more significance. Relevant biomarkers, objective measures that can be derived from body fluids such as blood or urine, are needed to diagnose and forecast OA in an earlier phase of the disease (14). To obtain representative biomarkers it is essential to better understand the role of local and systemic factors, which are involved in the pathogenesis of OA.

1.2 Local alterations; from cartilage to multiple tissue disease

OA has long been considered a wear and tear disease leading to loss of cartilage. During the past decades there have been significant developments in the scientific understanding of OA. These days OA is appreciated as a disease affecting the whole joint which involves complex interactions between several joint tissues (figure 2)(15, 16).

1.2.1 Cartilage

Articular cartilage is a highly specialized avascular connective tissue which provides smooth articulation and bending of the joints during movement. The extracellular matrix of cartilage consists mainly of collagen type II. Collagen type II provides a network in which other constituents, such as proteoglycans and chondrocytes, are

embedded. Collagens and proteoglycans give cartilage the capacity to absorb and distribute loads and to present a low-friction surface (18). Chondrocytes, the only cell type present in cartilage, have very low metabolic activity and are assumed to maintain the extracellular matrix by a low turnover replacement of matrix proteins. During OA development the chondrocytes become “activated”, a process which is characterized by cell proliferation and cluster formation. A disturbed equilibrium develops in which the rate of loss of collagens and proteoglycans goes beyond the rate of the deposition of newly synthesized molecules (19).

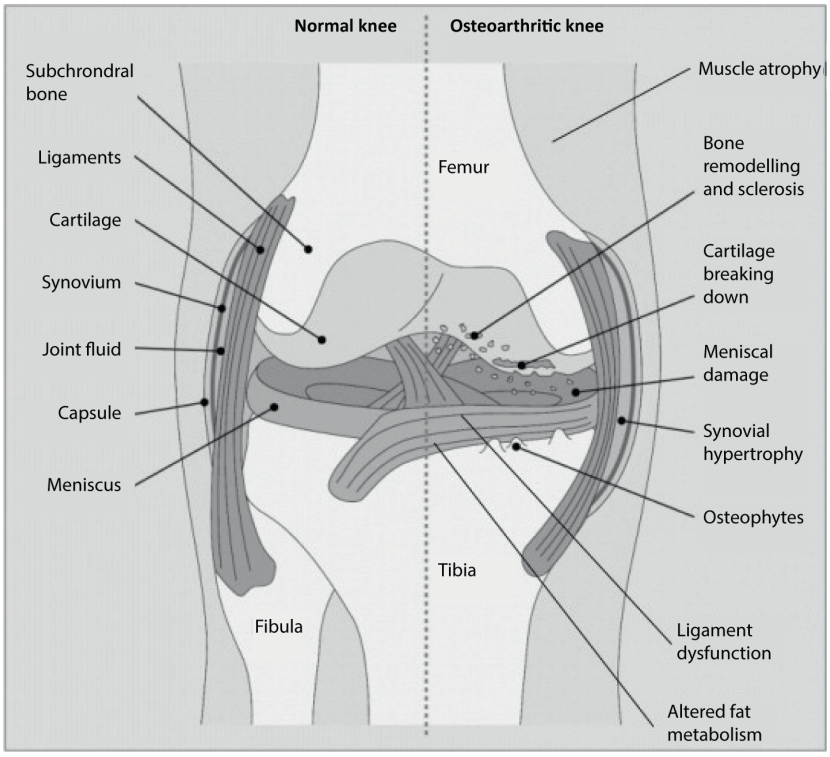


Figure 2. Schematic overview of the normal and OA knee joint depicting the joint tissues affected in osteoarthritis (17)

1.2.2 Synovial tissue

Synovial tissue is in direct contact with articular cartilage and primarily maintains the synovial cavity and synthesizes synovial fluid. Synovial fluid has a lubricating function and facilitates a smooth movement between joints. Under normal conditions, synovial tissue consists of only 1-2 cell layers of macrophage-like (type A) synoviocytes and fibroblast-like (FLS or type B) synoviocytes (20). The inflammation of the synovium (synovitis) has been shown to occur in a number of OA patients and may produce proteases and cytokines that contribute to the disease (21). It has been suggested that activated synovial macrophages play a key role in the processes leading to synovial inflammation. This inflammation may act as a trigger for several symptoms of OA via release of soluble mediators by synovial tissue, thus contributing to the breakdown of cartilage by promoting destruction and impairing the ability of repair (22).

1.2.3 Infrapatellar fat pad

The knee joint contains a special form of adipose tissue named the infrapatellar fat pad (IPFP), which until recently did not receive a lot of attention as a contributor to the OA process. IPFP is located intra-capsularly and extra-synovially in the knee joint, and is in close contact with synovial layers and articular cartilage. Its main role is to facilitate the distribution of synovial fluid and to absorb forces through the knee (23). Considering its location and with regard to OA as a multiple tissue disease it is likely that IPFP is also involved in the pathogenesis of OA (24). Several soluble mediators are locally produced in the knee joint by the IPFP (25, 26). However, the precise role of the IPFP still needs to be elucidated.

1.2.4 Bone, meniscus and ligaments

Subchondral bone, meniscus and ligaments are described to be involved in OA pathogenesis. During OA, bone remodeling takes place as a result of altered joint homeostasis. Hereby, new bone at the joint margins (osteophytes) is formed. In addition, there is evidence available suggesting a link between bone sclerosis and modifications in bone mineralization and the progression of OA. A role for transforming growth factor (TGF)-β, which for example is produced by synoviocytes, is appreciated

in the development of osteophytes (27, 28). Whether subchondral bone sclerosis precedes the onset of OA or is a change that occurs parallel to cartilage degradation is unknown (16, 29). Furthermore, a role for the meniscus and surrounding ligaments is proposed. Meniscal damage occurs in 63% of adults with symptomatic knee OA (30), and it was shown that it leads to a 7.4 times higher chance to develop radiographic knee OA 30 months later (31). These data suggest that bone and meniscus need to be incorporated in the search for new OA targets.

1.2.5 Local Inflammation

Although OA is conventionally not considered as an inflammatory disease, the production of several inflammatory soluble mediators by different tissues in the knee joint suggests that inflammation has a more important role in affecting cartilage homeostasis than originally thought. Until now the most studied cytokines in OA are interleukin (IL)-1β and tumor necrosis factor (TNF)-α. These cytokines are likely to be produced by articular chondrocytes, but synoviocytes may also very well be the source. In clinical trials attempting to block their activity, however, only minimal efficacy was found (32, 33). Both cytokines are mediated through the nuclear factor kappa B (NFκB) cascade, which is a pathway designated to have an important role in OA pathogenesis and a central regulator in catabolic actions in chondrocytes (34). Also, these cytokines are able to initiate other cytokines such as IL-8, IL-6, monocyte chemoattractant protein (MCP)-1 and RANTES (regulated on activation, normal T-cell expressed and secreted), which drive inflammation, inhibit matrix synthesis and promote cellular apoptosis by affecting, for example, aggrecanase (ADAMTS; a disintegrin and metalloproteinase with thrombospondin motifs) and collagenase activities (MMPs; matrix metalloproteinases) (22, 29, 35).

A special class of inflammatory mediators are oxylipins. These mediators are derived from fatty acids and can be found in all tissues in the body and may be interesting targets in relation to OA initiation and perpetuation. A balanced level of oxylipins is essential in maintaining joint homeostasis and alterations have widespread consequences. Prostaglandins are an example of such oxylipins and they have shown to be inhibited by non-steroidal anti-inflammatory drugs (NSAIDs), which leads to reduced OA pain. The precise role of other oxylipins, however, is unknown and merits more extensive research (36-38).

Exploring the role of the different oxylipins and soluble mediators involved and produced by several tissues in the joint, such as the synovial tissue and the IPFP, may lead to potential targets for disease-modifying interventions.

1.3 Systemic alterations; from mechanical to systemic disease

For several decades OA was regarded as a joint disease complicated by mechanical factors and age-related modifications. Limited attention to other factors influencing the disease was given. With the current knowledge, it is widely accepted that OA is more than only a mechanical disease and may be seen as a systemic disorder of multifactorial origin wherein genetic, environmental, hormonal and metabolic factors interact and contribute to OA pathogenesis (39). The metabolic syndrome comprises a profile including a combination of being obese, hypertension, dyslipidemia and impaired glucose tolerance (40). Systemic alterations may be induced by components of the metabolic syndrome. Recently, various studies presented a relation between OA and the prevalence of metabolic syndrome. For example, it was demonstrated that the prevalence of metabolic syndrome is over twofold higher in the OA population (41). In addition, having OA is associated with an over 5 times increased risk of having metabolic syndrome (42). These results have led investigators to contemplate common underlying pathologies in OA and metabolic syndrome related diseases (39, 43, 44).

1.3.1 Obesity

Obesity is an important and strong risk factor for OA and one of the components of the metabolic syndrome (45, 46). Obesity is becoming an increasing problem in the western world. In the Netherlands, 11 % of the adult population is obese (body mass index (BMI) >30) (47). For long, it was thought that the mechanical forces induced by obesity could explain the association between OA and obesity. However, it has been demonstrated that obesity is also associated with hand OA (48). As we do not walk on our hands, this suggests that systemic factors induced by obesity contribute considerably to the initiation and progression of OA (49). Obesity is associated with a mild chronic inflammation and the adipokines secreted by adipocytes and

macrophages within adipose tissue are suggested to be a metabolic link between obesity and OA (50). The relative contribution of these processes in the onset and progression of OA, however, remains unclear.

The association between obesity and the development of OA has been studied in several animal models. In the early 50's it was already discovered that mice receiving a high fat diet (HFD) developed features of OA twice as fast compared to mice fed a normal diet (figure 3) (51). In addition, mice of the STR/ort strain, which are susceptible to develop spontaneous obesity and certain aspects of the human metabolic syndrome, develop OA in a short period of time (52). Furthermore, it has been demonstrated that mice receiving HFD showed more OA cartilage degeneration than those fed a normal diet in a post-traumatic mouse model (53). A very HFD in mice induces OA, but when animals are placed on a wheel-running exercise plan progression of knee OA is inhibited without reduced body fat (54). Furthermore, leptin-deficient (ob/ob) and leptin receptor-deficient (db/db) mice, which have extremely high body weight on a normal diet, are protected from the development of OA (55), which excludes a role for mechanical factors. The fact that adipose tissue of mice can become inflamed under conditions of metabolic stress (e.g. HFD feeding) and secrete a broad spectrum of inflammatory mediators (56, 57), suggests that HFD-fed mice constitute a suitable model to study the role of (metabolic stress-induced) inflammation on the development of OA.

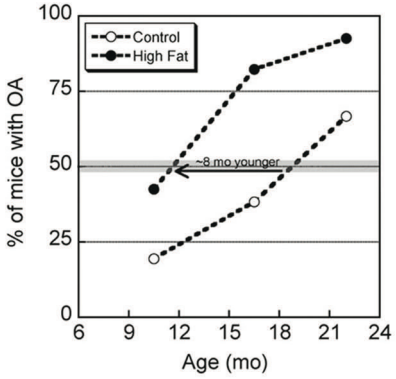


Figure 3. Male C57/Bl6 mice receiving a high fat diet developed osteoarthritis (OA) twice as fast as mice receiving a control diet. The similar slopes indicate that a high fat diet accelerates the onset but not the progression of OA (51).

1.3.2 Cholesterol

Hypercholesterolemia is associated with the metabolic syndrome. Increased levels of cholesterol in the plasma have shown to be associated with generalized OA (58). Furthermore, it has been demonstrated that genes regulating cholesterol efflux have a diminished expression in OA cartilage compared to normal cartilage (59). The underlying mechanism explaining the link between cholesterol and OA features are therefore very interesting. This is supported by the fact that atherosclerosis, which is an atheromatous vascular disease caused by elevated cholesterol intake, is independently associated with OA in women (60, 61). Whether atherosclerosis and OA have common underlying mechanisms or are causative for each other is uncertain.

With respect to animal models, the effects of hypercholesterolemia on OA development is difficult to examine, as wild type mice have a different lipoprotein metabolism compared to humans. The intake of a cholesterol-containing diet by wild type mice leads to elevated plasma cholesterol levels. However, as a result of high levels of anti-atherosclerotic high density lipoprotein (HDL) ('good' cholesterol) and low levels of very low (VL)DL and low (L)DL ('bad' cholesterol) no atherosclerosis develops. Experimental data on investigating the role of cholesterol, and the mechanism behind it in relation to OA development, are therefore scarce and more research needs to be done to elaborate on this association.

1.3.3 Systemic inflammation

The metabolic syndrome induces systemic inflammatory responses. This mechanism of action may also be held responsible for the association of metabolic syndrome with OA, either by a direct effect on articular cartilage, or by the modulation of several tissues in and around the joint. The precise role of inflammation in OA development is, as mentioned earlier, uncertain (chapter 1.2.4) (22, 62). Among the components of the metabolic syndrome, obesity was thought to be key initiator for OA. However, recently published epidemiologic data demonstrated that the metabolic syndrome rather than obesity in itself has the greatest impact on the severity of OA (63). OA donors showed several changed systemic mediators in their serum compared to those obtained from healthy persons (64, 65). Of these

mediators the adipokines, such as leptin, adiponectin, resistin and visfatin, have been extensively studied for their pro- and anti-inflammatory capacities in OA (50). It is found that serum adiponectin, leptin, and resistin concentrations are associated with OA severity and progression and with local synovial tissue inflammation (66-68). The effect of adipokines on the OA process is controversial. Leptin is thought to play a role in TGF-β activation (69) and, as mentioned, extremely obese leptin-impaired mice are protected from the development of OA (55). In addition, leptin induces collagen release from bovine cartilage explants and upregulates MMP-1 and MMP-13 in bovine chondrocytes (70). It has been demonstrated that adiponectin alters the balance by downregulating MMPs and upregulating TIMPs. However, it is also believed that it may act protective against OA by reducing the production of pro-inflammatory cytokines (71). Adiponectin-treated chondrocytes produce IL-6, MMP-3 and MMP-9 and this adipokine induces MMP-1 and IL-6 production in synovial fibroblasts (72, 73). A relatively newly discovered adipokine, visfatin, has been suggested as a promising target for treating OA. Visfatin synthesis is increased by IL-1β treatment in an *in vitro* culture of human chondrocytes. Furthermore, visfatin increases the synthesis and release of MMP-3, MMP-13, ADAMTS-4, and ADAMTS-5 and decreases aggrecan production in chondrocytes, suggesting a pro-inflammatory function in cartilage (74, 75). With regard to cholesterol, it is postulated that oxidized low density lipoprotein triggers pro-inflammatory cytokine production (76). It should be noted that these mediators are not solely originating from the blood and that synovial tissue (21) and IPFP (24) secrete a number of these inflammatory factors as well, which may contribute to OA. Whether targeting the inflammatory response induced by components of the metabolic syndrome, such as adipokines, will reduce OA development is unknown. The integration of knowledge of the metabolic syndrome and related diseases in OA research provides new leads to tackle the underlying mechanisms responsible for OA pathogenesis.

1.4 Outline of the thesis

The aim of this thesis is to provide insight in the role of multiple local and systemic factors contributing to the pathogenesis of OA. With the current knowledge, more advanced technologies, and novel animal models, we should be able to shed a different light on the tissues involved in OA pathogenesis. Hopefully, this will ultimately lead to the identification of mechanisms that provide targets for disease-modifying therapies and novel biomarkers to detect OA in an earlier phase. With respect to the future, we try to contribute to a rationale for a better stratification of OA patients and consequently to more personalized medicine.

The first three chapters include a description of local factors that possibly contribute to OA development. By investigating the secretion of several mediators by synovial tissue, IPFP and synovial fluid, a profile of OA and normal (non-OA) donors is provided.

In **Chapter 2** we aimed to get insight in the role of synovial tissue. We hypothesized that synovial tissue derived from end-stage OA patients is more inflamed than that from normal donors (21). The synovial tissue of OA donors may therefore secrete a wide array of factors that can alter cartilage degradation, which is not the case for synovial tissues derived from normal donors. To test this hypothesis, we used a multiplex approach (an advanced technology including multiplex bead-based immunoassays (77)). In addition, the ability of OA and normal synovial tissue to initiate degeneration of healthy cartilage was assessed by culturing synovial tissues and healthy cartilage explants in a complex *in vitro* co-culture transwell system.

The purpose of **Chapter 3** was to explore the role of IPFP in OA. Oxylipins, important signaling molecules involved in the modulation of inflammatory responses (78, 79), were detected using a mass spectrometry method to get an overview of what is secreted by the IPFP. Furthermore, differences in IPFP secretion between OA and normal donors were evaluated.

We profiled synovial fluid samples as representatives of the secretion of soluble mediators by different tissues involved in the joint (**Chapter 4).** Again we used a multiplex approach to get a wide and comprehensive overview of OA and normal donors. Data were further explored using multi-variate statistics to identify clusters of interrelated mediators.

Besides the involvement of local factors we examined the development of OA with regard to alterations on a systemic level. Considering the hypothesis that obesity might lead to metabolic stress, which possibly contributes to the development of OA (62), we used a HFD-induced OA mouse model which is described in **Chapter 5**. More specifically, we used the human C-reactive protein (hCRP) transgenic mice (80). CRP is an acute phase protein and an established marker for systemic inflammation in humans (81). Consequently, we were able to monitor the inflammatory state of the animals, using a human marker, during the development of OA. To perceive whether medicines applied for other targets in the metabolic syndrome were able to counteract OA-related changes, we included a prophylactic intervention with statins (cholesterol-lowering drug) and a peroxisome proliferator activated receptor (PPAR) agonist (anti-diabetic drug).

In **Chapter 6** we emphasized on lipid and systemic alterations induced by elevated cholesterol intake instead of HFD. We investigated whether a cholesterol-containing diet and, consequently, the development of atherosclerosis was sufficient to induce OA in APOE*3 Leiden.CETP transgenic mice. This is a well-established model for hyperlipidemia and atherosclerosis and resembles the human lipoprotein metabolism in contrary to wild type mice (82-84). Furthermore, we included cholesterol-lowering interventions, with a statin and ezetimibe, in groups receiving a high cholesterol diet, to assess their effects on OA development. Both drugs diminish cholesterol levels to a same extent but have a different mode of action, which allowed us to investigate possible additional effects of statin beyond its cholesterol-lowering capacities.

To elaborate more on the outcomes of chapters 5 and 6, in **Chapter 7** we extrapolated the HFD-induced OA mouse model to the APOE*3 Leiden.CETP mouse. We included an early ('prophylactic') and late ('therapeutic') intervention with a statin. Furthermore, we included a fenofibrate (cholesterol-lowering drug) intervention, since another study indicated that this type of drug has beneficial effects on OA development. To gain more insight into the mechanisms behind the HFD-induced OA model, the effect of a caspase-1 inhibitor, responsible for the conversion of pro-IL-1 to IL-1 (one of the key cytokines believed to be involved in OA), was studied. In addition, a group that started on HFD, but returned to control chow diet halfway the study, was included to evaluate the effect of a diet switch on OA development.

In the final chapters, **Chapter 8** and **Chapter 9** (in Dutch), a comprehensive overview of the performed work is given and discussed.

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2

Profiling the secretion of soluble mediators by end stage osteoarthritis synovial tissue explants reveals a reduced responsiveness to an inflammatory trigger

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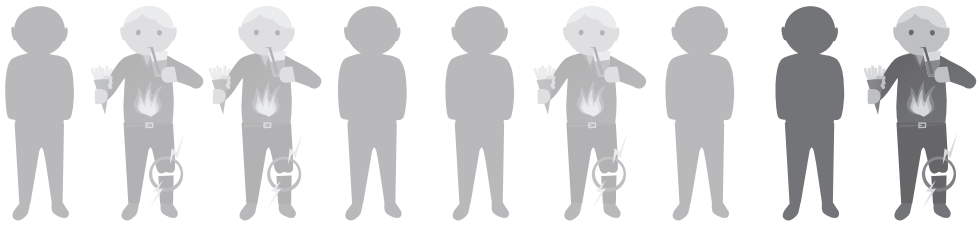

Abstract

Objective: Evidence is accumulating that synovial tissue plays an active role in osteoarthritis (OA), however, exact understanding of its contribution is lacking. In order to further elucidate its role in the OA process, we aimed to identify the secretion pattern of soluble mediators by synovial tissue and to assess its ability to initiate cartilage degeneration.

Methods: Synovial tissue explants (STEs) obtained from donors without history of OA (n=8) or from end stage OA patients (n=16) were cultured alone or together with bovine cartilage explants in the absence or presence of IL-1α. The secretion of 48 soluble mediators was measured and the effect on glycosaminoglycan (GAG) release and matrix metalloproteinase (MMP) activity was determined.

Results: Normal and OA STEs secreted comparable levels of almost all measured soluble mediators. However, in the presence of IL-1α these mediators were less secreted by OA than by normal STEs of which 15 differed significantly ($p<0.01$). No effect of normal or OA STEs on GAG release from the cartilage explants was observed, and no differences in MMP activity between OA and normal STEs were detected.

Conclusions: Unexpectedly, a comparable secretion profile of soluble mediators was found for OA and normal STEs while the reduced responsiveness of OA STEs to an inflammatory trigger indicates a different state of this tissue in OA patients. The effects could be the result of prolonged exposure to an inflammatory environment in OA development. Further understanding of the pro-inflammatory and inflammation resolving mechanisms during disease progression in synovial tissue may provide valuable targets for therapy in the future.

Osteoarthritis (OA) is one of the most frequently occurring rheumatic diseases. In Western populations OA is by far the most common form of joint disease, causing pain, loss of function and disability (1). The main characteristic of the disease is progressive loss of articular cartilage, which is thought to be due to an imbalanced interplay between anabolic, anti-catabolic, anti- and pro-inflammatory and anti- and pro-apoptotic activities (2, 3). Numerous risk factors for OA have been identified, however, the exact etiology, pathogenesis and progression of this disease have yet to be determined (4). As a consequence of the limited understanding of the disease complexity, no disease modifying treatments are currently available. The only existing therapeutic strategies are primarily aimed at reducing pain and improving joint function.

Traditionally, research on knee OA has been focused on cartilage degradation. Nowadays, however, it is generally accepted that the entire joint organ including synovium, synovial fluid, bone and infrapatellar fat pad can contribute to the disease (5). Inflammation, classically seen as a characteristic for rheumatoid arthritis (RA), has lately also been recognized for its role in OA development (6, 7). Inflammation of the synovium (synovitis) has been shown to occur in a number of knee OA patients (8) and may produce proteases and cytokines that contribute to the disease. However, its role in the onset and progression in OA has yet to be elucidated.

It has been suggested that activated synovial macrophages might play a key role in the processes leading to synovial inflammation. This inflammation may act as a trigger for several symptoms of OA via release of soluble mediators by synovium, thus contributing to the breakdown of cartilage by promoting destruction and impairing the ability of repair (6). The cytokines interleukin (IL)-1β and tumor necrosis factor (TNF)-α are likely to be one of those soluble mediators (9). The understanding of the role of synovium and inflammation in the process of OA development could provide leads for new targets for OA treatment.

Based on published literature we hypothesized that synovium from OA patients is more inflamed than synovium of normal donors and therefore plays an active role in the breakdown of cartilage. To provide insight in this hypothesis, synovial tissue explants (STEs) of normal donors and OA patients were collected and analyzed for the spontaneous secretion of soluble mediators as well as their response to a pro-

inflammatory trigger which was expected to be more increased in OA STEs than in normal STEs. We also used a complex *in vitro* co-culture model in which STEs were cultured together with cartilage explants in a Transwell system to assess the ability of STEs to initiate degeneration of healthy cartilage.

Methods

STEs collection

Synovium of the knee was obtained from *post-mortem* material of 8 donors with macroscopically healthy cartilage and no history of OA (normal STE), or from material obtained during joint replacement surgery of 16 OA patients (OA STE) (Articular Engineering, Northbrook, USA). Human tissues were obtained according to legal and ethical requirements approved by the institutional review board of the University of Pennsylvania including anonymous informed written consent from the donor or nearest relative. Normal donors' age ranged from 18 – 67 years and body mass index (BMI) from 19 – 37. OA donors' age ranged from 43 – 70 and BMI from 18 – 47. Within 24 hours, synovial tissue was carefully excised from surrounding fat. Explants of 3 mm in size and a weight of 22 ± 3.5 mg were placed in a 48-wells plate in Iscove's Modified Dulbecco's Medium (IMDM, Invitrogen, Paisley, UK) containing 10% v/v fetal calf serum (FBS) (GibcoBRL, Invitrogen) and 1% v/v of 10,000 units/ml Penicillin:10,000 units/ml streptomycin (Penstrep) (Biowhittaker, Verviers, Belgium). STEs were placed at 4°C until the start of the experiment. STEs were placed for 1 hour at 37°C in a humidified atmosphere of 5% CO_2 in air before the start of the experiment.

Immunohistochemistry of STEs

From each donor one STE was directly frozen in Tissue Tek O.C.T. compound (Sakura Finetek, Zoeterwoude, the Netherlands) and cut into 5 µm slices using a cryotome. Samples were thawed for 30 minutes and blocked with PBS containing 5% bovine serum albumin (BSA) (Sigma-Aldrich, St. Louis, USA). Sections were incubated overnight at 4°C with antibody CD68-biotin (mouse IgG2b 1:750) (E-bioscience, San Diego, USA). The next day sections were washed with PBS and blocked for 5 minutes

with peroxidase (Dako, Heverlee, Belgium). After a final wash step in PBS, sections were incubated with Novared for 10 minutes and counterstained with heamatoxylin. As a negative control an isotype matched control antibody was used (IgG2b-biotin, BD).

STEs culture

As a reflection of the whole synovium, for each condition 6 STEs from different anatomical locations in the joint per donor were cultured in a 24 wells plate in 700 µl Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen) supplemented with Insulin-transferrin-sodium selenite (ITS) (Roche Diagnostics, Basel, Switzerland), 1% v/v Penstrep, 1 mg/ml lactalbumin and 5 µg/ml vitamin C (Sigma-Aldrich) (serum free medium) and were incubated with or without 10 ng/ml IL-1α (Peprotech, Rocky Hill, USA). After 3 and 5 days 350 µl medium was refreshed and stored. After 7 days all medium was collected. Pooled supernatant samples were made representing the average of 6 different STEs per donor and the production over a time period of 7 days. All samples were stored at -80 °C.

Multiplex ELISA assay

By using 2 commercial kits (42- human cytokine-plex and 11-human adipokine-plex, catalogue number: MPXHCYTO60KPMX42 and HADCYT-61K-11, Millipore, Billerica, USA) a wide panel of soluble mediators was measured in pooled supernatants of the STEs cultured alone. These assays were performed with Luminex xMap Technology (Qiagen, Billerica, USA) according to the manufacturer's instructions. Liquichip analyzer software (Qiagen) was used for data analysis.

Preparation of cartilage explants

Due to the availability and heterogeneity of human cartilage explants, standardization for *in vitro* models is difficult and therefore we used bovine cartilage explants (10-12). Cartilage from the metacarpophanlangeal joints of 6 months old calves was obtained on the day of slaughter. Permission of the slaughterhouse (Ton Boer en zn., Nieuwerkerk a/d IJssel, the Netherlands) to use these joints in this experiment was given. Joints were aseptically opened and cartilage explants were obtained by using

a biopsy punch of 4 mm. The cartilage explants were cultured in serum free medium overnight at 37°C in a humidified atmosphere of 5% CO_2 in air.

STE-cartilage co-culture

Co-culturing of cartilage explants and STEs was performed in a 24-wells polycarbonate Transwell system with a pore size of 0.4 µm (Corning Incorporated, NY, USA). Each well contained a cartilage punch in the lower compartment and a STE in the upper compartment in 700 µl serum free medium with or without 10 ng/ml human IL-1α. Six STEs per donor per condition were used. As a control, cartilage was cultured alone. After 3 and 5 days 350 µl medium was refreshed and stored. After 7 days all medium was collected. Pooled supernatant samples were made representing the average of 6 different STEs per donor and the production over a time period of 7 days. All samples were stored at -80°C.

Analysis of glycosaminoglycans (GAGs)

Cartilage explants were digested 24 hours at 56°C in 3% v/v papain from papaya latex, 5 mM cysteine HCL, 50 mM EDTA, and 0.1 M sodium acetate (pH 5.53) (Sigma-Aldrich). The amount of glycosaminoglycans (GAGs), reflecting the amount of proteoglycans, was determined in cartilage explants as well as in culture medium using a commercial kit (Biocolor Ltd, Belfast, N. Ireland). The total GAG content was calculated by summing the amount of GAGs in the cartilage explant and the culture medium. Proteoglycan degradation was expressed as the percentage of GAG release into the medium compared to the total GAG content.

Analysis of matrix metalloproteinase (MMP) activity

Secreted MMP activity was assessed in the culture medium using a fluorogenic substrate as described previously (13). The secreted MMP activity was calculated by determining the difference in substrate conversion in the presence or absence of MMP inhibitor BB94 (10 µM). This approach detects only MMP-mediated substrate conversion and reflects the MMP activity in the culture conditions.

Data analysis

Statistical analyses were performed using SPSS software version 17.0. Individual differences were tested by non-parametric Mann-Whitney tests. For comparisons of normal and OA STEs in the co-culture conditions, the GAG release of the cartilage explants were subtracted from the co-culture condition to correct for differences in bovine donors (Δ). Differences were considered statistically significant at $p<0.01$ for the Multiplex Elisa data. This cut-off was chosen to reduce the chance of false-positives, since a large number of variables were tested in a relative small number of donors due to the uniqueness of this material. For GAG release and MMP activity significance levels were set at $p<0.05$.

Results

Secretion of soluble mediators by STEs

To assess differences between normal and OA STEs 48 soluble mediators were measured in the pooled supernatants. Without IL-1α stimulation, only 5 soluble mediators were secreted at significantly different levels ($p<0.01$). Remarkably, these mediators were excreted at a lower level by OA STEs than normal STEs (Figure 1A). Under pro-inflammatory (IL-1α-stimulated) conditions 15 of the measured mediators were significantly less secreted by OA STEs than by normal STEs ($p<0.01$). Of these 15 mediators, 4 were also significantly different in the absence of IL-1α (Figure 1B). IL-13, IL-17, IL-2, IL-4, IL-9, PDGF-AB, TGFα, TNF-β and IL-12p70 could not be detected in either condition.

To assess the response to an inflammatory trigger of STEs from normal and OA donors, soluble mediator excretion levels were analyzed in both the non-stimulated and the IL-1α-stimulated condition. For normal STEs, 16 mediators increased significantly ($p<0.01$) after stimulation with IL-1α (Figure 2A). Unexpectedly, the STEs obtained from OA donors secreted only 3 mediators at a significantly different level after IL-1α stimulation (Figure 2B). An overview of all measured mediators in these supernatants can be consulted in table S1.

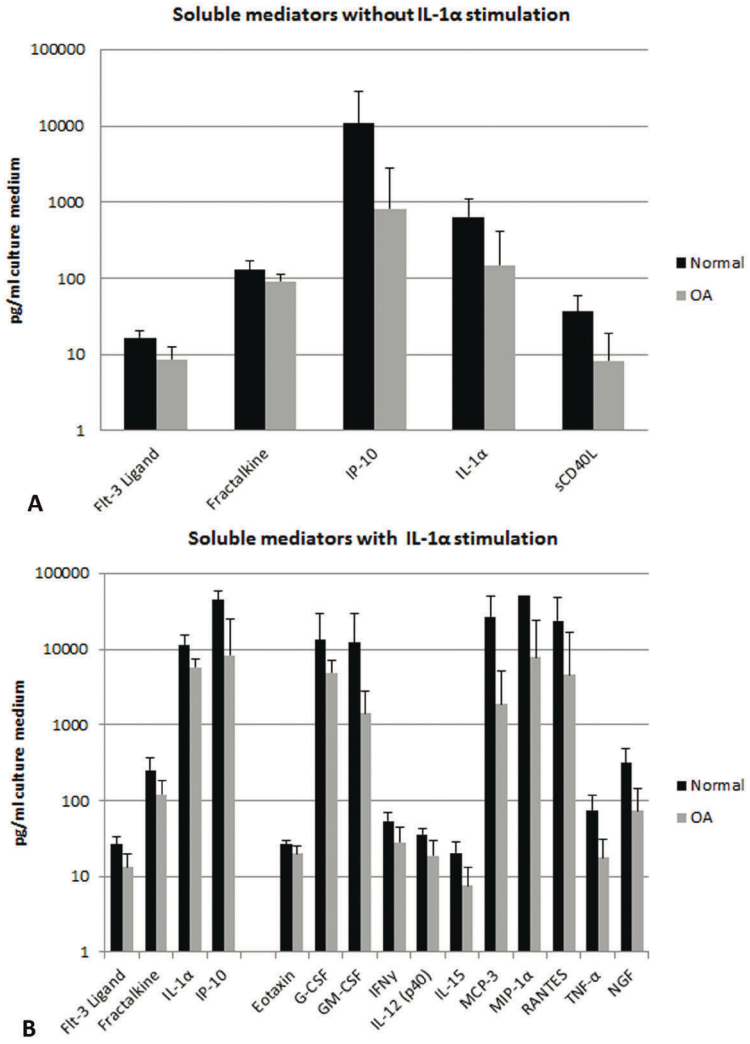

Figure 1. Soluble mediator secretion by normal and OA synovial tissue explants (STEs) with or without IL-1α. Graphs demonstrate all soluble mediators which were significantly ($p<0.01$) different between **A**. normal (black bars) and OA (grey bars) STEs and **B.** normal and OA STEs under pro-inflammatory (IL-1α) conditions. Data were obtained from pooled supernatants representing the average of 6 STEs per donor and the production in a time period of 7 days. Data are plotted on a log scale. Bars indicate mean concentration (pg/ml culture medium) ± SD.

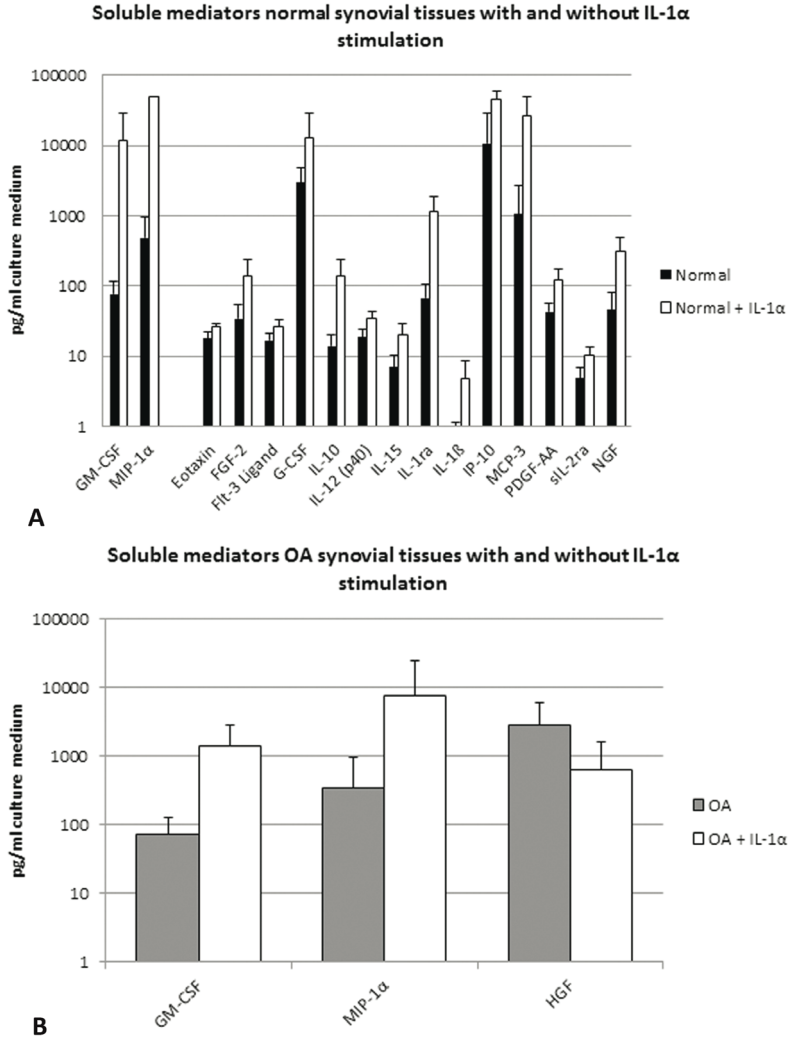

Figure 2. Soluble mediator secretion by non-stimulated and stimulated synovial tissue explants (STEs) from normal and OA donors. Graphs demonstrate the absolute levels of all soluble mediators, which were significantly ($p<0.01$) different between **A**. non-stimulated (black bars) and IL-1α-stimulated (white bars) normal STEs and **B.** non-stimulated (grey bars) and IL-1α-stimulated (white bars) OA STEs. Data were obtained from pooled supernatants representing the average of 6 STEs per donor and the production in a time period of 7 days. Data are plotted on a log scale. Bars indicate mean concentration (pg/ml culture medium) ± SD.

Immunohistochemistry on one STE from each donor showed well known characteristics of OA such as hyperplasia as observed by an increase in cell number and thickening of the synovial layer. CD68+ cells were present in both OA and normal STEs, indicating the presence of synovial macrophages during the culture period (Figure S1).

Co-culture effects on GAG-release and MMP-activity in cartilage explants

Based on multiplex analysis we questioned if there was also a different effect of normal and OA STEs on cartilage degradation. Hereto, we used an *in vitro* co-culture model in which cartilage explants were cultured together with STEs. Co-culturing with normal or OA STEs did not induce additional GAG release above the basal release of cartilage explants in non- and IL-1α-stimulated culture conditions (Figure 3). IL-1α stimulation led to significantly more GAG release in all culture conditions (table 1). When comparing absolute values, normal and OA donors differed significantly, however, this was due to a significant difference in basal release of the cartilage explants and not for additional GAG release induced by STEs (indicated in table 1 by the calculated delta (Δ)).

Table 1. Percentage GAG release from cartilage explants cultured alone or together with STEs under non-stimulated and IL-1α-stimulated conditions.

Condition	% GAG release				P-values			
	Normal	Normal (IL-1α)	OA	OA (IL-1α)	Normal vs Normal (IL-1α)	OA vs OA (IL-1α)	Normal vs OA	Normal (IL-1α) vs OA (IL-1α)
Cartilage	18.6 (2.6)	43.1 (9.3)	21.5 (3.4)	54.9 (4.7)	0.002	0.000	0.026	0.005
STE + cartilage	18.2 (2.5)	45.1 (8.8)	19.6 (3.5)	54.8 (8.1)	0.002	0.000	0.245	0.032
Δ STE + cartilage	-0.32 (2.9)	1.95 (2.5)	-1.9 (3.0)	-0.15 (6.4)	0.180	0.576	0.245	0.267

%GAG release is indicated as mean (SD).
Δ STE + cartilage: corrected value for GAG release in the cartilage explant.

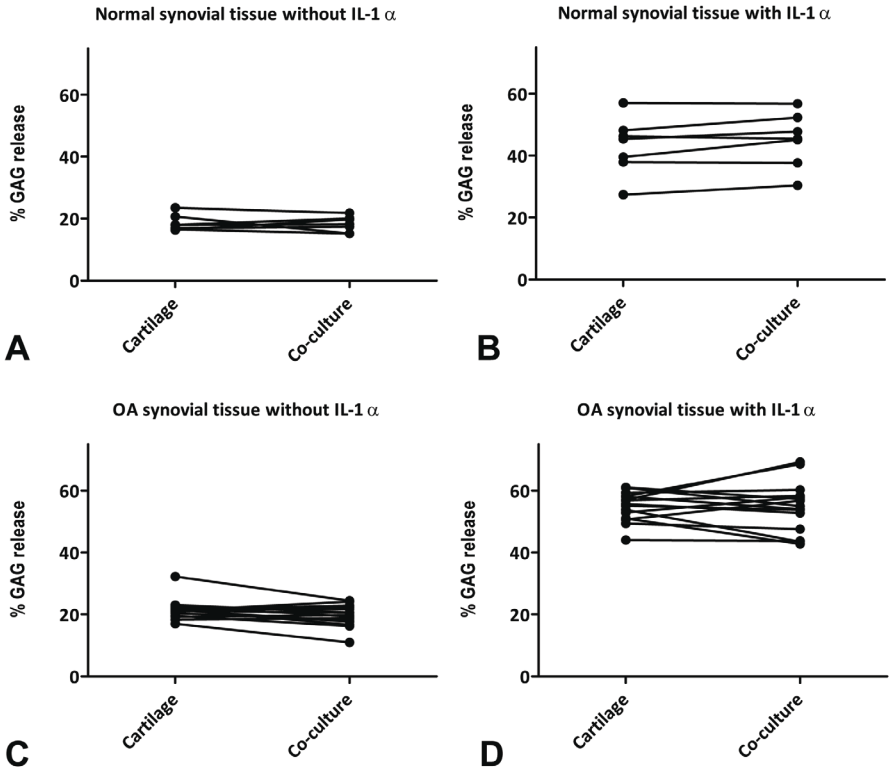

Figure 3. Proteoglycan degradation of healthy cartilage explants cultured alone or together with synovial tissue explants (STEs). Proteoglycan degradation was expressed as the percentage of glycosaminoglycans (GAG) released into the medium during 7 days of culture of cartilage alone or co-cultured together with normal **(A, B)** or OA **(C, D)** STEs without **(A, C)** or with **(B, D)** IL-1α stimulation. Each line represents an individual donor and connects the % GAG release of cartilage alone (left) with the matching co-culture condition of cartilage together with STEs (right). There were no significant differences between cartilage cultured alone or co-cultured with normal or OA STEs.

To assess for collagen degrading enzymes, MMP activity was measured in the different culture conditions. Remarkably, the MMP activity in the medium of non-stimulated normal and OA STEs cultured alone was significantly higher than in the co-cultures ($p<0.01$, Figure 4A,C). This was also observed for the IL-1α-stimulated OA condition (Figure 4D; $p<0.05$). A trend was observed in the IL-1α-stimulated normal condition (Figure 4B; $p<0.1$). IL-1α stimulation did not led to significantly more MMP activity when normal or OA STE was cultured alone (table 2). IL-1α stimulation yielded

significantly more MMP activity in the co-cultures with OA STE while this was not the case for normal STE culture conditions. This is probably due to the fact that bovine cartilage donors used in the OA STE conditions were more prone to IL-1α stimulation with respect to MMP activity than those used in normal STE conditions (table 2).

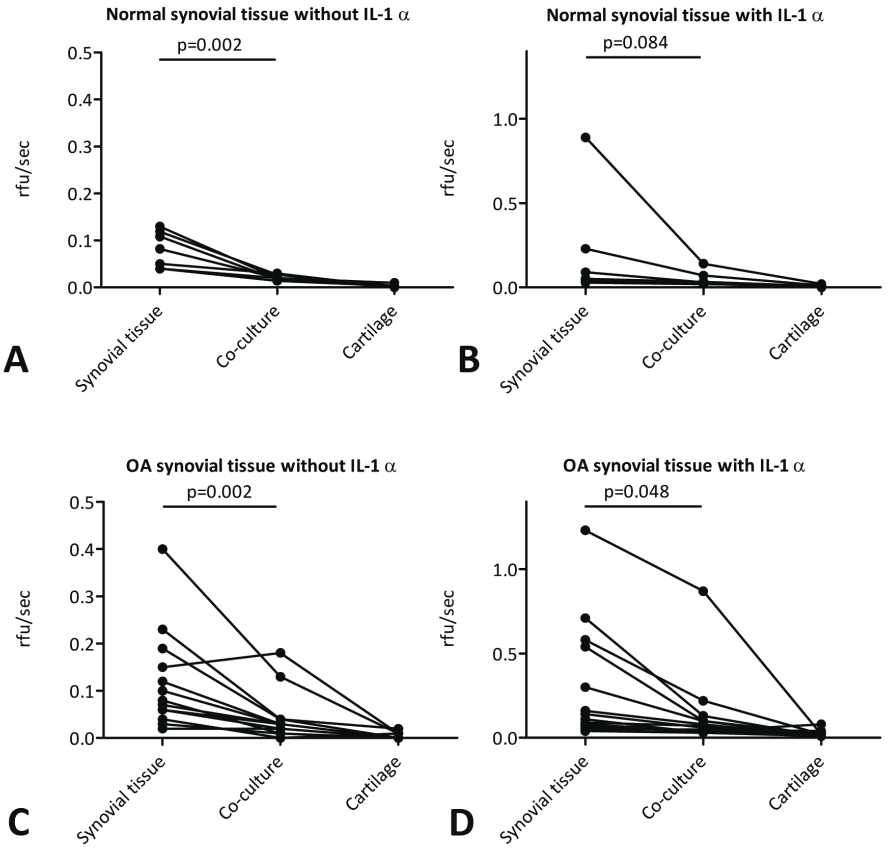

Figure 4. Secreted MMP activity of STEs and cartilage explants cultured alone or together. After 7 days, the MMP-activity (rfu/sec) was determined (in culture medium) of normal **(A, B)** and OA **(C, D)** STEs and healthy cartilage explants, cultured alone or together, without **(A, C)** or with **(B, D)** IL-1α stimulation. Each line represents an individual donor and demonstrates the activity of STEs alone (left), STEs together with cartilage explants (middle) or cartilage explants cultured alone (right). The MMP activity in the medium of STEs cultured alone was higher, compared to the MMP activity in the co-culture conditions. Significance levels are indicated.

Table 2. Secreted MMP activity (rfu/sec) by STE and cartilage alone and by the co-culture of STE and cartilage under non-stimulated and Il-1α-stimulated conditions.

Condition	Secreted MMP-activity (rfu/sec)				P-values			
	Normal	Normal (IL-1α)	OA	OA (IL-1α)	Normal vs Normal (IL-1α)	OA vs OA (IL-1α)	Normal vs OA	Normal (IL-1α) vs OA (IL-1α)
Cartilage	0.003 (0.003)	0.009 (0.007)	0.003 (0.006)	0.023 (0.018)	0.170	0.000	0.232	0.007
STE	0.081 (0.039)	0.196 (0.314)	0.115 (0.098)	0.280 (0.343)	0.898	0.245	0.596	0.244
STE + cartilage	0.021 (0.006)	0.047 (0.045)	0.042 (0.048)	0.126 (0.212)	0.174	0.004	0.121	0.061

Secreted MMP activity is indicated as mean (SD)

Discussion

Increasing evidence indicates that inflammation of the synovium is a feature of OA associated with the progression of disease (6). It remains, however, unclear in which way alterations in the synovium contribute to degenerative processes in the cartilage. By measuring the secretion of soluble mediators by the synovium and investigate its effect on cartilage break-down, we hoped to identify new mechanisms in OA and to provide new targets to intervene in the disease process. Unexpectedly, STEs from end stage OA patients showed comparable secretion levels as normal STEs for a wide panel of soluble mediators, suggesting that the inflammatory state of end stage OA-derived synovium is not different from normal. Differences between normal and end-stage OA STEs appeared after stimulation with IL-1α showing a diminished responsiveness of OA compared to normal STEs. Furthermore, STEs derived from end stage OA patients did not induce more GAG release from healthy cartilage explants or displayed more MMP activity than normal STEs in the presence or absence of a pro-inflammatory trigger. This suggests that synovium from end stage OA patients is not, or no longer capable of initiating cartilage degradation.

With regard to OA, the pro-inflammatory cytokines IL-1β and TNFα are most frequently studied and detected. Furthermore IFN-γ, IL-2, IL-4, IL-6, IL-8, TGFβ and IL-10 have received attention as important cytokines in the OA process (14-17). Of these soluble mediators all, except IL-2, were secreted by STEs. Unexpectedly, IL-

1α, one of the key cytokines in the pathogenesis of OA (18), was significantly less secreted by end stage OA STEs than by normal STEs.

The unexpected effects in mediator release and on GAG release seen in our study could be explained by the state of the STEs. All OA STEs were obtained from OA patients undergoing joint replacement surgery and are therefore in the end stage of the disease. Originally, it was thought that the condition of the synovium was correlated with OA severity and occurred gradually through the disease process (19, 20). However, Roemer et al. showed that baseline joint effusion and synovitis predicted the risk of cartilage loss after 30 months follow up (21). This indicates an important role for inflammation in an earlier stage of OA. Furthermore, Benito et al. demonstrated that early OA synovium has more features of inflammation (greater cell infiltration and overexpression of inflammatory mediators) than late OA synovium (8). In line with this, Ning et al. found a decreased expression of MMP-1, COX-2 and IL-1β expression in synovium depending upon the severity of OA (17). These previous observations are in agreement with our result that end stage OA STEs display an excretion profile that is rather similar to that of normal controls. The occurrence of inflammation resolving mechanisms in end stage OA STEs due to exposure to inflammatory triggers earlier in life might explain this observation. This is substantiated by its diminished responsiveness to a pro-inflammatory trigger. Potential molecular mechanism would be the down regulation of many receptors and a decreased secretion of resolving mediators to counter balance chronic inflammation. The fact that MMP activity seemed to be down regulated when co-cultured with cartilage explants also contributes to this idea. Furthermore, end-stage OA STEs were not able to induce cartilage degeneration in a co-culture model which endorses the idea that synovium is not capable of initiating cartilage degradation. However, it does not rule out the possibility that it plays a role in progression and aggravation of the cartilage destruction process in OA, since we have not tested OA and normal STEs on OA-derived cartilage.

Beekhuizen et al. have demonstrated in a comparable *in vitro* co-culture model with matched OA cartilage and OA STEs an effect on GAG production, but not on GAG release (22). Due to our design, we were not able to determine GAG synthesis by, for example, the rate of sulphate incorporation. However, based on total GAG content (data not shown) we do not expect an effect of OA or normal STEs on GAG synthesis.

Remarkably, it seems that IL-1α is not involved in the activation of STEs leading to cartilage destruction, since no increase in GAG release was observed even though numerous soluble mediators were elevated. The effect of the IL-1α trigger on GAG release and MMP activity can be subscribed to its direct effect on chondrocytes without any contribution of IL-1α- stimulated STEs. However, other explanations could be that IL-1α overrules the effect of the secreted soluble mediators or not all human mediators excreted by STEs cross-react with bovine receptors.

Minor points of the study need to be addressed. As the excretion of soluble mediators is not corrected for the amount of cells, the absolute levels can be discussed. To reach feasible standardization in this experiment the results were based on multiple explants per donor with comparable weights. Furthermore, the presence of small pieces of other tissues as well as the injury response at collection cannot be fully excluded. At last, we cannot exclude that hypoxia in *post mortem* obtained normal STEs influenced the outcome of the study although hypoxia inducible factor-driven VEGF levels were not different between normal and OA STEs (23, 24). Due to the fact that the procedures for OA and normal STEs were standardized we expect that the observed differences include comparable limitations.

In summary, we found the excretion profile of OA STEs to be comparable to that of normal donors. This is in contrast to what we had anticipated, namely more inflamed tissue with elevated secretion of pro-inflammatory mediators and MMPs. As such it is understandable that no contribution of the OA STEs was observed on cartilage destruction in the co-culture model. The responsiveness of the STEs to a pro-inflammatory trigger, however, indicates that there are differences in the state of the tissue between OA and normal donors. It suggests that end stage OA STEs are less sensitive to inflammatory stimulation which might be due to prolonged exposure to an inflammatory environment during disease progression. However, this does not rule out the involvement of STEs in an earlier phase of OA development. Further understanding of the pro-inflammatory and inflammation resolving mechanisms during disease progression in synovium may provide valuable targets for therapy in the future.

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Supplementary information

Table S1. Mean (SD) levels (pg/ml culture medium) of soluble mediators excreted by normal and OA synovial tissue explants without (Normal and OA) and with IL-1α stimulation (Normal (IL-1α) and OA (IL-1α)).

	Mean (SD) Normal	Mean (SD) OA	Mean (SD) Normal (IL-1α)	Mean (SD) OA (IL-1α)	p-value Normal vs OA	p-value Normal (IL-1α) vs OA (IL-1α)	p-value Normal vs Normal (IL-1α)	p-value OA vs OA (IL-1α)
EGF	2.1 (2.7)	1.1(1.6)	2.6 (3.4)	1.1(1.5)	0.56	0.46	0.83	0.93
Eotaxin	17.8(4.5)	14.0(7.0)	26.4(3.1)	20.0(5.5)	0.10	**0.005	**0.002	*0.01
FGF-2	34.7(19.8)	28.5(20.0)	137.0(103.1)	52.0(31.8)	0.35	** .016	**0.009	*0.011
Flt-3 Ligand	16.6(4.2)	8.7(3.8)	26.4(6.6)	13.3(6.2)	**0.001	***0.001	**0.007	*0.015
Fractalkine	128.7(42.5)	90.9 (20.0)	253.5(111.5)	121.6(62.9)	**0.005	**0.006	*0.018	*0.026
G-CSF	2946.2(1944.8)	3255.7(2656.4)	13218.7(16253.7)	4845.4(2343.5)	0.84	**0.008	**0.003	0.12
GM-CSF	76.2(42.7)	74.6 (54.0)	12072.6(16901.8)	1401.2(1423.8)	0.74	***0.001	**0.002	***0.001
GRO	21428.6(19518)	29079.7 (21731.6)	27142.9(21380.9)	15407.9(17573.6)	0.60	0.11	0.59	0.07
IFNa2	52.0(19.9)	40.2(15.0)	58.7 (20.4)	42.9 (24.5)	0.14	0.19	0.48	0.76
IFNy	34.7(17.0)	30.2 (14.6)	53.3(15.7)	28.0 (16.8)	0.64	**0.005	*0.048	0.84
IL-10	14.0(6.7)	17.0 (12.7)	138.3(98.4)	69.5(80.1)	0.71	0.06	**0.009	*0.019
IL-12 (p40)	18.8(5.0)	14.1(5.2)	35.5(7.8)	18.8(10.6)	0.06	**0.003	**0.002	0.21
IL-15	7.0(3.5)	4.1(2.7)	20.4(8.1)	7.6(5.7)	0.06	**0.002	**0.003	0.08
IL-1α	639.8(471.7)	149.1(256.9)	11528.7(4095.5)	5744.9(1685.4)	**0.008	**0.001	**0.002	***0.001
IL-1ra	67.2(39.0)	255.5 (414.4)	1168.0(697.3)	1059.1(1873.6)	0.32	0.08	**0.002	0.13
IL-1ß	0.5(0.6)	2.0 (3.4)	4.8(3.7)	4.4(6.5)	0.22	0.20	**0.002	0.15
IL-3	4.9(6.2)	1.1(2.2)	4.5(6.9)	0.6(1.0)	0.27	0.51	0.94	0.81
IL-5	0.1(0.0)	0.1(0.1)	0.2(0.1)	0.2(0.2)	0.52	0.35	0.10	0.37
IL-6	25450.7(22964.7)	20452.0(20574.9)	25436.9(22977.3)	8820.2(11234.0)	0.36	0.07	0.89	*0.092
IL-7	63.7(12.7)	61.4(20.0)	61.9(18.7)	53.1(21.3)	0.46	0.32	0.95	0.99
IL-8	24015.8(24309.1)	24325.3(23387.5)	17449.0(22238.5)	9505.2(15879.5)	0.78	0.35	0.47	0.07
IP-10	10792.7(17747.9)	823.3(1949.0)	44637.7(14187.2)	8301.9(16614.8)	**0.003	***0.001	**0.005	*0.038
MCP-1	13106.1(16350.2)	5940.7 (1750.2)	7776.2(3328.6)	6159.3(5842.8)	*0.045	0.14	0.41	0.49
MCP-3	1061.7(1612.9)	378.8 (706.3)	26633.4(22873.2)	1884.8(3176.7)	*0.027	**0.005	**0.009	0.16
MDC	10.0(12.3)	35.1(55.9)	17.6(10.9)	56.9(87.0)	0.32	0.74	0.18	0.56

MIP-1α	489.9 (451.4)	348.1(637.9)	50000.0(0.0)	7708.6(16573.8)	0.11	***0.001	**0.001	**0.002
MIP-1ß	216.9(101.4)	167.7(202.3)	1013.4(820.3)	410.1(386.8)	0.29	0.08	*0.013	0.15
PDGF-AA	43.5(12.6)	122.0(107.8)	122.1(54.1)	231.8(233.7)	0.07	0.74	**0.002	0.20
RANTES	7924.8(18582.4)	510.9(882.2)	23564.6(24730.1)	4586.5(12200.3)	*0.045	**0.003	*0.014	*0.016
sCD40L	36.4(22.9)	8.3(10.5)	54.0(10.7)	26.0	**0.002	*0.045	0.11	*0.016
sIL-2Ra	4.9(2.2)	3.9(2.0)	10.2 (3.3)	8.1	0.35	0.19	**0.006	*0.036
TNF-α	21.9(14.6)	7.3(6.1)	75.0(43.4)	17.6	*0.012	**0.001	0.01	*0.029
VEGF	251.5(202.1)	343.4(421.0)	775.4(523.3)	690.7	0.84	0.59	*0.018	*0.046
Adiponectin	10322.5(5338.3)	20991.6(9787.8)	11991.8 (10521.7)	20518.5	*0.021	0.50	0.57	0.52
HGF	4120.3(2662.5)	2791.5(3177.3)	1004.8 (641.0)	647.0	0.41	0.07	0.06	**0.002
Leptin	20.7(24.3)	92.3(181.3)	45.6 (50.4)	268.0	0.50	0.84	0.31	0.86
NGF	46.0	22.5(20.0)	316.1(168.8)	73.1	0.10	***0.001	**0.004	0.06
PAI-1 Total	1242.6	891.5(486.6)	1704.0 (578.9)	1429.3	0.25	0.38	0.17	0.16
Resistin	16.9	33.9(61.6)	405.6(1006.2)	65.9	0.30	0.33	0.41	0.69

****p<0.001, **p<0.01, *p<0.05.*

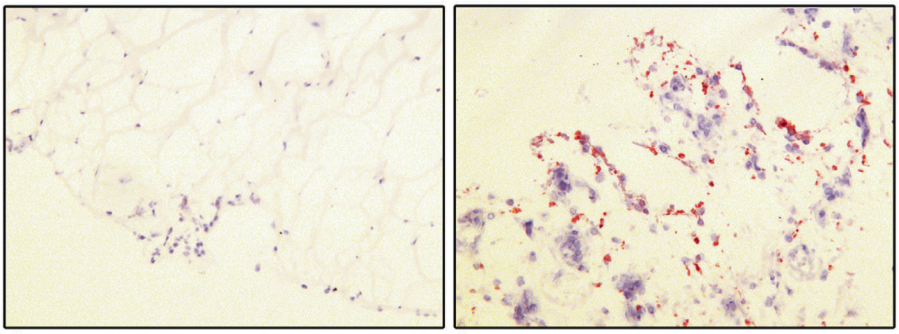

Figure S1. Immunohistochemical staining for CD68+ cells in representative synovial tissue explants directly after acquiring. **A.** normal donor and **B.** OA donor. Magnification 20x

3

Metabolic profiling reveals differences in concentrations of oxylipins and fatty acids secreted by the infrapatellar fat pad of end-stage osteoarthritis and normal donors

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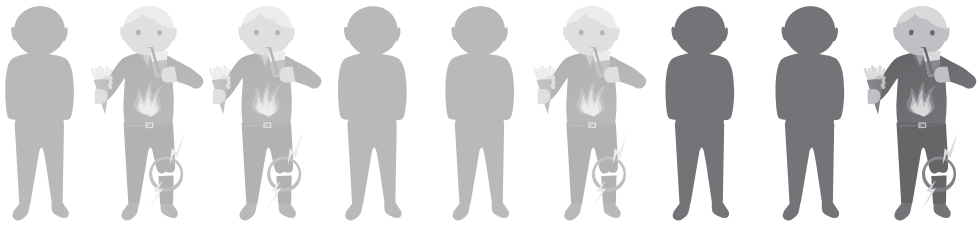
Submitted



Abstract

Objective: The infrapatellar fat pad (IPFP) in the knee joint is hypothesized to contribute to osteoarthritis (OA) development possibly by influencing inflammatory processes. Oxylipins are essential mediators in the inflammatory process. We aimed to identify the secretion of fatty acids and oxylipins derived thereof by the IPFP.

Methods: IPFP explants of 13 OA (joint replacement surgery) and 10 normal (*post-mortem*) donors were cultured for 24 hours and supernatants were collected (FCM). Liquid chromatography-tandem mass spectrometry detected fatty acids and oxylipins in FCM samples. Univariate and multivariate (PLS-DA; Partial Least Squares Discriminant Analysis) analyses were performed followed by pathway analysis. To validate these outcomes a second set of OA FCM samples was measured (n=23).

Results: Twenty nine oxylipins and fatty acids could be detected in FCM. Univariate analysis showed no differences between normal and OA FCM, however PLS-DA revealed an oxylipin/fatty acid profile consisting of 14 mediators associated with OA (accuracy rate 72%). Most important contributors to the model were lipoxin A4 (decreased), thromboxane B2 (increased) and arachidonic acid (increased). The statistical model predicted 64% of the second set of OA FCM samples correctly. Pathway analysis indicated differences on individual mediators rather than complete pathways.

Conclusion: IPFP secretes multiple and different oxylipins and a subset thereof provides a distinctive profile for OA donors. The observed changes are likely regulated by the OA process rather than a consequence of basal metabolism changes as the increase in fatty acid levels was not necessarily associated with an increase in oxylipins derived from this fatty acid.

Osteoarthritis (OA) is a complex joint disease with multiple risk factors leading to cartilage degradation (1). For a long time, the emphasis in OA research has primarily been on degeneration of articular cartilage. Nowadays it is considered a disease of the whole joint in which multiple joint tissues, such as synovial tissue, ligaments, muscles and bone, are involved or affected by the process (2, 3).

An important risk factor for OA is obesity, which was until recently related to increased mechanical load in obese individuals leading to cartilage degradation. However, recent findings, such as the association between obesity and OA in non-weight bearing joints, suggest that there are other connections between obesity and OA (4, 5). Adipocytes and infiltrating immune cells in the adipose tissue actively secrete numerous cytokines and adipokines that in turn can influence metabolism and inflammatory responses in the body (6). These factors derived from adipose tissue may therefore contribute to the development of OA.

A special form of adipose tissue, named the infrapattelar fat pad (IPFP), is located intracapsularly and extrasynovially in the joint and is in close contact with synovial layers and articular cartilage. Its main role is to facilitate the distribution of synovial fluid and to absorb mechanical forces through the knee (7). Given the fact that adipose tissue is a highly active metabolic and endocrine organ, IPFP possibly has an important role in OA pathogenesis which only recently received attention. Its exact role in OA development still needs to be elucidated (8). Several cytokines are locally produced in the knee joint by the IPFP (9) and it has been found that IPFP derived from OA patients secretes higher levels of inflammatory mediators and has a different cell composition compared to subcutaneous fat (10). On the other hand, IPFP conditioned medium from end stage OA patients had anti-catabolic effects on cartilage explants in an *in vitro* culture experiment (11).

Other important signaling molecules implicated in the modulation of inflammatory responses are oxylipins. Oxylipins are formed from essential fatty acids via oxygenation and their biosynthesis is initiated via three major pathways, cyclooxygenase (COX), lipoxygenase (LOX) and cytochrome P450 (CYP). These lipid mediators are found in all tissues in the body and are involved in the control of many physiological processes (12, 13). The activity of the COX, LOX and CYP pathways and the binding of their products to corresponding receptors are important drug targets

for multiple inflammatory diseases such as rheumatoid arthritis, cardiovascular diseases and psoriasis (14). Pro-inflammatory oxylipins such as prostaglandin (PG)E2 and leukotrienes (LT)B4 are produced by oxidation of omega (ω)-6 fatty acids such as arachidonic acid (AA) by the COX enzyme system. Oxidation of (ω)-3 fatty acids (such as EPA and DHA) give rise to the lesser inflammatory mediators as PGE3 following COX oxidation, and LTB5 following LOX activity (15).

The role of PGs in OA has been explored extensively (16, 17) and non-steroidal anti-inflammatory drugs (NSAIDs) which reduce the production of PGE2 are commonly used in medical management of OA aiming to reduce pain. These drugs interfere, however, not only with PGE2 but also with other mediators that are generated by the same enzymatic pathways (18). The individual role of other mediators in OA has not received full attention, but may provide valuable targets for OA therapy (19, 20).

The main aim of this study was to investigate which fatty acids and oxygenated derivatives thereof (oxylipins) are secreted by the IPFP. Furthermore, we questioned whether an oxylipin and fatty acid secretion profile of the IPFP could distinguish between normal and OA donors using a liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis approach (21-23). The results were used to construct biological networks, providing visualization of the interactions between identified markers. This integrated inspection might provide new insight in the role of IPFP in the OA process.

Materials & Methods

Chemicals and reagents

Indomethacin, paraoxon and butylated hydroxytoluene (BHT) were from Sigma (Steinheim, Germany). Phenylmethylsulfonyl fluoride (PMSF) was from Fluka (Steinheim, Germany). 12-[(tricyclo[3.3.1.13,7]dec-1-ylamino) carbonyl]amino]-dodecanoic acid (AUDA) was purchased from Cayman (Ann Arbor, MI, USA). Milli-Q water (MQ) (Milli-Q Advantage unit, Millipore, Amsterdam, The Netherlands) was used in all analyses. Ultra Liquid Chromatography (ULC)-grade acetonitrile (ACN), formic acid (FA) and trifluoro acetic acid (TFA) were obtained from Biosolve (Valkenswaard, The Netherlands). LC-MS grade methanol was from Riedel-de-Häen (Steinheim, Germany). Isopropanol and ethanol were from JT Baker (Deventer, The Netherlands). All analytical and internal standards were purchased from Cayman. For oxylipins, stock solutions were prepared in ethanol, aliquoted and stored at -80°C until analysis. Hydrophilic-lipophilic-balanced reversed-phase sorbent for acids, bases and neutrals (HLB) solid phase extraction (SPE) columns (Oasis, 60 mg, 3 mL) were from Waters (Etten-Leur, The Netherlands).

Preparation of fat-conditioned medium of infrapatellar fat pad

IPFP was obtained from 13 OA patients undergoing joint replacement surgery (OA-I) or from *post-mortem* material of 10 non-OA donors with macroscopically no signs and no clinical history of OA (normal). IPFP were obtained according to legal and ethical requirements approved by the institutional review board of the University of Pennsylvania including anonymous informed written consent from the donor or nearest relative (Articular Engineering, Northbrook, USA). Due to the anonymous collection, no information was available about these donors excluding age, sex and body mass index (BMI). Immediately after collection the inner parts of the fat pads, where no synovial tissue is present, were cut in small pieces and cultured in Dulbecco's Modified Eagle medium (DMEM) + glutamax (Invitrogen, Paisley, UK), 1:100 Insulin-transferrin-sodium selenite supplement (ITS) (Roche Diagnostics, Indianapolis, USA), 1% penicillin and streptomycin (Pen Strep) (Biowitthaker Verviers, Belgium) and 0.1% lactalbumine (Sigma-Aldrich, St. Louis, USA) in a concentration of 50 mg tissue/ml

and incubated at 37°C. After 24 hours, the supernatant, i.e. fat-conditioned medium (FCM) was harvested, centrifuged at 300 g for 8 minutes and frozen at -80˚C in aliquots until analysis (Articular Engineering, Northbrook, USA). This incubation time was chosen arbitrarily since each mediator has its own optimum regarding release kinetics Furthermore, 23 FCM samples from end-stage OA patients, obtained and processed with a comparable protocol but at another laboratory, were included and are called OA-II in this manuscript (Erasmus MC, Rotterdam, the Netherlands, MEC-2008-181). These OA-II FCM samples were used to validate the outcomes of the study. Demographic data of all included donors can be found in table 1.

Table 1. Demographic data of the normal and osteoarthritis (OA-I and OA-II) donors included.

	Normal (n=10)	OA-I (n=13)	OA-II (n=23)
Age	62.1 (11.9)	62.5 (6.9)	64.5 (9.5)
Sex, M/F	6/4	8/5	7/16
BMI	24.4 (4.5)	32.0 (10.5)	29.9 (3.9)

Data are presented as mean (SD). Number of donors (n), Age in years, male (M), female (F), Body Mass Index (BMI).

Extraction of oxylipins and fatty acids from fat-conditioned medium

Samples were precipitated with methanol and 50 µl internal standards (Table S1) were added to 1 ml of the FCM samples and put on ice for 30 minutes. Negative controls, containing culture medium used in the processing of normal, OA-I and OA-II samples which was not incubated with IPFP, were also included. Samples were subsequently centrifuged (5' at 3000 x g and 4°C) and the supernatant was transferred to a glass tube. Just before loading on activated HLB columns, 4.75 mL MQ water containing 0.1% v/v FA was added to the methanol extract, diluting the extract to 20% methanol. After loading, the columns were washed with 2 mL 20% methanol in MQ water containing 0.1% FA, and the columns were allowed to dry for 15 minutes. The SPE columns were eluted with 2 mL methanol and the samples were captured in tubes already containing 20 µL of 10% glycerol and 500 µM butylhydroxytolueen (BHT) in ethanol. The tubes were placed in a water bath at 40°C and the methanol was evaporated under a gentle stream of nitrogen, after which the samples were reconstituted in 100 µL ethanol containing internal standard (CUDA) (for monitoring the system performance) and immediately used for LC-MS/MS analysis.

Liquid chromatography-mass spectroscopy (LC-MS)/MS analysis of oxylipins and fatty acids

All analyses were performed on an ultra-performance liquid chromatography (UPLC) coupled to a Xevo TQ-S mass spectrometer (Waters). Five µl extract was injected on an Acquity C18 BEH (ethylene bridged hybrid technology) UPLC column (2.1 x 100 mm, 1.7 µm) and was separated using gradient elution with a stable flow of 600 µl/min. The gradient started with 95% A (MQ water with 0.1% FA) and 5% B (ACN with 0.1% FA) followed by a linear increase to 70% A and 30% B which was achieved at 5.0 minutes. This was followed by a linear increase towards 50% B which was achieved at 11.25 minutes and maintained until 13.25 minutes. The system was subsequently switched to 100% B, which was achieved at 15.75 minutes and maintained until 16.75 minutes, after which the column was left to equilibrate at 5% B for approximately 3 minutes. The column was maintained at 50°C during analysis, and the samples were kept at 10°C. The MS was operating in selective reaction mode using electrospray ionization in negative ion mode, with a capillary voltage of 3.3 kV, a source temperature of 150°C and a desolvation temperature of 600°C. Cone voltage and collision energy were optimized for each compound individually (consult table S1 for parent and product m/z values). Peak identification and quantification were performed using MassLynx software version 4.1. Calibration curves were run in duplicate from which one regression equation was generated after internal correction.

Statistical analysis and interpretation

Partial least square discriminant analyses (PLS-DA) (24) was used to identify oxylipins and fatty acids that differed between 13 OA-I and 10 normal FCM samples. Levels lower than the lowest level of the detection limit were replaced by 0.5 times the lowest level of detection. In PLS-DA, a y-variable containing class membership information is correlated to a data matrix (X-block). OA-I donors and normal donors were assigned to class 0 and class 1 respectively. The PLS-DA model was validated using a 10-fold double cross validation approach (25). The 23 OA-II samples were used to validate the statistical model for OA FCM samples. All multivariate data analyses were performed using Matlab Version 7.11.0 (R2010b, the Mathworks,

Inc). Based on the outcomes of the PLS-DA, a pathway analysis was performed using Pathvisio in which the observations were visualized (www.pathvisio.org). A custom made pathway was created based on literature searches. Univariate analyses were performed with the parametric student t-test or non-parametric Mann-Witney test depending on their normal distribution as assessed with Levene's test by using SPSS version 20.. Graphs were made by GraphPad Prism version 17.0.

Results

Lipid content of fat-conditioned medium

Metabolic profiling of oxylipins and fatty acids in FCM samples of 13 OA-I donors and 10 normal donors was performed. This screening approach led to the identification of the lipid mediators presented in table 2. Out of 52 oxylipins and fatty acids that can be measured by this approach, 29 could be detected in the FCM samples. Additionally, 23 OA-II FCM samples, processed with the same protocol but at another laboratory, were measured and revealed to contain the same lipid mediators as the OA-I FCM samples, but at different concentrations (table S2).

Most fatty acids were detected (with exception of linoleic acid), i.e. arachidonic acid (AA) (ω-6), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (both ω-3), in the FCM samples of OA-I, OA-II and normal donors. No significant differences were detected between OA-I and normal donors when applying univariate statistical analysis, although AA showed a tendency of higher concentrations in OA-I FCM than in normal FCM ($p=0.10$) (Figure 1). DHA also showed a tendency towards higher concentrations in OA-I FCM than in normal FCM ($p=0.10$).

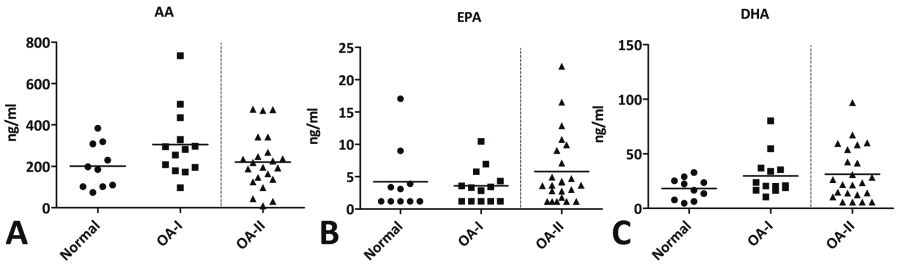


Figure 1. Concentrations of essential fatty acids detected in fat-conditioned medium (FCM) samples of normal (n=10) and osteoarthritis ((OA)-I, n=13) donors. Concentrations of independently isolated OA FCM samples are also presented (OA-II, n=23). Line indicates the mean. Arachidonic acid (AA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA).

Using multivariate statistical analysis (PLS-DA), a set of 14 out of 29 quantified oxylipins and fatty acids was retained in a model which distinguished OA-I and from normal FCM samples with a median error rate of 28% after 10-fold double cross validation. The model provided a ranking (Table 3), in which lipoxin A4 was the most discriminative oxylipin between OA-I and normal FCM. The essential fatty acid AA was ranked on the third position and DHA was ranked as fifth. The individual concentrations of the 3 most discriminating oxylipins are illustrated in figure 2. Levels of lipoxin A4 were lower in OA-I than in normal FCM. Thromboxane B2 (TBXB2), an inactive metabolite of the biologically active TBXA2, was ranked on the second position and was higher in OA-I FCM than in normal FCM, whereas prostaglandin PGD3 (ranked fifth), a derivative from the ω-3 fatty acid EPA, was found to be lower in OA-I FCM. Furthermore, the precursor for resolvin D1, 17(S)HDoHE (derived from DHA), was higher in OA-I FCM. AA-derived PGF2α and D2 were higher in OA-I FCM, whereas PGB2 and F2β levels were lower. Finally, linoleic acid-derived 9-HODE and 13-HODE were found to be lower in OA-I FCM. None of the resolvins, maresins and leukotrienes could be detected.

Table 2. Oxylipins and essential fatty acids detectable in fat-conditioned medium (FCM) of 13 osteoarthritis (OA-I) and 10 normal donors.

	LLOD (ng/ml)	OA-I				Normal			
		Mean (ng/ml)	SD	CV (%)	N	Mean (ng/ml)	SD	CV (%)	N
Essential fatty acids									
EPA	2.34	3.6	2.8	78	5	4.2	5.1	121	5
AA	46.85	305.8	168.7	55	0	200.8	107.5	54	0
DHA	11.70	29.9	19.2	64	0	18.2	9.9	55	0
EPA-derived oxylipins (ω-3)									
PGE3	0.06	0.1	0.2	175	10	0.2	0.6	238	8
PGD3	0.10	0.2	0.4	187	10	0.5	1.0	188	5
DHA-derived oxylipins (ω-3)									
17(S)_HDoHE	0.10	0.2	0.5	213	9	0.1	0.1	168	9
AA-derived oxylipins									
13,14-dihydro-15-keto-PGD2	0.01	0.04	0.1	280	11	0.05	0.1	259	8
13,14-dihydro-15-keto-PGE2	0.02	0.8	1.7	212	2	0.8	2.1	271	2
13,14-dihydro-15-keto-PGF2α	0.08	2.4	4.5	190	1	1.5	2.5	169	0
15(S)-HETE	0.01	0.2	0.1	81	0	0.2	0.1	73	0
11(S)-HETE	0.01	0.1	0.1	85	0	0.1	0.2	116	0
12(S)-HETE	0.01	0.2	0.2	81	0	0.1	0.1	45	0
5(S)-HETE	0.02	0.1	0.1	105	0	0.1	0.1	69	0
TBXB2	0.05	2.1	2.1	98	0	0.9	1.2	134	1
PGB2	0.02	0.2	0.3	153	2	0.6	1.3	206	3
PGF2β	0.02	0.1	0.1	159	7	0.2	0.2	128	1
PGD2	0.01	0.8	1.03	131	0	1.0	1.3	133	0
PGE2	0.02	74.6	133.3	179	0	90.0	207.2	230	0
Lipoxin A4	0.01	0.04	0.04	85	3	0.1	0.2	128	1
8-iso-PGF2α	0.02	0.3	0.6	161	1	0.3	0.6	189	0
11b-PGF2α	0.02	1.8	2.6	145	1	0.9	2.2	244	3

PGF2α	0.02	5.0	6.8	136	0	5.0	8.7	173	0
11,12-DiHETrE	0.01	0.05	0.05	101	0	0.04	0.03	76	1
14,15-DiHETrE	0.01	0.04	0.03	80	1	0.05	0.03	64	1
8,9-EET	0.08	0.05	0.03	63	1	0.05	0.03	54	1
8,9-DiHETrE	0.02	0.05	0.07	145	4	0.03	0.03	103	5
12(S)-HHTrE	0.16	0.2	0.24	102	7	0.3	0.3	115	1
LA-derived oxylipines									
9(S)-HODE	0.02	2.5	1.0	39	0	4.5	3.7	84	0
13(S)-HODE	0.04	3.2	0.8	25	0	4.8	3.5	72	0

Standard deviation (SD), coefficient of variance (CV), lower level of detection (LLOD), number of samples below the LLOD (N). Eicosapentaenoic acid (EPA), arachidonic acid (AA), docosahexaenoic acid (DHA), prostaglandin (PG), hydroxydocosahexaenoic acid (HDoHE), hydroxyeicosatetraenoic acid (HETE), thromboxane (TBX), dihydroxyeicosatrienoic acid (diHETre), eicosatrienoic acid(EET), hydroxyheptadecatrienoic acid (HHTrE), linoleic acid (LA), hydroxyoctadecadienoic acid (HODE).
Resolvin D1, Resolvin D2, Maresin, Leukotriene B4, E4, D4, n-acetyl-leukotriene E4. TBXB3, 11,12 EET, 5,6 EET, 14,15 EET, 10(S)-17(S) diHDoHE, 6-keto-PGF1α, 2,3-dinor-8-iso-PGF2α, 12(S)- 12S-hydroxyeicosapentaenoic acid (HEPE), 5(S)-HEPE, 20(S)-HEPE, 15-deoxy-d-12,14-PGJ2, 5,6-DiHETre, 19,20-dihydroxydocosapentaenoic acid (HDoPE), 17-keto, 4(z),7(z),10(z),13(z),15(E)),19(z)-DHA, 12,13-di-hydroxyoctadecenoic acid (HOME) and 9,10-diHOME could not be detected in these samples.

Validation of the osteoarthritis fat-conditioned medium samples

To assess whether the collected OA-I FCM samples were representative for OA, we included independently isolated FCM samples from 23 OA donors (OA-II) in the study. Regarding individual levels, the 14 lipid mediators obtained from the PLS-DA model had different absolute mean values in the OA-I samples compared to the OA-II samples, although the majority was changed in the same direction compared to normal FCM (Figure 1 and 2, table S2). For TBXB2, 9(S)HODE and 17(S) HODE different patterns were seen in the OA FCM samples from different laboratories; with respect to normal FCM samples the TBXB2 levels were higher in OA-I while lower in OA-II samples. For 9(S)HODE and 17(S) HODE lower levels were found in OA-I than in normal FCM samples, in contrast to higher levels in OA-II samples. Additionally, the OA-II FCM samples were analyzed within the built statistical PLS-DA model of OA-I and normal samples. Eight out of 23 OA-II FCM samples were not classified as OA FCM sample by using this approach (error rate of 36%).

Table 3. Ranking in partial least square discriminant analysis (PLS-DA) of 13 osteoarthritis (OA-I) and 10 normal donor derived fat-conditioned medium samples.

Rank	Name	Regression coefficient
1	Lipoxin A4	-0.144
2	TBXB2	0.135
3	AA	0.110
4	PGD3	-0.095
5	DHA	0.093
6	17(S)_HDoHE	0.087
7	PGB2	-0.073
8	14,15-DiHETrE	-0.067
9	11b-PGF2α	0.059
10	15(S)-HETE	-0.057
11	13,14-dihydro-15-keto-PGF2α	0.053
12	PGF2β	-0.037
13	13(S)-HODE	-0.034
14	9(S)-HODE	-0.026

The contribution of a variable in the PLS-DA model is expressed as its regression with a positive score indicating an increase and a negative score indicating a decrease in OA-I compared to control FCM samples. A greater absolute value means more contribution of this oxylipin to the measured differences between OA-I and normal FCM samples. Thromboxane B2 (TBXB2), arachidonic acid (AA), prostaglandin (PG), docosahexaenoic acid (DHA), hydroxy docosahexaenoic acid (HDoHE), dihydroxy eicosatrienoic acid (diHETrE), hydroxyl eicosatetraenoic acid (HETE), hydroxyl octadecadienoic acid (HODE).

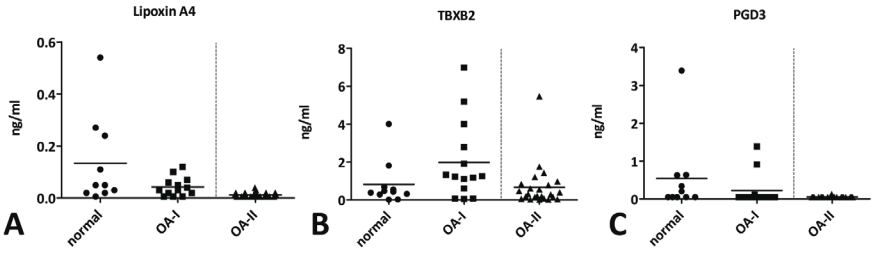


Figure 2. Concentrations of oxylipins which are high in the ranking in the contribution to the distinction between normal (n=10) and osteoarthritis ((OA)-I, n=13) donors derived fat-conditioned medium (FCM) samples after partial least square discriminant analysis (PLS-DA). Concentrations of independently isolated OA FCM samples are also presented (OA-II, n=23). Thromboxane B2 (TBXB2) and prostaglandine (PG)D3. Line indicates the mean.

Pathway analysis

To visualize the results from the quantified fatty acids and oxylipins in the OA-I FCM samples, all data were imported and analyzed using Pathvisio (Figure 3). In this overview, the quantified fatty acids and oxylipins are shown together with their main enzymes (LOX, COX, and CYP). Herein it is shown that higher concentrations of the fatty acids not necessarily lead to higher concentrations of its downstream products. AA was found in higher concentrations in OA-I FCM than in normal FCM, but for its downstream products both higher and lower concentrations were found. None of the main enzymes (COX, LOX or CYP) seemed to be consistently up or down regulated, although all LOX-derived oxylipins in the PLS-DA model showed lower concentrations in OA-I patients. However, 3 out of 7 quantified LOX-derived oxylipins did not contribute to the discrimination of OA-I from normal donors. Oxylipins involved in the synthesis of lipoxin A4 (lipoxin A4 and 15-HETE) and downstream derivatives of linoleic acid (9-HODE and 13-HODE) showed decreased concentrations in OA-I FCM.

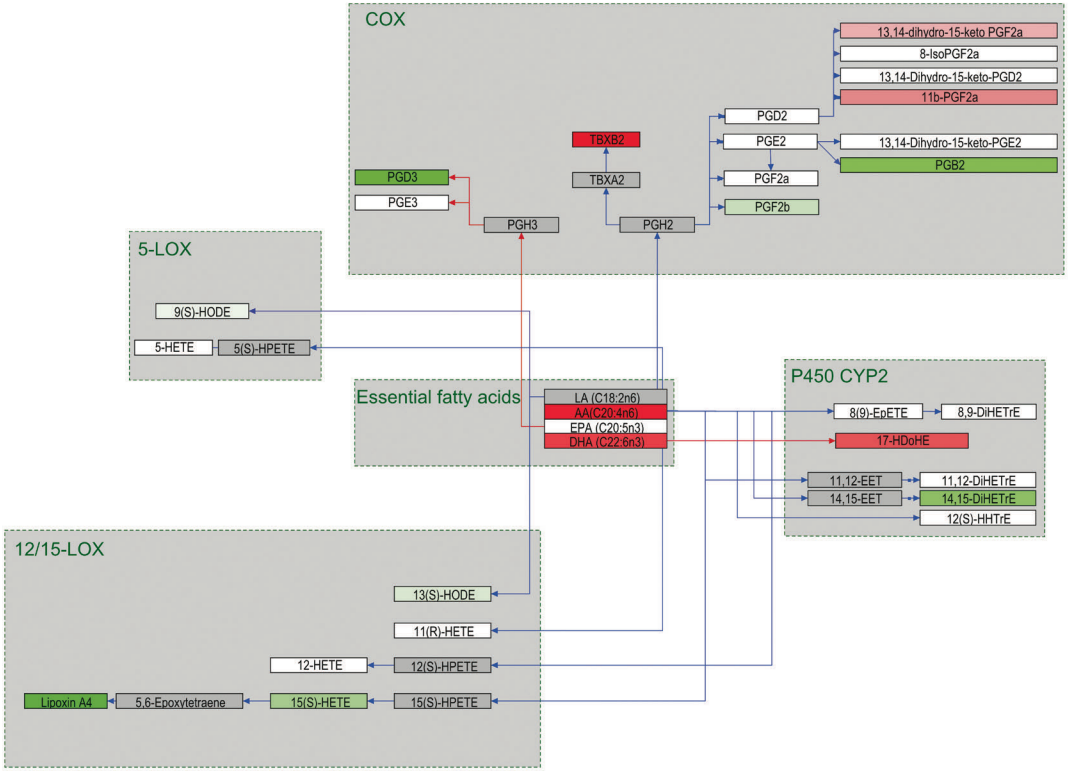


Figure 3. Overview of essential fatty acids and oxylipins differences in fat-conditioned medium (FCM) samples from osteoarthritis (OA-I) and normal donors as detected by partial least square discriminant analysis (PLS-DA). The following is indicated by the colors: grey, not measured or detected; white, quantified but not different between OA-I and normal FCM; red, increased concentrations in OA-I FCM; green, decreased concentrations in OA-I FCM. The more intense the color, the higher the ranking in the PLS-DA model.

Discussion

The IPFP located in the knee joint is considered as a tissue that may contribute to local inflammatory and destructive processes in OA (8). Important signaling molecules implicated in the modulation of inflammatory responses are oxylipins (14). In this study, we provide a comprehensive approach to identify oxylipins and fatty acids secreted by the IPFP in the knee joint. Using an LC-MS/MS approach, 29 out of the measurable 52 oxylipins and fatty acids could be detected in the FCM samples, indicating the presence of bioactive lipids in IPFP. None of these 29 lipid mediators differed significantly between OA-I and normal donors based on univariate statistics. By using multivariate analysis (PLS-DA), an oxylipin profile associated with OA-I was obtained with an accuracy rate of 72% after 10-fold double cross validation. The validation of the statistical model with independently isolated OA FCM samples (OA-II) revealed a comparable accuracy rate for OA samples (64%). These accuracy rates are acceptable given the relatively small included number of donors and the biological variety in these sample type. The measured changes visualized by pathway analysis demonstrated that the profile associated with OA FCM is likely to be specifically changed by the disease process rather than due to alterations in basal metabolism or generic enzymes as the increase of a fatty acid concentration was not necessarily associated with an increase in oxylipins derived from this fatty acid.

In addition to the profiling of essential fatty acids and oxylipins secreted by IPFP, we wondered whether the profile of IPFP from OA would differ from normal donors. A difference may reveal more insight in the role of IPFP in the OA disease process. Based on multivariate analysis lipoxin A4, which is derived from AA, was the most discriminative oxylipin between OA-I and normal FCM with lower levels in OA donors. Also 15-HETE, an intermediate in lipoxin A4 synthesis, was found in decreased concentrations in OA-I compared to normal FCM. Lipoxin A4 is known to have a role in the resolution of inflammation, is produced by cartilage explants (26) and is present in OA and rheumatoid arthritis (RA) synovial fluid (27). Lipoxin A4 inhibits interleukin (IL)-1β induced IL-6 and IL-8 release in fibroblast-like synoviocytes (FLS) and reduces matrix metalloproteinase (MMP)-3 release and increases tissue inhibitor of MMP (TIMP) release in IL-1β stimulated FLS (28, 29). Deletion of

12/15-lipoxygenase (12/15-LOX), a major enzyme involved in the generation of a subclass of oxylipins including lipoxin A4, in a mouse model for arthritis leads to uncontrolled inflammation and tissue damage (30). The lower concentrations of lipoxin A4 and 15-HETE in the OA-I compared to normal FCM samples contribute to the concept that lipoxin A4 may act as counter regulatory signal and has a potential to limit inflammation-induced tissue damage (31).

TBXB2 was ranked as the second oxylipin by PLS-DA that contributed to the distinction between OA-I and normal FCM samples. TBXB2 was elevated in OA-I compared to normal FCM samples. In FCM of the OA-II samples, however, this oxylipin was not different from normal FCM. This discrepancy may be explained by several reasons. Firstly, although the same protocol for the collection and processing of OA-I and OA-II FCM was applied, reliable detection of oxylipins may be disturbed by *ex vivo* degradation of lipid mediators due to rapid enzymatic conversion or chemical instability (32, 33). Therefore the generated absolute values and the differences between OA-I and OA-II samples should be interpreted with caution. Secondly, due to the anonymous collection of the FCM samples, the medical history of these patients is unknown. OA-I and OA-II FCM samples were collected in different countries and different protocols for the use of drugs prior to surgery may explain the discrepancy between OA-I and OA-II FCM samples. Especially, the use of pain killers such as NSAIDs or COX inhibitors, which interfere in pathways leading to the production of TBXB2, may explain these differences. It should as well be noted that the lack of information about the medical history of the donors also counts for the other observed differences between OA-I and normal donors and it cannot be excluded that this influences the detected levels. Most of the essential fatty acids were detected in the FCM samples. It has been postulated that the intake of dietary essential fatty acids can influence the development of OA (34). In a spontaneous OA guinea pig model an ω-3 rich diet was reported to improve histological and biochemical markers of OA (35). Unexpectedly, in our data DHA (ω-3) levels were higher in OA-I than normal derived FCM samples. EPA (ω-3) was not altered in OA-I compared to normal FCM samples. The higher secretion of the pro-inflammatory ω-6 fatty acid AA by OA IPFP was in line with our expectations. Notably, the different PGs derived from AA were either unchanged, increased and decreased in OA-I compared to normal FCM

samples. PGs exert diverse and complex modulatory roles during pathophysiologic conditions and are extensively investigated in relation to OA (16, 17, 20). PGE2, for example, is known to increase the productions of MMPs and pro-inflammatory soluble mediators which can lead to alterations in the cartilage, synovial membrane and bone. Remarkably, no differences between OA-I and normal FCM were observed for PGE2. Furthermore, PGD2 concentrations, which can augment the production of PGE2 (26), were not different between OA-I and normal FCM. The increase of the essential fatty acid AA but not necessarily their by-products supports the idea that the observed changes are specifically for the disease process and indicates the complexity of systemic equilibrium in these pathways.

Although essential fatty acids were detected, some apparent by-products from pathways instigated by these fatty acids were not measured in the FCM samples, such as the leukotrienes which are suggested to increase bone resorption and to induce IL-1 synthesis (20, 36). Resolvins, which play an important role in the resolution of inflammation, were also not identified using this approach (37). The absence of these oxylipins might be due to the detection limit of our analysis and more sensitive methods may reveal their presence. Furthermore, as mentioned previously, concentrations may be influenced by *ex vivo* degradation of lipid mediators due to rapid enzymatic conversion or chemical instability (32). For all detected oxylipins and fatty acids, having identical MS/MS transitions, counts that they were distinguished by retention time after optimization of the chromatographic conditions using commercially available standards, however it cannot be fully excluded that other isomers may be present in biological materials that co-elute with those tested.

There are few references to such a broad lipid mediator approach performed in tissues and fluids in the knee joint. Attur et al. analyzed the excretion of a selected panel of oxylipins and their related fatty acids by OA and normal cartilage explants. The main outcomes were the increased spontaneous secretion of PGE2 and leukotriene B4 by OA compared to normal cartilage explants (26). A study with equine synovial fluid reported a radical change of the oxylipin profile after experimental induction of transient acute synovitis (e.g. PGE2, PGD2, PGF2a, leukotriene B4 and TBXB2) (38). Our data contribute to the theory that during the OA process the essential fatty acids and oxylipin profiles in various joint tissues change. It remains the question, however,

whether these changes in profiles contribute to the process of cartilage degeneration or are a consequence thereof.

The extensive biological variance which was measured in the samples led to a multivariate statistical model with an error rate of 28%. This should be taken into account when interpreting the data. By the validation of the model with independently isolated OA FCM samples (OA-II) a comparable error rate for OA samples was obtained, which strengthens the observations measured in the OA-I FCM sample set. An expansion of the number of donors is needed to investigate whether the error rate can be reduced, thereby improving the reliability of the model and the validity that differences in oxylipin secretion are present between OA and normal IPFP.

This study demonstrates that OA and normal IPFP generate multiple and different oxylipins. OA patients can be distinguished from normal donors based on the secretion of lipid mediators involved in the oxylipin pathways by IPFP. Pathway analysis underlined the complexity of equilibrated mechanisms in the OA process as an increase in a fatty acid not necessarily led to an increase in a by-product of the instigated pathway. These data may provide a basis for development of therapeutics in OA.

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Supplementary information

Table S1. MS/MS parent and product ion m/z values

Compounds	Parent (m/z)	Product (m/z)
12-HHTrE	279.0	179.3
13-HODE	295.1	195.0
9-HODE	295.1	171.0
EPA	301.1	257.2
ARA	303.1	259.2
12,13-DiHOME	313.2	183.0
9,10-DiHOME	313.2	201.0
15-deoxy-d-12,14-PGJ_2	315.0	271.1
12-HEPE	317.2	179.0
5-HEPE	317.2	115.0
11,12-EET	319.1	167.0
12-HETE	319.1	179.2
5-HETE	319.1	203.1
14,15-EET	319.1	219.2
5,6-EET	319.1	191.3
11-HETE	319.1	167.0
8,9-EET	319.1	167.0
15-HETE	319.2	219.1
20-HETE	319.2	275.3
2,3-dinor-8-iso-$PGF_{2\alpha}$	325.1	237.2
DHA	327.1	283.1
9,10,13-TriHOME	329.2	171.1
9,12,13-TriHOME	329.2	211.1
PGB2	333.2	174.9
LTB4	335.1	194.8
14,15-DiHETrE	337.1	207.0
11,12-DiHETrE	337.1	166.9
5,6-DiHETrE	337.1	144.8
8,9-DiHETrE	337.1	127.0
17-keto-4(z),7(z),10(z),13(z),15(e),19(z) DHA	341.5	111.0
17-HDoHE	343.1	281.4
PGE3	349.0	269.1
PGD3	349.1	269.1
PGD2	351.1	271.1
Lipoxin A4	351.1	114.9
13,14-dihydro-15-keto-PGD2	351.1	175.0
13,14-dihydro-15-keto-PGE2	351.1	175.1
PGE2	351.1	271.2
13,14-dihydro-15-keto-PGF2	353.1	113.1

Compounds	Parent (m/z)	Product (m/z)
8-iso-PGF2α	353.1	193.0
11-PGF2α	353.1	193.0
PGF2α	353.1	193.0
PGF2α	353.2	193.1
10,17-DiHDoHE	359.1	152.9
Maresin	359.6	177.1
19,20-DiHDoPE	361.1	272.7
TBXB3	367.1	168.9
TBXB2	369.1	169.0
Resolvin D2	375.1	175.0
Resolvin D1	375.1	140.8
LTE4	438.0	351.0
n-acetyl LTE4	480.0	351.1
LTD4	495.1	142.9
Internal standards (quantification)		
13-HODE-d4	299.2	198.2
ARA-d8	311.2	267.2
20-HETE-d6	325.1	281.3
15-HETE-d8	327.2	226.1
14,15-EET-d11	330.2	268.3
PGB2-d4	337.1	178.9
LTB4-d4	339.1	197.1
8,9-DiHETrE-d11	348.2	127.0
PGE2-d4	355.1	275.1
13-14-dihydro-15-keto-PGF2α-d4	357.1	187.0
8-iso-PGF2α-d4	357.1	196.9
11β-PGF2α-d4	357.1	313.4
PGF2-d4	357.1	313.4
PGD2-d9	360.3	280.1
TBXB2-d4	373.1	173.0
LTD4-d5	500.0	142.9
Internal standard (system performance)		
CUDA	339.1	214.1

Table S2. Essential fatty acids and oxylipins concentrations in fat-conditioned medium samples of 10 normal, 13 osteoarthritis (OA)-I and 23 OA-II donors.

	Normal			OA-I			OA-II					
	Mean ng/ml	95 % CI		Mean ng/ml	95 % CI		Mean ng/ml	SD	CV (%)	N	95% CI	
Essential fatty acids												
EPA	4.2	1,049	7,398	3.6	2,052	5,087	6.8	4.6	68	5	4,923	8,690
AA	200.8	134,199	267,454	305.8	214,123	397,537	290.4	115.3	40	5	243,300	337,506
DHA	18.2	12,003	24,331	29.9	19,448	40,276	41.4	26.4	64	4	30,630	52,231
EPA-derived oxylipins (ω-3)												
PGE3	0.2	-0,110	0,572	0.1	0,007	0,266	0.1	0.1	148	19	0,027	0,109
PGD3	0.5	-0,091	1,182	0.2	-0,004	0,455	0.1	0.0	0	22	0,051	0,051
DHA-derived oxylipins (ω-3)												
17(S)_HDoHE	0.1	-0,003	0,170	0.2	-0,037	0,503	0.05	0.02	43	21	0,037	0,053
AA-derived oxylipins												
13,14-dihydro-15-keto-PGD2	0.05	-0,028	0,122	0.04	-0,020	0,096	0.02	0.03	152	12	0,007	0,031
13,14-dihydro-15-keto-PGE2	0.8	-0,529	2,081	0.8	-0,123	1,726	0.4	1.0	251	5	-0,010	0,782
13,14-dihydro-15-keto-PGF2α	1.5	-0,067	3,071	2.4	-0,077	4,863	1.1	2.7	253	8	-0,037	2,185
15(S)-HETE	0.2	0,112	0,296	0.2	0,090	0,232	0.1	0.05	73	6	0,044	0,081
11(S)-HETE	0.1	0,042	0,255	0.1	0,067	0,182	0.1	0.09	89	6	0,063	0,134
12(S)-HETE	0.1	0,102	0,182	0.2	0,114	0,291	0.1	0.1	121	3	0,056	0,165
5(S)-HETE	0.1	0,056	0,141	0.1	0,047	0,173	0.1	0.02	44	6	0,045	0,065
TBXB2	0.9	0,154	1,635	2.1	0,995	3,261	0.6	0.6	91	0	0,395	0,865
PGB2	0.6	-0,170	1,392	0.2	0,037	0,403	0.2	0.4	165	7	0,070	0,358
PGF2β	0.2	0,032	0,278	0.1	0,008	0,115	0.04	0.03	81	8	0,028	0,055
PGD2	1.0	0,169	1,741	0.8	0,225	1,346	0.2	0.3	103	4	0,142	0,350
PGE2	90.0	-38,358	218,440	74.6	2,168	147,083	33.6	71.6	213	1	4,293	62,807
Lipoxin A4	0.1	0,028	0,239	0.04	0,023	0,063	0.01	0.01	77	11	0,010	0,019
8-iso-PGF2α	0.3	-0,054	0,672	0.3	0,043	0,647	0.2	0.4	186	2	0,055	0,401
11b-PGF2α	0.9	-0,470	2,313	1.8	0,376	3,173	0.8	1.5	190	3	0,179	1,413

PGF2α	5.0	-0,373	10,381	5.0	1,308	8,683	3.3	5.3	160	2	1,146	5,484
11,12-DiHETrE	0.04	0,023	0,064	0.05	0,023	0,080	0.02	0.02	69	3	0,015	0,027
14,15-DiHETrE	0.05	0,028	0,066	0.04	0,024	0,060	0.01	0.01	95	9	0,008	0,018
8,9-EET	0.05	0,034	0,069	0.05	0,031	0,064	0.05	0.02	33	19	0,040	0,052
8,9-DiHETrE	0.03	0,009	0,041	0.05	0,010	0,080	0.02	0.02	92	9	0,015	0,033
12(S)-HHTrE	0.3	0,084	0,495	0.2	0,107	0,371	0.1	0.1	74	16	0,096	0,179
LA-derived oxylipins												
9(S)-HODE	4.5	2,660	6,949	2.5	2,730	3,608	11.6	4.3	37	2	9,844	13,352
13(S)-HODE	4.8	2,151	6,778	3.2	1,959	3,013	7.0	2.4	34	2	6,005	7,964

Standard deviation (SD), coefficient of variance (CV), number of samples below the lower level of detection (N) and 95 % confidence interval (CI). Eicosapentaenoic acid (EPA), arachidonic acid (AA), docosahexaenoic acid (DHA), prostaglandin (PG), hydroxydocosahexaenoic acid (HDoHE), hydroxyeicosatetraenoic acid (HETE), thromboxane (TBX), dihydroxyeicosatrienoic acid (diHETre), eicosatrienoic acid(EET), hydroxyheptadecatrienoic acid (HHTrE), hydroxyoctadecadienoic acid (HODE).
Resolvin D1, Resolvin D2, Maresin, Leukotriene B4, E4, D4, n-acetyl-leukotriene E4. TBXB3, 11,12 EET, 5,6 EET, 14,15 EET, 10(S)-17(S) diHDoHE, 6-keto-PGF1α, 2,3-dinor-8-iso-PGF2α, 12(S)- 12S-hydroxyeicosapentaenoic acid (HEPE), 5(S)-HEPE, 20(S)-HEPE, 15-deoxy-d-12,14-PGJ2, 5,6-DiHETre, 19,20-dihydroxydocosapentaenoic acid (HDoPE), 17-keto, 4(z),7(z),10(z),13(z),15(E)),19(z)-DHA, 12,13-dihydroxyoctadecenoic acid (HOME) and 9,10-diHOME could not be detected in these samples.

4

An explorative study comparing levels of soluble mediators in control and osteoarthritic synovial fluid

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Abstract

Soluble mediators, e.g. cytokines, chemokines and growth factors, are acknowledged as key players in the pathophysiology of osteoarthritis (OA) (1, 2). However, a wide-spectrum screening of such mediators in the joint environment is currently lacking. In this study, synovial fluid was collected from control donors and end-stage knee OA patients and analysed for 47 cytokines, chemokines and growth factors using several multiplex ELISAs. In addition, a principal component analysis (PCA) was performed to cluster the measured mediators. IL-6, IP-10, MDC, PDGF-AA and RANTES levels were found to be higher in OA compared to control synovial fluid ($p<0.001$). Leptin, IL-13, MIP-1β, sCD40L levels were higher and eotaxin and G-CSF levels were lower in OA synovial fluid than in control synovial fluid, albeit at borderline significance ($p<0.05$). Increased levels of inflammatory mediators and chemokines, such as MDC and IL-6, imply involvement of inflammatory processes in OA and might be associated with the influx of inflammatory cells in OA synovial tissue. Additionally, the PCA enabled identification of 6 different clusters, which explained 76% of the variance, and in this way could indicate different pathophysiological pathways in the joint. This dataset is valuable as a reference for future experiments to study pathophysiological pathways, and useful in more extensive profiling studies for OA.

Brief report

A control group of knee synovial fluid samples (n=16) were collected from *post mortem* donors within 24 hours after death. Control donors had no history of OA, other joint pathology and possessed macroscopic healthy cartilage. OA synovial fluid samples (n=18) were collected during total knee arthroplasty. All OA patients were diagnosed according to the ACR criteria for OA (3). Exclusion criteria were rheumatoid arthritis (RA) or infection. Synovial fluid samples were centrifuged at 3000 rpm for 3 minutes to spin down any cells or debris. The supernatant was stored at -80 °C until further analysis. The control synovial fluid samples were stored for 1 to 10 years and OA synovial fluid samples were stored for 1 to 3 years. None of the samples had ever been thawed before. Collection of the synovial fluid was done according to the Medical Ethical regulations of the University Medical Centre Utrecht and according to the guideline 'good use of redundant tissue for clinical research' constructed by the Dutch Federation of Medical Research Societies on collection of redundant tissue for research. As according to these guidelines, no information about the patients' characteristics could be obtained. Gender and age information was available for limited donors. Control donors had an average age of 39.6 ± 9.3 and consisted of 55% female. OA donors had an average age of 69.9 ± 7.9 and consisted of 64 % female. Due to the limited availability these data could not be linked to any of the outcomes.

Two hundred µl of each of the OA synovial fluid samples was pre-treated with 20 µl of hyaluronidase (Sigma, St, Louis, MO, USA; 10 mg/ml) for 15 min at 37 °C. Samples were spun down in a X-column (Corning, Amsterdam, Netherlands; Costar 8169). Finally, 150 µl of the synovial fluid sample was dissolved in 300 µl HPE-0.1375% Tween (Sanquin, Amsterdam, Netherlands). The pre-treated synovial fluid samples were used for all Multiplex ELISA assays mentioned below.

To determine a wide panel of soluble mediators the commercially available human inflammation 42-multiplex and the human adipokine 13-multiplex (Millipore, Bellirica, MA, USA) were used according to the manufacturer's protocol. Additionally, 12 different soluble mediators were measured with the Bio-Plex suspension system (Bio-Rad laboratories, Hercules CA, USA) as previously described (4). The levels of cytokines in the synovial fluid samples were expressed as pg/ml. All samples were

measured in the same plate and in duplo. Levels below the lower limit of quantification (LLOQ) were indicated as the value of the lowest point on the calibration curve divided by 2. The measured mediators are listed in table 1. Data are expressed as median ± interquartile range (IqR) as the data had a non-Gaussian distribution. IBM SPSS 20.0 software (IBM SPSS Inc. Chicago, IL, USA) was used for the statistical analysis.

For this study, a descriptive statistics approach was used for the interpretation of the data due to the limited sample size. A Mann Whitney-U test was used to assess differences between control and OA synovial fluid samples due to the predominantly non-Gaussian distribution of the data. As a descriptive statistic approach was used, values with a p-value less than 0.001 were considered significant. P values between 0.05 and 0.001 were considered borderline significant. No *post-hoc* correction for multiple testing was performed on the data, due to the descriptive approach.

PCA was performed to enable identification of clusters (i.e. components) of interrelated mediators within the complete dataset. Mediator levels were mean-centered to remove dependence on magnitude of levels. Control and OA patient data were combined in one single PCA analysis. Separate analysis of patients and controls was judged impossible due to the limited number of subjects. Only mediators with communalities > 0.3 were included. Loading factors were maximized using Direct oblimin rotation with Kaiser Normalisation. The optimum number of clusters was then decided based on the scree-plot and eigenvalues (> 1.0). Mediators were categorized per cluster when their loading scores were > 0.5. A Crohnbach's α was performed on each cluster to determine internal consistency.

This is one of the first studies in which such a comprehensive profile of soluble mediators was measured in synovial fluid from (end-stage) OA patients and control donors. With regard to previous studies, the pattern of the mediator levels was found to be compatible for OA donors. However, in these studies a small panel of mediators was measured and the inclusion of control donors was lacking (e.g. (5)). Table 1 provides an overview of the measured levels of each mediator (median ± IqR). The majority of the soluble mediators could be detected in the synovial fluid samples of both control and OA donors. Of the 47 measured mediators, 5 mediators were present at significantly different levels in OA compared to control synovial fluid. The levels of the chemokines MDC, RANTES and IP-10, the growth factor PDGF-AA and

the pro-inflammatory cytokine IL-6 were significantly higher in OA than in control synovial fluid ($p < 0.001$). In addition, levels of the adipokine leptin, the chemokine MIP-1β, and the pro-inflammatory cytokine sCD40L were higher and levels of the chemokine eotaxin and the growth factor G-CSF were lower in OA than in control synovial fluid, albeit all with borderline significance ($p < 0.05$).

Table 1 Overview of the measured mediator concentration (pg/ml) in control and osteoarthritic (OA) synovial fluid (SF).

	Healthy SF (Median ± IqR)	CV (%)	<LLOQ or 0 (n)	OA SF (Median ± IqR)	CV (%)	<LLOQ or 0 (n)	Detection limit	Mann-Whitney U (p-value)
EGF	4.8 ± 13.1	102.3	11	4.8 ± 0	189.3	14	5.3	0.65
Eotaxin	14.6 ± 39.6	96.0	6	0 ± 0	236.7	15	12.1	*0.02
FGF-2	37.2 ± 70.4	137.5	2	21.6 ± 231.4	134.1	4	16.0	0.67
Flt-3 ligand	135.9 ± 119.7	59.0	0	103.5 ± 76.4	53.1	0	6.1	0.23
Fractalkine	0 ± 0	387.7	14	0 ± 19	168.5	12	7.6	0.14
G-CSF	34.8 ± 151	105.9	1	16.9 ± 15	66.9	0	3.9	*0.03
GRO	59.2 ± 79.5	74.4	0	84.2 ± 96	81.6	0	11.4	0.38
IFNα2	7.2 ± 10.5	67.4	7	16.4 ± 46.8	89.3	7	27.2	0.09
IFNγ	40.7 ± 12.9	23.30	0	28.0 ± 21.0	136.74	2	2.14	0.1
IL-10	3.3 ± 6.3	127.6	6	2.1 ± 2.7	103.7	3	0.3	*0.04
IL-12 (p40)	4.8 ± 1.7	60.5	9	4.8 ± 11.9	137.7	10	12.4	0.30
IL-15	9.9 ± 6.0	37.1	0	12.8 ± 5.7	31.2	0	0.6	0.32
IL-1α	0 ± 1.8	217.8	9	0 ± 4.9	153.4	12	1.5	0.74
IL-1ra	0 ± 6.9	125.6	8	0 ± 0	288.4	14	2.3	0.14
IL-1ß	0 ± 1.5	205.4	11	4.8 ± 11.9	298.6	14	0.7	0.61
IL-3	0 ± 0	0.0	16	4.8 ± 0	179.3	16	9.8	0.18
IL-6	4.8 ± 0	196.9	13	135.8 ± 224.6	120.3	3	0.4	#0.001
IL-8	16.2 ± 43.5	92.2	0	30 ± 23.5	170.9	0	0.3	0.24
IP-10	302.1 ± 280.8	107.6	0	710.4 ± 597.1	93.1	0	1.3	#0.001
MCP-1	542.4 ± 839.2	102.1	0	824.8 ± 645.5	47.8	0	1.2	0.58
MCP-3	4.8 ± 36	123.5	10	4.8 ± 5	215.5	14	5.2	0.83
MDC	52.2 ± 38.4	43.9	0	189.5 ± 119.8	41.8	0	2.4	#0.001
MIP-1α	4.8 ± 0	26.7	16	4.8 ± 0	172.7	15	6.6	0.06
MIP-1ß	9.6 ± 24.0	83.7	5	21.8 ± 23.5	127.6	3	3.2	*0.04
PDGF-AA	0 ± 2.1	201.3	11	72.6 ± 116.9	72.8	0	0.3	#0.001
PDGF-AB/BB	43.2 ± 42.9	93.6	1	34.2 ± 69.7	137.8	0	12.2	0.44

RANTES	15.6 ± 28.1	152.7	0	408.2 ± 910.9	114.3	0	1.6	#0.001
sCD40L	0 ± 0	230.9	13	5.9 ± 45.1	144.6	7	5.2	*0.005
sIL-2ra	37.5 ± 76.8	90.8	1	63.0 ± 72.5	65.9	0	7.5	0.11
TGFα	0 ± 0.6	201.2	9	0 ± 0	401.5	17	1.4	0.4
HGF	2554.4 ± 3505.7	80.2	0	2303 ± 1695.2	38.12	0	1.6	0.32
Leptin	82.2 ± 1565.3	133.21	4	1637.5 ± 2414.1	125.33	0	27.4	*0.01
Resistin	2713.4 ± 1854.6	87.25	0	3824.8 ± 3550.7	72.71	0	4.5	0.12
Adiponectin	>250000	na	na	>250000	na	na	80.3	na
OSM	0 ± 0	447.83	16	0 ± 0	307.79	12	1.59	0.09

*Data are indicated as median ± Interquartile Range (IqR). The coefficient of variation (CV) in percentage and Lower Limited of Quantification (LLOQ) with the number of samples are given. Data are subjected to non-parametric statistical analysis Mann-Whitney U; *p<0.05 and # p<0.001. GM-CSF, IL-12(p70), IL-13, IL-17, IL-2, IL-4, IL-5, IL-7, IL-9, TNFα, TNFβ, VEGF and NGF could not be detected*

Table 2. Pattern matrix after Oblimin rotation with Kaiser Normalisation as obtained from PCA in the spectrum of synovial fluid mediators.

Action	Mediator	Cluster					
		1	2	3	4	5	6
Pro-inflammatory	IFNγ	0.988					
Pro-inflammatory	OSM	0.980					
Pro-inflammatory	IL-7	0.971					
Pro-inflammatory	IL-1β	0.947					
Anti-inflammatory	IL-1ra	0.851					
Chemokine	MCP-3	0.849					
Pro-inflammatory	IL-8	0.823					
Growth factor	TGFα	0.612					
Pro-inflammatory	IL-3		0.942				
Chemokine	MIP-1α		0.919				
Proliferation	EGF		0.888				
Adipokine	Leptin		0.871				
Proliferation	IL-12		0.702				
Chemokine	MCP-1			0.841			
Anti-inflammatory	IL-10			0.807			
Chemokine	Eotaxin			0.653			
Chemokine	G-CSF			0.622			
Chemokine	RANTES				-0.953		
Growth factor	PDGF-AB/BB				-0.935		
Growth factor	PDGF-AA				-0.914		
Chemokine	IP-10				-0.705		
Growth factor	sCD40L				-0.683		
Pro-inflammatory	IFNα				-0.589		
T-cell factor	Flt-3 ligand					-0.827	
Growth factor	HGF					-0.795	
T-cell factor	IL-15					-0.703	
T-cell factor	sIL-2ra					-0.565	
Adipokine	IL-6						0.922
Adipokine	Resistin						0.678
Eigenvalues		10.5	5.8	3.8	2.2	2.1	2.1
Variances explained (%)		30%	17%	11%	6%	6%	6%
Cronbach's alphas		0.835	0.059	0.114	0.615	0.070	0.115

Control and osteoarthritic samples are combined. Only loading factors > 0.5 are displayed.

The PCA was performed to gain insight into the associations between individual mediators that were assessed. The PCA showed communalities > 0.3, which as such, were included in the analysis. PCA enabled identification of 6 clusters of interrelated mediators among the spectrum of 47 mediators (Table 2). These clusters may reflect

important pathophysiological pathways in the joint. In the pattern matrix, the mediators IFNγ, OSM, IL-7, IL-1β, IL-8 and TGFα were clustered in the first cluster, and together explained 30.2 % of the total variance with an eigenvalue of 10.5. Cluster 2 included IL-3, MIP-1α, EGF, leptin and IL-12, (variance explained 16.8%, eigenvalue 5.9). Cluster 3, contained MCP-1, IL-10, eotaxin and G-CSF (variance explained 10.8%; eigenvalue 3.8). Cluster 4 contained RANTES, PDGF-AB/BB, PDGF-AA, sCD40L, IP-10, IFNα (variance explained 6.4%, eigenvalue 2.2). Cluster 5 included Flt-3 ligand, HGF, IL-15 and sIL-2ra (variance explained 6.1%, eigenvalue 2.1). Cluster 6 included the adipokines IL-6 and resistin (variance explained 6.0%, eigenvalue 2.1). In total, these 6 clusters explained more than 76% of the variance. The first two clusters mainly contained pro-inflammatory mediators and cluster 3 included predominantly chemokines. Cluster 4 contained predominantly growth factors and cluster 5 factors associated with T-cell proliferation and maturation. Finally, cluster 6 contained only adipokines. Mediators in all these 6 clusters are known to be part of important processes in the joint homeostasis. However, distinguishing between potential clusters was difficult, since these mediators are involved in adjacent and interrelated pathways.

Cytokines, chemokines, adipokines and growth factors play a major role in inflammatory diseases, such as OA, and are currently intensively studied. The adipokines are associated with obesity, which is in itself associated with low-grade systemic inflammation, one of the risk factors for the development and progression of OA (2, 6, 7). The different levels of IL-6, leptin and adiponectin between control and OA synovial fluid might indicate a role for certain adipokines in OA processes, which is in line with literature (7). However, these data were not corrected for BMI, which might influence the outcomes. Another group of mediators consisted of chemokines. The measured chemokines such as the related Chemokines-Chemokines (CC) mediators RANTES, MDC, MIP-1α and MIP-1β were higher in OA than in control synovial fluid. This group of chemokines share the same receptor complexes, e.g. CCR1, CCR2 and CCR5 (8) and their major function is to attract inflammatory cells, e.g. T-cells, macrophages and other inflammatory cells to sites of inflammation. Chemokines, such as RANTES, are also capable of activating (inflammatory) cells in the production of inflammatory mediators. As shown in previous studies, RANTES promotes the

production of IL-6 by synovial fibroblasts and enhances the inflammatory response in OA through the different CCR receptor in synovial tissue (9). Moreover, CCR receptors are present in chondrocytes and treatment of cartilage explants with RANTES was demonstrated to increase release of proteoglycans (10). The combination of multiple CC-chemokines in combination with IL-6 and leptin in the synovial fluid was not demonstrated earlier. Blocking these CC-chemokines or their receptors in OA might reduce IL-6 and leptin production and, consequently, the infiltration of inflammatory cells and the production of catabolic factors in the joint. Future research is necessary to elucidate this.

In none of the synovial fluid samples GM-CSF, IL-12(p70), IL-13, IL-17, IL-2, IL-4, IL-5, IL-7, IL-9, TNFβ, VEGF and NGF and TNFα, could be detected. IL-1 was only detected at very low levels. TNFα and IL-1 are pro-inflammatory cytokines, which are associated with cartilage degeneration, synovial inflammation and bone changes (11). Although in some *in vivo* animal models of OA, blocking IL-1 or TNFα gave promising results, this could not be validated in clinical studies (12). Our results likewise do not support a prominent role for IL-1 and TNFα in end-stage OA patients. Nonetheless, this does not exclude a role for IL-1 and TNFα in for example early OA, as OA has a heterogeneous character with multiple phenotypes.

For the interpretation of the data it should be mentioned that our results are based on a small sample size, due to donor availability, which were not paired on age, BMI and sex. Moreover, it should be taken into account that ex-vivo modifications cannot be excluded for the control donors as samples were taken after death. Also due to the small sample size, no further statistics were performed on the PCA to study differences between the clusters for OA and control synovial fluid samples. Therefore this pilot dataset should be regarded as a reference and more extensive profiling studies are necessary to confirm these data.

In summary, this is the first study measuring a wide panel of mediators in the synovial fluid of both control and end-stage OA donors. Increased levels of mediators such as MDC, IL-6 and RANTES once more confirm involvement of inflammatory processes and might be associated with the influx of inflammatory cells in OA synovial tissue. In addition, the PCA indicated 6 clusters, which reflect different processes in the joint. Due to the small samples size no hard conclusions can be drawn.

Nonetheless, this pilot dataset provides a valuable reference for future experiments to study pathophysiological pathways, and to be useful in more extensive profiling studies for OA.

Acknowledgements

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5

Metabolic stress–induced inflammation plays a major role in the development of osteoarthritis in mice

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Abstract

Objective: Obesity is associated with systemic inflammation and is a risk factor for osteoarthritis (OA) development. We undertook this study to test the hypothesis that metabolic stress–induced inflammation, and not mechanical overload, is responsible for the development of high-fat diet–induced OA in mice.

Methods: Human C-reactive protein (CRP)–transgenic mice received a high-fat diet without or with 0.005% (weight/weight) rosuvastatin or 0.018% (w/w) rosiglitazone, 2 different drugs with antiinflammatory properties. Mice fed chow were included as controls. After 42 weeks, mice were killed and histologic OA grading of the knees was performed. To monitor the overall inflammation state, systemic human CRP levels were determined.

Results: Male mice on a high-fat diet had significantly higher OA grades than mice on chow and showed no correlation between OA severity and body weight. In male mice, high-fat diet–induced OA was significantly inhibited by rosuvastatin or rosiglitazone to OA grades observed in control mice. Both treatments resulted in reduced human CRP levels. Furthermore, a positive correlation was found between the relative individual induction of human CRP evoked by a high-fat diet on day 3 and OA grade at end point.

Conclusion: High-fat diet–induced OA in mice is due to low-grade inflammation and not to mechanical overload, since no relationship between body weight and OA grade was observed. Moreover, the OA process was inhibited to a great extent by treatment with 2 drugs with antiinflammatory properties. The inflammatory response to a metabolic high-fat challenge may predict individual susceptibility to developing OA later in life. The use of statins or peroxisome proliferator–activated receptor γ agonists (e.g., rosiglitazone) could be a strategy for interfering with the progression of OA.

Osteoarthritis (OA) is a chronic degenerative joint disease with large consequences for the quality of life of patients. It is now generally accepted that OA is not only a disease of articular cartilage, but in fact involves the entire joint, including Hoffa's fat pad, synovium, subchondral bone, menisci, and ligaments. Insight into the different underlying processes leading to the clinical and pathologic outcomes of OA is crucial in the search for new therapies (1, 2).

Obesity is a risk factor for the development of OA and is classically seen as a biomechanical factor, suggesting that the increase of loading forces causes cartilage damage. However, from the association between obesity and OA of non–load bearing joints it is hypothesized that systemic factors induced by obesity contribute considerably to the initiation and progression of OA (3). Obesity is associated with a mild chronic inflammation, and adipokines secreted by adipocytes and macrophages within adipose tissue are suggested to be a metabolic link between obesity and OA (4, 5). However, the relative contribution of these processes in the onset and progression of OA remains unclear.

The association between obesity and the development of OA has been studied in several animal models. Previously, Sokoloff et al showed that high-fat diet–induced obesity caused OA in 2 different mouse strains and in a rat strain. In addition, STR/Ort mice, which are vulnerable to spontaneous obesity, developed OA in a short period of time (6). The incidence of knee OA was found to be twice as high in C57BL/6 mice on a high-fat diet than in the control group fed with a chow diet. Changes associated with the human metabolic syndrome such as abdominal obesity, hyperglycemia, hyperinsulinemia, and hypertension are also present in these animals (7, 8). Although some of these diet-induced OA mouse model studies support a role of mechanical factors in the progression of OA, a correlation between body weight and OA is lacking. The fact that adipose tissue of C57BL/6 mice can become inflamed under conditions of metabolic stress (e.g., high-fat diet feeding) and secrete a broad spectrum of inflammatory mediators (9) suggests that high-fat diet–fed C57BL/6 mice constitute a suitable model to study the role of (metabolic stress–induced) inflammation in the development of OA.

The human C-reactive protein (CRP)–transgenic mouse model enables us to study the role of inflammation in mice. CRP is an acute-phase protein and an established

marker for systemic inflammation in humans, but not in wild-type mice, where its serum levels never rise above 2–3 mg/liter (10). CRP is produced by hepatocytes and adipocytes and is regulated by proinflammatory cytokines. Human CRP–transgenic mice carry the entire human CRP gene, including the coding region and 17 kb of the 5'-flanking promoter region, and the expression of human CRP in these mice resembles its regulation in humans. Unlike its wild-type mouse counterpart, human CRP behaves as a major acute-phase reactant when introduced into the mouse genome (11). Obese individuals are found to have higher CRP levels, indicating a low-grade inflammation state (12, 13). Obese human CRP–transgenic mice on a C57BL/6 background are therefore an interesting model for analyzing the role of inflammation in the development of OA. Modulation of this metabolic stress–induced inflammation provides a tool to gain more insight into the relevant contribution of this process in OA.

Statins and peroxisome proliferator–activated receptor γ (PPARγ) agonists have been shown to have important pleiotropic antiinflammatory effects in a number of studies and are therefore interesting interventions for studying the role of inflammation in OA (14-16). Statins are inhibitors of the rate-determining enzyme in the biosynthesis of cholesterol and are considered the most effective drugs to reduce serum cholesterol levels (17). PPARγ receptors are nuclear hormone receptors and are expressed at high levels in adipose tissue (18). PPARγ agonists bind to and activate these receptors and are used for the treatment of type 2 diabetes mellitus. In recent studies, PPARγ ligands were shown to have a wide spectrum of actions in the treatment of metabolic disorders (19, 20).

Statin or PPARγ agonist therapy for OA has been suggested in the literature (19, 21, 22); however, in vivo data demonstrating the use of these drugs are scarce. Treatment with statins or PPARγ agonists in animals with experimentally induced OA resulted in reduced cartilage degradation, probably due to decreased inflammatory cell infiltration and matrix-degrading enzyme expression (23-25). However, their effect in high-fat diet–induced OA has not been investigated.

In this study, we hypothesize that high-fat diet–induced OA in mice is not due to mechanical overload, but is the consequence of metabolic stress–induced inflammation. We used the human CRP–transgenic mouse model to monitor the

overall inflammation state during the high-fat diet. Interventions with statins and PPARγ agonists were applied to suppress metabolic stress–induced inflammation to demonstrate that this process is involved in the pathogenesis of OA under conditions of obesity and the metabolic syndrome.

Methods

Animals

Human CRP–transgenic mice (11, 26) on a C57BL/6 background were characterized by polymerase chain reaction and enzyme-linked immunosorbent assay (ELISA) for human CRP expression. Mice were housed in groups under standard conditions on a 12-hour light/dark cycle and had free access to water and food. Experiments were approved by the Institutional Animal Care and Use Committee of TNO and were in compliance with European Community specifications regarding the use of laboratory animals.

Diets

Human CRP–transgenic 12-week-old male and female mice were fed standard lab chow (V1534; Ssniff Spezialdiäten) (time 0). After 4 weeks (time 4) the diets were changed, and mice were randomly distributed in 3 groups consisting of 9 male and 9 female mice. Group 1 consisted of control mice that remained on a standard chow diet. Mice in group 2 were switched to a high-fat diet (D12451, 23.1% fat; Ssniff Spezialdiäten), and mice in group 3 were fed a high-fat diet containing 0.018% (weight/weight) rosiglitazone (GlaxoSmithKline). Besides these 3 groups, another group (group 4) was added that received chow supplemented with 0.01% (w/w) rosuvastatin (AstraZeneca) (time 0), which was continued in a high-fat diet after 4 weeks but at a lower dosage of 0.005% (w/w) because of increased absorption of rosuvastatin on high-fat diets. Rosuvastatin and rosiglitazone doses were based on their antiinflammatory effects observed in previous studies (27, 28). Chow-fed control mice (group 1), untreated mice on a high-fat diet (group 2), and treated mice on a high-fat diet (groups 3 and 4) remained on their respective diets until the completion of the study at age 54 weeks (time 42).

Histologic examination

At end point (time 42), knee joints of the hind limbs were fixed in a 10% neutral buffered formalin solution (Sigma-Aldrich), decalcified in Kristensen's solution (29), dehydrated, and embedded in paraffin for histologic analysis. Serial coronal 5 µm sections were collected throughout the patella, medial and lateral joint. Sections were stained with hematoxylin, fast green, and Safranin O as well as with hematoxylin–phloxine–saffron. Sections were randomly scored in a blinded manner at 3 locations in the joint: the femorotibial joint at the lateral side, the femorotibial joint at the medial side, and the patellofemoral joint. The scoring system included changes in articular cartilage structure, proteoglycan depletion, and chondrocyte morphology. The whole joint was scored following the international guidelines of the Osteoarthritis Research Society International (OARSI) histologic grading system (30). Briefly, this scoring system is based on a combined assessment of severity (from grade 0 = surface and cartilage intact to grade 6 = deformation) and extent (from stage 0 = no OA activity to stage 4 = >50% OA activity) of OA in the articular cartilage (grade by stage).

Human CRP levels

To monitor metabolic stress–induced inflammatory changes and the antiinflammatory effects of rosuvastatin and rosiglitazone using human CRP levels, after 4 hours of food deprivation blood was obtained by tail incisions and collected in EDTA tubes for plasma preparation (Sarstedt) at times 0, 2, 4, 4.5, 5, 11, and 34 weeks. Tubes were centrifuged for 10 minutes at 6,000 revolutions per minute, and plasma was immediately stored at −80°C until analysis. Human CRP was quantified in the plasma samples by established ELISA (R&D Systems).

To characterize the inflammatory responsiveness of the mice and to verify the antiinflammatory effects of rosuvastatin and rosiglitazone, all groups on high-fat diets were injected intraperitoneally (IP) with 100 µl interleukin-1β (IL-1β; PeproTech) at a mild dose (~200 ng) 2 weeks before (time 2) and 8 weeks after (time 12) diet switch. The concentration used in this study is negligible compared with that used in previous models in which IL-1 was injected intraarticularly into the mouse knee joint (31). Plasma samples were collected prior to and 18 hours after this challenge and used to quantify the human CRP level.

Cytokine measurements

At end point, blood was collected by heart puncture and centrifuged for 5 minutes at 10,000 rpm. Serum samples were immediately stored at −80°C. Levels of the cytokines IL-6, leptin, resistin, and tumor necrosis factor α (TNFα) were measured in the serum samples using a multiplex bead immunoassay (Millipore) for mice with the Luminex 100 instrument. All samples were analyzed as recommended by the manufacturer.

Insulin levels

To determine the effects of high-fat diet and treatment on insulin, levels were measured at times 5 and 11 in plasma samples using an ELISA (Mercodia).

Statistical analysis

Statistical analysis was performed using the nonparametric Kruskal-Wallis and Mann-Whitney U tests. Pearson's correlation coefficient was used to assess the relationship between OA grade and human CRP levels. P values less than 0.05 were considered significant. Unless stated otherwise, data are shown as the mean ± SD.

Results

Body weight and body fat

Male mice in all the high-fat diet groups (treated and untreated) had significantly higher mean ± SEM body weights at the end of the study compared to the chow-fed control group. Male mice treated with rosiglitazone had the highest mean ± SEM body weight due to an increased fat accumulation, and this weight was significantly higher than that in the untreated high-fat diet group (untreated high-fat diet group 41.4 ± 1.6 grams, $P < 0.01$ versus chow-fed control group [35.1 ± 0.6 grams]; high-fat diet with rosuvastatin group 46.1 ± 2.3 grams, $P < 0.01$ versus chow-fed control group; high-fat diet with rosiglitazone group 57.7 ± 3.9 grams, $P < 0.001$ versus chow-fed control group and untreated high-fat diet group) (Figure 1A). Female mice on a high-fat diet also had increased body weights compared to the chow-fed control group (untreated high-fat diet group 33.2 ± 2.4 grams, *P* not significant versus chow-

fed control group [28.0 ± 1.0 grams]; high-fat diet with rosuvastatin group 37.7 ± 2.0 grams, *P* < 0.001 versus chow-fed control group; high-fat diet with rosiglitazone group 41.6 ± 2.5 grams, *P* < 0.001 versus chow-fed control group) (Figure 1B).

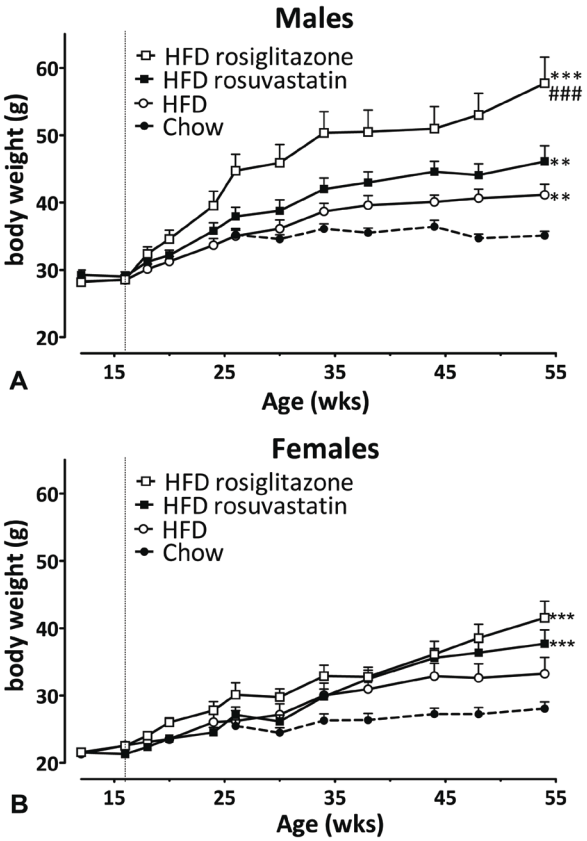

Figure 1. Body weights in each group during the study of male (A) and female (B) human C-reactive protein–transgenic mice. Male mice had a higher average body weight compared to female mice. Values are the mean ± SEM. ** = p < 0.01; *** = p < 0.001 versus chow-fed control group. ### = p < 0.001 versus untreated high-fat diet (HFD) group.

Gonadal, visceral, and subcutaneous fat content of each mouse was summed together to obtain the total fat content and was subsequently expressed as a percentage of the body mass of each mouse at end point. Control female mice on chow had a relatively higher mean ± SD percentage of fat than did male mice on chow (5.0 ± 4.1% versus

3.4 ± 1.0%; $P < 0.05$). High-fat diet groups (treated and untreated) had significantly higher percentages of fat than did the chow-fed control group, both for male mice (untreated high-fat diet group 7.1 ± 1.4%, high-fat diet with rosuvastatin group 8.5 ± 3.1%, high-fat diet with rosiglitazone group 7.7 ± 1.4%; all $P < 0.001$ versus chow-fed control group) and for female mice (untreated high-fat diet group 9.2 ± 3.0%, $P < 0.001$ versus chow-fed control group; high-fat diet with rosuvastatin group 12.4 ± 2.6%, $P < 0.001$ versus chow-fed control group; high-fat diet with rosiglitazone group 6.5 ± 1.5%, $P < 0.05$ versus chow-fed control group).

Effects of high-fat diet and antiinflammatory interventions on OA development

On macroscopic evaluation, male mice from the untreated high-fat diet group had red, swollen knee joints with malformed morphology, which was not present in male mice from the chow-fed control group. High-fat diet groups treated with rosuvastatin or rosiglitazone did not have such macroscopic alterations. No macroscopic abnormal joint morphologies were observed in any female mice in the chow-fed control group or in the untreated or treated high-fat diet groups.

To determine microscopic differences, hematoxylin–, fast green–, and Safranin O–stained slides as well as hematoxylin–phloxine–saffron–stained slides of all knee joints were scored on different components. All joints had at least some minor OA changes such as slight fibrillation, hypocellularity, and Safranin O loss (Figure 2). Using the OARSI grading system, male and female mice on chow had comparable low mean ± SD OA grades (4.4 ± 2.9 versus 2.9 ± 1.6, respectively; $P = 0.3$). Male mice receiving a high-fat diet instead of chow had significantly higher OA grades at end point. Severely affected mice had cartilage loss, bone deformation, and osteophyte formation (Figures 2C and D), while the joints of treated mice were comparable to those of their control counterparts (Figures 2E–H).

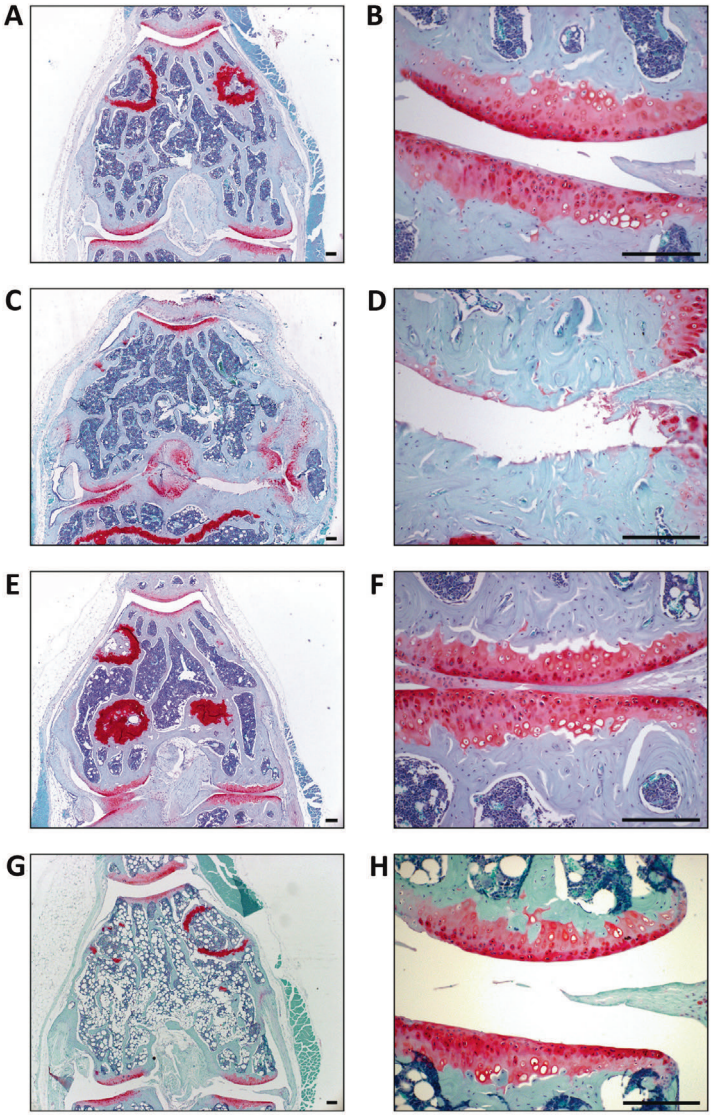

Figure 2. Representative Safranin O–, fast green–, and hematoxylin-stained coronal sections of the whole knee joint (A, C, E, and G) with a detailed picture of the medial femoral condyle and tibial plateau (B, D, F, and H) of human C-reactive protein–transgenic male mice in the chow-fed control group (A and B), the untreated high-fat diet group (C and D), the high-fat diet with rosuvastatin group (E and F), and the high-fat diet with rosiglitazone group (G and H). Bars = 2 mm. Original magnification × 4 in A, C, E, and G; × 20 in B, D, F, and H.

Modulation of metabolic stress–induced inflammation with rosuvastatin or rosiglitazone in male mice on high-fat diets resulted in completely reduced mean ± SD OA grades (high-fat diet with rosuvastatin group 3.8 ± 1.3, $P < 0.01$ versus untreated high-fat diet group [10.1 ± 6.0]; high-fat diet with rosiglitazone group 4.4 ± 0.9, $P < 0.05$ versus untreated high-fat diet group) (Figure 3A). Remarkably, OA grades observed in the treatment groups were comparable to those in mice fed with chow. In contrast to male mice, untreated female mice receiving high-fat diets hardly developed OA, as judged from their OA grades, which were comparable to those of their chow-fed controls. Treatment with rosuvastatin and rosiglitazone in female mice yielded mean ± SD OA grades similar to those of the untreated high-fat diet group (untreated high-fat diet group 4.7 ± 3.7, high-fat diet with rosuvastatin group 5.4 ± 2.7, high-fat diet with rosiglitazone group 2.0 ± 0.0) (Figure 3B). Analysis of the individual components (lateral and medial femorotibial joint, patellofemoral joint) for cartilage structure, chondrocyte morphology, and Safranin O staining summed together showed results similar to those of the OA score (OARSI grade by stage) for the whole joint.

To investigate a possible influence of mechanical forces, body weight and body fat were correlated with OA development. OA grades of male mice in the untreated high-fat diet group were not significantly correlated with body weight or body fat, ruling out mechanical stress and fat mass as important factors for OA in this model.

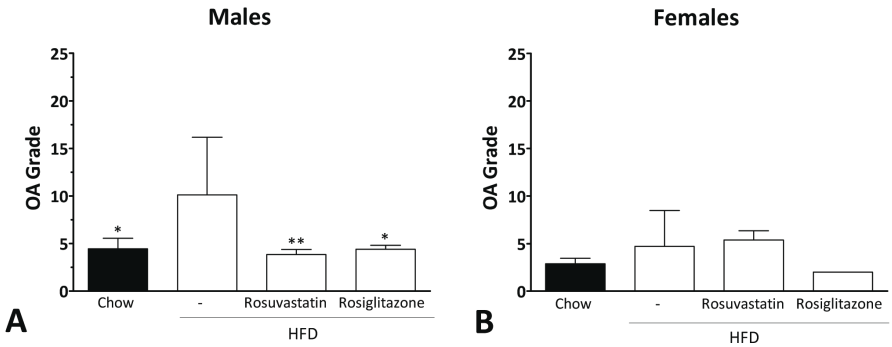

Figure 3. Histologic osteoarthritis (OA) grade of the knee joints of male (A) and female (B) human C-reactive protein–transgenic mice. Rosuvastatin and rosiglitazone decreased the OA grade in male mice significantly. There were no significant differences in female mice ($p = 0.13$ by Kruskal-Wallis test). Values are the mean ± SD. ∗ = $p < 0.05$; ∗∗ = $p < 0.01$ versus untreated high-fat diet (HFD) group.

Effects of high-fat diet and antiinflammatory interventions on human CRP, cytokine, and insulin levels

Human CRP levels were measured during the experiment to monitor the overall inflammation state during high-fat diets as well as the effects of intervention with rosuvastatin and rosiglitazone. Before diet switch, all male mice on chow had similar baseline human CRP levels. In all cases, the switch to high-fat diets resulted in an increase in human CRP levels. Although not significant at every analyzed time point, rosiglitazone- and rosuvastatin-treated mice had lower human CRP levels than those in untreated mice on a high-fat diet. At a later phase of the study (time 34), human CRP levels decreased back to baseline levels (Figure 4). All female mice had an ~1,000-fold lower level of human CRP compared to male mice, and there was no effect of rosuvastatin or rosiglitazone (data not shown).

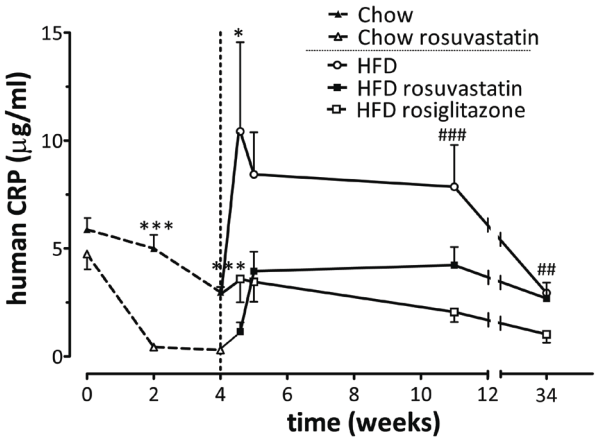

Figure 4. Human C-reactive protein (CRP) levels in male mouse plasma at several time points during the study. Values are the mean ± SD. Dashed lines represent groups before diet switch. Overall, rosiglitazone- and rosuvastatin-treated mice showed lower human CRP levels compared to untreated mice on a high-fat diet (HFD) after diet switch (vertical and horizontal dotted lines), although the difference was not significant at every time point. At times 2 and 4 weeks, untreated mice in the chow-fed control group showed significantly higher human CRP levels compared to rosuvastatin-treated mice in the chow-fed control group. ∗∗∗ = $p < 0.001$ versus rosuvastatin-treated mice in the chow-fed control group. At time 4.5 weeks, untreated mice on a high-fat diet showed significantly higher human CRP levels compared to rosuvastatin-treated mice on a high-fat diet. ∗ = $p < 0.05$ versus rosuvastatin-treated mice on a high-fat diet. At times 11 and 34 weeks, untreated mice on a high-fat diet showed significantly higher human CRP levels compared to rosiglitazone-treated mice. ## = $p < 0.01$; ### = $p < 0.001$ versus rosiglitazone-treated mice. Human CRP levels in female mice were 1,000-fold lower (data not shown).

To assess obesity-related cytokines, a multiplex assay was performed on serum samples collected from male and female mice at end point. Levels of IL-6 and TNFα were below the limit of detection. Leptin and resistin levels were high and increased in high-fat diet groups (treated and untreated) compared to those in the respective chow-fed control groups (Figures 5A and B). Treatment with rosuvastatin or rosiglitazone did not significantly alter leptin or resistin levels compared to those in the untreated high-fat diet group. No apparent differences between male and female mice were observed for leptin and resistin levels. No significant correlations were found in any of the groups between leptin or resistin levels and OA grade at end point (data not shown).

Insulin levels were measured to detect the effects of high-fat diet and intervention with rosuvastatin or rosiglitazone on the development of insulin resistance in this mouse model. At time 5, 1 week after diet switch, mean ± SD insulin levels were significantly suppressed in rosiglitazone-treated male mice on a high-fat diet compared to those in untreated male mice on a high-fat diet (0.5 ± 0.2 µg/liter versus 0.9 ± 0.5 µg/liter; $P < 0.004$). Treatment with rosuvastatin did not alter insulin levels (1.1 ± 0.6 µg/liter) compared to those in untreated mice on a high-fat diet. The same effects were still observed at time 11. Insulin levels in female mice were very low, and there were no significant differences between groups.

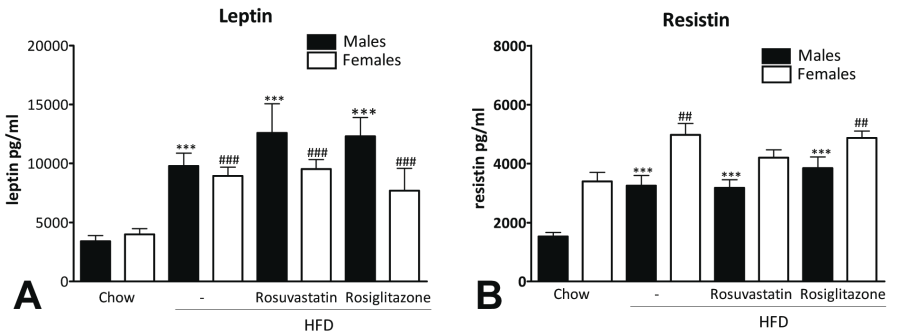

Figure 5. Serum cytokine concentrations of leptin (A) or resistin (B) at end point (time 42 weeks) in chow-fed control mice, untreated mice on a high-fat diet (HFD), and mice on a high-fat diet treated with rosuvastatin or rosiglitazone. Values are the mean ± SD. *** = P < 0.001 versus chow-fed control male mice. ## = P < 0.01; ### = P < 0.001 versus chow-fed control female mice.

Initial response to metabolic high-fat stress correlates with OA at end point

The initial response to metabolic high-fat stress (during the first days of high-fat diet feeding) was determined by calculating the difference in plasma levels of human CRP before and after diet switch. We correlated individual changes in human CRP level with individual OA grades at end point (Table 1). The initial response to high-fat diet feeding (3 days on a high-fat diet; time 4.5 weeks) was significantly correlated with OA. This relationship was lost for human CRP levels determined at later time points, indicating that the inflammatory response evoked by a metabolic stressor predicts susceptibility to developing OA at the level of an individual subject. This notion was further substantiated by demonstrating that no correlation was found between OA and change in human CRP level when human CRP was evoked by a nonmetabolic inducer of inflammation (200 ng IL-1 injected IP) (data not shown).

Table 1. Correlation between relative individual induction of huCRP levels (compared to t=4 weeks; diet switch) and individual end point OA grades.

		Δ huCRP level µg/ml weeks (t)				OA grade
Mouse	**t#**	**4.5**	**5**	**11**	**34**	**42**
1		8.8	3.9	17.6	1.2	9
2		16.3	16.2	13.5	4.1	15
3		37.5	5.5	4.9	1.1	20
4		-1.2	4.3	1.4	-0.8	4
5		9.0	11.2	3.7	-1.1	18
6		4.1	10.1	1.1	0.3	6
7		-0.6	0.8	3.3	-0.4	6
8		-0.6	2.5	2.1	0.6	9
9		-0.1	0.9	2.5	0.6	4
	Mean ± SEM	8.1 ± 4.2	6.1 ± 1.8	5.6 ± 2.0	0.6 ± 0.5	10.1 ± 2.0
	correlation					
	Δ CRP-OA grade	**0.83****	0.54	0.28	0.30	

*Values are tested with a 2-tailed Pearson correlation test. Only correlation directly after diet switch (t=4.5) was statistically significant **p < 0.01*

Discussion

Obesity is associated with low-grade systemic inflammation and is a risk factor for OA development. Whether this mild chronic inflammation or other factors such as mechanical stress are responsible for the development of OA is under debate.

This study provides evidence for a role of metabolic stress–induced systemic inflammation in the development of OA. High-fat diet feeding in human CRP–transgenic mice led to severe OA development compared to chow-fed control mice. We found no correlation between OA and individual body weight or fat mass, which excludes mechanical stress as a major trigger of OA in the present experimental setting. Instead, we demonstrated that antiinflammatory intervention with either rosuvastatin or rosiglitazone suppressed OA development to a great extent. This strongly suggests that metabolic stress–induced inflammation plays a major role in the onset and progression of high-fat diet–induced OA in mice.

In the clinic, statins are used as cholesterol-lowering drugs, and PPARγ agonists are administered as treatments for type 2 diabetes mellitus (17, 20). These different types of drugs only have antiinflammatory properties in common. Therefore, it is most likely that these drugs exert their inhibitory effect on high-fat diet–induced OA through suppression of low-grade systemic inflammation associated with obesity and metabolic stress. Indeed, both treatments resulted in decreased levels of human CRP, which served as a marker of inflammation in this study. This is in line with previously reported data demonstrating that statins reduce basal and IL-1β–induced human CRP expression independent of their cholesterol-lowering effect (12). This implies that systemic inflammatory factors play an important role in the development of high-fat diet–induced OA.

A primary role for inflammation in general in the pathogenesis of OA is also supported by the finding that a statin and a PPARγ agonist suppressed mechanically induced OA in animal models by interfering with local inflammatory processes. Intraarticular administration of mevastatin reduced inflammatory cell infiltration and matrix-degrading enzyme expression in a rabbit model of experimental OA (23). Furthermore, Boileau et al showed an inhibitory effect of pioglitazone (a PPARγ agonist) on the activation of signaling pathways of inflammation in a dog model of experimental OA (24). In addition to models of mechanically induced OA, Yudoh and Karasawa recently demonstrated significantly reduced cartilage degeneration due to statin treatment in STR/Ort mice, a strain that spontaneously develops OA (32). In vitro data suggest that statins and PPARγ agonists have several direct effects on chondrocytes or synoviocytes. For example, statins inhibited matrix metalloproteinase

3 (MMP-3) expression in stimulated human OA chondrocytes and also inhibited apoptosis of synoviocytes. Furthermore, PPARγ agonists suppressed the expression of inducible nitric oxide synthase and MMP-13 in human chondrocytes as well as the expression of MMP-1 in human synovial fibroblasts (21, 22, 33, 34). Stimulation with IL-1 in these in vitro experiments suggests that induction of inflammatory pathways is important for these outcomes and explains the effect of statins and PPARγ agonists on these OA-related processes through their antiinflammatory action.

The adipokines leptin and resistin are important systemic factors that are associated with obesity and suggested to be involved in the development of OA (35). Griffin et al showed in leptin-deficient (*ob*/*ob*) and leptin receptor–deficient (*db*/*db*) mice that body fat adiposity in the absence of leptin signaling is insufficient to induce systemic inflammation and knee OA (36). Consistent with this, we recently showed that quality of adipose tissue (and not its mass) is linked to systemic inflammation status (37). This confirms that proinflammatory factors induced by fat gain are more important in our model than mechanical overload due to an increase in body mass, although previously an association was suggested between body mass and fat and the loss of cartilage matrix proteoglycans in C57BL/6 mice on a high-fat diet (38). The proposed proinflammatory effect of leptin would match very well with increased serum leptin levels in the high-fat diet model and the involvement of inflammation in high-fat diet–induced OA. However, conclusive evidence for this is lacking, since we found no correlation between severity of OA and circulating leptin levels at end point. Furthermore, rosuvastatin and rosiglitazone treatment did not reduce leptin levels, indicating that if leptin is involved, the inhibitory effect of these treatments is not by acting directly on leptin, but probably by influencing downstream inflammatory processes regulated by leptin.

Obesity is also associated with the development of insulin resistance (39). All mice developed higher insulin levels after switching to a high-fat diet except for those treated with rosiglitazone. The antidiabetic property of rosiglitazone is thereby confirmed in this model, but the insulin-lowering capacity is most likely not the cause of the suppressive effect seen on OA development. Since rosuvastatin-treated mice display insulin levels comparable to those in untreated mice, insulin resistance as part of the metabolic syndrome cannot be used to clarify the inhibitory effect of these drugs on OA development in this model.

Besides variation in body weight, the human CRP levels, which were used to monitor inflammation status, also varied between mice of the same group. The variation seen in our study is typical for human CRP–transgenic mice, and similar variation has been reported previously (40, 41). In several human studies, human CRP level is correlated with various features of OA, such as extent and severity of knee OA, knee pain or markers of local synovial inflammation, and knee OA progression. However, human CRP level is not associated with incidence of knee or hip OA when possible confounding factors such as body mass index are taken into account, suggesting that human CRP is not a functional protein in the onset of OA (42, 43).

In our study, we found a correlation between OA grade at end point and change in human CRP level 3 days after diet switch, which suggests that the responsiveness to a metabolic challenge can be used as a marker to estimate the individual susceptibility to developing OA later in life. Consistent with this, we showed that the acute response to high-fat diet–induced stress results in a transient inflammatory response that normalizes as soon as metabolically active organs have adapted their glucose and lipid metabolism (44). Indeed, after several weeks, human CRP levels decreased to baseline levels, and the correlation with OA grade was lost. Since only 9 male mice were in this group, an increased number should be investigated to confirm these results. Our finding of no correlation between human CRP level and OA when an inflammatory response was evoked by IL-1 suggests that metabolic stress–induced inflammation encompasses more inflammatory signaling routes than only those triggered by IL-1.

It is known that in different inbred strains, male mice have a higher susceptibility to developing OA than do female mice (45-47). Concordant with this, in our study only male mice developed substantial and severe OA characteristics after 38 weeks on a high-fat diet. Also, it is well established that female mice on the C57BL/6 background are less sensitive to developing features of the metabolic syndrome (obesity, adiposity, metabolic inflammation, and the like) than are their male counterparts (48). Highlighting this is the fact that insulin levels, as a measure of diabetes, were very low in female mice compared to male mice on a high-fat diet. This indicates that metabolic stress is more important than age-dependent processes on the development of OA in this model.

The present study demonstrates that low-grade inflammation associated with obesity plays an important role in the development of high-fat diet–induced OA, since rosuvastatin and rosiglitazone suppress development of OA features via their antiinflammatory mode of action. Mechanical overload seems to be less important in the development of OA in this high-fat diet–induced disease model, since no correlation with body weight or body fat mass was observed. Individual susceptibility to a metabolic challenge may predict OA development at end point and needs to be further investigated. These findings suggest that the use of statins and PPARγ agonists could be a strategy for interfering with the progression of the disease, at least in those patients with metabolic disorders. Identification of the inflammatory mediators involved may provide leads to new treatment regimens in OA.

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6

Osteoarthritis development is induced by increased dietary cholesterol and can be inhibited by atorvastatin in APOE*3Leiden.CETP mice, a translational model for atherosclerosis

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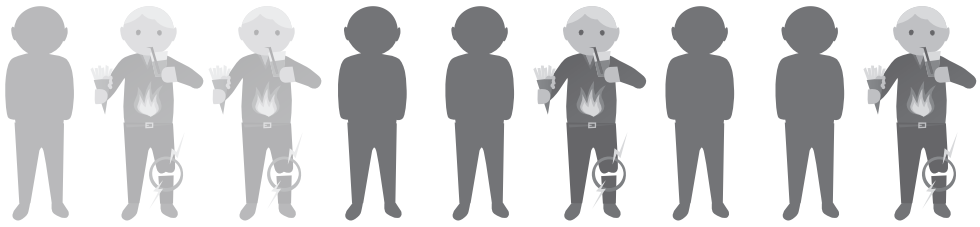

Abstract

Objective Hypercholesterolemia, a risk factor for atherosclerosis (ATH), has been suggested to have a role in osteoarthritis (OA) development. To test this hypothesis, the effect of cholesterol and different cholesterol-lowering treatments on OA was investigated in a mouse model resembling human lipoprotein metabolism.

Methods Female ApolipoproteinE*3Leiden.human Cholesteryl Ester Transfer Protein mice received a western type diet (WTD) with 0.1% (w/w) cholesterol (LC), 0.3% (w/w) cholesterol alone (HC) or treated with 3 mg/kg/day atorvastatin or 0.3 mg/kg/day ezetimibe. One group remained on chow (control). After 39 weeks, OA grades of the knees and the extent of ATH were determined. Plasma cholesterol levels were measured throughout the study.

Results LC and HC groups developed significantly more OA at the medial side than the control group in a dose-dependent manner. Atorvastatin but not ezetimibe treatment significantly suppressed OA development. As expected, features of ATH were significantly increased in the LC and HC groups compared to the control group, and suppressed by atorvastatin (48%) and ezetimibe (55%) treatment. There were significant correlations between the development of OA on the medial side of the joint and cholesterol exposure (r=0.4) or ATH features (r=0.3).

Conclusions Dietary cholesterol and accordingly increased plasma levels play a role in the development of OA. The correlation found between OA, cholesterol and ATH demonstrates that these variables are connected, but indicates the contribution of other ongoing processes in OA development. The suppressive effect on OA development of atorvastatin but not of ezetimibe, which had similar cholesterol exposure levels, corroborates these findings.

Osteoarthritis (OA) is a degenerative joint disease with major implications on the quality of life. Recently, various studies presented a relation between OA and the prevalence of metabolic syndrome (MetS) (1-3). MetS is generally considered a combination of obesity, hypertension, dyslipidemia and impaired glucose tolerance (4). Puenpatom *et al* demonstrated that prevalence of MetS was over twofold higher in the OA population and OA was associated with an increased risk of having MetS (5). These results have led investigators to contemplate common underlying pathologies in OA and MetS related diseases.

Related to MetS is hypercholesterolemia that can result as a consequence of abnormalities in the cholesterol uptake in the gut and/or improper regulation of plasma cholesterol levels. Interestingly, hypercholesterolemia was shown to be associated with generalized OA (6). Furthermore, serum cholesterol and triglycerides are linked with the incidence of bone marrow lesions, which are common in OA patients (7). Some studies indicate that cholesterol induces low grade inflammation in humans and in mice and that this is dependent on the amount of cholesterol in the diet (8, 9). Low grade inflammation is also designated to play a role in OA development, which suggests it could be the link between elevated cholesterol levels and OA (10).

An extended exposure to increased cholesterol levels can lead to the development of atherosclerosis (ATH) (11, 12). The question is raised if ATH and OA are linked by common underlying mechanisms (13). This suggestion is strengthened by the finding that ATH was associated with OA in several joints in women (14, 15). Furthermore, it has been hypothesized that atheromatous vascular disease is linked to OA and is probably more involved in the progression of OA than in its initiation (16). It is unclear whether OA and ATH occur as concurrent diseases due to common etiology or are causally related (13).

Cholesterol lowering interventions are used to prevent harmful consequences caused by high cholesterol exposure (e.g. ATH) (17). Statins, hydroxymethylglutaryl (HMG) Co-A reductase inhibitors, are commonly used and have been reported to possess pleiotropic effects independent of their cholesterol lowering capacity. Statins are also suggested to have important therapeutic value in the context of OA (18-20). Another drug used to control cholesterol levels, but with a different mode of

action to that of statins, is ezetimibe which inhibits the uptake of cholesterol in the intestine.

Given the emerging evidence that in man cholesterol levels are linked to OA features, we questioned whether increased intake of cholesterol would be sufficient to induce OA. We used the ApolipoproteinE*3Leiden.Human Cholesteryl Ester Transfer Protein (APOE*3L.CETP) transgenic mouse which is a well-established model for hyperlipidemia and ATH and resembles human lipoprotein metabolism in contrary to wild type mice (21). Statin and ezetimibe treatments were included to further understand the contribution of cholesterol in the development of OA. The dosages of the treatments were chosen to result in similar plasma cholesterol levels as obtained in mice receiving low cholesterol concentrations in their diet. This enabled us to study the effects of the drug interventions on OA and ATH independent of but also in addition to their cholesterol lowering properties (22, 23).

Methods

Mice

Female APOE*3Leiden mice, characterized by an enzyme-linked immunosorbent assay (ELISA) for human APOE, were crossbred with human CETP transgenic mice which express CETP under control of its natural flanking regions in our animal facility to obtain heterozygous APOE*3L.CETP mice (24-28). Mice were housed in groups under standard conditions with a 12h light-dark cycle and had free access to water and food. Body weight (BW) and food intake were monitored during the study. Experiments were approved by the institutional Animal Care and Use Committee of TNO and were in compliance with European Community specifications regarding the use of laboratory animals.

Experimental design and diets

Mice (n=78) were fed standard lab chow (V1534 Ssniff Spezialdiäten GmbH, Germany) until the start of the study at the age of 10-16 weeks. At t=-4 weeks 66 mice received a semi-synthetic western type diet (WTD) (AB diets, the Netherlands) supplemented with 0.1% (w/w) cholesterol (low cholesterol, LC group). This resulted

in moderately elevated plasma cholesterol levels of mean ± SD 10.1 ± 1.1 mmol/L. Twelve mice remained on chow (control group) (plasma cholesterol level mean ± SD 3.5 ± 0.9 mmol/L). After 4 weeks (t=0) mice receiving LC (n=66) were randomized into 4 groups of 12 animals with on average comparable age, BW, plasma cholesterol and triglyceride levels. Of these 4 groups one continued on LC (LC group) and the others switched to a WTD containing 0.3% (w/w) cholesterol (high cholesterol HC group) or HC supplemented with 3 mg/kg/day (0.0036% (w/w)) atorvastatin (*Lipitor*, Pfizer, the Netherlands) (HC atorvastatin group) or 0.3 mg/kg/day (0.0003% (w/w)) ezetimibe (*Ezetrol*, OPG Pharma, the Netherlands) (HC ezetimibe group). Mice which not responded to the LC diet were sacrificed immediately (n=18). Atorvastatin and ezetimibe dosages were chosen to reduce plasma cholesterol to the same extent as levels in the LC group. Mice were sacrificed after 39 weeks (t=39), reaching an age of 53-59 weeks.

Lipid and lipoprotein analysis

Blood samples were collected after 4h of food deprivation by tail incisions in EDTA tubes (Sarstedt, Germany) at t=-4, 0, 2, 6, 10, 14, 18, 22, 26, 30, 34 and 38 and by heart punction at t=39 weeks. After centrifuging for 10 minutes at 6000 rpm plasma was collected. Total plasma cholesterol (Roche Diagnostics, No-1489437) was determined immediately. For lipoprotein profiles, pooled plasma of each group was fractionated using an Äkta FPLC system (Pharmacia, the Netherlands) and analyzed for their cholesterol containing fractions. Thereafter plasma was stored at -80 °C. Total cholesterol exposure (mmol/l * time in weeks) was calculated for each mouse.

Plasma inflammation markers

Alanine Aminotransferase (ALT) was determined spectrophotometrically in pooled plasma samples at t=0, 18 and 38 weeks using a reflotron system (Roche diagnostics, USA). Serum Amyloid A (SAA) (Tridelta development, Ireland) (t=0, 18 and 38 weeks), E-selectin and monocyte chemoattractant protein-1 (MCP-1) (both R&D Systems Inc., USA) (t=39 weeks) levels were determined in the individual plasma samples by ELISA according to the manufacturers' instruction. IL-1, IL-6, TNF-α and IFN-γ levels were determined in plasma samples (t=39 weeks) using multiplex analysis according to the manufacturers' instruction (Millipore, Billerica, USA and Invitrogen, Paisley, UK).

Histological examination of OA and ATH

The knee joints of the left hind limbs were fixed in formalin (Sigma-Aldrich, USA), decalcified in Kristensen solution (29), dehydrated and embedded in paraffin. Serial coronal 5 µm sections were collected and stained with hematoxylin, fast green and safranine-O and with hematoxylin, phloxine and saffron (HPS). Sections were scored by 2 independent observers, who were blinded for group assignment, at 6 locations in the joint; femoral condyle and tibia plateau at the lateral and medial side, trochlear groove and the patella (score 0-6) (30). The whole joint was scored following the guidelines of the OARSI grading system including cartilage structure, proteoglycan depletion and chondrocyte morphology (31).

Hearts were isolated, formalin-fixed, embedded in paraffin, sectioned and assessed for ATH features (Figure S1). ATH lesion areas were calculated and classified into five categories according to the American Heart Association (AHA) (32). Type I–III were classified as mild and IV–V lesions as severe lesions. For each mouse, the percentage of all lesions found in the respective categories were calculated (25).

Statistical analysis

Data are presented as mean ± SD. Statistical differences were assessed using the non-parametrical Kruskal Wallis test followed by Mann Whitney U test (OA scores, ATH lesion area, total cholesterol exposure and MCP-1 and e-selectin levels). BW, cholesterol and SAA levels were tested with repeated measure analysis followed by Dunnett T3 *post hoc* test. OA scores, ATH lesion area and cholesterol exposure correlations were calculated with the Spearman Correlation test. A partial correlation was performed for OA scores and ATH lesion area after correction for cholesterol exposure. Inter-observer correlation for OA scores was measured with Spearman Correlation test. SPSS 20.0 for Windows (SPSS, USA) was used for statistical analyses. For all analyses $p<0.05$ was considered significant.

Results

Mice characteristics

Every 2 weeks, BW of all mice were measured and revealed no significant differences between groups during the course of the study (Figure 1A).

To assess the effects of cholesterol intake, plasma cholesterol levels were determined every 4 weeks (mmol/l). The LC and HC groups had significantly higher cholesterol levels over time than the control group (both $p<0.001$) (Figure 1B). This was confirmed by the total cholesterol exposure (mmol/l * time in weeks) (Figure 1C); the LC group had 3.1 and the HC group had 5.6 times more cholesterol exposure than the control group (both $p<0.001$).

Elevated cholesterol levels due to HC were significantly reduced by atorvastatin and ezetimibe treatment (both $p<0.001$) to similar levels as the LC group (Figure 1B). Atorvastatin reduced the total cholesterol exposure levels by 35% and ezetimibe by 38% (both $p<0.001$), to a level comparable as in the LC group (Figure 1C). In general, lipoprotein profiling revealed that the reduction in total cholesterol by the atorvastatin and ezetimibe treatments was mainly confined to the (very-)low-density lipoprotein (VLDL)/LDL fractions which is demonstrated by representative data obtained at t=34 (Figure 1D).

Inflammation markers

The acute phase protein SAA, the liver damage marker ALT, the adhesion molecule E-selectin and the pro-inflammatory chemokine MCP-1 were measured as functional markers for inflammation. SAA levels were higher in the LC and HC compared to the control group during the course of the study (non-significant). The LC and HC groups showed a tendency for higher ALT levels, but significance could not be tested since this parameter could only be measured in pooled plasma. At end-point, MCP-1 levels in the LC and HC group were significantly higher than in the control group ($p<0.001$). E-selectin levels were significantly higher in the HC group than in the control group($p<0.001$).

After atorvastatin and ezetimibe treatment, mice had lower SAA and ALT levels compared to the untreated HC group (not significant). Both treatments resulted in

significantly reduced E-selectin levels ($p<0.01$). MCP-1 levels were not affected by the treatments (Table 1).

IL-1, IL-6, TNF-α and IFN-γ levels in the plasma samples at end point were below the lowest level of detection in the multiplex assays.

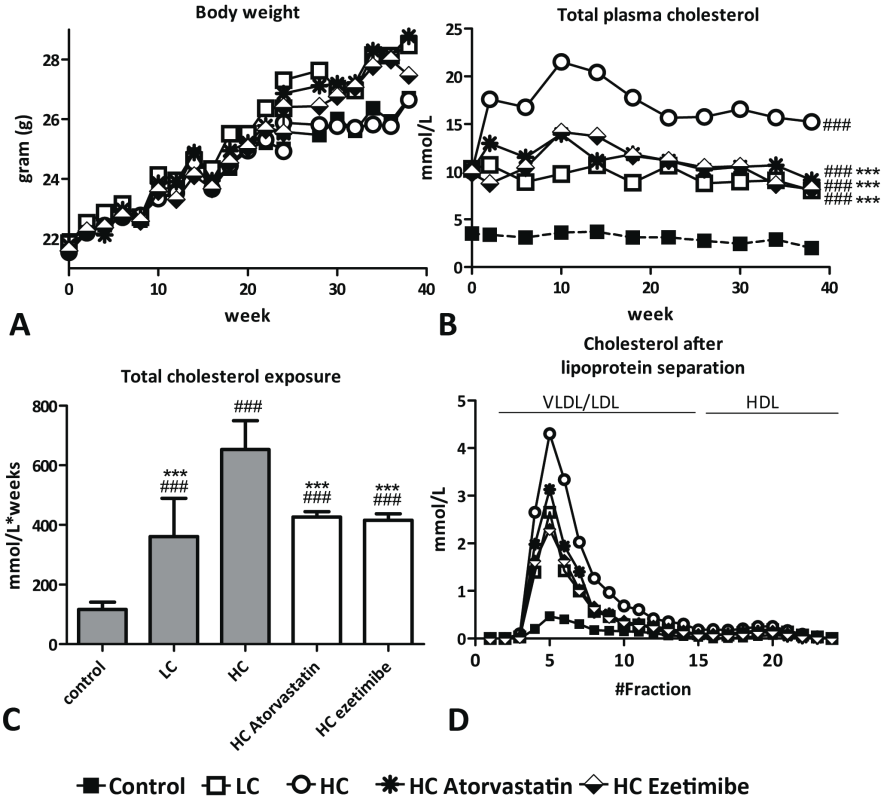

Figure 1. Characteristics of APOE*3Leiden.CETP mice on a control, low and high cholesterol diet A. Body weight (grams), B. Total plasma cholesterol levels (mmol/L), C. Total cholesterol exposure (mmol/L*weeks) in the complete study, D. Representative cholesterol distribution after lipoprotein separation (mmol/L) at t=34 weeks. Fractions 4-15 contain the ApoB containing lipoproteins (VLDL/LDL) and fractions 16-24 contain the HDL particles. Control (n=12), low cholesterol (LC) (n=10), high cholesterol (HC) (n=12) or HC group treated with atorvastatin (n=12) or ezetimibe (n=11) (HC atorvastatin and HC ezetimibe), ###$p<0.001$ vs control; ***$p< 0.001$ vs HC.

Table 1. Functional plasma markers for inflammation.

	SAA µg/ml			ALT µg/ml			E-selectin ng/ml	MCP-1 pg/ml
	t=0	t=18	t=38	t=0	t=18	t=38	t=39	t=39
Control	4.6 (4.6)	2.3 (0.9)	7.0 (4.5)	59.7	56.9	51.3	40.6 (9.4)	26.1 (7.7)
LC	3.4 (0.8)	3.1 (1.0)	7.4 (4.2)	43.9	68.3	169	41.8 (6.8)**	45.5 (12.2)$^{###}$
HC	3.75 (0.61)	5.9 (2.1)	9.1 (5.0)	50.2	109	192	52.9 (7.3)$^{###}$	51.4 (11.0)$^{###}$
HC Atorvastatin	3.9 (3.2)	3.3 (1.2)	6.4 (1.8)	48.9	64.5	164	42.9 (8.2)**	51.4 (17.4)$^{###}$
HC Ezetimibe	3.5 (3.0)	3.0 (0.9)	8.6 (5.4)	40.3	52.2	174	43.9 (12.0)**	46.2 (15.2)$^{###}$

Serum amyloid A (SAA) and Alanine Aminotransferase (ALT) levels at t=0, t=18 and t=38 weeks and E-selectin and monocyte chemoattractant protein-1 (MCP-1) levels at t=39 weeks. Data are indicated as mean (SD). SAA, E-selectin and MCP-1 levels were determined in individual plasma samples. ALT levels were measured in pooled plasma samples. Control (n=12), low cholesterol (LC) (n=10), high cholesterol (HC) (n=12) or HC treated with atorvastatin (n=12) or ezetimibe (n=11) (HC atorvastatin and HC ezetimibe) groups. $^{###}$p<0.001 vs control, **p<0.01 vs HC.

Osteoarthritis development

The knee joints were analyzed to assess the effect of increased dietary cholesterol on OA development (Inter observer rate R=0.8, p<0.001). Representative histological pictures are presented in Figure 2A. Mice developed significantly more OA features at the medial side of the joint in the LC (p=0.03) and HC (p=0.01) groups than in the control group (Figure 2B). No effects of increased cholesterol intake were seen on the lateral side or on the trochlear groove. When giving an integrated score of the entire knee joint (including all components, i.e. total OA grade), effects of increased cholesterol intake on OA development were also observed, although not significant (Table 2).

To investigate whether cholesterol-lowering drugs can diminish the development of OA, treatment with atorvastatin or ezetimibe was included. Atorvastatin suppressed the development of OA at the medial side significantly compared to the HC group (p=0.046), whereas ezetimibe did not have this effect (Figure 2A). No significant effects of these treatments were seen on the lateral side, the trochlear groove or the total OA grade (Table 2).

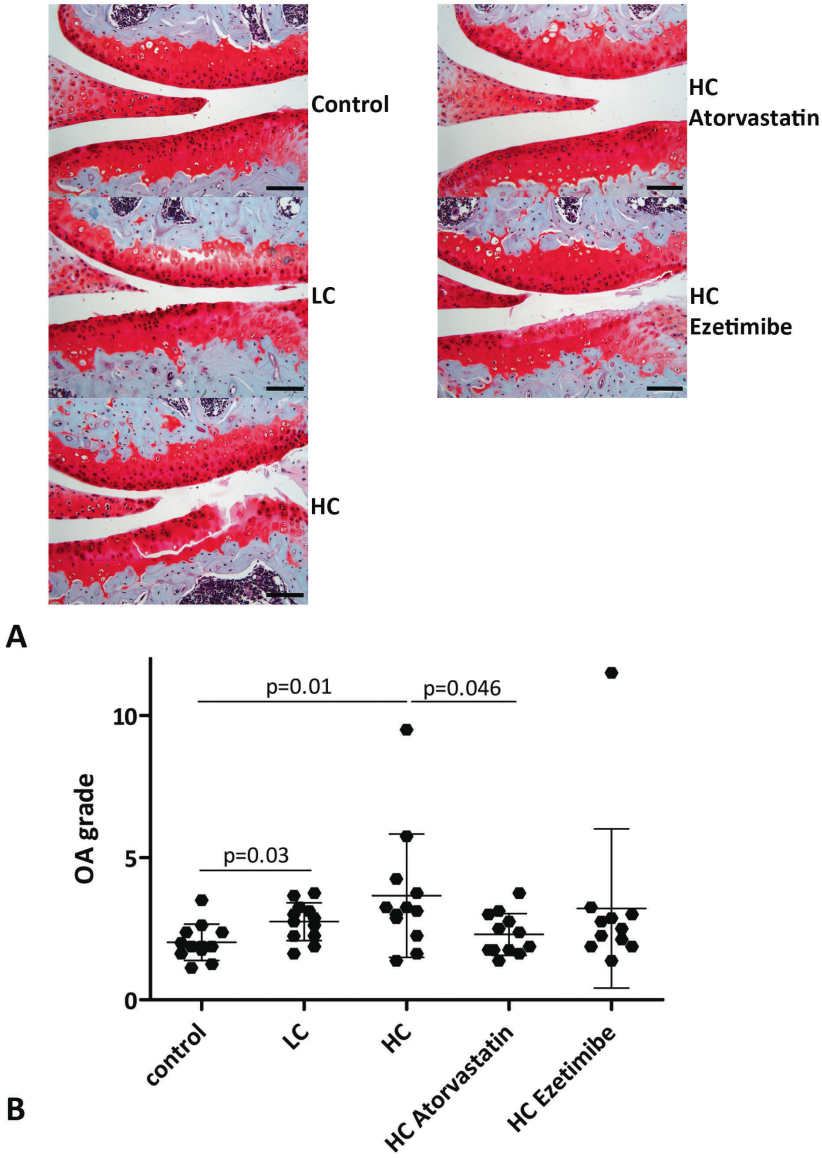

Figure 2. Effect of dietary cholesterol on osteoarthritis (OA) development in the knee joints of APOE*3Leiden.CETP mice A. Representative pictures of hematoxylin, fast green and safranine-O stained medial knee joints. Scale bar indicates 100 µm, B. Sum total of the OA grades at the medial femur and tibia of the knee joint. Each dot is an individual mouse. Line indicates the mean ± SD. Control (n=12), low cholesterol (LC) (n=10), high cholesterol (HC) (n=12) or HC group treated with atorvastatin (n=12) or ezetimibe (n=11) (HC atorvastatin and HC ezetimibe).

Table 2. Osteoarthritis (OA) grades

		Control	LC	HC	HC Atorvastatin	HC Ezetimibe
Medial	Femoral condyle	0.73 (0.37)	1.20 (0.49)$^{\#}$	1.63 (1.23)$^{\#}$	0.84 (0.34) ^	1.36 (1.57)
	Tibia plateau	1.29 (0.42)	1.51 (0.30)	2.04 (1.02)$^{\#}$	1.46 (0.54)	1.85 (1.26)
	Total	2.02 (0.64)	2.71 (0.65)$^{\#}$	3.67 (2.17)$^{\#}$	2.30 (0.73)*	3.22 (2.80)
Lateral	Femoral condyle	1.72 (0.26)	1.68 (0.35)	1.33 (0.61)	1.45 (0.47)	1.49 (0.67)
	Tibia plateau	1.61 (0.55)	1.59 (0.67)	1.81 (0.81)	1.83 (0.94)	1.70 (0.53)
	Total	3.33 (0.76)	3.26 (0.94)	3.15 (0.97)	3.28 (1.27)	3.19 (1.03)
Patella	Patella	0.83 (0.71)	1.20 (1.69)	1.26 (1.42)	1.09 (0.83)	1.09 (1.01)
	Trochlear groove	1.01 (0.61)	1.11 (1.14)	1.23 (0.88)	0.98 (0.56)	1.24 (0.89)
Total OA grade		5.85 (1.57)	7.00 (2.96)	7.35 (3.46)	5.68 (2.03)	7.11 (3.71)

OA grades were scored at the medial, lateral side and patella in control (n=12), low cholesterol (LC) (n=10), high cholesterol (HC) (n=12) or HC treated with atorvastatin (n=12) or ezetimibe (n=11) (HC atorvastatin and HC ezetimibe) groups. Data are indicated as mean (SD). $^{\#}$*p<0.05 vs control;* **p<0.05, ^p<0.1 vs HC.*

Atherosclerosis development

The origin of the aorta in the hearts were analyzed for ATH features. Regular ATH studies in APOE*3L.CETP mice last on average 19-21 weeks (21, 24, 25), however in this study the exposure to high cholesterol was almost twice as long. After 39 weeks the LC and HC groups developed severe ATH in the aortic root (Figure 3A). The total lesion area per cross-section was 33.5 time higher in the LC group and 91.8 times higher in the HC group than the control group (both p<0.001) (Figure 3B). The LC and HC group had significantly more severe lesions than the control group (both p<0.001) (Figure 3C).

Atorvastatin and ezetimibe treatment led to significantly reduced lesion areas compared to the untreated HC group (48% and 55% respectively; both p<0.001) (Figure 3B). Mice receiving atorvastatin treatment had significantly less severe (V-IV) lesions (p<0.05) compared to the untreated HC group which was not the case for ezetimibe.

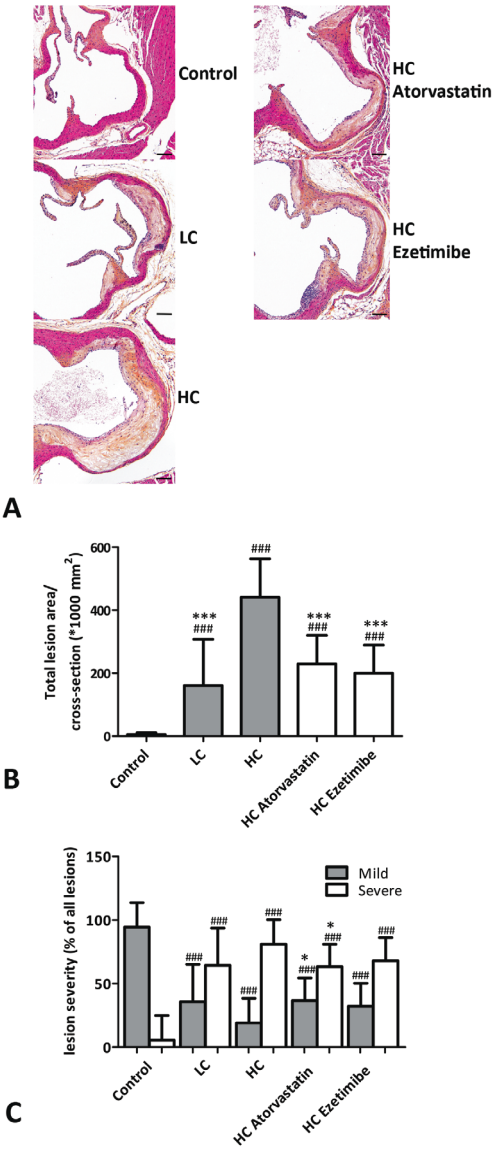

Figure 3. Effect of dietary cholesterol on atherosclerotic development in the aortic root area of APOE*3Leiden.CETP mice A. Representative pictures of atherosclerotic lesions in the root stained with hematoxylin, phloxine and saffron (HPS). Scale bar indicates 100 µm, B. Total lesion area per cross section * 1000 µm2, C. Lesion severity as a percentage of all lesions (mild: I-III and severe: IV-V). Control (n=12), low cholesterol (LC) (n=10), high cholesterol (HC) (n=12) or HC group treated with atorvastatin (n=12) or ezetimibe (n=11) (HC atorvastatin and HC ezetimibe), ###p<0.001, #p<0.05 vs control; ***p<0.001, *p<0.05 vs HC.

Correlations

To explore how and to which extent OA was related to cholesterol exposure and ATH lesion area, linear regression analysis was performed. OA grades at the medial side of the knee joint in the control, LC and HC groups were significantly associated with total cholesterol exposure ($R=0.43$; $p<0.01$) (Figure 4A). Cholesterol exposure levels of each mouse in the control, LC and HC group were strongly and significantly correlated with the corresponding lesion area ($R=0.91$; $p<0.001$) (Figure 4B), and therefore it was consistent that lesion area and OA grades were also significantly correlated ($R=0.34$; $p=0.03$) (Figure 4C). To evaluate the contribution of ATH features in addition to cholesterol exposure, partial correlation correcting for cholesterol exposure was performed and revealed no significant correlation between ATH features and OA grades ($R=0.09$, $p=0.6$).

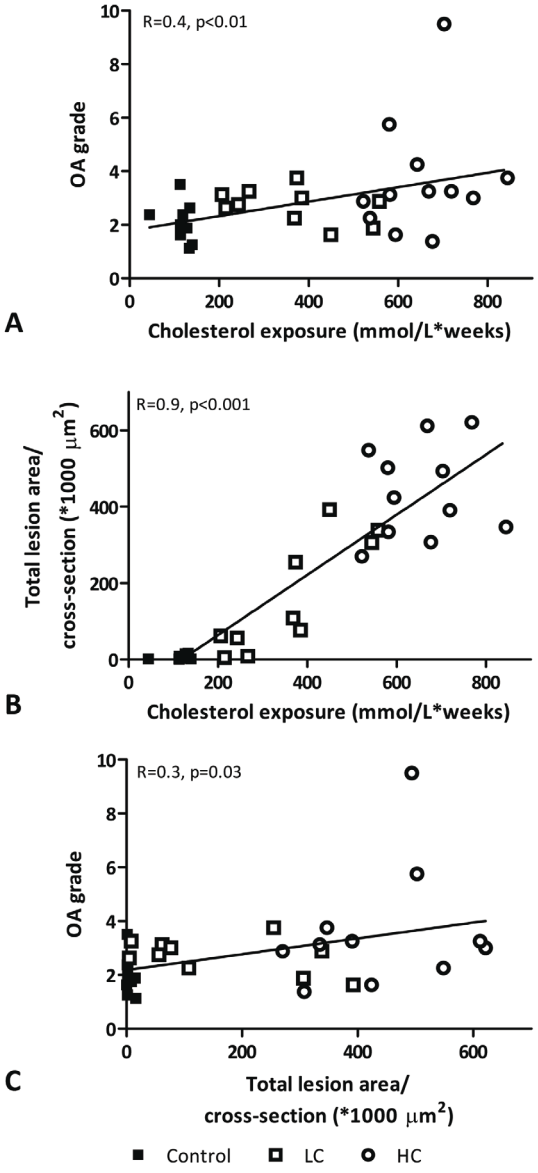

Figure 4. Overview of correlations in the control, low cholesterol (LC) and untreated high cholesterol (HC) groups A. OA grade (sum total of the OA grades at the medial femur and tibia of the knee joint) and cholesterol exposure (mmol/L*weeks), B. Cholesterol exposure (mmol/L*weeks) and atherosclerosis (ATH) development (total lesion area/cross-section (*1000 μm2)), C. OA grade (sum total of the OA grades at the medial femur and tibia of the knee joint) and ATH development (total lesion area/cross-section (*1000 μm2)).

Discussion

There is currently mounting evidence that the MetS and OA are associated with one another (2, 3). Hypercholesterolemia is related to MetS and a risk factor for ATH. Associations between OA and ATH suggest similarities in their underlying pathologies (11, 15, 33, 34). In this study using the APOE*3L.CETP mouse model, we demonstrate that hypercholesterolemia indeed plays a role in the development of OA since an increased intake of cholesterol led to more severe OA features in the knee joints. Furthermore, a significant correlation between cholesterol exposure and OA features on the medial side of the knee joint indicates that high levels of cholesterol in the blood contribute to the OA process. However, correlation coefficients suggest that other processes, evoked by the intake of cholesterol, are also involved in the pathogenesis of OA. The suppressive effect on OA development by atorvastatin but not by ezetimibe, which had similar effects on cholesterol exposure levels, corroborates these findings.

Impaired lipid metabolism has been suggested an important factor in OA development. We used the APOE*3Leiden.CETP mice as translational model for human lipoprotein metabolism and development of ATH. To our knowledge this is the first study in which the effect of solely cholesterol, without interference of other variables such as BW, on OA development has been investigated. Since elevated cholesterol intake led to more advanced OA features, these data suggest a role for cholesterol in OA development. Compared to other spontaneous models, such as high fat diet (HFD)-induced OA, the development of OA is mild, although it is difficult being conclusive due to differences in strains, gender, and lengths of the studies (10, 35-37). The differences were only seen at the medial side of the joint which is known to be most susceptible in mice models and therefore in line with our expectations (38).

Whether the development of OA after cholesterol intake is due to a direct effect of cholesterol (local effects), other mechanisms induced by cholesterol (systemic effects), or a consequence of ATH is uncertain. With respect to local effects, several mechanisms of altered lipid mechanisms on OA development are suggested. For example, in human OA chondrocytes the expression of genes regulating

cholesterol efflux is impaired, resulting in a toxic accumulation of lipid droplets in the chondrocyte (39, 40). In addition, an association of OA pathogenesis with SREBP-2, a gene that plays a key role in cholesterol homeostasis, was demonstrated (41). These findings suggest a direct role for cholesterol in triggering degenerative processes in the cartilage by altered cholesterol homeostasis in the chondrocytes. However, in our *in vivo* mouse model only a moderate correlation between plasma cholesterol exposure and OA development was observed which suggests that cholesterol is not solely responsible for the development of OA. Altered underlying systemic processes such as inflammation that are the result of increased cholesterol load, may therefore be more relevant. This is substantiated by the fact that SAA, ALT, E-selectin and MCP-1 levels in the mice were increased when fed a cholesterol diet. Kleemann *et al.* performed a gene expression analysis which indicated 4 key inflammatory pathways consisting of IFNγ, TNFα, IL-1 and platelet derived growth factor (PDGF) to play a central role in the evolution of cholesterol-induced inflammation in the liver (8). These pathways are also critical for lesion development in the vessel wall and known to be important in OA development (42). Circulating levels of cytokines thought to be important in OA, e.g. TNFα. IFNγ, IL-6 and IL-1 were not detectable in this study which is consistent with previous findings in our lab with these transgenic mice on a cholesterol diet (unpublished data).

Alterations due to ATH may provide an alternative explanation for the development of OA in this model. For example, effects of a reduced blood flow in the small vessels in the subchondral bone may modify the cartilage homeostasis and cause OA changes (7, 13, 14, 16). This notion is supported by analysis of human data that revealed a significant association between intima media thickness and knee OA after adjustment for e.g. cholesterol levels (14). Moreover, hand OA was linearly associated with ATH features after adjustment for potential confounders (15). In our model, the lack of correlation between OA and ATH, when corrected for cholesterol exposure, debilitate ATH as main driving force for OA. This is further substantiated by the fact that ezetimibe, in contrast to atorvastatin, did not led to significant suppressive effects on OA development whereas these drugs had comparable effects on plasma cholesterol levels and ATH characteristics. Atorvastatin is known to have several pleiotropic effects, in contrary to ezetimibe, including anti-

inflammatory immunomodulation, plaque stabilization, decreased activation of the blood coagulation cascade, and inhibition of platelet aggregation which may be responsible for the different effect of atorvastatin compared to ezetimibe (43). In a previous study, we have shown that rosuvastatin suppressed the development of HFD-induced OA through its anti-inflammatory capacities (10). Moreover, it has been shown that intraarticular administration of mevastatin reduced inflammatory cell infiltration and matrix-degrading enzyme expression in a rabbit model of experimental OA indicating local effects of atorvastatin (19, 20). Our data suggest that statins may be an important candidate for the management of OA and additional studies are warranted.

In conclusion, elevated levels of cholesterol play a role in OA as mice receiving cholesterol in their diet develop OA features on the medial side of the knee joint. The moderate correlation between OA and cholesterol exposure suggests that processes independent of cholesterol exposure also contribute to development of OA features. This is further supported by the differences in effect of atorvastatin and ezetimibe on OA. Both drugs reduced cholesterol levels to the same extent, but only atorvastatin significantly diminished OA severity which may be attributed to its anti-inflammatory properties. This study supports the notion that components related to MetS such as hypercholesterolemia could significantly contribute to the development, progression and severity of OA however further research is needed to elucidate the underlying molecular mechanisms.

Supplementary Information

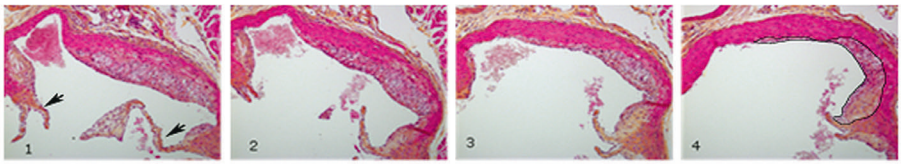

Figure S1. Representative photos of consecutive slices used for quantification of atherosclerosis (ATH). Valves were used as anatomical landmarks to start quantification of ATH development as indicated by arrows. Once the aortic root was identified by the appearance of aortic valve leaflets, serial cross-sections (5 µm thick with intervals of 50 µm) were collected and stained with hematoxylin, phloxine and saffron. For each mouse, the lesion area (as illustrated in photo 4) was measured in 4 subsequent sections (three segments/section). Therefore, ATH development area was measured over a length of 150 µm in the aortic origin. Photos represent one of the three segments.

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7

Exploring high fat diet-induced osteoarthritis in APOE*3Leiden.CETP mice

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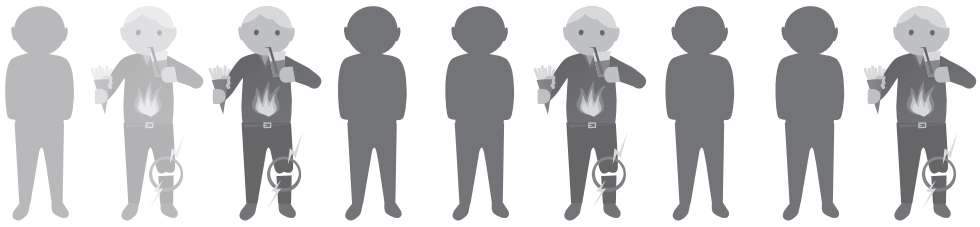

Abstract

Objective: Obesity, a major risk factor for OA, has been proposed to induce an inflammatory component in the pathogenesis of osteoarthritis (OA). We aimed to assess high fat diet (HFD)- induced OA development in APOE*3Leiden.cholesteryl ester transfer proteïne (CETP) mice, a mouse model with a human-like lipoprotein metabolism.

Methods: Male APOE*3Leiden.CETP mice (n=150) were switched to a HFD at the start of the study. After a 12 weeks run-in period, mice were matched based on body weight, plasma cholesterol, triglycerides and insulin levels in 8 different groups (n=12/group); 1 HFD control group, a HFD group which was switched back to a chow diet after 22 weeks, 2 HFD groups treated with rosuvastatin from either t=12 or 22 weeks onward, 2 HFD groups treated with fenofibrate from either t=12 or t=22 weeks onward, 1 HFD group receiving caspase-1 inhibitor from 14 weeks onward and a group of low responders on HFD (with respect to above-mentioned parameters). A group receiving control chow diet (n=12) was included as well. All mice were sacrificed 32 weeks after the start of the study and knee joints were histologically assessed for OA development. Serum amyloid A (SAA) levels in the plasma at the start of the study were measured as marker for systemic inflammation.

Results: In contrary to a previous study, OA grades in the HFD group were low. Furthermore, the HFD group tended to display a lower overall OA grade than the control group (p=0.081). Low responders on a HFD as well as mice switched from HFD to a chow diet developed OA features to a comparable extent as the high responders. There was no significant effect of the various interventions on OA grades compared to the untreated HFD group. No anomalies were seen on body weight, plasma cholesterol and triglycerides levels. SAA levels were highly variable at the start of the study.

Conclusion: These results indicate that improved understanding of the susceptibility of APOE*3Leiden.CETP mice strain towards OA development is necessary to understand the outcomes of this study as well as the mechanisms leading to HFD-induced OA.

The prevalence of obesity is worldwide rapidly accumulating (1). Severe medical consequences as well as negative effects on the quality of life due to obesity are widely recognized (2). Obesity is an important component of the metabolic syndrome and a major risk factor for the development of osteoarthritis (OA) (3, 4). Since recently, no longer only the mechanical forces due to obesity are considered as instigator of OA but also the inflammatory responses triggered by obesity are believed to be important in the development (5-7). This was confirmed in a previous study in which we demonstrated in a high fat diet (HFD)-induced OA mouse model that the metabolic stress response played a major role in the development of OA instead of body weight. In the same study, treatment with rosuvastatin (a cholesterol lowering intervention) or rosiglitazone (peroxisome proliferator activator receptor (PPAR) gamma agonist, anti-diabetic drug) inhibited the development of OA possibly by their anti-inflammatory mode of action (8). This provided the foundation for further investigation into HFD-induced OA.

The apolipoprotein E*3Leiden.human Cholesteryl Ester Transfer Protein (APOE*3Leiden.CETP) transgenic mouse is a model which resembles human lipoprotein metabolism and is sensitive to lipid modulating therapies in contrary to wild type mice (9-11). Feeding a HFD to these mice leads to a profile of anomalies quite similar to those that are present in the majority of the patients having metabolic syndrome (12). In an unpublished study which was designed for lipid research purposes we found that APOE*3Leiden.CETP mice receiving a HFD and fructose in their drinking water developed severe features of OA. Moreover, we observed a strong inhibitory effect on OA development when mice were treated with fenofibrate (figure 1). Fibrates are agonists for the PPAR-α receptors and used in the clinic as drugs to reduce plasma triglycerides and cholesterol levels (13). Fibrates are, like statins, recognized for their pleiotropic effects (14). With respect to OA it has been shown in *in vitro* experiments that a PPAR-α agonist counteracts IL-1-induced proteoglycan degradation and increases MMP-activities in rabbit articular chondrocytes (15). Furthermore, Clockaerts et al. showed that a PPAR-α agonist inhibited inflammatory and destructive responses in human OA cartilage explants, while collagen type II or aggrecan mRNA expression remained unaffected (16). In addition, PPAR-α agonists have an inhibitory effect on inflammatory cytokines in infrapatellar fat pad explants and to a lesser extent in synovium explants (17).

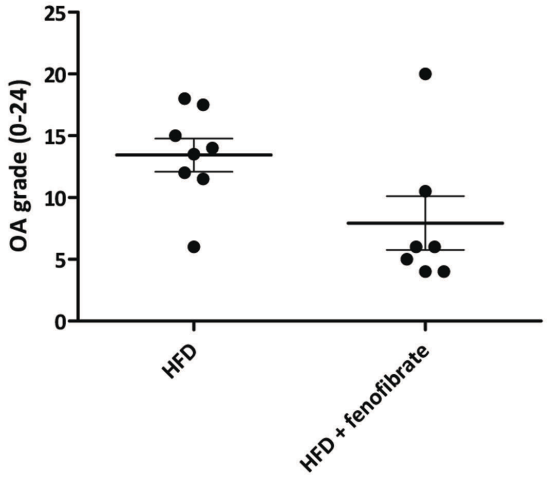

Figure 1. OA grades of male APOE*3Leiden.CETP mice included in a study designed for lipid research purposes. Mice received high fat diet (HFD) for 32 weeks or HFD supplemented with 0.0212% w/w fenofibrate for 9 weeks. Fructose (10%) was added to the drinking water during the treatment period. Each point represents the value of an individual mouse. Line indicates mean ± SEM (n=8/group).

As mentioned, statins proved to inhibit the development of OA in a HFD-induced OA mouse model (8). Statins are HMG Co-A reductase inhibitors and used to reduce serum cholesterol levels in humans. There are indications from several *in vitro* and *in vivo* studies that statins have beneficial effects on the development of OA, probably by their anti-inflammatory mode of action (18-22). In an epidemiological study statin use was associated with more than 50% reduction in overall progression of knee OA (23). These findings are promising and warrant further investigation of fibrates and statins as potential therapeutic strategies for OA.

The pro-inflammatory cytokine interleukin (IL)1-β is suggested to have an important role in OA pathogenesis (24-26) as well as in obesity (27). IL-1β is synthesized as an inactive precursor and needs to be converted by the enzyme caspase-1 to become active. Caspase-1 itself requires activation via a molecular platform called the inflammasome (28). The activation of inflammasome-mediated caspase-1 plays a key role in the enhanced inflammatory state characteristic of obesity and has a central role in the pathogenesis of type 2 diabetes (27). Although recently it has been demonstrated that OA cartilage may be degraded independently

of any inflammasome activity (29), the role of inflammasome in relation to obesity-induced OA merits more investigation.

A more pragmatic way to intervene in the OA process are weight-losing programs. Increasing evidence exists that irrespective of the weight-loss method (e.g. exercise, diet, bariatric surgery), a reduced body fat contributes to a reduced OA development (30). In relation to inflammation, substantial weight loss in obese subjects with type 2 diabetes was shown to reduce expression levels of IL-1β and inflammasome in adipose tissue (31). Furthermore, weight loss decreases leptin and C-reactive protein (CRP) levels, reduces the synthesis of IL-6 and tumor necrosis factor (TNF)-α and increases the production of anti-inflammatory cytokines by subcutaneous adipose tissue (32, 33). These biochemical changes would, beside the reduction of mechanical stressors, attribute to improved clinical outcome of OA.

The aim of this study was to assess the sensitivity of the APOE*3Leiden.CETP mouse as a model for HFD-induced osteoarthritis (OA) as a more predictive model for the human situation. Furthermore, we aimed at a better understanding of the mechanisms underlying HFD-induced OA to identify new potential targets for therapy. Different treatment regimens (early *versus* late) with rosuvastatin and fenofibrate were included to evaluate the efficacy of these drugs at different stages of the OA process. To investigate whether the inflammasome plays a role in HFD-induced OA a treatment with caspase-1 inhibitor was included. At last, we examined if losing weight at a stage where there is already significant progression of OA, could prevent further advancing of or delay the process of OA development.

Methods

Mice

Male APOE*3Leiden mice, characterized by an enzyme-linked immunosorbent assay (ELISA) for human APOE, were crossbred with human CETP transgenic mice which express CETP under control of its natural flanking regions in our animal facility to obtain heterozygous APOE*3Leiden.CETP mice (10-12). Mice were housed in groups under standard conditions with a 12 h light-dark cycle and had free access to water and food. Body weight (BW) and food intake were monitored during the study. Mice

received a standard lab chow (V1534 Ssniff Spezialdiäten GmbH, Germany) until the start of the study at the age of 10-16 weeks (t=0 weeks). Experiments were approved by the institutional Animal Care and Use Committee of TNO and were in compliance with European Community specifications regarding the use of laboratory animals.

Pilot Study

A pilot study ran 5 weeks ahead of the main study to investigate OA development over time. APOE*3Leiden.CETP mice (n=27) were switched to a HFD (60% kcal from fat; Research Diets Inc. art. No. 12492) at the start of the study. After 12 weeks mice were matched based on BW, plasma cholesterol, triglycerides (TG) and insulin levels in 4 different groups (n=5/group, group 1-4) and low responders with respect to the above-mentioned parameters were removed from the study. Groups were sacrificed after either 12, 16, 20 or 24 weeks.

Main study

APOE*3Leiden.CETP mice (n=150) were switched to a HFD at t=0 weeks. After a 12 weeks run-in period (t=12), mice were matched based on BW, plasma cholesterol, TG and insulin levels in 8 different groups (n=12/group) and low responders were removed from the study, except for a group of n=12 mice that continued on HFD (HFD Low Responders) to investigate whether the low response on above-mentioned parameters is indicative for less OA progression. All other groups continued on HFD with or without treatment (HFD), except for one group (HFD_Chow) which was switched back to standard lab chow after 22 weeks (t=22 weeks). Two groups received a HFD supplemented with 0.005% w/w rosuvastatin (*Provisacor*®, Astrazeneca, Zoetermeer, the Netherlands) in which the first group received rosuvastatin from 12 weeks onward (HFD + Rosu t=12) while the other received this drug from 22 weeks onward (HFD + Rosu t=22). The same treatment schedule was applied for 2 groups receiving HFD supplemented with 0.0212 % w/w fenofibrate (art no F6020, Sigma-Aldrich, St. Louis, USA) (HFD + Feno t=12 and HFD + Feno t=22). Furthermore, one group received a daily intra-peritoneal (i.p) injection with 10 mg/kg caspase-1 inhibitor (AC-YVAC-CMK art.no. N-1330, Bachem, Bubendorf, Switzerland) dissolved in PBS/0.6% DMSO from 14 weeks (t=14) onward (HFD + Casp.inh). A control group

of n=12 mice received standard lab chow diet until the end of the study (Control). All mice were sacrificed after 32 weeks (t=32).

Cholesterol, triglyceride and insulin analyses

At t=12, 16, 20, 24, 28 and 32 weeks blood was collected 4 hours after food deprivation by tail vein incision to determine plasma levels of cholesterol and TG. Total plasma cholesterol (Roche Diagnostics, No-1489437) and TG (Roche diagnostics, No-1488872) levels were determined immediately after plasma collection to avoid influence of freeze-thawing on these parameters. Insulin was measured at t=12 for randomization purposes using an immunoassay ((Cat. no. 10-1113-01, Mercodia, Spain). Assays were performed according to the manufacturers' instruction.

Serum Amyloid A analysis

Serum Amyloid A (SAA), an acute phase protein, was determined in the individual plasma samples collected at the start of the main study (t=0) by an ELISA (tridelta development, Ireland, distributed by Invitrogen) according to the manufacturers' instruction.

Histopathology

All mice were sacrificed and the knee joints of the hind limbs were fixed in a 10% formalin neutral buffer solution (Sigma-Aldrich), decalcified in Kristensen's solution (34), dehydrated and embedded in paraffin for histological analysis. Serial coronal 5 µm sections were collected throughout the patella, medial and lateral side of the left knee joint. Sections were stained with hematoxylin, fast green and safranin O as well as with hematoxylin, phloxine and safran (HPS). Two sections per mouse were scored in a blinded fashion by 2 observers. The joint as a whole was scored following the international guidelines of the OARSI histological grading system. Briefly this scoring system is based on a combined assessment of severity ("grade 0; surface and cartilage intact" – "grade 6; deformation") and extent ("stage 0; no OA activity" – "stage 4 >50% OA activity") of OA in the articular cartilage (grade x stage, total score of 24)(35). In addition, 6 locations in the joint; femoral condyle and tibia plateau at the lateral and medial side, trochlear groove and the patella were individually scored (score 0-6)(36).

Statistical analysis

Data are presented as mean ± standard deviation (SD) unless stated otherwise. All statistical analyses were performed using the SPSS 17.0 statistical software package for Windows (SPSS Inc. Chicago, USA). Statistical differences were assessed using the non-parametrical Kruskal Wallis test followed by the *post hoc* Mann Whithey U test or the parametrical (repeated measures) ANOVA test followed by Dunnett's *post hoc* tests. For statistical analysis the study was divided according to the various research questions in HFD *versus* Control or HFD_Chow, HFD Low Responders *versus* HFD, or HFD + Rosu t=12/22, HFD + Feno t=12/22 *versus* HFD or HFD + Casp.Inh. *versus* HFD. $P<0.05$ was considered statistically significant.

Results

Pilot study

BW, plasma cholesterol and TG levels were assessed during the study. All parameters increased gradually (Table 1). Mice were sacrificed at t=12, 16, 20 and 24 weeks on HFD to investigate the development of OA in the knee joint over time. After 12 weeks on a HFD, the OA score was relatively homogeneous. The longer the mice received a HFD, the more variation in OA score was observed (Figure 2A). Based on the severity of OA score, it was decided to start the late treatments in the main study at t=22 weeks and to run the main study till 32 weeks. Plasma SAA levels at the start of the study were comparable between the different pilot groups with an average of 29.6 ± 37.3 µg/ml (Figure 2B).

Table 1. Body weight, plasma cholesterol and triglyceride levels of APOE*3Leiden.CETP mice in the pilot study.

	t=12			t=16			t=20			t=24		
Group	**BW**	**Chol**	**TG**	**BW**	**Chol**	**TG**	**BW**	**Chol**	**TG**	**BW**	**Chol**	**TG**
1	47.5 (3.1)	4.2 (0.8)	1.6 (0.3)									
2	46.4 (3.5)	4.5 (0.8)	1.6 (0.3)	47.4 (4.6)	4.3 (0.7)	2.8 (0.9)						
3	46.0 (2.0)	4.7 (1.1)	1.8 (0.6)	48.0 (3.4)	4.5 (0.8)	2.9 (1.6)	50.9 (4.8)	4.4 (0.4)	2.0 (0.5)			
4	47.6 (3.5)	4.5 (1.2)	1.5 (0.8)	47.4 (4.5)	4.3 (0.4)	2.1 (0.6)	49.2 (4.7)	4.6 (0.4)	2.0 (0.5)	50.9 (5.3)	4.8 (1.3)	2.4 (0.8)

*Body weight (BW), plasma cholesterol (Chol) and triglycerides (TG) levels of the different groups of APOE*3Leiden.CETP mice in the pilot study. Data are indicated as mean(SD)at t=12, 16, 20 and 24 weeks. BW in grams, Chol en TG in mmol/L.*

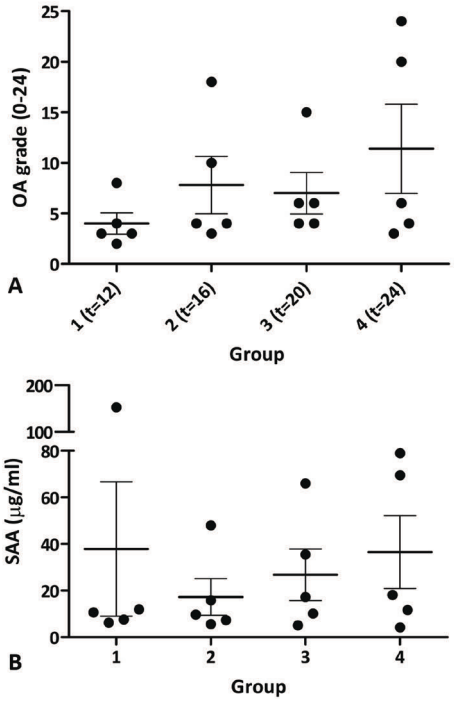

Figure 2. Osteoarthritis (OA) grades and Serum Amyloid A (SAA) levels **A.** Time course of OA development and **B.** Serum amyloid A (SAA) levels at the start of the study (t=0) in APOE*3Leiden.CETP mice on a high fat diet (HFD) in the pilot study. Groups were sacrificed after t=12, 16, 20 and 24 weeks (group 1-4). Each point represents the value of an individual mouse. Line indicates mean ± SEM (n=5/group).

Main study

Mice characteristics

BW of the mice in the HFD group were significantly higher than those of mice in the control group during the course of the study (t=12 till t=32 weeks) (HFD vs Control $p<0.001$) (Figure 3A). A tremendous effect on BW was observed when the mice were switched from a HFD to a control chow diet at t=22 weeks. Within 6 weeks after the diet switch BWs of these mice were comparable to mice which were continuously fed a chow diet (HFD_Chow vs HFD $p<0.001$). Mice in the low-responder group gained more BW than the chow group, but never reached BW of the HFD group (HFD Low Responders vs HFD $p<0.001$). Rosuvastatin and fenofibrate treatment had no effect on BW (Figure 3B). BW was also not affected by a treatment with caspase-1 inhibitor (Figure 3C).

To evaluate the effects of a HFD-intake plasma cholesterol and TG levels were determined every 4 weeks. Plasma cholesterol levels in the HFD group were significantly higher than in the control group (HFD vs Control $p<0.001$) (Figure 4A). No such significant effect was observed for the TG levels, which already reached substantial levels in the control group (Figure 4D). A switch back from a HFD to a control chow diet decreased cholesterol levels immediately, returning to almost the levels in the control group (HFD_Chow vs HFD $p=0.002$). This was accompanied by a reduction in TG levels. The HFD Low Responder group had significantly lower cholesterol ($p<0.001$) and TG ($p<0.01$) levels than the HFD group. Rosuvastatin, fenofibrate and caspase-1 inhibitor treatments did not significantly influence these parameters (Figure 4BCEF).

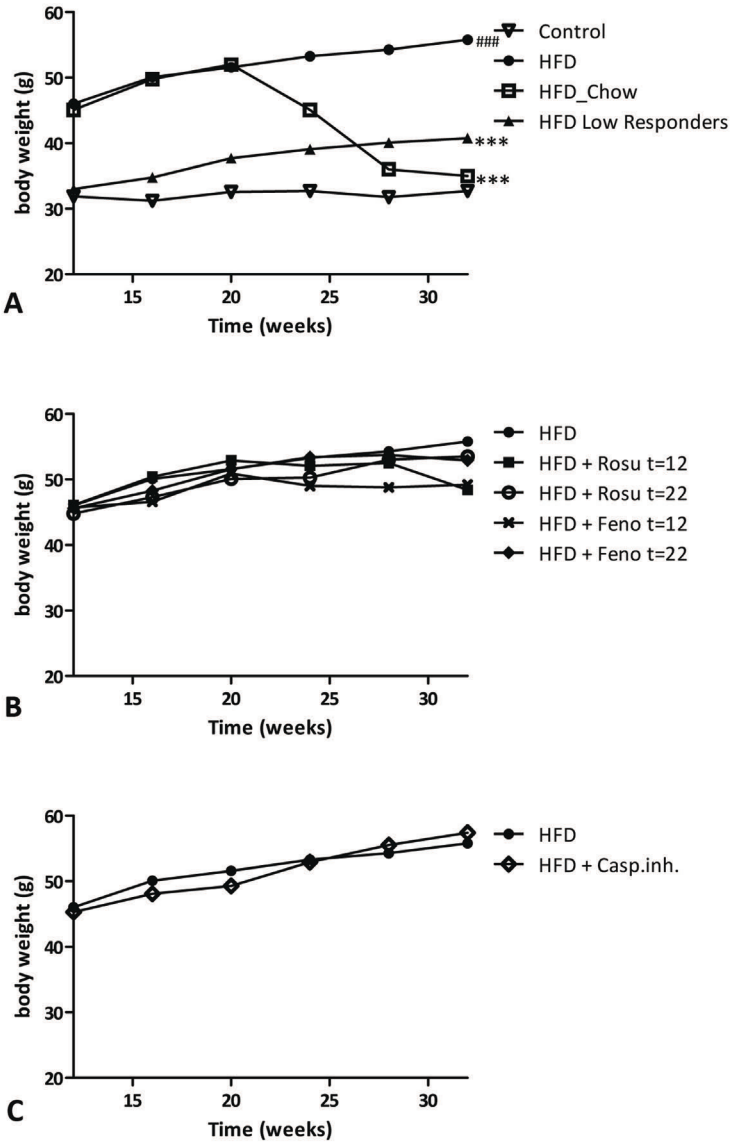

Figure 3. Body weight (BW). Time course of BW of APOE*3Leiden.CETP mice in the **A.** chow (Control), high fat diet (HFD), HFD_Chow and HFD Low Responders groups, **B.** HFD, HFD treated with rosuvastatin started from t=12 or t=22 onwards (HFD + Rosu t=12/ 22) and HFD treated with fenofibrate started from t=12 or t=22 weeks onwards (HFD + Feno t=12/22) groups and **C.** HFD and HFD treated with caspase-1 inhibitor (HFD Casp.inh.) groups. Each point represents the mean of the group (Control, HFD, HFD_Chow, HFD Low Responders, HFD + Rosu t=12/22, HFD + Feno t=12: n=12/group, HFD + Feno t=22 and HFD Casp.Inh.: n=11/group).***P<0.001 vs HFD,$^{###}$p<0.001 vs Control.

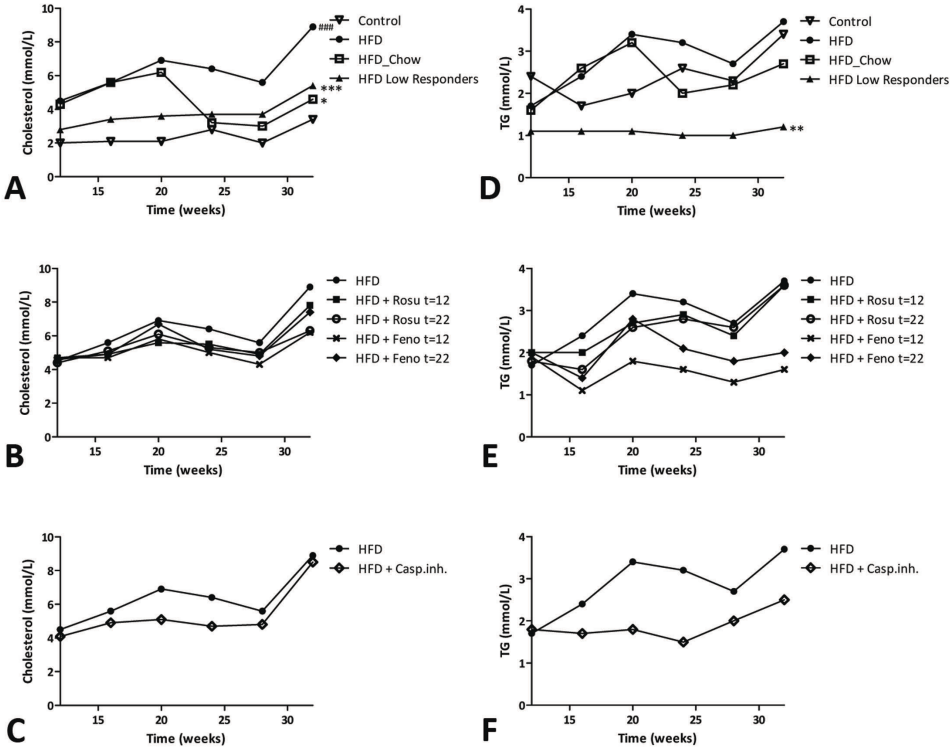

Figure 4. Plasma cholesterol en triglycerides (TG) levels. Time course of plasma cholesterol (mmol/L) and triglycerides (mmol/L) levels of APOE*3Leiden.CETP mice in the chow (Control), high fat diet (HFD), HFD_Chow and HFD Low Responders groups (**A,D**) or the HFD, HFD treated with rosuvastatin started from t=12 or t=22 weeks onwards (HFD + Rosu t=12/22) and HFD treated with fenofibrate started from t=12 or t=22 weeks onwards (HFD + Feno t=12/22) groups (**B,E**) or HFD and HFD treated with caspase-1 inhibitor (HFD Casp.inh.) groups (**C,F**). Each point represents the mean (Control, HFD, HFD_Chow, HFD Low Responders, HFD + Rosu t=12/22, HFD + Feno t=12: n=12/group, HFD + Feno t=22 and HFD Casp.Inh.: n=11/group).***P <0.001,**p<0.01,*p<0.05 vs HFD and $^{###}$p<0.001 vs Control.

OA grades

The knee joints of the APOE*3Leiden.CETP were analysed for the effects of HFD as well as different interventions on the development of OA. Surprisingly, the control group tended to display an higher overall OA grade than the HFD group (Control vs HFD p=0.081). The HFD Low Responders group as well as the group which was switched from a HFD to a control diet halfway the study had no significantly different OA grades than the HFD group (Figure 5A). In line with this, no significant effect of the

various treatments on HFD-induced OA were observed (Figure 5BC). With respect to the individual components of the knee joint (femoral condyle and tibia plateau at the lateral and medial side, trochlear groove and the patella) no significant effects were observed either (data not shown).

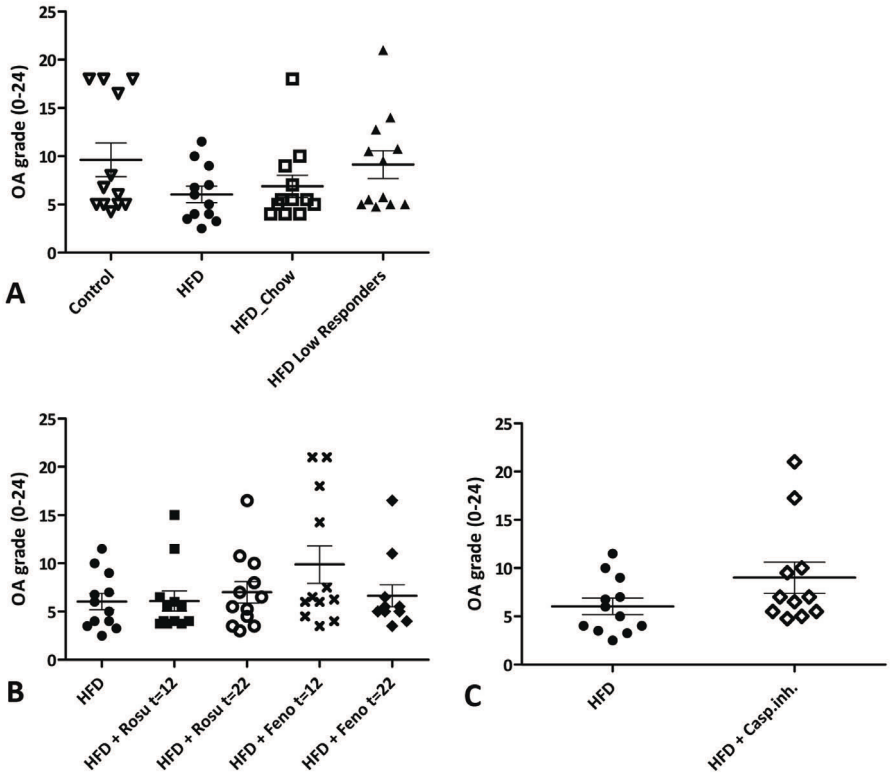

Figure 5. OA development in the hind knee joint of APOE*3Leiden.CETP mice in the main study (t=32 weeks). An overall OA grade was determined in the **A**. chow (Control), high fat diet (HFD), HFD_Chow and HFD Low Responders group, **B.** HFD, HFD treated with rosuvastatin started from t=12 or t=22 weeks onwards (HFD + Rosu t=12/22) and HFD treated with fenofibrate started from t=12 or t=22 weeks onwards (HFD + Feno t=12/22) groups and **C.** HFD and HFD treated with caspase-1 inhibitor (HFD Casp.inh.) group. Each point represents the value of an individual mouse (Control, HFD, HFD_Chow, HFD Low Responders, HFD + Rosu t=12/22, HFD + Feno t=12: n=12/group, HFD + Feno t=22 and HFD Casp.Inh.: n=11/group). Line indicates mean ± SEM.

Serum Amyloid A levels

As there were unexpected observations with regard to OA grades we assessed the level of inflammation by measuring the acute phase protein SAA in plasma samples collected at the start of the study (t=0 weeks). Mice in all groups demonstrated a great variation in their SAA levels (Figure 6).

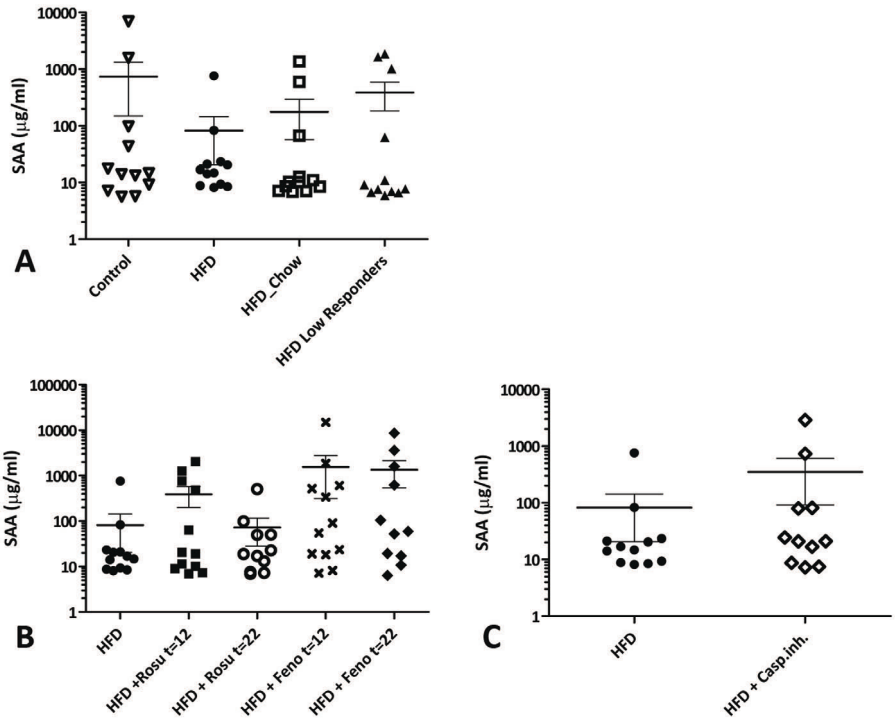

Figure 6. Serum Amyloid A levels (SAA) in the APOE*3Leiden.CETP mice at the start of the study (t=0 weeks). **A**. chow (Control), high fat diet (HFD), HFD_Chow and HFD Low Responders group, **B.** HFD, HFD treated with rosuvastatin started from t=12 or t=22 weeks onwards (HFD + Rosu t=12/22) and HFD treated with fenofibrate started from t=12 or t=22 weeks onwards (HFD + Feno t=12/ 22) groups and **C.** HFD and HFD treated with caspase-1 inhibitor (HFD Casp. inh.) group. Each point represents the value of an individual mouse (Control, HFD, HFD_Chow, HFD Low Responders, HFD + Rosu t=12/22, HFD + Feno t=12: n=12/group, HFD + Feno t=22 and HFD Casp.Inh.: n=11/group). Line indicates mean ± SEM. Data are plotted on a log scale.

Discussion

Obesity is a major risk factor for OA and low grade systemic inflammation associated with obesity have been suggested to contribute to the development of OA (4, 8). Unraveling the precise mechanism of how obesity influences OA development has the potential to find new targets for the development of disease modifying therapies for OA. We studied the effects of a HFD on the development of OA in male APOE*3Leiden.CETP mice, a mouse model with a human-like lipoprotein metabolism. The data in this study revealed that after 32 weeks of HFD features of OA were present, however less severe than expected. As a matter of fact, OA development in the HFD group tended to be lower than in lean mice receiving a chow control diet. As a consequence the data of the various treatments are difficult to interpret.

The current study was based on an unpublished study designed for lipid research purposes. Herein we found promising results for the APOE*3Leiden.CETP mouse as a model for HFD-induced OA. Interestingly, in that experiment treatment for 9 weeks with fenofibrate significantly suppressed OA grades (HFD 13.4 ± 3.8 vs HFD fenofibrate 7.9 ± 5.7, p=0.04), thereby substantiating *in vitro* observations that fenofibrate has the potential to interfere with degenerative and inflammatory processes in OA. Unexpectedly, we could not validate these data in the current study. We found an overall OA score of 6.0 ± 2.9 in the HFD group which was substantially lower than the OA score of 13.4 ± 3.8 found in the HFD group of the lipid study. With respect to the in-life parameters BW, plasma cholesterol and TG levels no anomalies were observed during the study. Moreover the pilot study, which ran 5 weeks ahead of the main study, gave no indication which would explain the lack of effect of HFD on OA development. These unexpected results may potentially be explained by several observations. At first, although a comparable protocol with regard to strain, length of the study, HFD and gender was applied as in the lipid study, the addition of fructose to the drinking water was omitted. Addition of fructose to the drinking water results in a switch from HDL to ApoB containing lipoprotein ((V)LDL) in the lipoprotein profile of APO*3Leiden.CETP mice inducing a more human-like cholesterol distribution (12). Fructose consumption in human subjects has been linked to features of the metabolic syndrome and can induce hepatic TG overproduction and accumulation which leads

to the activation of classical inflammatory pathways such as nuclear factor kappa B (37). In a previous publication, OA development and progression was accelerated in the human C-reactive protein (hCRP) transgenic mice, a translational model to monitor inflammation, on HFD (8). As these mice have no VLDL fraction, these data suggest that VLDL particles are not contributing to the process of HFD-induced OA. The HFD-induced OA mouse model in combination with a fructose component has never been investigated (38). At this moment we cannot exclude that fructose water is required to induce OA in the APOE*3Leiden.CETP strain in combination with a HFD. If the hypothesis that fructose is essential for inducing OA is prospectively validated then a highly specific pathway for induction of OA emerges.

Secondly, SAA levels, a marker for inflammation, in the plasma levels collected at the start of the study were highly variable. This may have influenced their response to the HFD. Whether these highly variable SAA levels could be correlated to the lack of OA development 32 weeks later on is uncertain and further research on the inflammatory status of these mice is required.

It is remarkable that lean mice on a chow control diet developed more advanced OA grades than previously observed in the hCRP mice (9.6 ± 6.0 vs 4.4 ± 2.9) (8). This may be attributed to differences in the lipoprotein profile between these strains, since the hCRP strain has a wild-type lipoprotein profile with cholesterol mostly contained in HDL, and the APOE*3Leiden.CETP strain has a human-like cholesterol distribution. To assess for the different lipoprotein profiles on OA development it would be very interesting to perform a study in which identical protocols are applied to APOE*3Leiden.CETP transgenic mice and their wild type littermates. Mice on a control chow diet tended to display even more OA than mice on a HFD. Four mice on a control diet developed severe OA while the others developed comparable OA grades to the other groups. We do not have an explanation for this cluster forming yet and more research is required.

In summary, from unpublished data we can conclude that the male APOE*3Leiden.CETP mouse strain develops knee OA on a HFD with fructose water and that fenofibrates can possibly interfere in this process. However data from the current experiment indicate that improved understanding of the used APOE*3Leiden.CETP mouse model towards OA development is necessary to understand the outcomes of this study as well as the mechanisms leading to HFD-induced OA.

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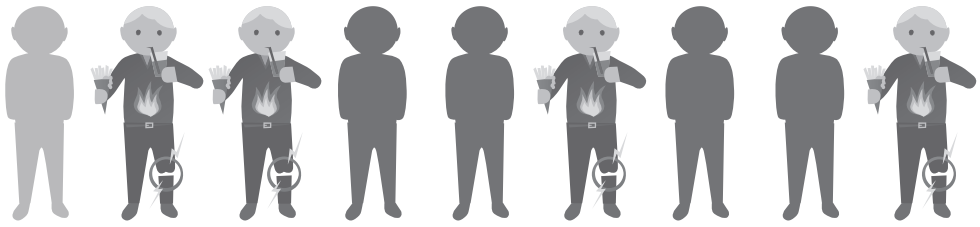
8

Summary & general discussion



Osteoarthritis (OA) is a frequently occurring joint disorder with great impact on the quality of life. In general, OA is described as a heterogeneous disease with degeneration of articular cartilage as main outcome (1-3). Despite extensive research on the pathogenesis of OA, there is until now no cure and treatments are primarily aimed at reducing pain. The heterogeneity of the patient population might be one of the reasons for the absence of appropriate treatments. Although the clinical symptoms are the same, i.e. structural damage and symptomatic features such as pain, the underlying processes for the development of OA may be different. The inclusion of all OA patients, instead of stratification for different types of OA patients, may lead to a 'contamination' of clinical trials and may explain the observed lack of efficacy of candidate drugs. Evidence starts to appear that mild inflammation and obesity-related biochemical changes are involved in OA pathology (4). It is uncertain what the relative contribution of these processes is and if they characterize a certain type of OA patients. By understanding the underlying disease mechanism, having a better patient stratification and an improved prediction of disease outcome, the efficiency of clinical trials of novel drugs can be optimized. We aim to provide insight in the role of multiple local and systemic factors contributing to the pathogenesis of OA.

Osteoarthritis as a multiple tissues disease

For a long time, OA research has concentrated on articular cartilage. Nowadays, however, a role for other tissues of the joint is appreciated as well (5-7). In the first three chapters of this thesis we discuss the possible involvement of multiple knee joint tissues in the OA process. One of these tissues is the synovium, which is in direct contact with articular cartilage. Increasing evidence indicates that inflammation of the synovium contributes to degenerative processes in the cartilage and that inflammatory cell types, such as macrophages, present in this tissue may secrete factors modifying joint homeostasis (8). In **chapter 2** we investigated if the secretion of soluble mediators (e.g. cytokines, chemokines, adipokines) is different between OA and normal synovial tissue and if OA synovial tissue is able to initiate cartilage degradation. Surprisingly, the levels of secreted mediators by OA synovial tissue were comparable to that of normal synovial tissue, whereas we expected a more elevated

excretion of pro-inflammatory cytokines from OA synovial tissue. Moreover, we demonstrated that the responsiveness of OA synovial tissue to the pro-inflammatory trigger IL-1 was diminished compared to normal. An explanation of these unexpected results could be that the OA synovial tissue was derived from end stage OA patients. The long-lasting exposure to an inflammatory environment during disease progression might have led to ongoing inflammation-resolving mechanisms in the synovial tissue of late phase OA patients and may explain why the synovial tissues do not respond to a pro-inflammatory trigger anymore (9). Furthermore, we found no additional effects of the synovial tissues on the basal release of glycosaminoglycans (GAG) from the matrix, nor on the matrix metalloproteinase (MMP) activity, when co-cultured with cartilage explants. We conclude that synovial tissue from end stage OA patients is not or no longer capable of initiating cartilage degradation, however these results do not rule out the involvement of synovial tissue in an earlier phase of OA development. It should also be noted that the effect of OA and normal synovial tissue on OA derived cartilage was not investigated for its role in the progression of OA. Other groups have shown that synovitis is correlated with symptom severity, rate of cartilage degeneration and osteophytosis (10). However, the synovial response in OA is complex and data in literature are contradictory. The data in chapter 2 emphasize this complexity, which may be attributed to time-dependent changes in the immune response during disease development.

Another neglected tissue in the knee joint is the infrapatellar fat pad (IPFP). This special form of adipose tissue is located intracapsularly and extrasynovially in the joint (11). The relevance of this joint tissue in the OA process is uncertain, but it is hypothesized that IPFP contributes to the development of OA by the secretion of inflammatory mediators (12-14). In **chapter 3** we examined the secretion of a special class of inflammatory mediators, named oxylipins, by the IPFP. These mediators are derived from essential fatty acids and are important signaling molecules involved in inflammatory processes and the maintenance of physiological processes through the whole body (15, 16). With an LC-MS/MS approach, a wide panel of essential fatty acids and oxylipins (e.g. prostaglandins) involved in pro-inflammatory and inflammation-resolving processes in IPFP-conditioned medium (FCM) was detected. Partial least squares discriminant analysis (PLS-DA), a multivariate statistical method,

demonstrated that OA FCM could be distinguished from normal FCM with regard to the detected essential fatty acids and oxylipins. OA FCM samples obtained with the same protocol but at another location revealed comparable error rates for OA samples in the PLS-DA model, which strengthened our findings. The observed changes were probably specifically due to OA-related alterations instead of basal systemic changes, as the increase of essential free fatty acids (precursors) levels were not necessarily associated with an increase in precursor-derived oxylipins. Although it is tempting to conclude that these changes are causative for OA development, it is very plausible that these disturbances occur as a consequence of OA and may contribute to the amplification of the disease process only. These data indicate the relevance of balanced mechanisms in the joint and provide a valuable tool for further research to the role of IPFP in the OA process.

In **chapter 4** we presented a comprehensive overview of soluble mediators (e.g. cytokines, chemokines, adipokines) in knee synovial fluid (SF) of OA and normal donors. The mediators in SF can be considered as a representative of processes in the knee joint as they are in close contact with synovial tissue, IPFP and articular cartilage. We showed with multiplex analysis that SF from OA donors contains increased levels of mediators, such as macrophage-derived chemokine (MDC), interleukine (IL)-6 and 'regulated and normal T cell expressed and secreted chemokine' (RANTES), compared to SF from normal donors, which is in agreement with the increased involvement of inflammatory processes in OA. In addition, principal component analysis (PCA), a multivariate statistical analysis, indicated various clusters of cytokines that probably reflect the involvement of different processes in the joint. We consider this data set valuable as a reference for future experiments to study pathophysiological pathways.

The use of more advanced technologies, such as applied in chapters 2-4, demonstrated the application of wide screening opportunities of joint tissues, which until recently did not gain a lot of attention in OA research, and emphasized the involved processes on a very small scale. The strength of these studies is that the results obtained with OA donors could be compared to that of normal donors. Tissues from normal donors are difficult to obtain and most studies in literature include a comparison with rheumatoid arthritis patients to assess for OA specific alterations. We experienced practical drawbacks, such as that all tissues were *ex vivo* material

and were obtained from OA patients at the end stage of the disease. The data in chapter 2 did not support the hypothesis that alterations in synovial tissue of OA patients is one of the potential mechanisms that leads to the initiation of cartilage degradation. Surprisingly, the synovial tissue obtained from OA patients turned out to secrete levels of mediators comparable to normal donors and was less responsive to a pro-inflammatory trigger. This makes us consider whether the end-phase of the disease is the most representative stage of OA for studying initiation of cartilage degeneration. It has been shown that end stage OA synovial tissue exacerbates OA features in OA-derived cartilage explants, suggesting its effect in the perpetuation of the disease (17). We realize that to study effects of synovial tissue on the initiation of cartilage degradation, tissue from an earlier stage would be more representative, but in practice this is very hard to obtain. It should be noted that the mediators secreted by IPFP and SF, which are described in chapter 3 and 4, are indicative for the end stage of the disease as well, but this may be different for earlier stages. Furthermore, the tissues may be altered due to *ex vivo* modifications. The data should therefore be interpreted with caution. Nevertheless, we detected differences in synovial tissues, IPFP and SF between normal and OA donors, indicating altered mechanisms in OA.

New questions are raised by the data obtained in chapters 2-4. For example, are IPFP and synovial tissue primarily active contributors to the OA process or do they get inflamed due to alterations in the joint or body? Do modifications of the tissues lead to a more pronounced stage of OA or are these modifications just indicative for the current state of the joint? Do these different types of tissues interact? Is it sufficient to target one of these tissues, or is a multiple target approach needed? To provide answers to these questions, additional research is required and we propose an integrated approach to study different potential active tissues in the OA process to understand the mechanisms and disturbed pathways. An expansion of the number of donors is needed for more extensive profiling. This possibly leads to the discovery of new targets for drug development. The results in chapter 2-4 are the first steps in the direction of approaching OA pathogenesis in a different way.

Osteoarthritis as a disease of the whole body

With regard to the above mentioned alterations on a local level, we speculated whether these changes could be induced by alterations on a systemic level. This hypothesis is encouraged by the fact that the metabolic syndrome is getting more and more attention in the Western world and is regarded as an instigator for several diseases, such as arthritis, cardiovascular diseases and psoriasis (4, 18). The metabolic syndrome comprises a profile including a combination of obesity, hypercholesterolemia, hypertension, dyslipidemia and impaired glucose tolerance. It has been demonstrated that the metabolic syndrome is associated with features of OA and, therefore, may be relevant in search for new targets (19-21). Since decades it is known that obesity, an important component of the metabolic syndrome, is a strong risk factor for the development and the progression of OA. It has been argued that the gain of fat mass leads to an increased loading of the knee joint, resulting in cartilage degradation. The fact that obese people also have an increased risk to develop OA in non-weight bearing joints, gives reason to consider features of the metabolic syndrome to be involved (22). This interesting new concept was studied in **chapter 5.** The intake of a high fat diet (HFD) by human c-reactive protein (hCRP, a marker for inflammation (23, 24)) transgenic mice led to an increased OA development compared to chow-fed control mice, which was not correlated with body weight or fat mass. Interventions with rosuvastatin (cholesterol-lowering) and rosiglitazone (a peroxisome proliferator-activated receptor (PPAR)-γ agonist, anti-diabetic) suppressed OA to a level comparable to that of control mice. As these drugs have different modes of action, but do have in common that they exert anti-inflammatory actions, we conclude that low grade inflammation (metabolic stress) plays a major role in the development of OA in this mouse model. Additionally, we found a correlation between OA grade at end point and the change in hCRP levels shortly after the start of the study. Extrapolation of these data to the human situation suggests that the responsiveness to a metabolic challenge can be used as a marker to estimate the individual susceptibility to develop OA later in life. Therefore, correlations between OA development and the metabolic susceptibility of a person should be explored in further investigations.

In **chapter 6** we demonstrated the effect of hypercholesterolemia, another feature of the metabolic syndrome, on the development of OA. Several epidemiological studies have shown an association between OA and elevated cholesterol levels or atherosclerosis (a cardiovascular disease for which elevated cholesterol levels display an increased risk of disease development) (25-28). The elevated intake of cholesterol has been suggested to cause a certain grade of inflammation in mice and men (29). In this study, a dose-dependent effect of the intake of cholesterol on the development of OA in female APOE*3Leiden cholesteryl ester transfer protein (CETP) transgenic mice (a mouse model which resembles human lipoprotein metabolism, in contrary to wild type mice, and develops hyperlipidemia and atherosclerosis (30-32)) was observed. A moderate association between OA, cholesterol levels and atherosclerosis indicated that cholesterol is involved. However, the data also suggested that other processes evoked by the intake of cholesterol are involved in the pathogenesis of OA. Indeed, levels of some inflammatory markers were elevated due to the high intake of cholesterol in the diet. Again, we demonstrated that statins had a suppressive effect on the development of OA features. This cannot be fully subscribed to their cholesterol lowering capacities, as ezetimibe treatment (a cholesterol-lowering drug with a different mode of action) reduced OA development to a lesser extent while its lowering of cholesterol was comparable. Like in chapter 5, there was no role for increased mechanical forces due to body weight, as mice receiving cholesterol in their diet had body weights comparable to control mice.

In the study in **chapter 7,** we aimed to better understand the mechanism of HFD-induced OA. We showed that the intake of a very HFD by male APOE*3LeidenCETP mice led to the development of OA, but less severe than expected. Moreover, OA progression in the HFD group was even less than in control mice receiving chow diet. There were no effects of various interventions on OA development compared to the HFD group. We have several indications that can explain these results. The first is that there was no fructose in the drinking water, in contrast to a previous study with male APOE*3LeidenCETP mice on HFD, which developed severe OA. Fructose leads to a shift from HDL (‘good’ cholesterol) to VLDL (‘bad’ cholesterol). The hypothesis that a shift in lipoprotein profile is essential for the induction of OA, deserves more investigation as it would indicate a highly specific pathway for the pathogenesis of

OA. Furthermore, serum amyloid A levels (an acute phase protein) in the plasma of these mice were highly variable at the start of the study, which may have influenced their response to the HFD. An interesting observation in this study is that mice on a control diet already developed substantial OA. This gives reason to believe that an altered lipoprotein profile, compared to wild type mice, increases the sensitivity of these mice to develop OA.

The susceptibility of male and female mice also needs to be addressed. In general, male mice are more prone to develop features of the metabolic syndrome and to develop features of OA, which is confirmed in the hCRP mice described in chapter 5 (33-35). However, female APOE*3Leiden.CETP mice are more susceptible to develop high serum cholesterol levels and consequently more features of atherosclerosis (chapter 6), which is not observed in their male counterparts, even though they had an even higher cholesterol intake (data not shown). Based on these observations, it would be interesting to examine the effects of a HFD in combination with cholesterol on the development of female APOE*3Leiden.CETP mice.

In chapters 5 and 6 we demonstrated that features of the metabolic syndrome (obesity and hypercholesterolemia) induced the development of OA in mice. The low grade systemic inflammation evoked by these metabolic alterations may be held responsible for the initiation or progression of OA processes in the joint, but we cannot exclude the involvement of other factors. The effects of statins on the development of OA in mice contribute to the idea of a beneficial effect of statins in human OA. Although limited human data are available, it has been demonstrated that the use of statins is associated with a substantial reduction in knee, but not hip, OA progression (36). A plausible mode of action for the effect of statins is the inhibition of the formation of atherosclerotic plaques, as a consequence of high cholesterol exposure, and therewith improving the blood flow of subchondral bone. Furthermore, a direct effect on chondrocytes by targeting MMPs (37) is suggested. The only moderate correlations between cholesterol levels, features of atherosclerosis and the development of OA we found (chapter 6) do not contribute to these hypotheses. Based on our data, we propose that the main effect of statins can be subscribed to their anti-inflammatory properties, possibly by the inhibition of nuclear factor kappa B-driven processes.

Unraveling the pathways involved in obesity-associated low-grade systemic inflammation leading to OA may reveal new targets for intervention. Adipokines, which are secreted by adipose tissues as well as by several joint tissues, may be interesting targets. It has been demonstrated that leptin expression is higher in OA cartilage than in normal cartilage (38). Furthermore, visfatin, resistin and adiponectin levels are associated with features of OA (39). A study in leptin-deficient mice shows that these mice become very obese, but do not develop features of OA, indicating a possible role of leptin in the development of OA (40). With the knowledge that, until now, regular anti-cytokine therapies failed in the treatment of OA, these relatively newly discovered adipokines should be considered as promising targets for interference. We measured a significant increase in leptin and resistin levels in mice fed a HFD compared to mice receiving a control diet (chapter 5). However, these levels (measured at end point) were not correlated with the development of OA and were not affected by statins or rosiglitazone. This suggests that, if leptin is involved in HFD-induced OA, the inhibitory effect of rosuvastatin and rosiglitazone treatments is not mediated through direct acting on leptin, but possibly by influencing the downstream inflammatory processes that are regulated by leptin.

To link the local (chapters 2-4) and systemic (chapters 5 and 6) alterations on OA development, the following model can be proposed (figure 1): the elevated intake of, for example, fat or cholesterol in the diet leads to systemic alterations, giving rise to features of the metabolic syndrome and resulting in low-grade systemic inflammation. Due to the increased release of pro-inflammatory mediators in the blood, cells in the different joint tissues are activated and stimulated to secrete more pro-inflammatory mediators and/or matrix-degrading enzymes, therewith influencing cartilage homeostasis and contributing to OA development. The local inflammation and degradation products of cartilage can accordingly amplify the inflammatory response. In addition, different features of the metabolic syndrome may directly lead to modification of the different joint tissues, for instance by the accumulation of lipid droplets in the cells. This proposed paradigm strongly suggests considering OA as part of a bigger circle and deserves more investigation in patients sensitive to develop features of the metabolic syndrome (4).

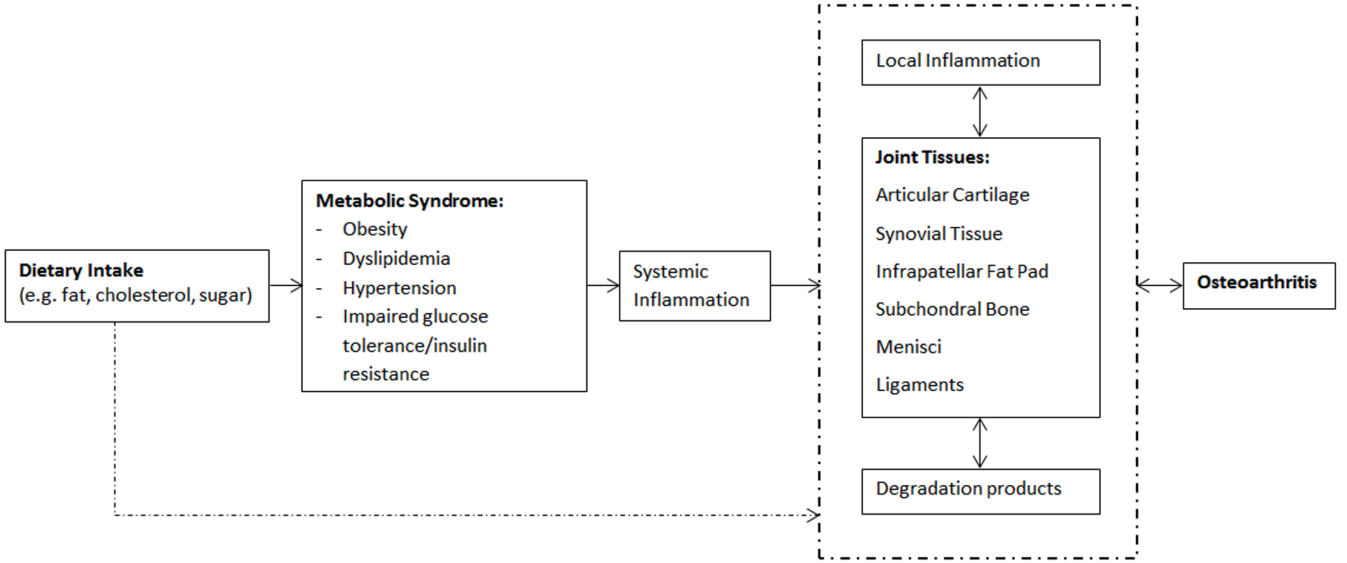


Figure 1. Proposed paradigm of osteoarthritis (OA) development based on data in this thesis, including both systemic and local factors. An increased dietary intake of e.g. fat, cholesterol or sugar leads to the development of features of the metabolic syndrome, such as obesity and hypercholesterolemia, which concurrently cause a systemic inflammation. This state of the body will in turn induce several tissues in the joint to secrete inflammatory mediators and matrix-degrading enzymes, leading to the development of OA. Consequently, the inflammatory mediators and the degeneration products produced by the joint tissues will amplify the OA process. We cannot exclude that the elevated dietary intake of e.g. fat, cholesterol or sugar has a direct effect on the joint tissues and contributes to the progression of OA as well.

Concluding remarks

OA is a frequently occurring disease and multiple factors, such as genetic, environmental, mechanical, and diet contribute to the development, progression and severity. We identify obesity, high cholesterol and systemic inflammation associated with these conditions as major players in OA development, which may activate joint tissues to secrete inflammatory mediators and contribute to the initiation and progression of OA. Our work suggests that a stratification of OA patients with (features of) the metabolic syndrome as underlying mechanism is recommendable, to optimize the efficacy of clinical trials. Approaching OA as a disease induced by whole body metabolism, and integrating knowledge about different potentially active tissues in the OA process, will provide new insights for possible pharmacological interventions.

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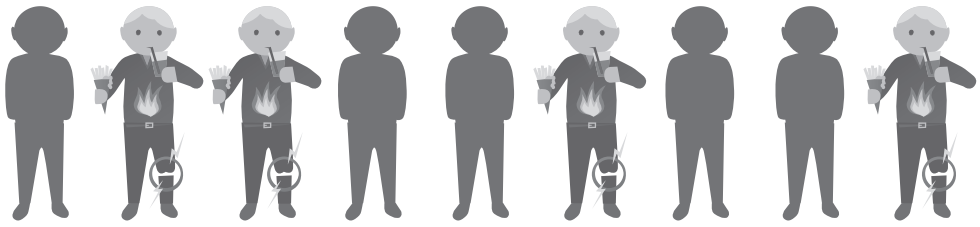
9

Nederlandse samenvatting



Artrose is een ziekte aan de gewrichten die wereldwijd veel voorkomt. In een gezonde situatie bedekt een laagje kraakbeen de botten, wat er onder andere voor zorgt dat je gewrichten soepel kunnen bewegen. Wanneer er artrose ontstaat breekt het kraakbeen langzaam af en dit leidt tot vervelende symptomen zoals pijn, stijfheid en problemen met bewegen. Ondanks dat er veel onderzoek wordt gedaan naar het ontstaan en de ontwikkeling van artrose, is er tot op heden nog geen geschikte behandeling beschikbaar die het ziekteproces kan stoppen of, beter nog, kan genezen. Patiënten zijn aangewezen op pijnmedicatie of moeten in een ernstiger stadium een knie- of heupvervangende operatie ondergaan. Het ziekteproces van artrose verloopt, ondanks vergelijkbare symptomen, niet bij iedere patiënt op de zelfde wijze. De 'heterogeniteit' van de patiëntenpopulatie kan een reden zijn voor de lage effectiviteit die nieuwe kandidaatmedicijnen veelal vertonen in klinische studies. Wellicht worden medicijnen ten onrechte afgekeurd, terwijl een subgroep van artrosepatiënten er wel baat bij kan hebben. Door het begrijpen van het onderliggende mechanisme van artrose, door een betere voorspelling van de ziekte te kunnen doen, en door een betere patiëntstratificatie te hebben, zouden klinische studies optimaler ingericht kunnen worden. In dit proefschrift onderzoeken wij het effect van verschillende factoren die bij kunnen dragen aan het ziekteproces van artrose.

Artrose als een ziekte van het gewricht

In de eerste drie hoofdstukken bestuderen wij de rol van verschillende factoren in het gewricht (lokale factoren) op het artroseproces. Tot nu toe lag de focus in wetenschappelijk onderzoek met betrekking tot artrose vooral op het kraakbeen. Veranderingen in andere weefsels in je gewricht zouden echter ook de oorzaak kunnen zijn voor het ontstaan (initiatie) of het verergeren (progressie) van artrose. Eén van deze weefsels is het synovium. Het synovium zorgt onder andere voor de productie van synoviale vloeistof, wat dient als een soort smeerolie van de gewrichten. Bij sommige artrosepatiënten komt ontsteking van het synovium voor. Er wordt gesuggereerd dat ontstekingsmediatoren (stoffen) die door de cellen in het synovium worden uitgescheiden, invloed kunnen hebben op het artroseproces.

In **hoofdstuk 2** laten we, tegen onze verwachting in, zien dat synoviaal weefsel van artrosepatiënten juist minder ontstekingsmediatoren uitscheidt dan synoviaal weefsel van gezonde personen. Ook demonstreren wij dat synoviaal weefsel van gezonde personen meer reageert op een ontstekingsstimulus dan het synoviaal weefsel van artrosepatiënten. Met behulp van een complex kweeksysteem laten wij zien dat het synovial weefsel niet in staat is kraakbeenafbraak te initiëren. Echter, onze data sluiten niet uit dat synovium wel betrokken is bij de progressie van de ziekte. Belangrijk om te weten is dat het synovium van de artrosepatiënten in deze studie afkomstig is van patiënten die een knievervangende operatie hebben ondergaan en dus in de eindfase van de ziekte zaten. Het synovium was wellicht al langdurig blootgesteld aan ontstekingsstimuli en er daardoor aan 'gewend' geraakt. Dit heeft mogelijk geleid tot de onverwachte uitkomsten in deze studie.

In **hoofdstuk 3** kijken wij naar de uitscheiding van ontstekingsmediatoren door het infrapatellaire vet. Het infrapatellaire vet is een speciaal soort vetweefsel gelegen in het gewricht, dat mogelijk bijdraagt aan het ziekteproces van artrose. Wij onderzoeken de uitscheiding van een specifiek soort mediatoren, oxylipines en vetzuren, door gezond en artrotisch infrapatellair vetweefsel. De gemeten mediatoren zijn statistisch niet verschillend tussen gezond en artrotisch infrapatellair vetweefsel. Multivariate analyse (een statische methode waarmee in plaats van één, meerdere variabelen worden vergeleken) laat echter zien dat de concentraties mediatoren die het infrapatellaire vet uitscheidt wel degelijk een specifiek profiel hebben in artrosepatiënten. Met behulp van een zogenaamde 'pathway analyse' demonstreren wij wáár in de onderliggende mechanismes de verschillen tussen gezonde en artrose donoren te detecteren zijn.

In **hoofdstuk 4** laten wij zien welke mediatoren er in de synoviale vloeistof van artrose en gezonde donoren zitten. We vinden hogere niveaus van ontstekingsmediatoren in synoviale vloeistof van artrosepatiënten ten opzichte van gezonde donoren.

De resultaten in hoofdstuk 2 tot en met 4 suggereren dat niet alleen het kraakbeen, maar ook andere weefsels in het gewricht betrokken zijn in het artroseproces en wellicht bijdragen aan de ziekte. Door artrose op deze manier te onderzoeken, kunnen wij nieuwe aangrijpingspunten voor interventie ontdekken. De eerste

stappen zijn gezet, maar er is meer onderzoek nodig om onderliggende mechanisme te kunnen begrijpen.

Artrose als een ziekte van het hele lichaam

In hoofdstuk 2 tot en met 4 onderzoeken wij de bijdrage van lokale factoren in het artrose proces. In de hoofdstukken die daarop volgen hebben wij het effect van processen die in het hele lichaam plaatsvinden (systemische factoren) bekeken. Specifiek hebben wij gekeken naar factoren die associëren met het metabool syndroom. Het metabool syndroom is een overkoepelende term voor aandoeningen zoals obesitas, diabetes en verstoorde lipiden levels. Het metabool syndroom komt in de westerse wereld steeds meer voor en studies hebben laten zien dat mensen met het metabool syndroom meer kans hebben op de ontwikkeling van artrose. Hoe dit precies komt is nog onduidelijk. Eén van de theorieën is dat het metabool syndroom wordt gezien als een aanstichter van ontstekingsprocessen in het lichaam en daardoor verschillende ziektes veroorzaakt. In hoofdstuk 5 tot en met 7 hebben wij onderzoek gedaan naar de relatie tussen verschillende componenten die geassocieerd worden met het metabool syndroom en artrose. Zo hebben wij onder andere gekeken naar de invloed van obesitas op de ontwikkeling van artrose. Obesitas wordt gezien als een belangrijke risicofactor voor het krijgen van artrose. Lang dachten onderzoekers dat de mechanische druk, veroorzaakt door de hoeveelheid vet bij zwaarlijvige mensen, hier verantwoordelijk voor is. Er is gebleken dat mensen met obesitas ook meer kans hebben op handartrose. Aangezien we niet op onze handen lopen, kunnen we deze associatie niet verklaren vanuit de mechanische belasting. De hypothese is daarom dat de lage graad van chronische ontsteking die gepaard gaat met obesitas ook een rol kan spelen in het ontstaan van artrose. Deze hypothese onderzoeken wij in **hoofdstuk 5** waarin muizen na het eten van een dieet met veel vet meer artrose laten zien dan muizen die een controle dieet te eten krijgen. Wij demonstreren ook dat de medicijnen statine (een cholesterol verlager) en rosiglitazone (een anti-diabetica) een remmend effect hebben op de ontwikkeling van artrose, ondanks dat deze muizen veel zwaarder zijn dan controlemuizen. Beide medicijnen hebben een ander werkingsmechanisme, maar gaan allebei ontsteking tegen. Daarnaast laten onze data zien dat de muizen die heftig op een dieet met veel vet reageren, gevoeliger zijn

om artrose te ontwikkelen in een later stadium. Als je dit zou vertalen naar de mens, zou dat betekenen dat de 'metabole' gevoeligheid wel eens voorspellend zou kunnen zijn of je artrose ontwikkelt. Hier moeten wij echter nog meer onderzoek naar doen.

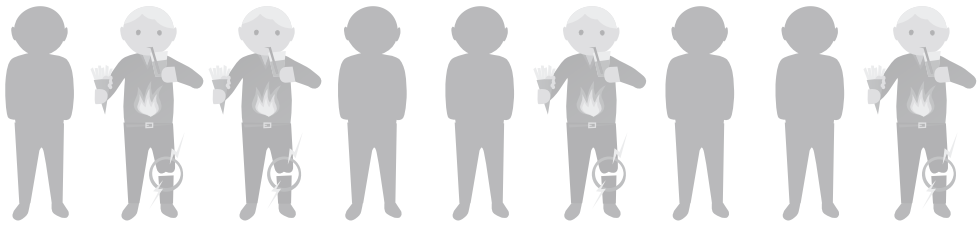
In **hoofdstuk 6** hebben we de focus gelegd op hypercholesterolemia (te hoge cholesterolwaarden in het bloed). Met behulp van speciale muizen, met de bijzondere eigenschap dat ze vergelijkbare cholesterol profielen hebben als de mens, kijken wij naar het effect van teveel cholesterol in het dieet op de ontwikkeling van artrose in de knieën. De muizen laten hele hoge cholesterolwaarden in hun bloed zien en ze ontwikkelen, zoals verwacht, heftige atherosclerose (aderverkalking). Wij zien dat muizen die een dieet met veel cholesterol ontvangen meer karakteristieken van artrose laten zien dan muizen die een controle dieet krijgen. Ook behandelen wij muizen met de medicijnen statine en ezetimibe. Deze behandelingen verlagen allebei cholesterol (en dus atherosclerose ontwikkeling), maar statine kan, in tegenstelling tot ezetimibe, ook ontsteking tegengaan. Wij zien wederom een onderdrukkend effect van statine op de ontwikkeling van artrose. De cholesterolwaarden in het bloed zijn niet heel sterk geassocieerd met de ontwikkeling van artrose. Wij concluderen daarom dat cholesterol in het bloed niet alleen verantwoordelijk is voor de ontwikkeling van artrose, maar dat er ook andere factoren geïnduceerd door cholesterol, zoals ontsteking, bij betrokken kunnen zijn.

In **hoofdstuk 7** proberen wij meer inzicht te krijgen in het mechanisme van het ontstaan van artrose gerelateerd aan de inname van een dieet met veel vet. Naar aanleiding van goede resultaten in een pilot studie, bestuderen wij twee interventies met een statine en een fenofibraat (beide cholesterol verlagend) bij muizen die een dieet met veel vet innemen. Deze behandelingen hebben wij zowel vroeg ('profylactisch') als laat ('therapeutisch') gegeven om daarmee te onderzoeken of wij ook in een later stadium van artrose het ziekteproces zouden kunnen remmen. Daarnaast onderzoeken wij muizen die niet dik worden van een dieet met veel vet, muizen die halverwege de studie van een dieet met veel vet naar een normaal dieet wisselen, en muizen die een caspase 1 remmer ontvangen (remt de omzetting van een mogelijk belangrijke stof in het artroseproces). De positieve controlegroep (muizen die alleen een dieet met veel vet ontvangen) laten niet de ernst van artrose zien die wij hadden verwacht en daarom kunnen wij de effecten van de interventies

niet analyseren. Een mogelijke reden hiervoor kan zijn dat de muizen geen suiker in hun water hebben ontvangen (dit leidt tot 'slechtere' cholesterolprofielen) in tegenstelling tot de muizen in de pilot studie. Daarnaast hebben de muizen in het beginstadium al hoge ontstekingsmarkers in hun bloed, wat mogelijk de onverwachte resultaten kan verklaren. Meer onderzoek is nodig om dit op te kunnen helderen.

Conclusie

Artrose is een gewrichtsziekte die veel voorkomt en meerdere factoren kunnen bijdragen aan de ontwikkeling, progressie en ernst ervan. In deze thesis hebben wij obesitas, hoog cholesterol, en daaraan gerelateerde ontsteking geïdentificeerd als mogelijk belangrijke spelers in het artrotische ziekteproces. De ontsteking kan op zijn beurt lokale weefsels in het gewricht activeren en daarmee de balans verstoren. Ons werk suggereert dat een stratificatie van artrosepatiënten, met als onderliggend mechanisme het metabool syndroom, kan bijdragen aan het optimaliseren van klinische studies. Door artrose als een ziekte vanuit het hele lichaam te bestuderen in plaats van alleen lokaal te kijken, verkrijgen wij mogelijk nieuwe inzichten voor behandelingen.



Dankwoord

Iedereen bedankt die op wat voor een manier dan ook heeft bijgedragen aan het tot stand komen van dit proefschrift.

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List of publications

(1) **L.M. Gierman**, S. Wopereis, B. van El, , E.R. Verheij, B.J.C. Werff-van der Vat, Y.M. Bastiaansen-Jenniskens, G.J.V.M. van Osch, M. Kloppenburg, V. Stojanovic-Susulic, T.W.J. Huizinga, A.-M. Zuurmond. Metabolic profiling reveals differences in concentrations of oxylipins and related free fatty acid precursors secreted by the infrapatellar fat pad of end-stage osteoarthritis patients and normal donors. *Submitted*

(2) M. Beekhuizen*, **L.M. Gierman***, W.E. van Spil, G.J.V.M. Van Osch, T.W.J. Huizinga, D.B.F. Saris, L.B Creemers, A.-M. Zuurmond. An explorative study comparing levels of soluble mediators in healthy and osteoarthritic synovial fluid.*Both authors contributed equally. Osteoarthritis Cartilage. 2013 Apr 15. [Epub ahead of print]

(3) **L.M. Gierman**, S. Kühnast, A. Koudijs, E. J. Pieterman, M. Kloppenburg, G.J.V.M. van Osch, V. Stojanovic-Susulic, T.W.J. Huizinga, H.M.G. Princen, A.-M. Zuurmond. Osteoarthritis development is induced by increased dietary cholesterol and can be inhibited by atorvastatin in APOE*3Leiden.CETP mice, a translational model for atherosclerosis. Ann Rheum Dis. 2013 Apr 26. [Epub ahead of print]

(4) **L.M. Gierman**, B. van El, F. van der Ham, A. Koudijs, R. Stoop, J.H. Verheijen, M. Kloppenburg, G.J.V.M. van Osch, V. Stojanovic-Susulic, T.W.J. Huizinga, A.-M. Zuurmond. Profiling end stage osteoarthritis synovial tissue explants reveals a reduced responsiveness to an inflammatory trigger. PLoS One. 2013 May;8:e62634

(5) **L.M. Gierman**, F. van der Ham, A. Koudijs, P. Wielinga, R. Kleemann, T. Kooistra, R. Stoop, M.Kloppenburg, G.J.V.M. van Osch, V. Stojanovic-Susulic, T.W.J. Huizinga, A.-M. Zuurmond. Metabolic stress-induced inflammation plays a major role in the development of osteoarthritis in mice. Arthritis Rheum. 2012; 64:1172-1181

(6) L. Garay, M.C. Gonzalez Deniselle, **L.M. Gierman**, M. Meyer, A. Lima, P. Roig, A.F. De Nicola. Steroid protection in the experimental autoimmune encephalomyelitis model of multiple sclerosis. Neuroimmunomodulation. 2008;15(1):76-83.

Curriculum Vitae

Lobke Marijn Gierman (roepnaam: Lobke) werd geboren op 27 december 1983 in Velp (gemeente Rheden). In juni 2002 behaalde zij haar VWO diploma aan het Arentheem College in Arnhem. In september van datzelfde jaar startte zij met de opleiding Biomedische Wetenschappen aan de Radboud Universiteit in Nijmegen. Zij behaalde in 2006 haar bachelor diploma. Tijdens haar masterfase specialiseerde zij zich in de richtingen pathobiologie en toxicologie. Haar eerste stage voerde zij uit in het laboratorium van Dr. A.F. De Nicola in Buenos Aires, Argentinië. Tijdens deze stage deed zij onderzoek naar de effecten van progesteron en oestrogeen op de ontwikkeling van multiple sclerose. Haar tweede stage voerde zij uit bij Organon NV te Oss onder begeleiding van Dr. W. Schoonen. In deze stage heeft zij een *in vitro* bioassay ontwikkeld voor de vitamine A receptor in humane lever HEPG2 cellen (een vroege biomarker voor de identificatie van embryotoxiciteit). In juni 2008 behaalde zij haar master diploma's. Na haar afstuderen werd Lobke vanaf september 2008 aangesteld als promovenda bij de afdeling reumatologie van het LUMC onder de supervisie van Prof. Dr. T.W.J. Huizinga. Voor haar promotie onderzoek werd zij gedetacheerd op de afdeling Metabolic Health Research van TNO in Leiden onder de supervisie van Dr. A.-M. Zuurmond. Het promotie onderzoek, waarvan de resultaten zijn beschreven in dit proefschrift, is afgerond in december 2012. Aansluitend is Lobke aangesteld als wetenschappelijk medewerker op de afdeling Metabolic Health Research van TNO in Leiden.