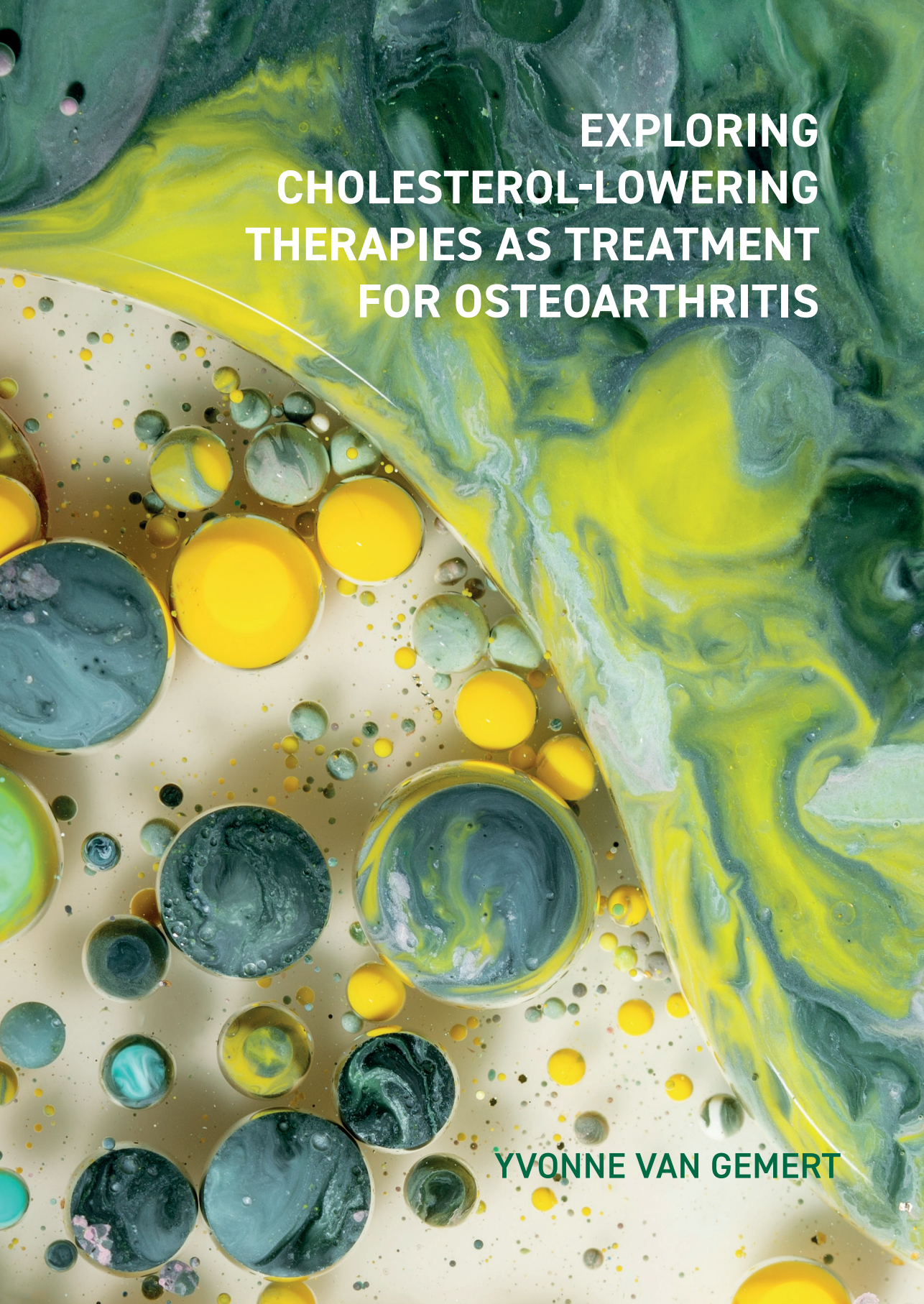

EXPLORING CHOLESTEROL-LOWERING THERAPIES AS TREATMENT FOR OSTEOARTHRITIS

YVONNE VAN GEMERT

EXPLORING CHOLESTEROL-LOWERING THERAPIES AS TREATMENT FOR OSTEOARTHRITIS

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Voor mijn moeder

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CHAPTER 1

Introduction and thesis outline

OSTEOARTHRITIS

Osteoarthritis (OA) is the most common disabling joint disease worldwide. OA mostly affects the elderly population and the disease is progressive in nature and comprises several tissue structures including cartilage, synovium, subchondral bone and ligaments[1] (**Figure 1**). There are many risk factors associated with OA development, including age, obesity, genetic predisposition and metabolic syndrome (Mets)[2, 3]. In the last decades, the prevalence of OA has increased, which is most likely caused by the ageing of the population and altered physical activity and weight gain. Although the knees, hips and hands are most frequently affected, OA can affect any joint. Previously, OA was seen mainly as a wear-and-tear disease. Over the last decades the paradigm of OA has been shifting towards that of a disease where, next to involvement and degradation of articular cartilage, local and systemic inflammatory processes have an important role in the development and progression of the disease[4, 5]. Due to the ageing population and increasing obesity, the incidence of OA and thus the impact on worldwide public health and the financial burden of OA is expected to rapidly increase in the next decades. Currently, there are no curative or disease-modifying treatments available and OA is generally managed by lifestyle interventions, analgesics and joint replacement.

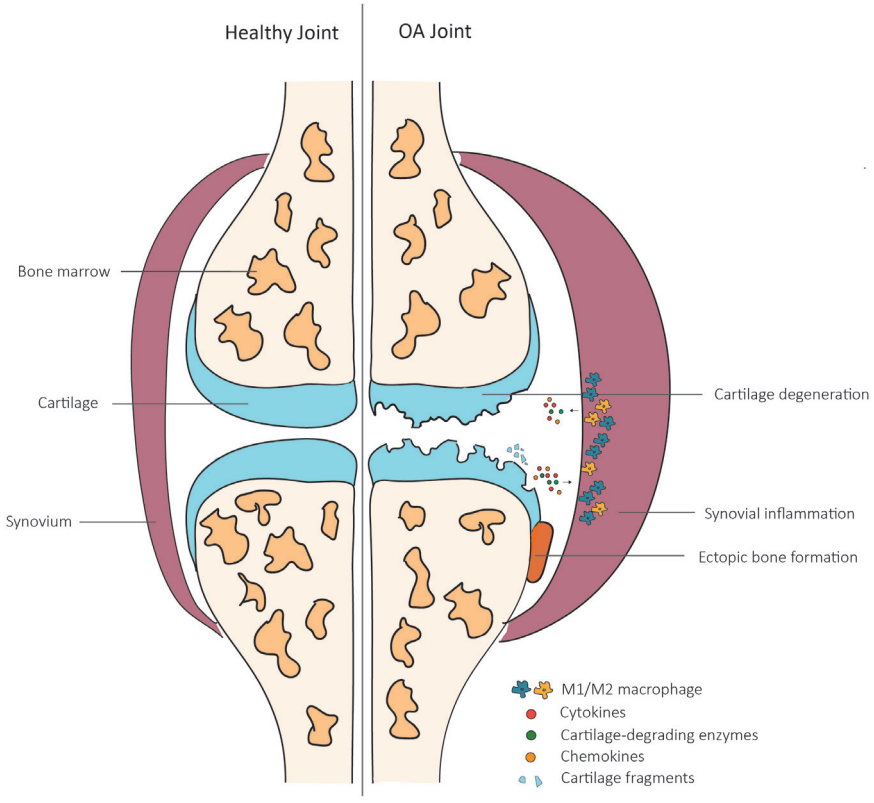

Figure 1. Schematic overview of pathological changes in an OA joint.

On the left, a healthy joint is depicted with the main structures present in a joint. On the right, an osteoarthritic joint is depicted which shows cartilage degeneration, synovial inflammation and ectopic bone formation.

Although it has become clear that OA is a highly multifactorial and heterogenous disease, the underlying mechanisms that drive OA pathology remain poorly understood. Articular cartilage is the connective tissue that covers articulating bone surfaces, facilitates movement and acts as a shock absorber. Articular cartilage is a very specialized tissue and does not contain blood vessels, resulting in a poor repair capacity[6]. Articular cartilage primarily consists, next to water, of proteoglycans like aggrecan and collagen type II fibers, the extracellular matrix (ECM), where chondrocytes are embedded[7, 8]. In healthy cartilage, there is a balance in the delicate interplay between anabolic and catabolic processes. In OA, this balance is deregulated. During OA, chondrocytes produce an increased amount of cartilage degrading enzymes, such as several matrix metalloproteinases (MMPs) and A Disintegrin and Metalloproteinase with Thrombospondin motifs (ADAMTSs)[9, 10]. MMPs and ADAMTSs are able to cleave components of the ECM, leading to degradation of the cartilage matrix. Although cartilage degeneration is the most notable hallmark of OA, other tissues such as synovium and subchondral bone play an important role in the pathology of OA.

The synovium is a specialized connective tissue that lines the entire joint cavity. The main function of the synovium is the production of synovial fluid, which acts as a lubricant for the joint and provides a source of nutrition for the articular cartilage[11]. The inner lining of the synovium contains type A and B synoviocytes, which are macrophages and fibroblasts, respectively[12]. During OA, the synovium shows characteristics like increased vascularity, fibrosis and cell infiltration[13]. Macrophages are the most common cell type found in OA, but other leukocytes like T cells are also present in OA synovium[14]. Macrophages have a highly plastic phenotype and can adapt quickly to changes in their microenvironment. Usually, macrophages are classified in two main groups, representing the two extremes of macrophage polarization states, the more pro-inflammatory M1-like macrophages and the more anti-inflammatory M2 like macrophages. During OA, the synovium produces an increased amount of pro-inflammatory factors, like interleukin-1β (IL-1β), IL-6 and IL-8, chemokines and cartilage degrading enzymes[15]. Moreover, activated macrophages produce high levels of the alarmin S100A8/A9, which we believe is an important mediator of the inflammatory process in OA[16]. Next to inflammatory mediators, the synovium produces high amounts of MMPs and ADAMTSs that contribute to the degradation of articular cartilage. In addition, uptake of degraded cartilage by synovial macrophages results in increased production of pro-inflammatory factors, creating a positive feedback loop. Next to joint inflammation, increased systemic inflammation has also been associated with OA development[5]. Low-grade systemic inflammation associated with metabolic syndrome, which is also referred to as metabolic inflammation, is thought to contribute to OA development and has been suggested as a connection between MetS and OA[17]. Metabolic inflammation is mainly caused by metabolic factors and nutrients, such as fatty acids or glucose, that induce inflammation in metabolic tissues like adipose tissue or hepatocytes[18, 19]. Increased levels of circulating inflammatory markers and adipokines are observed in OA patients and have been associated with increased joint inflammation[20, 21].

METABOLIC SYNDROME AS A RISK FACTOR FOR OSTEOARTHRITIS

As mentioned above, MetS is an important identified risk factor for OA development. MetS comprises a cluster of multiple metabolic conditions including visceral obesity, high glucose levels and insulin resistance, hypertension and dyslipidemia. The prevalence of MetS has increased rapidly over the last decades, leading to increasing rates of associated diseases like obesity and type 2 diabetes worldwide. An increased prevalence of MetS in the OA population was observed compared to the non-OA population[22] and MetS has been associated with the progression and development of OA[2, 3]. Moreover, OA patients with MetS in general develop OA at earlier ages than patients without MetS[22, 23]. Previously, it was thought that MetS mainly increased OA development via loading on the joint due to obesity. However, the observation that metabolic syndrome also increased the risk of developing general OA or OA in non-weight bearing joints, such as the hands, indicated that systemic mechanisms were most likely involved[24-27].

DYSLIPIDEMIA

Dyslipidemia, one of the main characteristics of MetS, results in an increase in circulating lipid levels, such as cholesterol and triglycerides. Usually, systemic low-density lipoprotein cholesterol (LDL-C) is increased while high-density lipoprotein cholesterol (HDL-C) is decreased in people with dyslipidemia. Cholesterol regulates membrane fluidity, permeability and cell signaling. Additionally, cholesterol is the precursor of bile acid, oxysterols, steroid hormones and is needed for the production of vitamin D. There are two main sources of cholesterol, (1) intracellular production in the liver by the cholesterol synthesis pathway, also known as the mevalonate pathway and (2) dietary cholesterol intake with receptor mediated endocytosis via the bloodstream. Cholesterol is transported in LDL and HDL particles. LDL is known as the 'bad' cholesterol and mainly transports cholesterol from the liver to peripheral organs, while HDL is known as 'good' cholesterol as it mainly transports cholesterol particles from the periphery to the liver. Cholesterol can be converted into bile acids in the liver, after which it enters the intestine and is ultimately excreted from the body. An increased intake of dietary fats and cholesterol leads to an overload of the system, resulting in increased hepatic cholesterol production and an increase of circulating cholesterol.

INTRACELLULAR ACCUMULATION OF CHOLESTEROL

High circulating levels of LDL-C can result in an increased exposure and uptake of LDL-C in peripheral tissues, which is linked to systemic diseases such as obesity and cardiovascular disease (CVD). Lipoproteins such as LDL-C are susceptible to various modifications, like oxidation and enzymatic cleavage. The scavenger receptors SR-A, CD36, LOX-1 and SR-B1 are a type of pattern recognition

receptor (PRR) expressed on macrophages that recognize and internalize modified LDL[28]. Macrophages are thought to play an important role in the development and the progression of OA via the release of cartilage degrading enzymes like MMPs and pro-inflammatory mediators including IL-1β, IL-6 and S100A8/A9 proteins[29-32]. Next to being ligand for scavenger receptors, modified LDL particles can act as a ligand Toll-like receptors (TLRs), directly inducing pro-inflammatory pathways leading to NLRP3 inflammasome activation and production of IL-1β, which is believed to play an important role in cartilage degradation[33, 34]. In atherosclerosis, oxLDL uptake by macrophages induces lipid accumulation and foam cell formation[35]. The uptake of modified lipids induces macrophages with a more pro-inflammatory phenotype, represented by increased production of cytokines like TNFα and IL-6. Other factors produced by oxLDL-trained macrophages include cartilage degrading enzymes MMP-2 and MMP-9. We have previously shown that increased oxLDL uptake by the synovium was associated with an increased production of S100A8/A9[36].

The reduction of LDL-C levels is one of the most important treatment options to reduce the risk of CVD, especially for atherosclerosis. Of note, OA and atherosclerosis share multiple risk factors, such as obesity, age and hypercholesterolemia, which could point to similar pathobiological mechanisms. In the Rotterdam study, it was shown that plasma levels of atherosclerotic markers were increased in women with knee OA which indicates a positive association of OA with CVD[37]. Additionally, it was shown that postmenopausal women with CVD have a higher chance of developing OA[38] and it was shown that there was an association between atherosclerosis and the severity of hand OA in women[39]. Importantly, several studies have shown that high systemic cholesterol by itself is a separate risk factor for OA development[40-42].

EVIDENCE FOR A ROLE OF CHOLESTEROL IN THE PATHOGENESIS OF OSTEOARTHRITIS

One of the main causes of cholesterol accumulation in tissues is a disbalance in cholesterol influx and efflux of the cells. Cholesterol accumulation in cells can induce cytotoxicity[43]. Cholesterol can accumulate in OA cartilage and is mainly distributed in the superficial layer of the cartilage compared to healthy samples[44]. Several studies have shown that the pathways involved in lipid metabolism are deregulated in OA chondrocytes[45, 46]. Additionally, mRNA expression of LXRs, which initiates genes that regulate cholesterol efflux, like ABCA1 and ABCA2, were decreased in OA cartilage compared to normal cartilage, which hampers cholesterol efflux and increases accumulation of cholesterol in OA chondrocytes[45].

The effect of high cholesterol levels on OA development has also been studied in pre-clinical models. Gierman et al. demonstrated that high dietary cholesterol increased cartilage damage compared to controls fed a regular chow diet[47]. Farnaghi et al. reported that *Apoe*$^{-/-}$ mice and diet-induced hypercholesterolemia rats exhibited OA-like changes when fed a high cholesterol diet[48]. Previous studies in our lab have shown that high systemic LDL-cholesterol increases ectopic bone formation and synovial

activation during inflammatory collagenase-induced OA[36, 49]. Others have reported that OA pathology was most severe when injury-induced OA models were used in dyslipidemic mice[50, 51]. Whereas some studies reported that hypercholesterolemia resulted in cartilage damage, multiple studies did not observe a detrimental effect of high cholesterol on cartilage pathology while joint inflammation was increased[36, 49, 52, 53]. Several clinical studies have shown that increased circulating lipid levels are associated with an increased incidence and progression of OA[40-42]. In the Chingford study, serum cholesterol was associated with increased knee OA[54]. Hypercholesterolemia has been associated with generalized OA[55] and increased cholesterol levels are associated with bone marrow lesions[56]. Although not all studies observed a link between high cholesterol levels and OA development[57], several recent meta-analyses have concluded that high cholesterol is evidently linked to OA development[58, 59].

CHOLESTEROL ACCUMULATION ACTIVATES PRO-INFLAMMATORY PATHWAYS

Inflammatory effects of cholesterol on macrophages

Human synovial fluid from OA and rheumatoid arthritis patients has been shown to contain cholesterol, although these levels are lower compared to plasma or serum levels. The levels of cholesterol and cholesterol crystals are increased with inflammation[60, 61], and is thought to be influenced by the permeability of the synovium. As has been shown in atherosclerotic plaques, oxLDL uptake and accumulation leads to an increase of lipid-laden macrophages. This increased oxLDL uptake is associated with a higher presence of pro-inflammatory M1-like macrophages and subsequent cytokine production. In contrast, presence of M2-like macrophages is more pronounced in atherosclerotic models of plaque regression[62, 63]. Similar to atherosclerotic plaques, uptake of oxLDL by macrophages in the synovial lining could increase the local inflammatory response and therefore aggravate OA development.

Systemic inflammation

Low-grade systemic inflammation, associated with metabolic disease, is thought to contribute to OA development and has been suggested as a connection between MetS and OA[17]. This metabolic inflammation is mainly caused by metabolic factors that induce oxidative stress and inflammation, such as lipids and glucose[18, 19]. Although inflammation is believed to be involved in the pathogenesis of OA, clinical trials targeting inflammatory factors proved disappointing in ameliorating disease progression. IL-1β, for example, is an important driver of inflammation and is believed to be involved in OA pathology. Despite the fact that IL-1β was thought to play a critical role in the pathogenesis of OA, several pre-clinical and clinical studies were not able to demonstrate any disease-modifying effects after inhibition of IL-1β[64-67]. However, these anti-inflammatory treatments might be more suitable for specific OA endotypes, as in most OA clinical trials no specific patient stratification was used. Therefore, IL-1β could play a more central role in OA development in patients with metabolic syndrome. Metabolic inflammation can increase activation of the NLRP3 inflammasome[33, 68], making IL-1β a target of interest in diseases associated with MetS, such as cardiovascular disease and OA. Recently, a secondary analysis of the Canakinumab Anti-inflammatory Thrombosis Outcome Study (CANTOS) trial showed that systemic

inhibition of IL-1β resulted in a decreased incidence of total knee and hip replacements in patients with CVD[69]. As the CANTOS trial was designed to study the effects on cardiovascular events, data on structural outcomes or radiographic evidence were not collected, and it would be of interest to investigate if inhibition if IL-1β in OA patients with high inflammatory risk reduces structural progression of pathology such as cartilage damage or joint space narrowing.

CHOLESTEROL-LOWERING TREATMENT AS A THERAPEUTIC STRATEGY FOR METS-ASSOCIATED OA DEVELOPMENT

Since both pre-clinical and clinical studies point towards an association between high systemic cholesterol levels and the development of OA as described above, research into the possible protective effects of cholesterol-lowering treatment is of interest for OA patients.

Statins

Systemic cholesterol levels can be reduced via a reduced intake of cholesterol or by using cholesterol-lowering therapy. Statins are the most commonly used and most investigated cholesterol-lowering drugs and there are multiple statins approved by the European Medicines Agency, including atorvastatin, simvastatin, lovastatin and pravastatin. Statins reduce systemic cholesterol levels by acting as competitive inhibitors of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, a key enzyme of the cholesterol synthesis pathway **(Figure 2**). Statins decrease hepatic cholesterol production and in addition, this reduction in intracellular cholesterol levels increases the uptake of cholesterol via increased expression or the low-density-lipoprotein receptor (LDLR). Moreover, statins have multiple pleiotropic effects independent of their cholesterol-lowering function and were shown to improve endothelial dysfunction, have antioxidant properties and reduce smooth muscle cell proliferation. In rheumatoid arthritis, statins were shown to reduce systemic cholesterol levels and inflammatory markers[70]. One of the additional benefits of statin treatment that can be beneficial for OA patients is the inhibition of several MMPs. It has been shown that statins can inhibit MMP production by chondrocytes[71-73] and promote apoptosis of synovial fibroblasts[74].

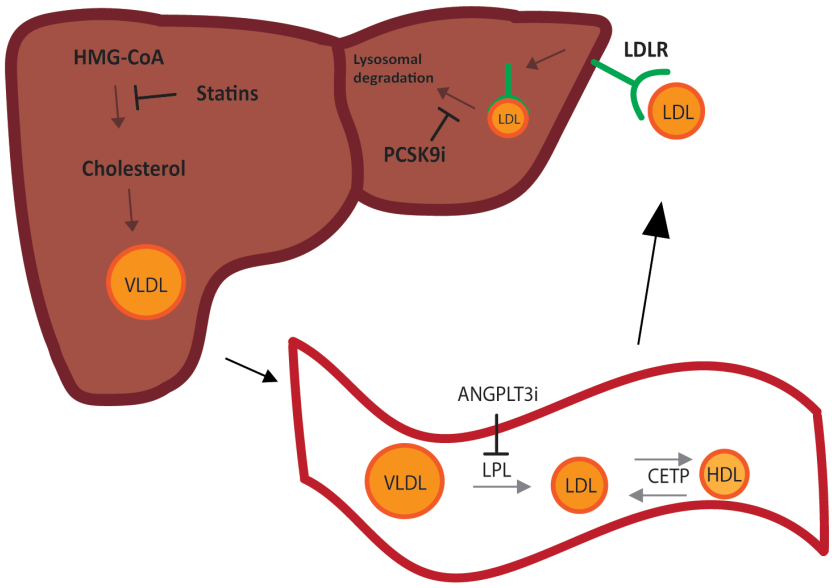

Figure 2. Schematic overview of cholesterol metabolism and the mechanism of cholesterol-lowering therapies.

Statins reduce the intracellular production of cholesterol. PCSK9 inhibitors prevent lysosomal degradation of the LDLR. ANGPTL inhibitors reduce the hydrolysis of lipids. *HMG-CoA: 3-hydroxy-3-methylglutaryl coenzyme A, LDLR: low-density lipoprotein receptor, PCSK9: Proprotein convertase subtilisin/kexin type 9, ANGPTL3: angiopoietin-like protein 3, CETP: Cholesteryl ester transfer protein, VLDL: very low-density lipoprotein, LDL: low-density lipoprotein, HDL: high-density lipoprotein.*

The use of statins show variable results with regard to progression of OA

In the last years, multiple studies have been conducted that evaluated if statin use could reduce the incidence and/or progression of OA. The use of statins have shown variable results concerning the development or progression of OA. In a longitudinal study cohort, statin use was associated with a decrease in clinical OA versus non-statin users and a higher cumulative dose was associated with an increased reduction[75]. In another cohort it was shown that statin use could reduce the incidence and the progression of radiographic OA over 50%[76]. In the Rotterdam study, statin use was associated with a decreased risk of the progression of knee OA with more than 50%[76]. A recent study showed a lower incidence of degenerative joint disease in statin users compared to non-users[77]. The requirement for total hip and total knee arthroplasty were decreased in statin users in two studies[78, 79]. In contrast, several studies showed that statin use was not associated reduced OA development, demonstrating no reduction in the incidence or symptomatic OA in a longitudinal study with a 4-year follow-up[80, 81]. However, increased statin use and atorvastatin treatments were associated with a reduced risk of developing pain[80]. Statin use did not reduce the risk of progression of knee OA compared to non-users[82],

also for the incidence of hand OA there was no association between the initiation of statin treatment compared to non-initiators[83]. Some studies even showed an increased OA incidence in statin user versus non-statin users[84, 85].

The effect of cholesterol-lowering treatment on OA development has also been studied in more detail in pre-clinical studies. Gierman et al. showed that preventative cholesterol-lowering treatment was able to significantly decrease OA development in transgenic E3L.CETP mice fed a cholesterol-supplemented Western-type diet (WTD)[47]. In contrast to cholesterol metabolism in humans, mice with a WT background have high HDL levels and low (V)LDL levels because they lack cholesteryl ester transfer protein (CETP) which transports cholesterol esters from HDL to VLDL and LDL. Transgenic E3L.CETP mice have a more humanized cholesterol metabolism as most of the cholesterol is transported in LDL particles and not HDL particles due to the introduction of the human ApoE3*Leiden (E3L) and CETP gene. The introduction of the human E3L mutation results in a reduced clearance of ApoB-containing lipoproteins, such as LDL and VLDL, leading to increased levels in the circulation. As mentioned before, introduction of the CETP gene results in increased (V)LDL levels due to the transports of cholesterol esters from HDL to VLDL and LDL. Gierman et al. observed that atorvastatin, but not ezetimibe, a cholesterol-lowering drug that reduces cholesterol uptake in the intestine, was able to reduce OA development. The authors indicated that this could possibly be explained by the anti-inflammatory effects of statins[47].

Anti-inflammatory effects of statins

Some studies have reported that the beneficial effects of statin use on OA development could be explained by the anti-inflammatory pleiotropic effects associated with statin use[47, 86]. One of the possible mechanisms by which statins can reduce inflammation, is by inhibiting the effects of monocyte migration and differentiation. Statin treatment reduced the expression of IL-6, IL-8 and MCP-1 in peripheral blood mononuclear cells in hypercholesterolemic patients[87] and reduced the production of inflammation-induced secretion of pro-inflammatory cytokines and monocyte chemotaxis[88]. Moreover, simvastatin was shown to inhibit expression of chemokine receptor CCR2 and in human circulating monocytes[89] and reduced expression or chemokine receptors in healthy volunteers as well as patients with acute coronary syndrome. It was shown that atorvastatin reduced activation of M1-like macrophages while promoting M2-like macrophage activation, thereby reducing the expression of pro-inflammatory cytokines. Also in human macrophages and monocytes, statin therapy skewed macrophages into an M2-like phenotype[90] and reduced expression of cytokines such as MCP-1 and MIP-1a[88]. These pleiotropic anti-inflammatory effects of statin therapy could lead to a reduction of joint inflammation and could be of additional benefit for OA patients.

Together, the above shows that statin therapy has variable results regarding the progression of OA and there are several potential mechanisms that can account for these conflicting results. Statin intolerance is estimated to occur in 20-30% of patients and often leads to discontinuation of statins[91]. Also, the use of statins does not always result in the desired reduction of systemic cholesterol levels. Moreover, the

presence of statin-associated muscle pain could interfere with the beneficial effects on OA symptoms such as pain and confound the results.

NOVEL CHOLESTEROL-LOWERING THERAPIES

In recent years, several novel cholesterol-lowering treatments have become available that are promising in the treatment for cardiovascular disease. The shared pathological risk factors between atherosclerosis and OA could provide new targets for the modulation of cholesterol in OA patients.

PCSK9 inhibitors

Recently, proprotein convertase subtilisin/kexin 9 (PCSK9) was discovered and identified as a new cholesterol-lowering target. PCSK9 is one of the key proteins involved in cholesterol homeostasis and targets the LDL-receptor (LDLR) for lysosomal degradation, thereby decreasing its membrane expression and the ability to clear LDL-cholesterol from the circulation[92, 93] (**Figure 2**). Also, PCSK9 was shown to promote degradation of several other receptors involved in lipid uptake, such as the very low-density lipoprotein receptor (VLDLR), LDLR-related protein-1 (LRP-1), apolipoprotein E receptor (ApoER), and CD36[94-96]. PCSK9 is expressed in several tissues such as the liver, small intestine, pancreas, kidney and in the brain[92]. In addition, PCSK9 was shown to be expressed in endothelial cells and macrophages present in atherosclerotic plaques[97-99]. The antibodies alirocumab and evolocumab are newly developed cholesterol-lowering drugs which target PCSK9. These antibodies have been shown to be very effective cholesterol-lowering drugs in both humans and mice and reduce LDL-C levels up to 50-60%[100-102]. The PCSK9 antibody alirocumab was shown to further reduce cardiovascular events in patients with cardiovascular disease and hypercholesterolemia on top of statin treatment[103-105]. Moreover, statin therapy combined with PCSK9 inhibition resulted in stabilization and regression of atherosclerotic plaques in both humans and mice[102, 106]. These data obtained from cardiovascular diseases suggest that the use of combined cholesterol-lowering therapies as a treatment for OA could be more efficient compared to statin therapy only.

Involvement of PCSK9 in inflammation

Besides playing a pivotal role in cholesterol homeostasis, PCSK9 is suggested to have a direct role in inflammation[107]. Various inflammatory stimuli are able to increase the expression of PCSK9, such as oxLDL or LPS. In mice, several inflammatory factors such as LPS, zymosan or bacteria upregulated PCSK9 expression levels in liver or blood. In mice that underwent cecal ligation and puncture, *Pcsk9*$^{-/-}$ mice showed a reduced inflammatory response, while overexpression of PCSK9 increased release of inflammatory cytokines[108]. Several studies have demonstrated that PCSK9 has a pro-inflammatory effect on macrophages, possibly via increased activation of the TLR4/NF-κB signaling pathway[107, 109, 110]. In line with these findings, in vitro studies showed that knockdown of PCSK9 reduced the LPS- and oxLDL-induced inflammatory response in macrophages, indicated by reduced levels of the pro-inflammatory

factors IL-1β, MCP-1 and TNFα, which are implicated in the development of OA[107]. Additionally, PCSK9 induced an inflammatory response in macrophages via an increased production of IL-6, MCP-1, TNFα and IL-1β, strengthening the idea that PCSK9 can directly influence inflammation[107, 110, 111]. The use of PCSK9 antibodies reduced systemic inflammation and endothelial dysfunction in familial hypercholesterolemia patients, via leukocyte-endothelium interactions[112]. Inhibition of these anti-inflammatory effects of PCSK9 could provide an additional benefit, next to cholesterol lowering, for patients with metabolic inflammation.

ANGPTL3 inhibitors

Angiopoietin-like 3 (ANGPLT3) regulates systemic triglycerides and HDL-C levels via inhibition of lipoprotein lipase and endothelial lipase. ANGTPL3 inhibitors interfere with the hydrolysis of triglycerides and phospholipids and the use of ANGPLT3 inhibitors results in decreased levels of triglycerides, LDL-C and HDL-C[113, 114] (**Figure 2**). The ANGPLT3 blocking antibody evinacumab seems a promising cholesterol lowering drug and has recently been approved by the FDA. Small molecule inhibitors for ANGPTL3 are also in development, Vupanorsen is an antisense oligonucleotide which inhibits ANGPLT3 production in the liver. Vupanorsen is now in phase II clinical trials and effectively reduces systemic atherogenic lipoproteins[115].

AIMS AND OUTLINES OF THIS THESIS

In recent years it has become evident that OA is a highly heterogeneous disease. Stratification of OA patients in subgroups could point towards more patient-driven therapy. Several studies have shown that high systemic cholesterol is a separate risk factor for OA development and more investigation into the effects of cholesterol-lowering treatments in OA is needed, which are discussed in Chapter 1[40-42].

The aim of this thesis was to investigate if high-intensive cholesterol-lowering therapies could reduce disease development in dyslipidemic mice. In the light of the varying results of statin-induced cholesterol lowering on OA and based on the promising results from combining various cholesterol-lowering therapies in CVD patients, we explored whether combinational treatment options that aimed at more intensive lowering of cholesterol and inflammation also could improve the treatment of OA.

In **Chapter 2** we investigated the role of PCSK9 in OA development in more detail using *Pcsk9*$^{-/-}$ and WT mice fed a chow or a cholesterol-supplemented WTD in the pre-clinical collagenase-induced OA (CiOA) model. The CiOA model is an injury-induced OA model with a strong local inflammatory response within the joint. We studied the effects of PCSK9 deficiency on cholesterol-associated OA pathology and investigated whether this affected joint inflammation, which is important for the development of this experimental OA model. We additionally determined whether PCSK9 protein affected cartilage damage and ectopic bone formation.

In **Chapter 3** we determined the effects of several cholesterol-lowering treatments on spontaneous OA development in transgenic E3L.CETP mice. We used atorvastatin alone or combined with PCSK9 inhibitor alirocumab and ANGPLT3 inhibitor evinacumab in E3L.CETP mice fed a cholesterol-supplemented WTD. We determined osteoarthritis development using histological analysis of the knee joints, where we analyzed synovial inflammation, cartilage damage, ectopic bone formation and enzymatic activity in articular cartilage.

Next to the effects of cholesterol-lowering treatment in a spontaneous model, we additionally investigated if lowering of cholesterol levels could reduce OA development in a model with a strong inflammatory component. Therefore, in **Chapter 4** we investigated if the PCSK9-inhibitor alirocumab combined with atorvastatin therapy could ameliorate OA development after induction of CiOA in E3L.CETP mice fed a cholesterol-supplemented WTD. We determined local and systemic amounts of inflammatory factors. Cartilage degeneration, synovial inflammation and ectopic bone formation were analyzed using histological sections of the knee joints.

Recently, a secondary analysis the CANTOS trial showed that systemic inhibition of IL-1β resulted in a 40-47% decrease in incidence of total knee and hip replacements in patients with CVD where over 93% of patients received cholesterol-lowering therapies[69]. This study could point towards a subgroup of OA patients where a combination treatment of cholesterol-lowering with inflammatory therapies could be of benefit. As the CANTOS trial was not designed to study OA pathology, the main read-out was endpoint joint replacement and data on structural outcomes was not collected. Therefore, we next investigated whether anti-inflammatory treatment showed additional effects on lowering OA pathology when combined with cholesterol-lowering treatment. In **Chapter 5** we first investigated if the inhibition of IL-1β could reduce OA development during a CiOA model in mice that were fed either a chow or cholesterol-supplemented WTD. We determined the effects on local and systemic inflammation using analysis of cytokines and flow cytometry to evaluate the effects on innate immune cells. Cartilage degeneration, synovial inflammation and ectopic bone formation were analyzed using histological sections of the knee joints.

It was shown in the CANTOS trial that patients with high levels of systemic inflammation showed a reduced incidence of cardiovascular events when treated with anti-IL-1β therapies. Therefore, we next investigated if these treatments were effective in ameliorating OA development using models with different degrees of systemic inflammation. In **Chapter 6** we investigated if inhibition of IL-1β combined with cholesterol-lowering therapy, consisting of statin therapy and the PCSK9-inhibitor alirocumab, could decrease spontaneous OA pathology in dyslipidemic mice E3L mice, which have a humanized lipoprotein metabolism. We quantified systemic levels of MCP-1, E-selectin and SAA to determine the effect of the treatments on systemic inflammation. We determined osteoarthritis development using histological analysis of the knee joints, where we analyzed synovial inflammation, cartilage damage and ectopic bone formation.

We summarized and discussed all described data in **Chapter 7** and provide future directions for cholesterol-lowering therapies in osteoarthritis.

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CHAPTER 2

PCSK9 deficiency reduces synovial inflammation in Western-type diet-fed mice but does not affect end-stage pathology during experimental osteoarthritis

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ABSTRACT

Introduction

The association between metabolic syndrome (MetS) and osteoarthritis (OA) development has become increasingly recognized. The presentation with high cholesterol levels is a hallmark of MetS. However, studies have shown contradictory results and the exact role of cholesterol and cholesterol-lowering therapies in OA development are currently under debate. Recently, monoclonal antibodies against proprotein convertase subtilisin/kexin 9 (PCSK9) have been developed. PCSK9 is one of the key proteins involved in cholesterol homeostasis and *Pcsk9*$^{-/-}$ mice have reduced systemic cholesterol levels. Here we used *Pcsk9*$^{-/-}$ mice to study the effects of PCSK9 deficiency in post-traumatic OA development in dyslipidemic mice.

Materials and Methods

In this study, female PCSK9$^{-/-}$ and WT mice were fed either regular chow or a cholesterol-supplemented Western-type diet (WTD). Collagenase-induced OA (CiOA) was induced 4 weeks after the start of the diet. We determined the effects of dietary cholesterol and PCSK9 deficiency on monocyte subsets using flow cytometry and synovial inflammation using histology during early-stage disease. We quantified cartilage degradation and osteophyte formation in histological sections at end point.

Results

5 weeks of WTD feeding significantly increased systemic cholesterol levels in both WT and *Pcsk9*$^{-/-}$ mice (TC: 53.1%, 66.3%, respectively). PCSK9 deficiency resulted in reduced cholesterol levels in both chow and WTD-fed mice (47.8%, -26.4%, respectively). *Pcsk9*$^{-/-}$ mice showed a reduction in synovial inflammation when only fed a cholesterol-supplemented WTD. We did not observe any differences caused by the diet or the genotype on cartilage degeneration nor ectopic bone formation at end-stage disease.

Conclusion

This study shows that PCSK9 deficiency reduces synovial inflammation in WTD-fed mice during collagenase-induced OA, but this is not sufficient to affect end stage pathology.

INTRODUCTION

Osteoarthritis (OA) is the most common joint disease worldwide and is characterized by joint pain and stiffness. Currently, no disease-modifying treatments are available and treatment is mostly focused on relieving symptoms and prevention of the disease.. OA is a highly heterogeneous disease and several risk factors are associated with disease development which include ageing, obesity, injury and metabolic syndrome (MetS)[1-3]. MetS is characterised by multiple metabolic phenotypes, including obesity, high glucose levels, hypertension and dyslipidaemia. Dyslipidaemia has been described as a separate risk factor for OA development[3-5]. Studies have shown that mice fed a cholesterol-supplemented diet showed increased spontaneous OA development compared to chow-fed controls[6]. Others showed that high cholesterol levels increased joint destruction in several induced OA animal models as well[7, 8]. In previous studies in our lab we have observed that increased systemic cholesterol levels exacerbates synovial activation and ectopic bone formation during experimental collagenase-induced OA[9-11]. Hence, cholesterol-lowering could be beneficial for a subgroup of OA patients with metabolic disease.

Statins are a class of cholesterol-lowering drugs that are frequently prescribed to lower systemic cholesterol levels. Use of statins was associated with a reduced incidence and progression of OA[12, 13]. Not all studies, however, demonstrated a protective effect of statin use on OA development and further investigation into the role of cholesterol-lowering drugs in OA is warranted[14, 15]. Recently, monoclonal antibodies against proprotein convertase subtilisin/kexin 9 (PCSK9), one of the key proteins involved in cholesterol homeostasis, were developed. PCSK9 targets the low-density lipoprotein receptor (LDLR) for lysosomal degradation and thereby decreases recycling of the LDLR back to the membrane. Reduced LDLR expression leads to a reduced uptake of cholesterol from the circulation by hepatocytes which subsequently results in increased levels of systemic cholesterol levels. Pharmacological inhibition of PCSK9 can reduce systemic cholesterol levels up to 60% in both humans and mice[16, 17]. The PCSK9 antibody alirocumab was shown to further reduce cardiovascular events in patients with cardiovascular disease and hypercholesterolemia on top of statin treatment[18-20]. Moreover, statin therapy combined with PCSK9 inhibition resulted in stabilization and regression of atherosclerotic plaques in both humans and mice[21, 22]. The use of combined cholesterol-lowering therapies as a treatment for OA could be more efficient compared to statin therapy only, as was shown in cardiovascular disease.

Pcsk9$^{-/-}$ mice have reduced systemic cholesterol levels and can be used to study the effects of diet and cholesterol on disease development[23]. In this study, *Pcsk9*$^{-/-}$ mice were fed either regular chow or a cholesterol-supplemented Western-type diet (WTD) to study the effects of PCSK9 deficiency in post-traumatic OA development. We determined the effects of dietary cholesterol and PCSK9 deficiency on cartilage degradation, osteophyte formation and synovial inflammation.

MATERIALS AND METHODS

Animals and experimental OA model

Pcsk9$^{-/-}$ (JAX 005993) mice[23] and B6129SF2/J control mice (JAX #101045) were obtained from the Jackson Laboratory. Mice were backcrossed with B6129SF2/J mice and wild type (WT) littermates were used as a control. Mice were housed with 6 animals in regular cages and received food and water *ad libitum*. Male and female mice (n=15 mice per group) were switched to a WTD (15% cacao butter, 40.5% sucrose, +1% cholesterol) or remained on regular maintenance chow at eight and nine weeks old. Mice were weighed regularly to monitor response to the diet. Four weeks after the start of the diet, experimental OA was induced via intra-articular injections of bacterial collagenase (1 unit) into the right knee joint on day 0 and day 2 of the experiment. On day 7 and day 42, mice were sacrificed (**Figure 1A**). Due to the high number of dislocations in the male mice we focused on the results in female mice in this chapter. Knee joints were collected for histological analysis and synovial explants were collected to produce washouts. Serum samples were collected for analysis of cholesterol and triglyceride levels and S100A8/A9 protein measurements. Bone marrow (BM) and blood were used for flow cytometry analysis. All animal studies were approved by the local ethics committees of the Radboud university medical center (Nijmegen, the Netherlands) and were performed according to the related codes of practice.

Histological processing and analysis

Murine knee joints were fixed in 4% paraformaldehyde and decalcified using formic acid. Subsequently, joints were embedded in paraffin and cut in 7µm coronal sections. Sections were stained using Safranin-O/Fast Green (SafO) or Haematoxylin/Eosin (H&E) for histological analysis. Cartilage damage was quantified in SafO stained sections using a more detailed version of the OARSI scoring system adapted for mice, as described previously (0 = no damage, 30 = maximal damage)[24, 25]. Five sections taken from different depths within the joint were scored and averaged per joint in a blinded fashion. Ectopic bone formation and maturation were scored at multiple locations in both the medial and the lateral side of the joint[26], the locations scored are represented in **Supplementary Figure 6A**. Osteophyte size was determined in three locations with a high prevalence of ectopic bone formation in all groups. Ectopic bone formation was quantified using the Leica Application Suite image analysis software (Leica Microsystems); three sections per joint were averaged[26]. Synovial inflammation was scored arbitrarily using H&E stained sections and averaged for three sections per joint with a scoring range from 0-3 (0 = no inflammation, 1 = mild inflammation, 2 = moderate inflammation; 3 = severe inflammation). All histological analyses were performed in a blinded fashion.

Flow cytometry analysis

Blood was collected in PBS containing 5 mM EDTA and kept at 4 ˚C. Erythrocytes were lysed using red blood cell lysis buffer (155 mM NH4Cl / 12 mM KHCO3 / 0.1 mM EDTA pH 7.3) at 4˚C before antibody staining. For flow cytometry analysis of bone marrow cells, the left femurs of the mice were crushed and cells were passed through a cell strainer and centrifuged. After red blood cell lysis, cells were washed

and resuspended in FACS buffer (PBS containing 0.5% BSA and 2 mM EDTA). For flow cytometry analysis, the following antibodies were used. CD3-fluorescein isothiocyanate (FITC) (1:200, clone: RA3-6B2, Biolegend), B220-FITC (1:200, clone: 145-2C11, Biolegend), CD49b-FITC (1:100, clone: DX5, Biolegend), Ter119-FITC (1:400, clone: TER-119, Biolegend), CD45-phycoerythrin (PE) (1:200, clone: 30-F11, Biolegend) CD11b-Allophycocyanin (APC) (1:800, clone: M1/70, Biolegend), Ly6G-AlexaFluor700 (1:400, clone 1A8, Biolegend), Ly6C-Brilliant Violet 421 (1:200, clone: HK1.4, Biolegend) and Fixable Viability Dye eFluor780 (1:10.000, eBioscience). Cells were incubated with Fc-blocking antibody at 4˚C (BD Pharmingen anti-mouse CD16/CD3, clone: 2.4G2, BD Biosciences), washed and stained with antibody mixtures for 30 minutes at 4˚C. Cells were washed with PBS before staining with viability dye. Cells were fixed in 1% paraformaldehyde before flow cytometry measurements. Data was acquired using the Gallios flow cytometer (Beckman Coulter Life Sciences) and analysed using Kaluza Analysis Software (Beckman Coulter Life Sciences). Classical monocytes were defined as $CD11b^{high}$ (B220/CD3/CD49b/Ter119) $^{neg}Ly6G^{low}Ly6C^{pos}$ and further subdivided into pro-inflammatory $Ly6C^{high}$ and anti-inflammatory $Ly6C^{low}$ monocytes. Fluorescence minus one and single stains were used as staining controls.

Synovial wash-outs and S100A8/A9 measurements

Synovium was collected from knee joints seven days after the first injection of intra-articular collagenase. Synovium was placed in RPMI medium supplemented with penicillin-streptomycin and 0.1% bovine serum albumin (BSA) for approximately 1 hour at room temperature and weighed afterwards to correct the protein concentrations for weight of the synovial explant. S100A8/A9 complexes were measured in the wash-outs or serum of mice using sandwich enzyme-linked immunosorbent assay (ELISA) as described previously[27].

Quantitative real-time polymerase chain reaction

Blood and bone marrow cells were collected for RNA analysis. Gene expression levels were determined using qPCR with specific primers and the SYBR Green Master Mix using the StepOnePlus RT-PCR System (Thermo Fisher Scientific). Expression levels are presented as $-\Delta Ct$, which is calculated by correcting for the household gene GAPDH. The following primer sequences were used: *mGapdh:* gcaaattcaacggcaca (forward), gttagtggggtctcgctcctg (reverse); *mS100a8:* tgtcctcagtttgtgcagaatataaat (forward), tttatcaccatcgcaaggaactc (reverse); *mS100a9:* ggcaaaggctgtgggaagt (forward), ccattgagtaagccattccctttta (reverse); *mTnfa:* cagaccctcacactcagatcatct (forward), cctccacttggtggtttgcta (reverse); *mIl-1β*: ggacagaatatcaaccaacaagtgata (forward), gtgtgccgtctttcattacacag (reverse); *mIl-10*: atttgaattccctgggtgagaa (forward), acaccttggtcttggagcttattaa (reverse); *mMcp-1*: ttggctcagccagatgca (forward), cctactcattgggatcatcttgct (reverse).

Statistical analysis

Statistical differences between groups were analysed using two-way analysis of variance (ANOVA) including a test for an interaction between the diet and the genotype of the mice. All statistical analyses

were performed using GraphPad Prism 9 (GraphPad software). *P*-values below 0.05 were considered significant. Data are depicted as mean ± 95% confidence interval (CI).

RESULTS

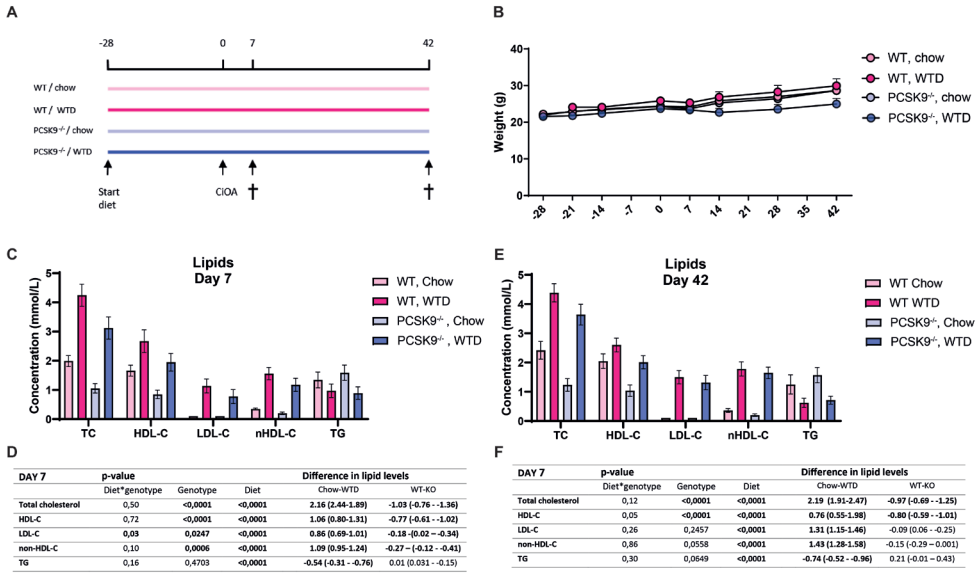
PCSK9 deficiency reduces systemic cholesterol levels in chow and WTD-fed mice

First, we determined the effect of PCSK9 deficiency on systemic cholesterol levels in both chow and WTD-fed mice. 5 weeks of WTD feeding significantly increased systemic cholesterol levels in both WT and *Pcsk9*$^{-/-}$ mice (TC: 53.1%, 66.3% increase, respectively), while triglyceride (TG) levels were decreased (-38.4%; -78.2%, respectively) (**Figure 1C,D**). 5 weeks after the start of the diet (D7 of CiOA), we observed a reduction in total cholesterol (TC) in *Pcsk9*$^{-/-}$ mice compared to WT mice on both a chow and a cholesterol-supplemented WTD. We observed that LDL-C levels were reduced in WTD-fed mice (chow TC; -47.8%, LDL-C: undetectable; WTD: TC: -26.4%, LDL-C:-31.7%, respectively) (**Figure 1C**). 10 weeks after the start of the diet (D42 of CiOA), total cholesterol levels were reduced in *Pcsk9*$^{-/-}$ mice compared to WT mice, although the difference between both genotypes was less pronounced compared to day 7 (Chow: TC: -49.0%; WTD: TC: -17.0%) (**Figure 1E, F**). Whereas LDL-C could not be detected in the chow-fed mice, levels did not differ significantly between both genotypes in the WTD-fed mice (WTD: LDL-C: -12.2%). Weight gain was monitored regularly throughout the study. All mice gained weight during the study, it was observed that WTD-fed *Pcsk9*$^{-/-}$ mice gained less weight compared to the other groups in the study (**Figure 1B**).

PCSK9 deficient mice show increased Ly6C^{high} monocytes in bone marrow and decreased ly6C^{low} monocytes in the circulation

As it was shown that high cholesterol levels can lead to monocytosis, we investigated the effect of WTD feeding and PCSK9 deficiency on different subsets of monocytes in the bone marrow and blood. Flow cytometry analysis showed that WTD feeding did not significantly alter various monocyte subsets in bone marrow as compared to a chow diet. In the blood, we did observe a significant increase in Ly6C^{high} monocytes of mice fed a cholesterol-supplemented WTD compared to chow fed controls (WT: 25.6%; *Pcsk9*$^{-/-}$: 49.6%) (**Figure 2A-F**). *Pcsk9*$^{-/-}$ mice showed a significant increase in total monocytes and pro-inflammatory Ly6C^{high} monocytes in bone marrow compared to WT mice (Chow: 19.2%; WTD: 16.9%, $P = 0.01$), while no differences were observed for Ly6C^{low} monocytes. The total amount of monocytes in the blood was increased in *Pcsk9*$^{-/-}$ mice compared to WT controls (Chow: 11.8%; WTD: 66.7%, $P = 0.02$). In contrast to the observations in the bone marrow, we found a significant increase in the more anti-inflammatory Ly6C^{low} monocytes in blood of *Pcsk9*$^{-/-}$ mice compared to WT mice (Chow: 15.5%; WTD: 44.6%, $P = 0.006$) (**Figure 2A-F**). We additionally determined gene expression of several inflammatory mediators in bone marrow cells isolated from femurs. Here, we detected a significant interaction between the genotype and the diet for S100A8 ($P = 0.0008$) and IL-1β ($P= 0.0001$) gene expression levels. WTD feeding resulted in an increase of S100A8 and IL-1β gene expression in WT mice. In contrast,

gene expression of S100A8 and IL-1β were decreased in *Pcsk9*$^{-/-}$ mice. WTD feeding decreased gene expression of IL-10 while PCSK9 deficient mice showed an increase of expression levels of TNFα and MCP-1 and decreased expression of IL-10 (**Figure 3A-F**).



D

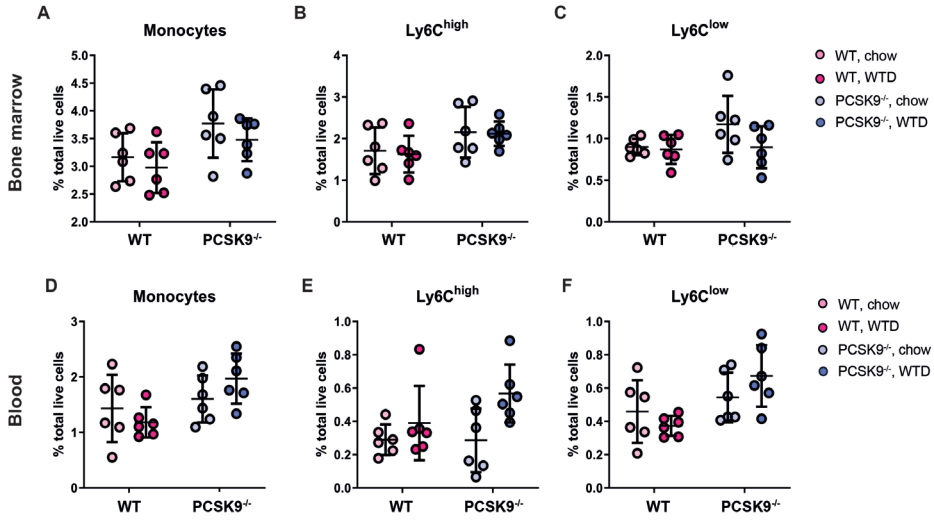
DAY 7	p-value			Difference in lipid levels	
	Diet*genotype	Genotype	Diet	Chow-WTD	WT-KO
Total cholesterol	0,50	**<0,0001**	**<0,0001**	**2.16 (2.44-1.89)**	**-1.03 (-0.76 - -1.36)**
HDL-C	0,72	**<0,0001**	**<0,0001**	**1.06 (0.80-1.31)**	**-0.77 (-0.61 - -1.02)**
LDL-C	**0,03**	**0,0247**	**<0,0001**	**0.86 (0.69-1.01)**	**-0.18 -(0.02 – -0.34)**
non-HDL-C	0,10	**0,0006**	**<0,0001**	**1.09 (0.95-1.24)**	**-0.27 – (-0.12 - -0.41)**
TG	0,16	0,4703	**<0,0001**	**-0.54 (-0.31 - -0.76)**	0.01 (0.031 - -0.15)

F

DAY 7	p-value			Difference in lipid levels	
	Diet*genotype	Genotype	Diet	Chow-WTD	WT-KO
Total cholesterol	0,12	**<0,0001**	**<0,0001**	**2.19 (1.91-2.47)**	**-0.97 (-0.69 - -1.25)**
HDL-C	0,05	**<0,0001**	**<0,0001**	**0.76 (0.55-1.98)**	**-0.80 (-0.59 - -1.01)**
LDL-C	0,26	0,2457	**<0,0001**	**1.31 (1.15-1.46)**	-0.09 (0.06 - -0.25)
non-HDL-C	0,86	0,0558	**<0,0001**	**1.43 (1.28-1.58)**	-0.15 (-0.29 – 0.001)
TG	0,30	0,0649	**<0,0001**	**-0.74 (-0.52 - -0.96)**	0.21 (-0.01 – 0.43)

Figure 1. PCSK9 deficiency reduces serum lipid levels in mice fed either a chow or cholesterol-supplemented WTD.

PCSK9$^{-/-}$ and WT mice were fed either a regular chow diet or a cholesterol-supplemented WTD. 4 weeks after the start of the diet, CiOA was induced via intra-articular injections in the right knee joint. Mice were sacrificed 7 or 42 days after the induction of CiOA. **(A)** Schematic overview of the study and the experimental groups. **(B)** Weight of the mice was monitored throughout the study. No significant differences were observed between all groups. **(C, D)** Serum lipid levels were determined 7 days after the induction of CiOA. WTD feeding significantly increased systemic cholesterol levels in both genotypes, while triglyceride levels were decreased. PCSK9 deficiency resulted in reduced lipid levels in both chow and WTD-fed mice compared to WT mice. **(E, F)** Serum lipid levels were determined 42 days after the induction of CiOA. WTD feeding significantly increased systemic lipid levels in both genotypes. PCSK9 deficiency resulted in a significant reduction of total cholesterol and HDL-C levels, LDL-C levels were not significantly reduced compared to WT mice.



G

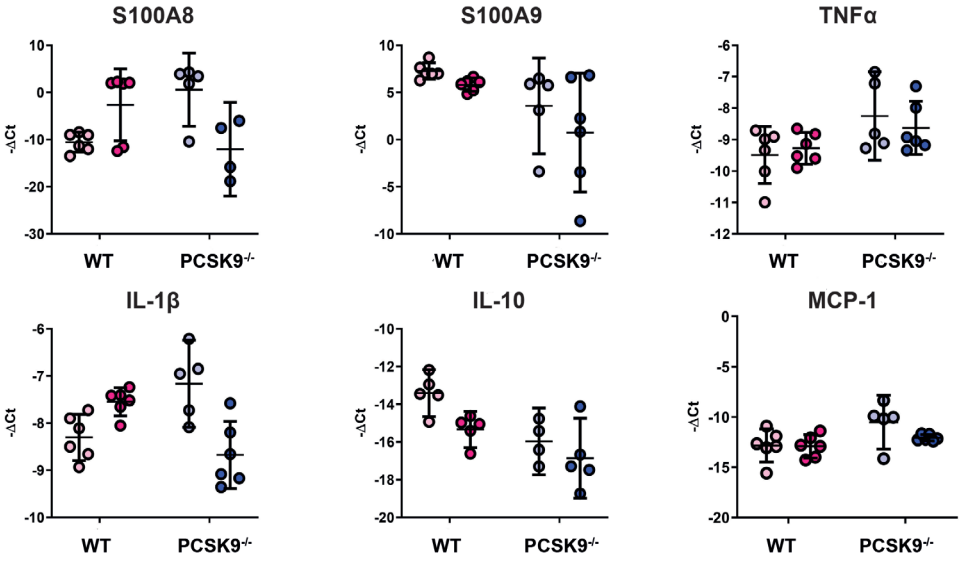
Bone marrow	p-value			Difference in % of live cells	
	Diet*genotype	Genotype	Diet	Chow-WTD	WT-KO
Monocytes	0.79	**0.01**	0.23	-0.24 (-0.065 – 0.64)	**0.055 (0.015-0.096)**
Ly6C^{high}	0.91	**0.03**	0.77	-0.06 (-0.48 - 0.36)	**0.47 (0.25-0.89)**
Ly6C^{low}	0.21	0.14	0.13	-0.15 (-0.35 -0.05)	0.15 (-0.05 – 0.35)

H

Blood	p-value			Difference in % of live cells	
	Diet*genotype	Genotype	Diet	Chow-WTD	WT-KO
Monocytes	0.11	**0.02**	0.76	0.06 (-0.33 – 0.44)	**0.48 (0.09-0.86)**
Ly6C^{high}	0.22	0.25	**0.02**	**0.19 (0.04-0.34)**	0.09 (-0.06 – 0.24)
Ly6C^{low}	0.10	**0.01**	0.73	0.02 (-0.11 – 0.15)	**0.19 (0.06-0.32**

Figure 2. PCSK9 deficiency and WTD feeding alter systemic monocyte subsets in blood and bone marrow.

PCSK9$^{-/-}$ and WT mice were fed either a regular chow diet or a cholesterol-supplemented WTD. 4 weeks after the start of the diet, CiOA was induced via intra-articular injections in the knee joint. Mice were sacrificed 7 days after the induction of CiOA and bone marrow and blood were collected for flow cytometry analysis. **(A)** PCSK9 deficiency increases total monocytes in bone marrow. **(B)** PCSK9 deficiency significantly increases Ly6C^{high} cells in bone marrow, while **(C)** Ly6C^{low} cells were not affected by PCSK9 deficiency or WTD feeding. **(D)** PCSK9 deficiency increases monocytes in blood compared to WT mice. **(E)** WTD-feeding deficiency significantly increases circulating Ly6C^{high} cells in blood, while **(F)** Ly6C^{low} cells are significanty decreased in PCSK9 deficient mice. **(G)** Two-way anova results of monocyte subsets in bone marrow. **(F)** Two-way anova results of monocyte subsets in blood. *Results are expressed as individual data points with mean ± 95% confidence intervals.*



Gene expression	p-value		
	Diet*genotype	Genotype	Diet
S100A8	**0.0008**	0.73	0.36
S100A9	0.66	**0.01**	0.18
TNFα	0.41	**0.01**	0.82
IL-1β	**0.0001**	0.98	0.13
IL-10	0.36	**0.002**	**0.02**
MCP-1	0.22	**0.01**	0.18

Figure 3. PCSK9 deficiency and WTD feeding alter gene expression of inflammatory cytokines in bone marrow of mice.

PCSK9$^{-/-}$ and WT mice were fed either a regular chow diet or a cholesterol-supplemented WTD. 4 weeks after the start of the diet, CiOA was induced via intra-articular injections in the knee joint. Bone marrow was isolated from femur 7 days after the induction of CiOA. (A) A significant interaction between diet and genotype was observed for expression of S100A8 in bone marrow cells. (B) PCSK9 deficient mice showed a significantly reduced expression of S100A9 in bone marrow cells. (C) PCSK9 deficient mice showed a significant increase in expression levels of TNFα in bone marrow cells. (D) A significant interaction between diet and genotype was observed for expression of IL-1β in bone marrow cells. (E) Both WTD feeding and PCSK9 deficiency significantly decreased gene expression of IL-10. (F) PCSK9 deficient mice showed a significant increase in expression levels of MCP-1 in bone marrow cells. *Results are expressed as individual data points with mean ± 95% confidence intervals.*

PCSK9$^{-/-}$ mice show reduced synovial inflammation when fed a cholesterol-supplemented WTD

We next determined if the inflammatory state of the synovium was altered by WTD feeding or PCSK9 deficiency 7 days after the induction of CiOA. Here, we observed a significant interaction between the genotype and the diet ($P = 0.03$) (**Figure 4A, D**). Synovial inflammation was reduced in PCSK9 deficient mice compared to WT mice when fed a WTD. Unexpectedly, we did not observe similar effects in mice fed a regular chow diet (Chow: +1.8% WTD: -53.7%, $P = 0.04$). To investigate the inflammatory state of the synovium in more detail we determined protein levels of the alarmin S100A8/A9, a marker for activated macrophages, in washouts of synovial explants. Here, we did not observe an effect of both the diet or PCSK9 deficiency on the amount of S100A8/A9 protein produced by synovial tissue (**Figure 4B**). Additionally, we measured S100A8/A9 levels in serum of mice, where we found a significant reduction of S100A8/A9 protein levels as a result of both the WTD ($P = 0.05$) and PCSK9 deficiency ($P = 0.04$). No statistically significant interaction was observed between the diet and genotype ($P = 0.06$) (**Figure 4C**).

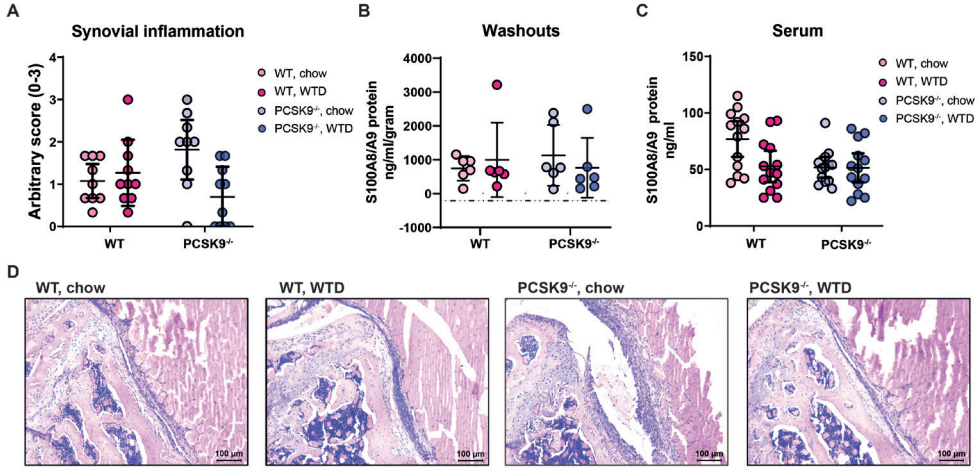
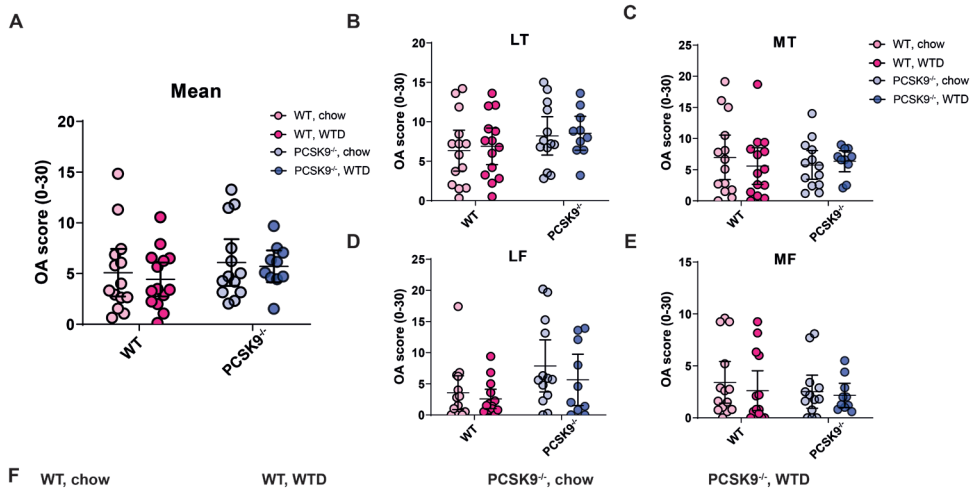


Figure 4. PCSK9$^{-/-}$ mice fed a cholesterol-supplemented WTD show decreased synovial inflammation.

PCSK9$^{-/-}$ and WT mice were fed either a regular chow diet or a cholesterol-supplemented WTD. 4 weeks after the start of the diet, CiOA was induced via intra-articular injections in the knee joint. Mice were sacrificed 7 days after the induction of CiOA. **(A)** A significant interaction between diet and genotype was observed in synovial inflammation, showing a reduction in synovial inflammation in PCSK9$^{-/-}$ mice fed a cholesterol-supplemented WTD. **(B)** Protein levels of the alarmin S100A8/A9 were determined in washouts of synovial explants. No significant differences were observed between all groups. **(C)** Protein levels of S100A8/A9 were determined in serum of mice. We observed a significant reduction in serum levels by both WTD-feeding and PCSK9 deficiency, while no significant interaction was observed. **(D)** Representative pictures of synovial inflammation in H&E stained sections. *Results are expressed as individual data points with mean ± 95% confidence intervals.*



Cartilage damage	p-value		
	Diet*genotype	Genotype	Diet
Mean	0.88	0.24	0.59
Lateral tibia	0.93	0.13	0.69
Lateral femur	0.66	**0.02**	0.27
Medial tibia	0.46	0.87	0.77
Medial femur	0.79	0.42	0.49

Figure 5. WTD-feeding and PCSK9 deficiency do not affect cartilage damage 42 days after the induction of collagenase-induced OA.

PCSK9$^{-/-}$ and WT mice were fed either a regular chow diet or a cholesterol-supplemented WTD. 4 weeks after the start of the diet, CiOA was induced via intra-articular injections in the knee joint. Mice were sacrificed 42 days after the induction of CiOA. **(A)** OA pathology was determined in SafO stained sections. WTD feeding or PCSK9 deficiency did not result in statistical differences in OA pathology. **(B-D)** OA scores are shown for each cartilage surface scored. **(F)** Representative pictures of cartilage damage 42 days after the induction of CiOA on the medial side of the joint. *LT= lateral tibia, LF= lateral femur, MT= medial tibia, MF= medial femur. Results are expressed as individual data points with mean ± 95% confidence intervals.*

PCSK9 deficiency does not reduce end stage OA pathology in chow and WTD-fed mice

We next determined if WTD feeding or PCSK9 deficiency affected OA pathology at end stage CiOA. We determined cartilage degeneration at end point in SafO stained sections. No effects of the WTD was observed in both WT and *Pcsk9*$^{-/-}$ mice compared to chow controls. Also PCSK9 deficiency did not affect cartilage degeneration in neither chow- nor WTD-fed mice (**Figure 5A-E**). Additionally, we determined the effect of WTD feeding and PCSK9 deficiency on ectopic bone. Ectopic bone formation was determined throughout the joint, no differences were observed in the prevalence of ectopic

bone formation caused by either the diet or genotype of the mice (**Figure 6**). We next determined the maturation stage and the size of ectopic bone formation in locations where we observed a high prevalence of ectopic bone formation. The size of ectopic bone formation was scored at three different locations in the joint. Here, we did not observe changes in size of ectopic bone caused by both WTD feeding or PCSK9 deficiency (**Figure 7A-C**). Osteophyte maturation remained similar between groups at (**Figure 8**).

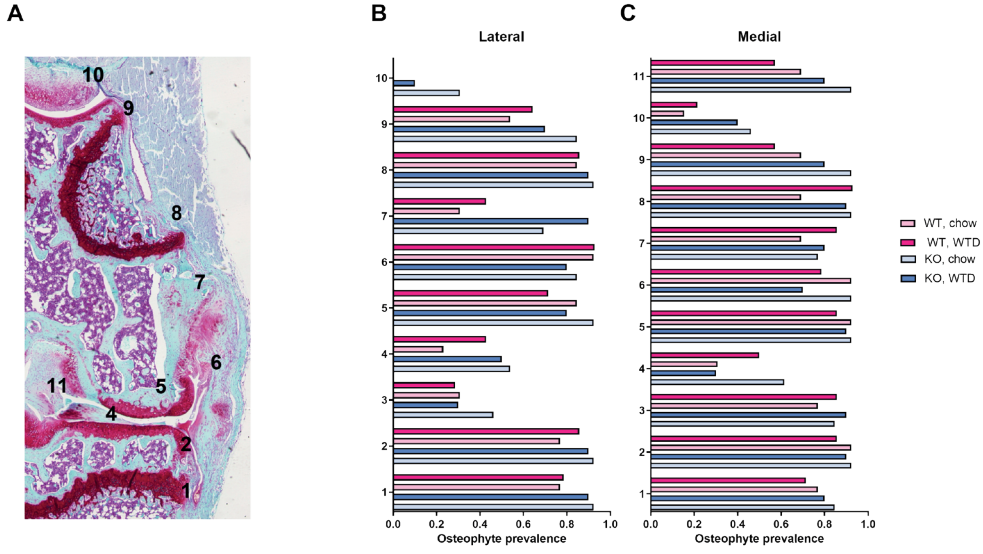
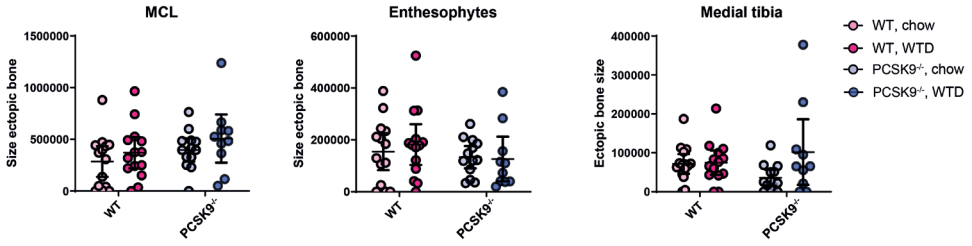


Figure 6. Incidence of ectopic bone formation at several locations in the joint.

PCSK9$^{-/-}$ and WT mice were fed either a regular chow diet or a cholesterol-supplemented WTD. 4 weeks after the start of the diet, CiOA was induced via intra-articular injections in the knee joint. Mice were sacrificed 42 days after the induction of CiOA. **(A)** Ectopic bone formation was determined at several locations in the joint on both the lateral and the medial side as described previously[120]. **(B,C)** We observed a high prevelance of ectopic bone formation in all groups on both the medial and lateral side of the joint.



Ectopic bone	p-value		
	Diet*genotype	Genotype	Diet
MCL	0.87	0.09	0.19
Enthesophyte	0.59	0.25	0.19
Medial tibia	0.09	0.82	0.07

Figure 7. WTD-feeding and PCSK9 deficiency do not affect affect ectopic bone formation at end-stage collagenase-induced OA

PCSK9$^{-/-}$ and WT mice were fed either a regular chow diet or a cholesterol-supplemented WTD. 4 weeks after the start of the diet, CiOA was induced via intra-articular injections in the knee joint. Mice were sacrificed 42 days after the induction of CiOA. Ectopic bone formation was determined by tracing the ectopic bone margins. PCSK9 deficiency or WTD feeding did not affect the size of ectopic bone formation at the **(A)** MCL, **(B)** enthesophytes or the joint margin of the **(C)** medial tibia. *Results are expressed as individual data points with mean ± 95% confidence intervals.*

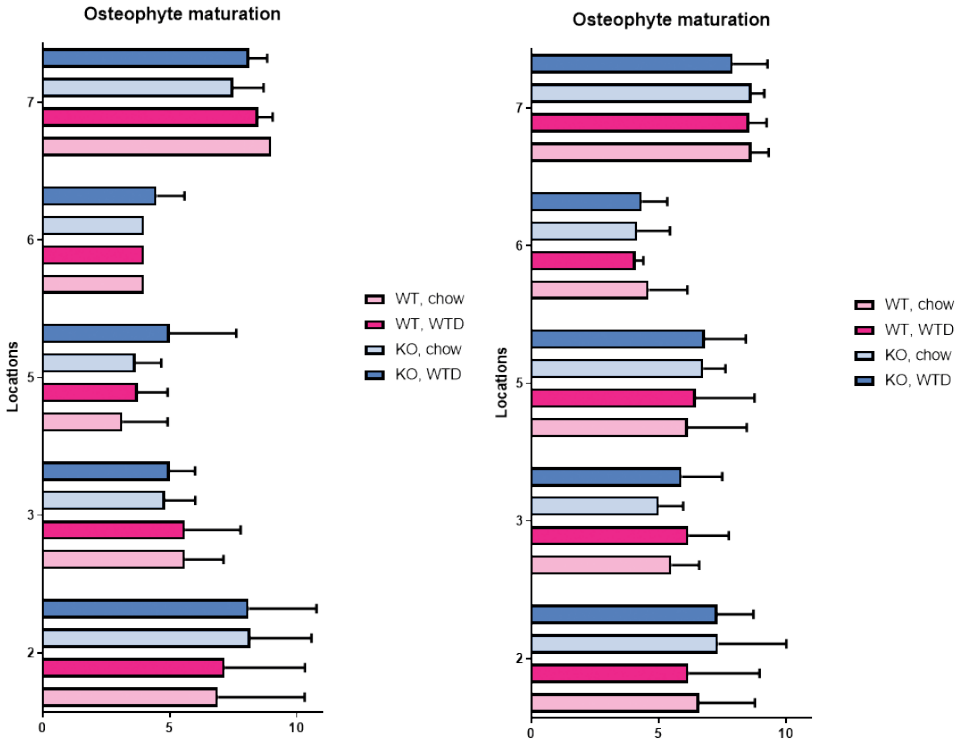


Figure 8. No difference in the maturation stage of ectopic bone formation at several locations in the joint.

PCSK9$^{-/-}$ and WT mice were fed either a regular chow diet or a cholesterol-supplemented WTD. 4 weeks after the start of the diet, CiOA was induced via intra-articular injections in the knee joint. Mice were sacrificed 42 days after the induction of CiOA. Osteophyte maturation was determined at several joint locations on the medial and lateral side of the joint. No differences were observed between groups at several locations in the joint.

DISCUSSION

Although a clear link between metabolic syndrome and OA development has been established, the exact role of cholesterol and cholesterol-lowering treatments on OA development remains to be elucidated. Statins are often prescribed to reduce systemic cholesterol levels, but the effects of statins on OA development have remained inconclusive. Recently, novel cholesterol-lowering drugs that target PCSK9, one of the key proteins in cholesterol homeostasis, have been developed. *Pcsk9*$^{-/-}$ mice have reduced plasma LDL-C levels via an increased hepatic LDLR expression, resulting in increased clearance of LDL-C[23]. In this study, we show that synovial inflammation was reduced in WTD-fed *Pcsk9*$^{-/-}$ mice. However PCSK9 deficiency in combination with WTD feeding did not result in differences in pathology at end-stage collagenase-induced OA.

High cholesterol levels have been associated with increased incidence and progression of OA development[3-5]. Hence, it was thought that cholesterol-lowering therapies could be a possible treatment for diet-induced OA development. Statins are often prescribed to reduce systemic cholesterol levels and several clinical studies have shown that statins reduced the progression of knee OA and reduced pain in OA patients[12, 13]. Also in animal models, multiple studies showed that statin treatment could ameliorate diet-induced OA pathology[6, 7]. However, others have reported results that contradict these findings and observed no beneficial effects of statins in both clinical and pre-clinical studies[14, 15, 28]. The antibodies alirocumab and evolocumab are newly developed cholesterol-lowering drugs which target PCSK9, one of the key proteins involved in cholesterol-homeostasis. PCSK9 targets the LDL-receptor for lysosomal degradation, thereby decreasing its membrane expression and the ability to clear cholesterol from the circulation. Additionally, PCSK9 was shown to promote degradation of several other receptors involved in lipid uptake, such as the very low-density lipoprotein receptor (VLDLR), LDLR-related protein-1 (LRP-1), ApoER, and CD36[29-31]. PCSK9 antibodies have been shown to be very effective cholesterol-lowering drugs in both humans and mice and reduce LDL-C levels up to 50-60%[16, 17, 22]. The PCSK9 antibody alirocumab was shown to further reduce cardiovascular events in patients with cardiovascular disease on top of statin treatment[18-20]. Moreover, statin therapy combined with PCSK9 inhibition resulted in stabilization and regression of atherosclerotic plaques in mice[21, 22]. The use of combined cholesterol-lowering therapies as a treatment for OA could be more efficient compared to statin therapy only, as was shown in cardiovascular disease.

Pcsk9$^{-/-}$ mice have reduced plasma LDL-C levels via an increased hepatic LDLR expression and have mainly been used to study the effects of PCSK9 deficiency on atherosclerosis development, as high cholesterol is one of the main risk factors for the development of cardiovascular disease. *Pcsk9*$^{-/-}$ mice fed a WTD were shown to be protected from atherosclerosis development via a reduction of systemic LDL-C[32]. Absence or overexpression of PCSK9 in mice with an LDLR-deficient background showed no effects on systemic cholesterol levels and atherosclerosis development, confirming that the cholesterol-lowering effect is indeed mainly LDLR-dependent[32]. In our study, PCSK9 deficiency resulted in reduced systemic

cholesterol levels in mice fed a chow or cholesterol-supplemented WTD. The cholesterol-lowering effect of PCSK9 deficiency was most effective in mice fed a chow diet compared to WTD-fed mice. In addition, we observed that the cholesterol lowering effect of *Pcsk9*$^{-/-}$ mice was reduced after prolonged WTD feeding, specifically for LDL. Similar to females, male mice fed a chow diet showed a more efficient cholesterol-lowering effect of PCSK9 deficiency compared to WTD-fed mice (**Figure 9**). Some studies have observed a similar decrease in cholesterol-lowering efficacy in WTD-fed mice *Pcsk9*$^{-/-}$ mice[33-35], while others did not confirm our findings[32, 36, 37]. Possibly, other pathways involved in cholesterol metabolism could become more important in *Pcsk9*$^{-/-}$ mice that are metabolically challenged, resulting in a lower cholesterol-lowering capacity compared to mice fed a chow diet. For instance, other pathways that regulate LDLR expression, such as the inducible degrader of LDLR (IDOL)[38], or pathways involved in cholesterol efflux could become more active and act as a compensatory mechanism in mice lacking the PCSK9 gene. In addition, studies have shown that the composition of the diet could drastically alter the effects on circulating lipids. Desmarcheller *et al*. observed that mice which were fed a cholesterol-supplemented WTD exhibit major adaptive changes in cholesterol and phospholipid metabolism in response to the increased cholesterol levels[39]. Use of PCSK9 inhibitors such as the antibody alirocumab or the small molecule inhibitor Inclisiran were shown to be very effective cholesterol-lowering drugs and should overcome these problems in future studies[17, 19, 22, 40-42].

The lipoprotein metabolism of mice differs compared to human lipoprotein metabolism. Mice have high HDL levels and lower (V)LDL levels because they lack cholesteryl ester transfer protein (CETP) which transports cholesterol esters from HDL to VLDL and LDL particles in exchange for triglycerides. This can explain why we mainly observed differences in total cholesterol levels instead of LDL-C in our study, as most of the cholesterol in mice is transported in HDL particles due to the absence of the CETP gene. Therefore, high dietary cholesterol is needed to increase LDL and VLDL particles in mice with a WT background. This could possibly explain why we observed little effect of the WTD on OA development in the current study. Using mice with a more translatable lipoprotein metabolism, such as ApoE3*Leiden.CETP mice, would increase the translatability of cholesterol-associated research on OA development[43]. ApoE*3Leiden and ApoE*3Leiden.CETP transgenic mice have a more humanized lipoprotein metabolism and they respond to lipid-lowering drugs such as statins or PCSK9-inhibitors in a human-like manner. Moreover, studies using PCSK9 inhibitors such as the monoclonal antibody alirocumab or the small molecule inhibitor Inclisiran are shown to be effective cholesterol-lowering strategies in mice and are more suitable for future studies aimed to investigate the effect of PCSK9 inhibition on OA development[17, 40, 42].

In contrast to previous experiments performed in dyslipidemic *Apoe*$^{-/-}$ *and Ldlr*$^{-/-}$ mice, a cholesterol-supplemented WTD did not increase OA pathology during experimental collagenase-induced OA in WT or *Pcsk9*$^{-/-}$ mice[9, 11]. Difference in the duration or the composition of the diet possibly explain these discrepancies. In previous experiments, mice were fed a cholesterol-supplemented WTD for 12 weeks before the induction of CiOA[9], while in the current study mice were fed a WTD for 4 weeks before the

induction of CiOA. However, in another study, using a cholesterol-rich diet increased OA pathology in both WT and *Apoe*$^{-/-}$ after only 2 weeks of WTD feeding[11]. *Apoe*$^{-/-}$ *and Ldlr*$^{-/-}$ mice have much higher systemic cholesterol levels compared to WT mice when fed a (cholesterol-supplemented) WTD. Although in these previous studies mouse models were used that develop supraphysiological cholesterol levels, increased OA pathology such as ectopic bone formation and synovial activation was also observed in WT mice fed a cholesterol-supplemented WTD.

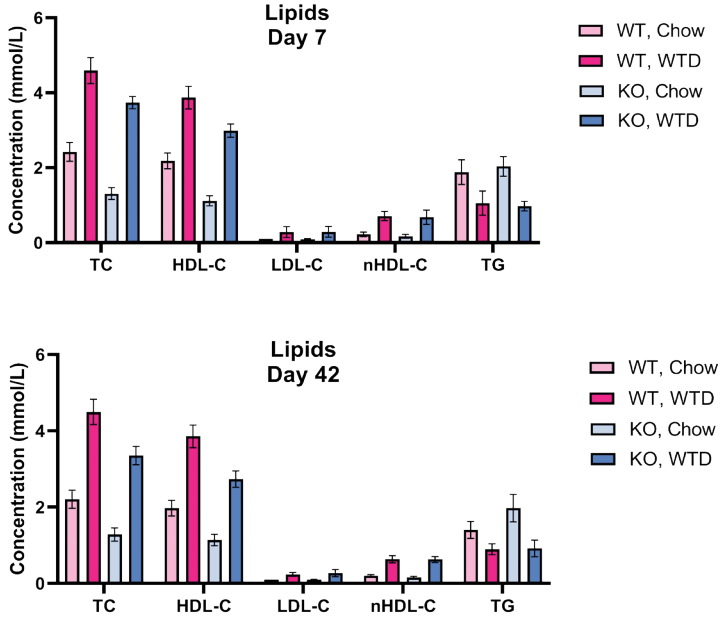


Figure 9. Lipid profile of male PCSK9$^{-/-}$ and WT mice

PCSK9$^{-/-}$ and WT mice were fed either a regular chow diet or a cholesterol-supplemented WTD. **(A)** Serum lipid levels were determined 7 days after the induction of CiOA. WTD feeding significantly increased systemic cholesterol levels in both genotypes, while triglyceride levels were decreased. PCSK9 deficiency resulted in a reduction of total cholesterol (TC) levels in both chow and WTD-fed mice compared to WT mice. LDL-C levels were not reduced in PCSK9$^{-/-}$ mice. **(B)** Serum lipid levels were determined 42 days after the induction of CiOA. WTD feeding significantly increased systemic lipid levels in both genotypes. PCSK9 deficiency resulted in a significant reduction of total cholesterol (TC), LDL-C levels were not significantly reduced compared to WT mice.

In this study, we observed an interaction between diet and genotype on synovial inflammation. While synovial inflammation was reduced in WTD-fed *Pcsk9*$^{-/-}$ mice, *Pcsk9*$^{-/-}$ mice fed a regular chow diet had higher levels of synovial inflammation compared to WT mice, indicating that WTD feeding has a different effect on synovial inflammation in *Pcsk9*$^{-/-}$ mice compared to WT mice. Although synovial inflammation was reduced in *Pcsk9*$^{-/-}$ mice fed a cholesterol-supplemented WTD, this difference did not result in differences in end stage pathology such as cartilage damage or ectopic bone formation. In contrast to earlier experiments where CiOA was induced in dyslipidemic mice, WTD feeding by itself did not result in an increase in OA pathology. In several animal studies, a high-fat diet resulted in increased cartilage damage[6, 7, 44]. However, some studies have shown that a WTD increased macrophage infiltration[45] and inflammation in the synovium, while no effects on cartilage damage were observed[45, 46]. The different outcomes of high cholesterol on OA development indicate that other mechanisms are likely involved in MetS-associated OA development as high cholesterol alone seems insufficient to induce joint pathology. Approximately half of OA patients show synovial inflammation[47] and the role of joint inflammation in OA development has become increasingly recognised[47, 48]. In an inflammatory environment LDL is transformed into oxidised LDL (oxLDL) via reactive oxygen species (ROS) which are produced under the influence of inflammatory mediators. We have previously shown that mainly oxLDL, and not LDL, is responsible for the OA pathology associated with high systemic cholesterol in mice[10].

Besides playing a pivotal role in cholesterol homeostasis, PCSK9 is suggested to have a direct role in inflammation[49]. Several studies have shown that PCSK9 has a pro-inflammatory effect on macrophages, possibly via increased activation of the TLR4/NF-κB signalling pathway[42, 49, 50]. Therefore, deletion or inhibition of PCSK9 could have additional beneficial effects via modulation of the inflammatory response. We additionally determined monocyte subsets in bone marrow and blood and determined gene expression levels of inflammatory mediators in bone marrow cells, as it has been described that high cholesterol levels van lead to an increase of circulating monocytes. Consistent with previous experiments, a cholesterol-supplemented WTD increased circulating Ly6C^{high} monocytes in the blood[51]. This confirms that increased levels of cholesterol can induce monocytosis and alter the inflammatory status of mice. Whereas *Pcsk9*$^{-/-}$ mice had increased pro-inflammatory Ly6C^{high} monocytes in bone marrow, in the circulation we observed an increase in the more anti-inflammatory Ly6C^{low} monocytes compared to WT mice. These findings could explain why we observed decreased gene expression of the pro-inflammatory mediators S100A8, S100A9 and IL-1β in *Pcsk9*$^{-/-}$ mice fed a cholesterol-supplemented WTD. However, these changes in monocyte subsets and inflammatory mediators did not result in changes in end-stage pathology during CiOA.

This study has some limitations, such as the use of only female mice for our analyses. We originally performed the experiment in both female and male mice. However, male mice showed a high incidence of dislocations. Mice with dislocations are excluded from the analyses, since these mice develop very severe OA pathology. This resulted in very small groups for the male mice, which does not allow for a decent statistical analysis. We did not observe differences between the groups for both early stage

synovial inflammation and late stage OA pathology such as cartilage damage or ectopic bone formation in male mice (**Figure 10**). Another limitation is the limited cholesterol-lowering efficacy of PCSK9 deficiency in mice fed a cholesterol-supplemented WTD. Although the setup of this study allows us to study the role of the PCSK9 protein during OA development, the main function of PCSK9 is its role in cholesterol metabolism. Pharmacological inhibition of PCSK9 was shown to be very effective in mice fed a cholesterol-supplemented WTD and should overcome this problem in future studies[17,40].

Taken together, our study shows that whereas absence of PCSK9 resulted in a reduction of synovial inflammation when fed a WTD, this did not lead to a reduction in end-stage pathology.

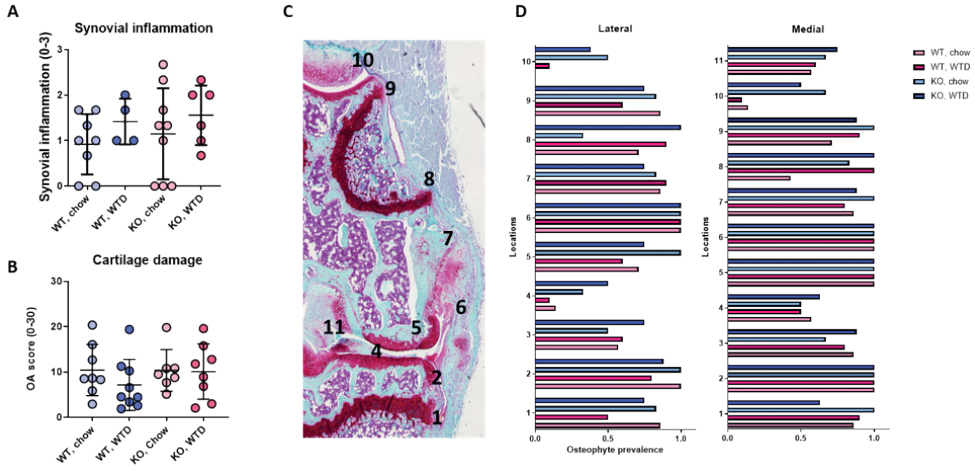


Figure 10. WTD-feeding and PCSK9 deficiency do not affect OA pathology in male mice after the induction of collagenase-induced OA

PCSK9$^{-/-}$ and WT mice were fed either a regular chow diet or a cholesterol-supplemented WTD. 4 weeks after the start of the diet, CiOA was induced via intra-articular injections in the knee joint. Mice were sacrificed 7 or 42 days after the induction of CiOA. **(A)** Synovial inflammation during early stage collagenase-OA did not show any significant differences between groups. **(B)** OA pathology was determined in SafO stained sections. WTD feeding or PCSK9 deficiency did not result in statistical differences in OA pathology. OA scores represent an average of all cartilage surfucases that were scored. **(C)** The locations where ectopic bone formation was determined. **(D)** The prevalence of ectopic bone formation on the different joint locations showed similar results for all groups.

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CHAPTER 3

Novel high-intensive cholesterol-lowering therapies do not ameliorate knee OA development in humanized dyslipidemic mice

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ABSTRACT

Objective

High systemic cholesterol levels have been associated with osteoarthritis (OA) development. Therefore, cholesterol lowering by statins has been suggested as a potential treatment for OA. We investigated whether therapeutic high-intensive cholesterol-lowering attenuated OA development in dyslipidemic APOE*3Leiden.CETP mice.

Methods

Female mice (n=13-16 per group) were fed a Western-type diet (WTD) for 38 weeks. After 13 weeks, mice were divided into a baseline group and 5 groups receiving WTD alone or with treatment: atorvastatin alone, combined with PCSK9 inhibitor alirocumab and/or ANGPTL3 inhibitor evinacumab. Knee joints were analysed for cartilage degradation, synovial inflammation and ectopic bone formation using histology. Aggrecanase activity in articular cartilage and synovial S100A8 expression were determined as markers of cartilage degradation/regeneration and inflammation.

Results

Cartilage degradation and active repair were significantly increased in WTD-fed mice, but cholesterol-lowering strategies did not ameliorate cartilage destruction. This was supported by comparable aggrecanase activity and S100A8 expression in all treatment groups. Ectopic bone formation was comparable between groups and independent of cholesterol levels.

Conclusions

Intensive therapeutic cholesterol lowering per se did not attenuate progression of cartilage degradation in dyslipidemic APOE*3Leiden.CETP mice, with minor joint inflammation. We propose that inflammation is a key feature in the disease and therapeutic cholesterol-lowering strategies may still be promising for OA patients presenting both dyslipidemia and inflammation.

key words: osteoarthritis (OA), cholesterol, mouse model, dyslipidemia, Western-type Diet

INTRODUCTION

Hypercholesterolemia, or increased systemic levels of low-density lipoprotein cholesterol (LDL-C), is a cardiometabolic risk factor associated with cardiovascular disease (CVD) and osteoarthritis (OA)[1, 2]. Although pathophysiological grounds for a causal relationship between OA and CVD have not yet been established in humans, common cardiometabolic risk factors may indicate shared biochemical pathways. Still, epidemiological studies are divided over the relationship between both conditions. A recent meta-analysis found a significantly increased prevalence and risk of overall CVD in OA patients compared to non-OA controls[3], while others did not observe this association[4]. Although some studies show that OA patients have significantly higher levels of low-density lipoprotein cholesterol (LDL-C)[5, 6], the role of an impaired lipid metabolism in OA pathology remains unclear. Increased total cholesterol levels were recently associated with increased risk of generalized OA[1, 7], while others found no association with LDL-C or total cholesterol[8] and even describe protective effects of high-density lipoprotein cholesterol (HDL-C). Although cholesterol-lowering therapy effectively reduces the risk of CVD, the effects on OA development remain to be elucidated. Use of statins, a class of drugs that inhibits cholesterol synthesis and is often prescribed to lower systemic LDL-C levels, was associated with reduced incidence and progression of knee OA in some studies[9], while not in others[10, 11]. Also in hand OA, often associated with inflammation, statin use did not affect disease incidence[12]. Recently, new therapies that lower LDL-C levels in CVD patients by different mechanisms as statins were introduced but have not yet been evaluated in patients with OA. These novel treatments include monoclonal antibodies against proprotein convertase subtilisin/kexin 9 (PCSK9), one of the key players involved in clearance of LDL, which reduce LDL-C levels alone and on top of a statin by up to 50-60% in humans[13]. And evinacumab, a monoclonal antibody directed against angiopoietin-like protein 3 (ANGPTL3), a circulating protein that inhibits the hydrolysis of triglycerides (TG) by lipoprotein lipase in TG-rich lipoproteins. Evinacumab was shown to decrease plasma triglycerides and LDL-C in humans by more than 70% and 25%, respectively[14, 15].

The efficacy of cholesterol-lowering treatments on OA incidence and progression can be evaluated in a more controlled manner in preclinical models. APOE*3Leiden.CETP mice are a translational model for human lipoprotein metabolism that responds to all registered lipid-lowering drugs in a human-like manner[14, 16-20]. In APOE*3Leiden.CETP mice, both alirocumab and evinacumab administration successfully reduced cholesterol levels by 40-50%[14, 21]. We and others have demonstrated that cholesterol-supplemented Western-type diet (WTD) feeding aggravated OA features in the knee joint[22-25]. APOE*3Leiden.CETP mice showed increased spontaneous development of mild articular cartilage degradation on a cholesterol-rich WTD[22, 25]. High cholesterol levels provoked synovial activation and ectopic bone formation in an inflammatory collagenase-induced OA model[23]. Hypercholesterolemia was shown to trigger OA development through oxidative stress and chondrocyte apoptosis[24]. Atorvastatin treatment ameliorated OA outcome in these models[22, 24]. These observations indicate that high cholesterol levels may contribute to OA pathogenesis and that lowering of cholesterol levels may have a favourable effect on OA.

In a previous study we showed that alirocumab and evinacumab—monoclonal antibodies against cholesterol-regulating PCSK9 and ANGPTL3[14]—, and atorvastatin triple therapy regresses atherosclerotic plaque lesions and improves lesion composition in APOE*3Leiden.CETP mice fed a cholesterol-supplemented WTD[26]. In the present study we used knee joints from the latter study to evaluate the effects of high-intensive cholesterol lowering on cartilage degradation, ectopic bone formation and synovial inflammation.

MATERIALS AND METHODS

Animals

The experiment was carried out in female APOE*3Leiden.CETP transgenic mice on a C57BL/6 background (8-12 weeks of age), obtained from the in-house breeding colony (TNO Metabolic Health Research, Leiden, The Netherlands). The study was initially designed to investigate the effect of high intensive cholesterol-lowering triple therapy on regression of pre-existent atherosclerosis[26]. Female mice were used as they are more susceptible to cholesterol-containing diets by having higher plasma cholesterol and TG levels relative to males, and therefore develop more pronounced atherosclerotic lesions[25, 27]. The reason for the higher plasma cholesterol and TG levels is that estrogen increases VLDL production and testosterone increases the VLDL clearance rate[27]. Group size was calculated for atherosclerosis development using a power of 0.80. An expected variance of 23% (a standard deviation of 47%) in atherosclerosis, a minimal difference of 40%, and a two-sided t-test test with 95% confidence interval, resulted in 16 animals per group. Based on previous experiments, alirocumab does not cause auto-antibody development in mice, while around 25–40% develop such a response after administration of evinacumab[14]. Therefore, additional mice were originally included in groups treated with evinacumab. All other groups had 16 mice per group at the beginning of the study. Mice that developed auto-antibodies were excluded from all analyses and a few mice died during the course of the study, resulting in 13–16 mice per group for osteoarthritis evaluation. The experiment was 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.

Diet and treatments

Metabolic OA was induced by switching the diet of the mice from standard chow to WTD with 0.30% cholesterol and 15% saturated fat. At t=13 weeks, mice were matched into 6 groups based on age, body weight, plasma total cholesterol (TC), plasma total triglycerides (TG) and cholesterol exposure (mmol/L*weeks) before the start of cholesterol-lowering treatment. Sixteen mice were sacrificed as the baseline control group and the other 5 groups continued to receive WTD alone or with treatment for 25 weeks (**Supplementary Figure 1**). Treatments comprised atorvastatin (4-13 mg/kg/d; concentrations based on food intake), atorvastatin and alirocumab (10 mg/kg), atorvastatin and evinacumab (25 mg/kg) or atorvastatin, alirocumab and evinacumab. Atorvastatin (mixed in the diet) and dietary cholesterol

concentrations were adapted during the study to reach the non-HDL-C lowering goal of 1 mM (**Supplementary Figure 1**). However, the increase in atorvastatin dose led to increases in TG levels in all groups (starting from week 16), and, therefore, we decided to lower the atorvastatin dose at week 24. Dietary cholesterol concentrations were decreased from 0.30% to 0.15% in week 24 for counterbalance. Lowering of dietary cholesterol resulted in TC levels of 11.5 mmol/l in control, which is a pro-atherogenic condition in APOE*3-Leiden.CETP mice[26, 28]. Alirocumab and evinacumab were administered by weekly subcutaneous injections. We refer to Pouwer *et al*[26] for a more detailed description of experimental design, treatments and sample size calculations.

Assessment of metabolic dysfunction

Plasma cholesterol levels were monitored throughout the study period. Peripheral blood (5 drops/animal) was drawn via tail incision using EDTA-coated tubes (Sarstedt) after 4h of food deprivation and by heart puncture at sacrifice. Total cholesterol levels were determined throughout the study with an enzymatic assay (Roche Diagnostics) according to manufacturer's instructions and total cholesterol exposure was calculated as mmol/L*weeks. For lipoprotein profiles, pooled plasma of each group was fractionated using an Äkta FPLC system (Pharmacia) and analyzed for their cholesterol-containing fractions.

Histological analysis of OA development

In most induced models of osteoarthritis (e.g. the collagenase-induced osteoarthritis model), predominantly the medial compartment of the joint is affected. Here we studied spontaneous cartilage degeneration, which was relatively mild and mainly developed in the lateral joint compartment. Murine knee joints were fixed in formalin and decalcified using formic acid. Subsequently, the joints were embedded in paraffin and cut in 7µm sections. Sections were stained using Safranin-O/Fast Green and Hematoxylin/Eosin for histological analysis. Cartilage damage in the joint was quantified using a more detailed version of the OARSI scoring system, as described previously[29, 30] (0 = no damage, 30 = maximal damage). Five sections were scored and averaged per joint after blinding. Osteophyte formation and maturation were determined using an arbitrary scoring system as described previously[31]. Ten different locations were scored for osteophyte formation and maturation on both the medial and lateral side of the joint. The total amount of osteophytes in the knee joints was determined throughout the whole joint. Synovial inflammation was scored using three sections per joint and a scoring range from 0-2 (0 = no inflammation, 1 = mild inflammation, 2 = moderate inflammation; **Supplementary Figure 2**).

Immunohistochemistry

For immunohistochemical analysis, knee joint sections were deparaffinized and endogenous peroxidase blocking was performed using H_2O_2 in methanol. Antigen retrieval was performed in citrate buffer pH 6.0. Sections were stained with polyclonal antibodies against S100A8 (kindly provided by Thomas Vogl, Institute of Immunology, University of Muenster, German), NITEGE (kindly provided by John Mort, Shriners Hospital for Children, Montreal, Canada) or non-relevant rabbit IgG control (R&D systems). Biotinylated anti-rabbit IgG was used as a secondary antibody. Subsequently, sections were stained with

avidin-streptavidin-peroxidase (Elite kit, Vector Laboratories) and diaminobenzidine (Sigma-Aldrich) was used for visualization of peroxidase staining. Counterstaining was performed using haematoxylin (Merck). NITEGE staining as a marker of repair was determined using the Leica Application Suite (Leica Microsystems), three sections were scored and averaged per joint in the superficial non-calcified layer of articular cartilage. Positive staining area was corrected for the total area that was analyzed. NITEGE staining was determined in a blinded fashion.

Statistical analysis

We determined the statistical power of our study based on the main readout parameter, cartilage degradation. The mean differences between groups detectable with a power of 0.8 for each analysis was determined using 16 mice per group, a two sided t-test, a 95% confidence interval and the observed SD in the WTD control group, which is 1.9 for our main read-out parameter cartilage degeneration. This resulted in a detectable difference of 1.9. Statistical analysis was performed using SPSS Statistics Data Editor (IBM). Normality was assessed using a Kolmogorov-Smirnov test. Differences between groups were analyzed using a parametric One-Way ANOVA followed by a Bonferroni post hoc test to correct for multiple comparisons. For synovitis and NITEGE scores, the nonparametric Mann-Whitney U-test was used for comparisons of the control group with the baseline and different treatment groups. *P*-values below 0.05 were considered significant. Results are expressed as mean ± 95% confidence intervals.

RESULTS

Intensive cholesterol lowering treatment reduces systemic cholesterol levels in dyslipidemic APOE*3Leiden.CETP mice

Systemic cholesterol levels were determined to assess the effectiveness of standard and high-intensive cholesterol-lowering treatments. All treatments induced an intervention-dependent gradual decrease of cholesterol levels over the course of the study, the combination treatments being most effective (**Figure 1A,B**). The decrease in systemic cholesterol levels coincided with a reduced body weight gain in the treated groups compared to WTD controls (**Supplementary Figure 3**). Analysis of lipoprotein profiles showed that all treatments induced a significant reduction of atherogenic VLDL/LDL cholesterol whereas no differences in HDL-C levels were observed **(Figure 1C**). To determine the immunomodulatory effects of WTD-feeding and cholesterol-lowering treatments, we measured systemic levels of SAA, E-selectin and MCP-1 as functional markers of inflammation at endpoint. SAA levels were slightly reduced in mice treated with atorvastatin alone and combined with evinacumab compared with the WTD control (A; $p<0.001$, 95% CI 4.5 to 5.4, AE: $p<0.05$, (95% CI 5.0 to 6.0 **Table 1**). E-selectin and MCP-1 levels were low and not affected by cholesterol-lowering treatment (**Table 1**).

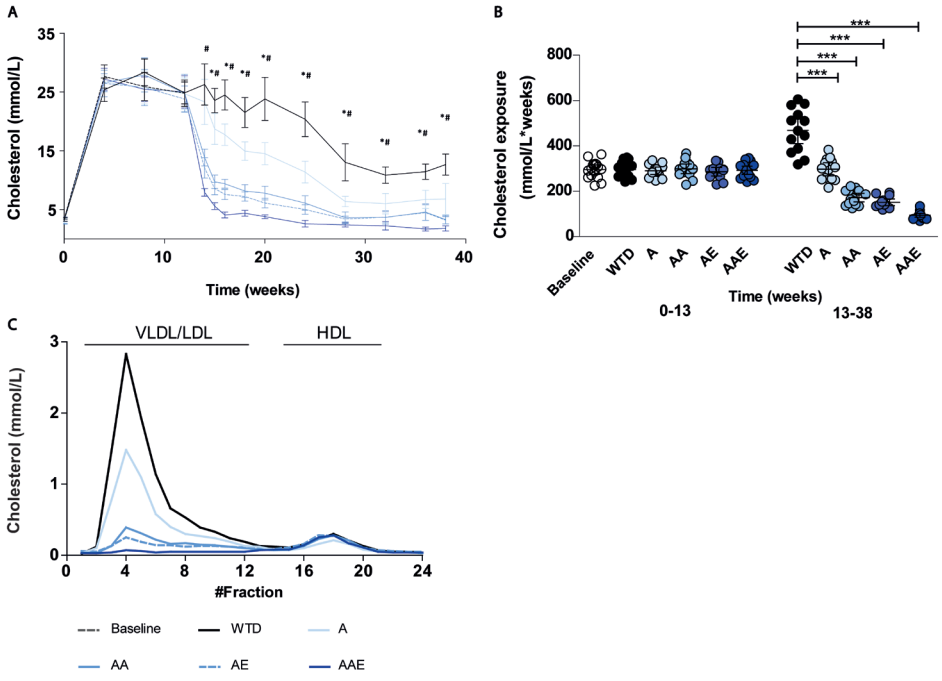

Figure 1. High intensive cholesterol lowering on top of atorvastatin treatment gradually reduces cholesterol levels

APOE*3Leiden.CETP mice received a Western Type Diet for 38 weeks with double or triple treatment with alirocumab and evinacumab on top of atorvastatin treatment. (Data depicted in figure 1A-C are given as background information. This research was originally published in the *Journal of Lipid Research* Pouwer, M. G. *et al.* Alirocumab, evinacumab, and atorvastatin triple therapy regresses plaque lesions and improves lesion composition in mice. J. Lipid Res. 2020, **61**, 365-375)[156] describing the effects on the regression of atherosclerosis. **(A)** WTD feeding significantly increased systemic cholesterol levels and all cholesterol-lowering treatments induced a significant gradual reduction in systemic cholesterol levels. **(B)** Plasma total cholesterol exposure (millimoles per liter x weeks) confirmed a further increase in the control group and an intervention-dependent decrease over the course of the study. **(C)** Cholesterol-lowering interventions resulted in a significant decrease of VLDL/LDL (fractions 4-15) levels while no changes in HDL (fraction 16-24) levels were observed. *A= atorvastatin, AA= atorvastatin + alirocumab, AE= atorvastatin + evinacumab, AAE= atorvastatin + alirocumab + evinacumab. n=13-16 per group. * P < 0.05, ** P < 0.01, ***P < 0.001 versus WTD; * P<0.001 WTD versus A, # P <0.001 WTD versus AA, AE and AAE in figure 1A.*

Table 1. Levels of inflammation markers in plasma

Group	SAA (µg/ml)	E-selectin (ng/ml)	MCP-1 (pg/ml)
Baseline	7.4 (6.7-8.1)	48.5 (45.3-51.7)	91.0 (76.5-105.6)
WTD	7.3 (6.1-8.5)	40.6 (36.9-44.3)	92.1 (73.9-110.3)
A	4.9 (4.5-5.4) ***	43.1 (37.0-49.3)	97,3 (75.7-118.9)
AA	6.1 (5.5-6.8)	43.1 (38.4-47.7)	97.8 (84.2-111.4)
AE	5.5 (5.0-6.0) **	41.0 (37.5-44.6)	125.8 (96.7- 155.0)
AAE	6.5 (6.0-7.1)	46.3 (42.4-50.2)	103.1 (89.6-116.6)

APOE*3Leiden.CETP mice received a Western Type Diet for 38 weeks with double or triple treatment with alirocumab and evinacumab on top of atorvastatin treatment. SAA, E-selectin and MCP-1 were determined in individual plasma samples at end point. SAA is significantly reduced after treatment with atorvastatin with or without evinacumab. Data are depicted as mean (95% CI) *A= atorvastatin, AA= atorvastatin + alirocumab, AE= atorvastatin + evinacumab, AAE= atorvastatin + alirocumab + evinacumab. n=13-16 per group. * P < 0.05, ** P < 0.01, ***P < 0.001 versus WTD.*

Therapeutic cholesterol lowering does not ameliorate cartilage degradation in APOE*3Leiden.CETP mice fed a cholesterol-supplemented WTD

As previously observed[22,25] cholesterol-supplemented WTD feeding coincided with a mild but significant increase in cartilage degeneration after 38 weeks (WTD, 7.8 ± 1.9) compared to 13 weeks (baseline, 3.8 ± 1.8; 2.1-fold increase; 95% CI 2,0 to 6,2; **Figure 2A**). The observed decline in systemic cholesterol levels did not attenuate progression of cartilage destruction in treatment groups as compared to WTD controls (**Figure 2A**). Cartilage degradation was mainly observed at the lateral tibia and femur (**Supplementary Figure 4A**). Proteolytic activity in the cartilage, as a measure of matrix degradation and repair (cartilage turnover activity) , was determined by immunohistochemical analysis of NITEGE and VDIPEN neo-epitopes[32]. VDIPEN staining, induced by MMPs and a marker of advanced cartilage degradation, was not observed in articular cartilage (**Figure 2D**). NITEGE staining, as marker of both early was significantly increased at 38 weeks in WTD-fed mice compared to baseline controls ($p<0.01$; **Figure 2B**). We observed a significant increase in NITEGE staining after double or triple cholesterol-lowering treatment, which, since VDIPEN was absent, is indicative of a more active repair process (AA, 2.2 fold-increase, $p<0.001$; AE, 1.9 fold-increase, $p<0.05$; AAE, 1.9 fold-increase, $p<0.01$; **Figure 2B**) when compared to WTD controls**.** Representative pictures of cartilage degeneration and NITEGE and VDIPEN staining are shown (**Figure 2C-E**). The combined data indicate that increased cartilage degradation also induces an active repair process in articular cartilage.

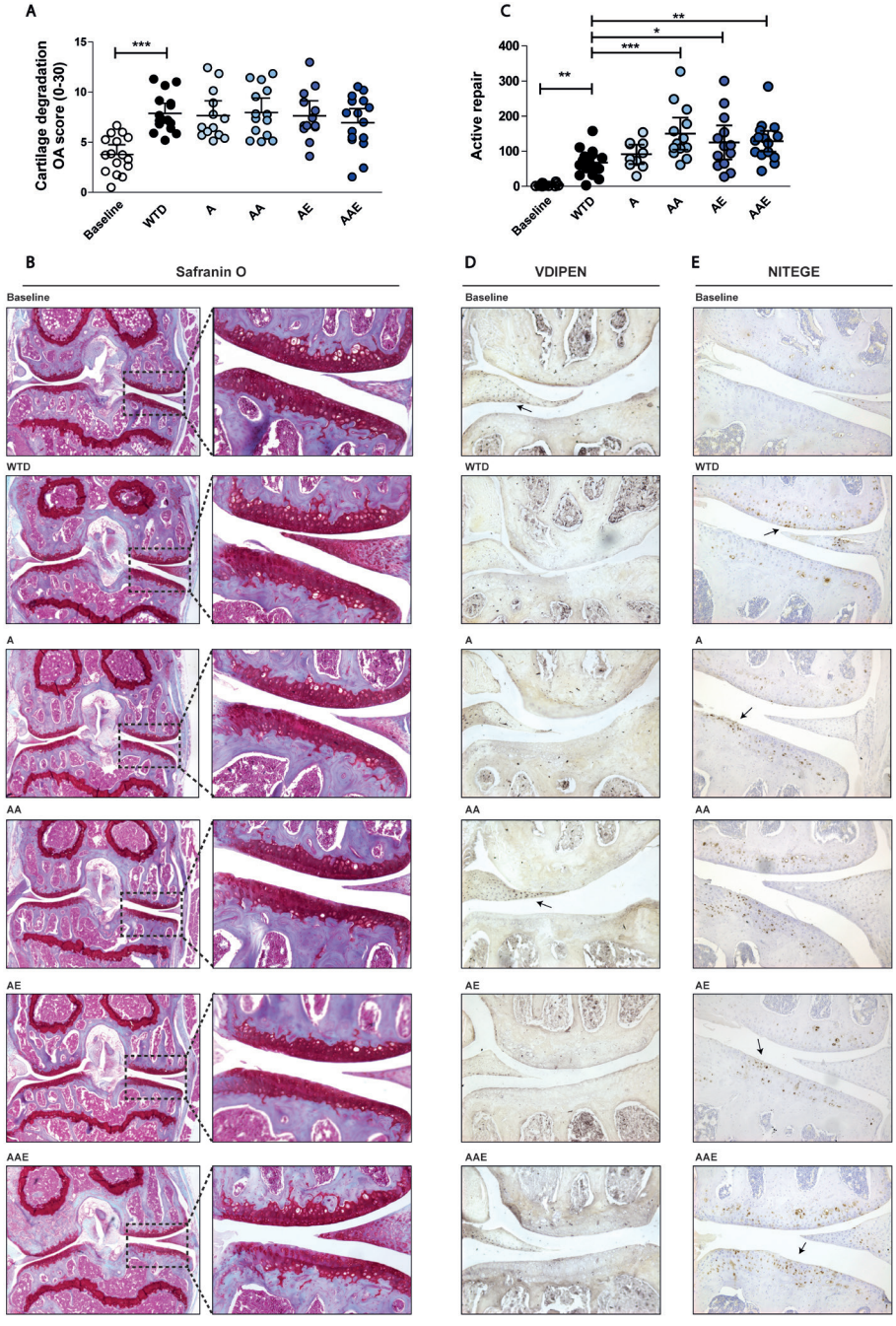

Figure 2. Cholesterol-lowering interventions do not ameliorate cartilage degradation in dyslipidemic mice

APOE*3Leiden.CETP mice received a Western Type Diet for 38 weeks. **(A)** Cartilage degradation was determined using histological analysis and revealed a significant increase in cartilage degeneration after 38 weeks of WTD-feeding compared to baseline controls. **(B)** Immunohistochemical analysis of NITEGE staining revealed a significant increase in WTD-fed mice receiving double or triple treatment (AA, p<0.001; AE, p<0.05; AAE, p<0.01). **(D)** Representative pictures of VDIPEN staining showing no expression in articular cartilage (20x magnification). Representative pictures of cartilage degradation and NITEGE staining are depicted in figures **(B)** and **(E)** (20x magnification) Arrows were used to indicate positive staining. *A= atorvastatin, AA= atorvastatin + alirocumab, AE= atorvastatin + evinacumab, AAE= atorvastatin + alirocumab + evinacumab. n=13-16 per group. * P < 0.05, ** P < 0.01, ***P < 0.001 versus WTD*

Modulation of systemic cholesterol levels does not affect synovial inflammation or ectopic bone formation

Previously, we reported that high systemic cholesterol levels aggravated synovial inflammation and ectopic bone formation in a collagenase-induced OA model[23]. Therefore, we examined the effect of therapeutic cholesterol-lowering therapies on these OA features. All groups developed minor synovial inflammation, which was independent of systemic cholesterol levels (**Figure 3A,B**). The alarmin S100A8, a marker for activated macrophages, also showed only minor expression in the synovial lining and was comparable between all groups (**Figure 3C**). Finally, we determined whether high plasma cholesterol promoted ectopic bone formation in this model. The total number of osteophytes per knee joint and the maturation stage remained comparable between all groups upon reduction of systemic cholesterol levels (**Figure 4A**). Most osteophytes occurred at the anterior side of the medial femoral condyle (**Figure 4B**).

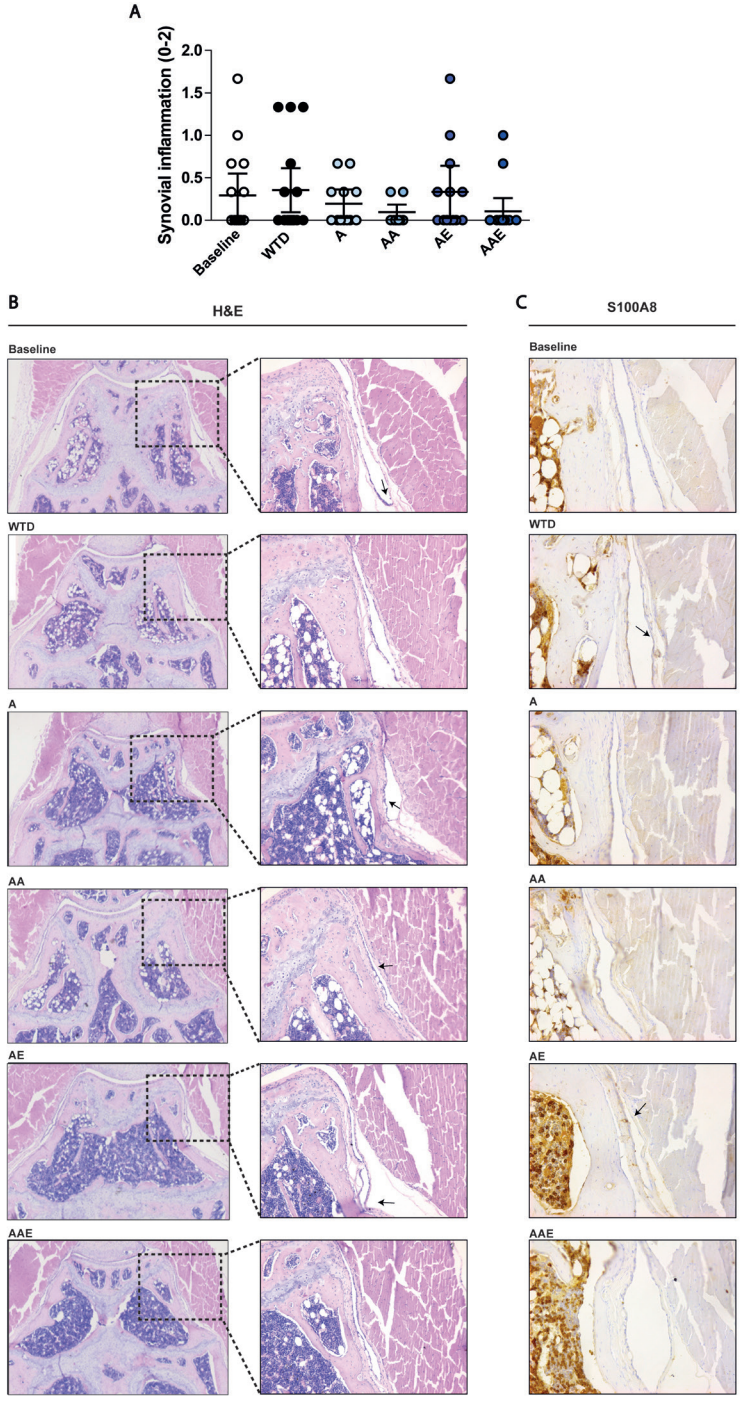

Figure 3. No effect of lowering systemic cholesterol on synovial inflammation

Synovial inflammation was determined using histological analysis. **(A)** Synovial inflammation was measured using an arbitrary score (0-2). Synovial inflammation was in general mild and independent of cholesterol levels. Representative pictures of synovial inflammation are shown in figure **(B)** (left: 5x magnification, right 10x magnification)**. (C)** Sections were stained for the pro-inflammatory alarmin S100A8, which was only expressed to a minor extent in the lining of the synovium (10x magnification). Arrows indicate positive staining. *A= atorvastatin, AA = atorvastatin + alirocumab, AE= atorvastatin + evinacumab, AAE= atorvastatin + alirocumab + evinacumab.*

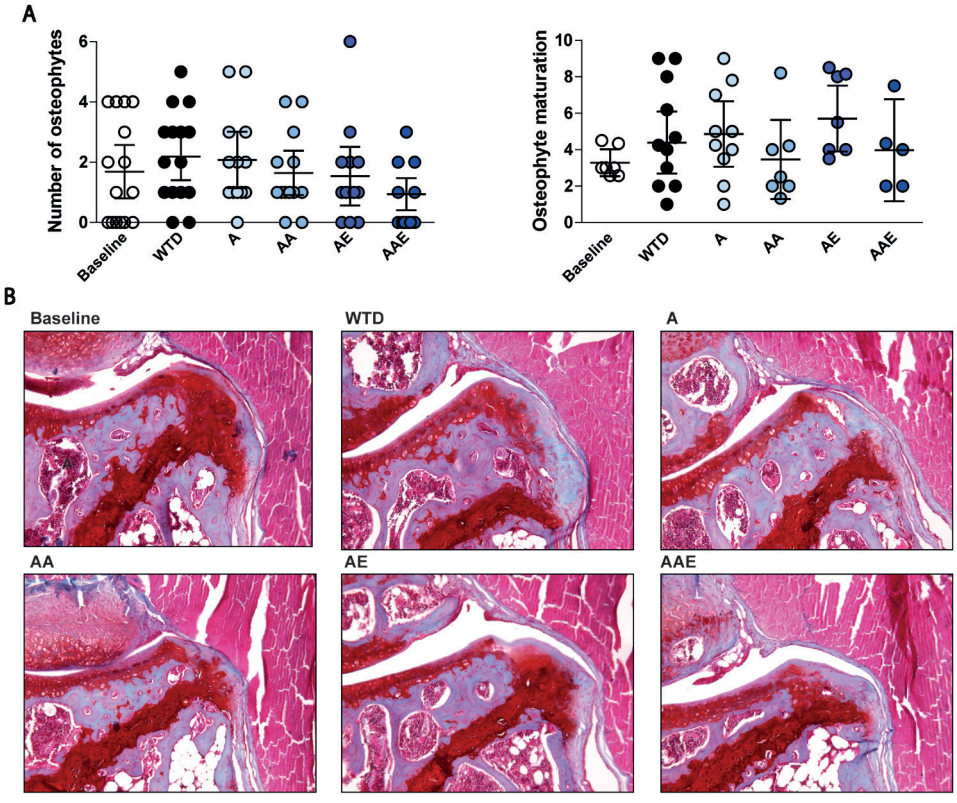

Figure 4. No effect of lowering systemic cholesterol on ectopic bone formation in dyslipidemic mice

Ectopic bone formation and maturation stage were determined after 13 and 38 weeks of WTD-feeding on Safranin-O/FastGreen-stained sections. **(A)** No differences were observed in the total number of osteophytes as well as maturation between all different groups**.** Figure **(B)** shows representative pictures of ectopic bone formation (10x magnification). *A= atorvastatin, AA = atorvastatin + alirocumab, AE= atorvastatin + evinacumab, AAE= atorvastatin + alirocumab + evinacumab.*

DISCUSSION

The association of CVD with OA has become increasingly recognized and understanding their interrelationship is imperative for improving therapeutic approaches. Cholesterol, with its crucial role in CVD, could be a potential link. Our study demonstrates that therapeutic cholesterol-lowering therapies proved insufficient in reducing progression of cartilage degradation, in contrast to earlier findings with cholesterol lowering in a prevention design[22]. Minor synovial inflammation and ectopic bone were formed independent of systemic cholesterol levels, while aggrecanase activity, as marker of the dynamic process of proteoglycan turnover in articular cartilage, was increased after cholesterol-lowering treatment. The absence of synovial activation suggests a minor role of joint inflammation in our model. Taken together, our findings demonstrate that therapeutic cholesterol lowering does not slow the progression of cartilage degradation in dyslipidemic APOE*3Leiden.CETP mice.

This is the first study to show the effects of novel, therapeutic cholesterol-lowering interventions on the progression of development of OA pathology in dyslipidemic mice. Compared to previous studies[22], the translational and clinical value is improved by the therapeutic experimental design. The APOE*3Leiden.CETP strain has high translatability in lipoprotein metabolism and metabolic diseases, showing human-like responses to hypolipidemic treatments[14, 16-20]. In this study, diet-induced dyslipidemia was distinct and manifested itself in cartilage degradation as well as atherosclerosis development. Cholesterol-lowering interventions reduced plasma cholesterol levels similarly as in humans and successfully induced regression of atherosclerosis, while mild cartilage degeneration progressed despite of therapy. Although OA and atherosclerosis may share overlapping pathophysiological processes[3], the role of an impaired lipid metabolism and the effects of cholesterol-lowering therapies on OA progression in humans remain unclear. A systematic literature review and meta-analysis revealed a clear association between dyslipidemia and OA, suggesting that lipid disturbances are a risk factor for OA Yet results from clinical studies have been diverse, showing beneficial[9, 33] or no[10-12] effects of statin use on OA incidence or progression. Different methods of analysis, treatment effect or the lack of patient stratification could explain these different outcomes.

In the current study, we used female APOE*3Leiden.CETP mice to study the effects of novel lipid-lowering therapies on diet-induced OA development. In contrast to an earlier study, where protective effects of preventive atorvastatin monotreatment in a prophylactic design on cholesterol-induced OA in APOE*3Leiden.CETP females were investigated[22], atorvastatin treatment did not protect against OA development in the current study where a therapeutic approach was applied. These collective results seem to indicate that statins can be beneficial pre-onset[22] but cannot modify disease course. Possibly, increased weight gain in response to the diet could already induce initiation of OA development, thereby limiting the beneficial effects of cholesterol-lowering therapy. One limitation of the present study is the absence of a chow control group, as this would have elucidated whether the observed pathology was directly caused by the cholesterol-supplemented WTD or by other mechanisms such

as ageing or weight of the mice. However, it has been reported previously that both ageing together with weight gain and cholesterol and fat containing diet contributes to the development of OA[22, 25]. The divergent effectiveness of statin treatment suggests that other mechanisms, additional to lipoprotein disturbances, are important in cholesterol-induced OA pathogenesis. We determined the effect of cholesterol-lowering treatment on systemic markers of inflammation. E-Selectin and MCP-1 levels were not affected by cholesterol-lowering treatments. Although systemic SAA levels were slightly reduced in mice treated with atorvastatin with or without evinacumab, did this not result in reduced OA pathology. These data show that systemic inflammation was only mildly affected by cholesterol-lowering treatment and did not contribute to OA pathology in the current study. Systemic dyslipidemia may induce a lipid imbalance within the synovial fluid of OA patients that could affect chondrocyte homeostasis. Although we were unable to investigate in detail the mechanistic pathways involved in cartilage pathophysiology, we have analysed the activity of catabolic mediators in the cartilage matrix using immunohistochemistry. Important catabolic mediators involved in cartilage degradation are aggrecanases and matrix metalloproteases (MMPs). Both cleave aggrecan at a specific site, leaving behind the neo-epitopes NITEGE and VDIPEN, respectively[32]. VDIPEN-epitopes are expressed during advanced cartilage degradation. NITEGE-epitopes, however, are expressed during early cartilage degradation and are also observed during regeneration of proteoglycan content in articular cartilage leading to cartilage repair[32]. In the present study, NITEGE staining in articular cartilage was increased after 38 weeks of WTD feeding compared to baseline controls. Cholesterol-lowering treatments resulted in a significant increase in NITEGE staining, whilst no coinciding increase in cartilage degradation was observed. The cartilage damage observed in our study was mild, which is supported by the absence of VDIPEN staining in articular cartilage. This finding is consistent with previous studies of arthritis models, which showed that NITEGE and VDIPEN neo-epitopes were not observed simultaneously and VDIPEN only in late severe cartilage destruction[32]. By breaking down the cartilage matrix, aggrecanases enable chondrocytes to proliferate or restore proteoglycan content in articular cartilage[32]. Therefore, we propose that the observed aggrecanase activity indicates an active repair mechanism in articular cartilage after cholesterol-lowering treatment. Possibly, continued cholesterol-lowering treatment could protect against cartilage degradation after prolonged cholesterol exposure.

As inflammatory involvement is increasingly recognized in OA, cholesterol-lowering treatments that have pleiotropic immunomodulatory effects – such as statins and anti-PCSK9 antibodies – may be beneficial for OA patients in multiple ways. We have previously reported that high cholesterol levels enhanced ectopic bone formation and synovial activation during pro-inflammatory collagenase-induced OA[23, 34]. Also in other post-traumatic OA models, high fat diets were shown to increase OA pathology after induction of joint injury[35, 36]. In contrast, we observed little ectopic bone formation or macrophage activation in the current study with minor synovial inflammation, both of which were independent of systemic cholesterol levels as well. This strongly suggests that high cholesterol alone is insufficient to induce joint pathology and that only in combination with a substantial joint inflammation a strong aggravation of joint pathology is observed. This implies that cholesterol lowering could still be

efficient under those circumstances. Local inflammation occurs in approximately 50% of the OA patients and has been associated with the development and progression of joint pathology. Inflammation is essential for the oxidation of LDL, which is taken up by synovial lining cells and drives joint pathology via pro-inflammatory mechanisms[34]. In previous studies we have shown that mainly oxLDL, and not LDL, was able to induce activation of the synovium[34]. Taken together, these findings imply that local joint injury inducing synovial inflammation is required for cholesterol-driven OA pathology.

In conclusion, our results show that cholesterol-supplemented WTD feeding disturbed lipoprotein metabolism and increased cartilage degradation in APOE*3Leiden.CETP mice. However, therapeutic, high-intensive cholesterol-lowering interventions per se did not attenuate the progression of cartilage degradation. We propose that local joint inflammation as a result of injury is a prerequisite in cholesterol-induced OA pathology. Therapeutic cholesterol-lowering strategies may still be promising for OA patients presenting both dyslipidemia and joint inflammation.

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AUTHOR CONTRIBUTIONS

MGP, EJP and HMGP have designed the study. MGP, EJP, AEK and YG have carried out experimental procedures. YG and AEK have been the primary persons responsible for writing the manuscript. NNLK, MHJB, ABB, EJP, HW, RS, HMGP and PLEML were involved in drafting the work or revising it critically for important intellectual content. All authors approved the final version to be published.

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ROLE OF THE FUNDING SOURCE

The funding sources had no role in study design, in collection, analysis or interpretation of data, or in writing the manuscript and decision to submit the manuscript.

COMPETING INTERESTS

Alirocumab (Praluent®) and evinacumab (REGN1500) are developed by Regeneron Pharmaceuticals and evinacumab is currently in clinical trials. EJP, RS and HMGP are employees of the Netherlands Organization for Applied Scientific Research (TNO).

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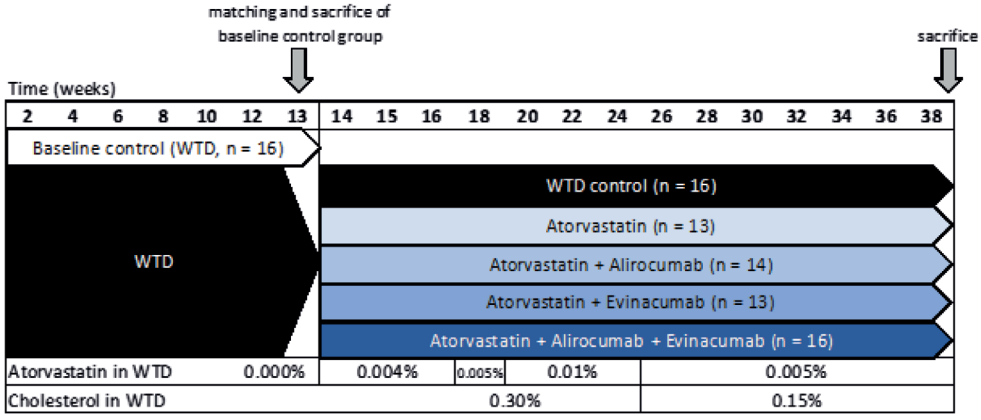
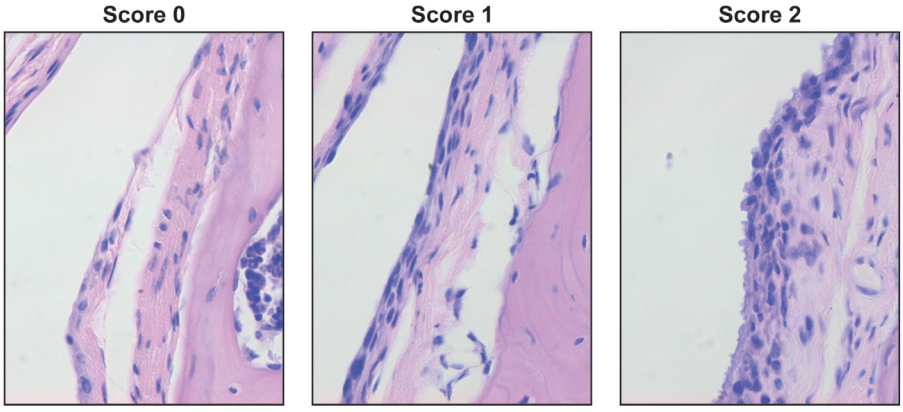
SUPPLEMENTARY MATERIAL



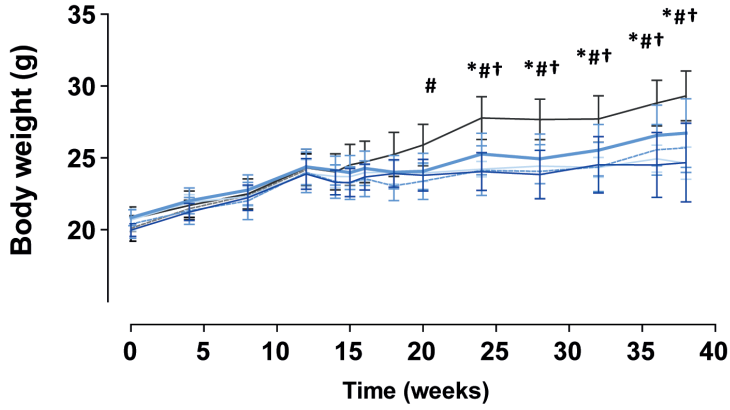
Supplementary Figure 1. Schematic overview of the study design

Metabolic OA was induced by switching the diet of the mice from standard chow to WTD with 0.30% cholesterol and 15% saturated fat. At t=13 weeks, mice were matched into 6 groups based on age, body weight, plasma total cholesterol (TC), plasma total triglycerides (TG) and cholesterol exposure (mmol/L*weeks) before the start of cholesterol-lowering treatment was started. Sixteen mice were sacrificed as the baseline control group and the other 5 groups continued to receive WTD alone or with treatment for 25 weeks. Treatments comprised atorvastatin (4-13 mg/kg/d; concentrations based on food intake), atorvastatin and alirocumab (10 mg/kg), atorvastatin and evinacumab (25 mg/kg) or atorvastatin, alirocumab and evinacumab. Alirocumab and evinacumab were administered by weekly subcutaneous injections.



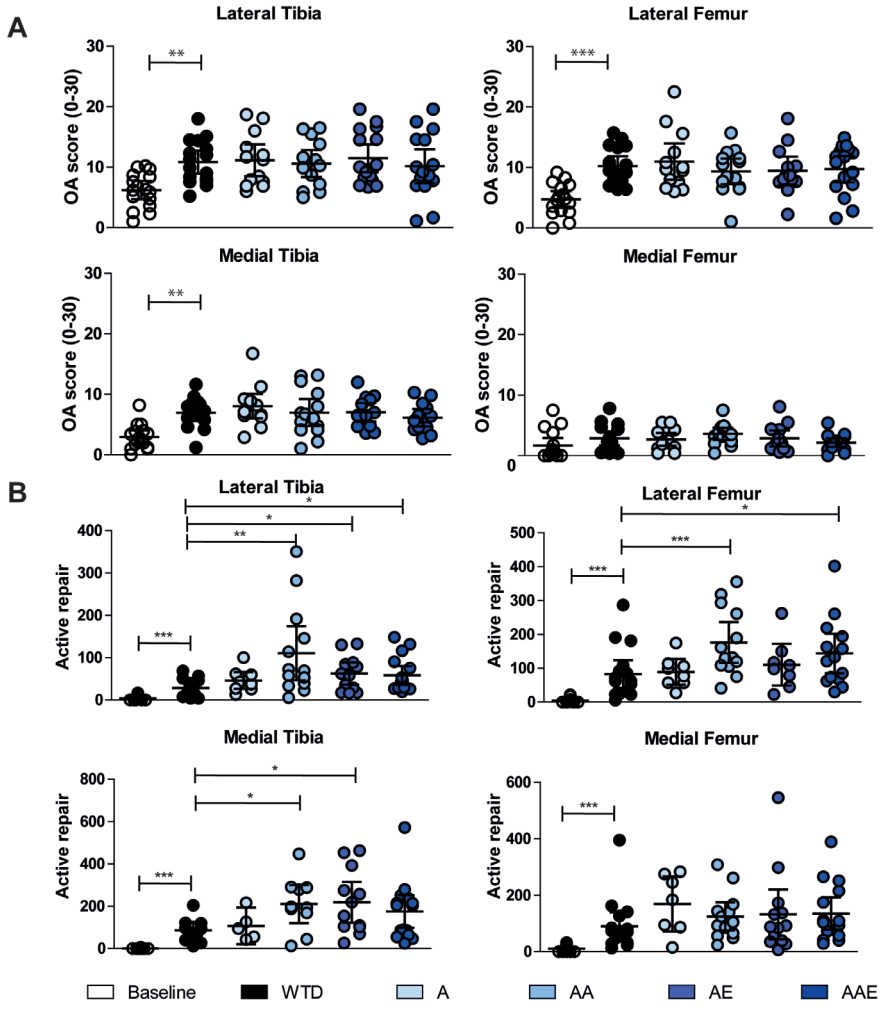
Supplementary Figure 2. Arbitrary score used to quantify synovial inflammation

Synovial inflammation was quantified by evaluating synovial thickening and cell infiltration in the synovial lining using H&E stained sections of the joint. Synovial inflammation was scored using an arbitrary score of 0-2 (0 = no inflammation, 1 = mild inflammation, 2 = moderate inflammation), three sections were scored and averaged per joint.



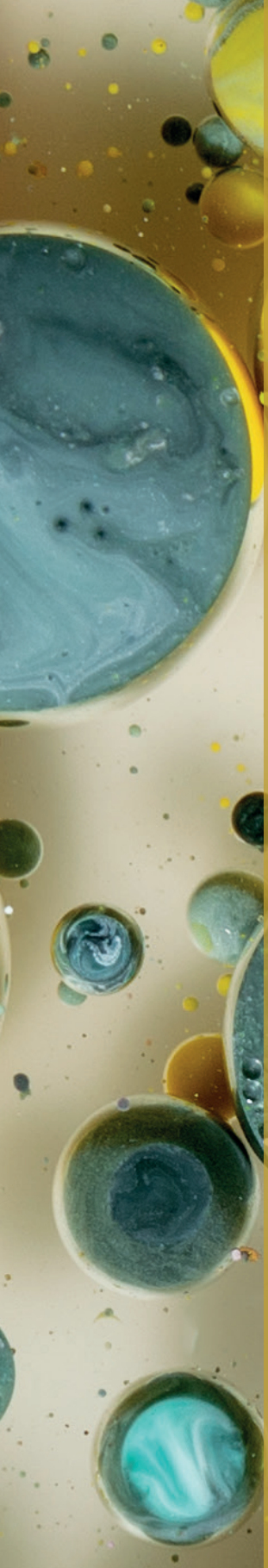
Supplementary Figure 3. Reduced body weight gain upon cholesterol-lowering treatment

Administration of different cholesterol-lowering interventions resulted in a reduced body weight gain. *A= atorvastatin, AA= atorvastatin + alirocumab, AE= atorvastatin + evinacumab, AAE= atorvastatin + alirocumab + evinacumab. n=13-16 per group. * P<0.001 WTD versus A, # P <0.001 WTD versus AA, AE and AAE, † P <0.001 WTD versus AAE*



Supplementary Figure 4. Cholesterol-lowering does not reduce cartilage degeneration in dyslipidemic mice.

(A) Sections were stained using Safranin-O/Fast Green for histological analysis. Cartilage damage in the joint was quantified using a detailed version of the OARSI score (0 = no damage, 30 = maximal damage). Five sections were scored and averaged per joint. A significant increase in cartilage degradation was observed between baseline and WTD at the lateral tibia, lateral femur and medial tibia side of the joint. **(B)** Immunohistochemical analysis of NITEGE staining revealed significant differences in aggrecanase activity after cholesterol-lowering treatments at the lateral tibia, lateral femur and medial tibia side of the joint. *A= atorvastatin, AA= atorvastatin + alirocumab, AE= atorvastatin + evinacumab, AAE= atorvastatin + alirocumab + evinacumab. n=13-16 per group. * P < 0.05, ** P < 0.01, ***P < 0.001.*



CHAPTER 4

Intensive cholesterol-lowering treatment reduces synovial inflammation during early collagenase-induced osteoarthritis, but not pathology at end-stage disease in female dyslipidemic E3L.CETP mice

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ABSTRACT

Introduction

The association between metabolic syndrome (MetS) and osteoarthritis (OA) development has become increasingly recognized. In this context, the exact role of cholesterol and cholesterol-lowering therapies in OA development has remained elusive. Recently, we did not observe beneficial effects of intensive cholesterol-lowering treatments on spontaneous OA development in E3L.CETP mice. We postulated that in the presence of local inflammation caused by a joint lesion, cholesterol-lowering therapies may ameliorate OA pathology.

Materials and Methods

Female ApoE3*Leiden.CETP mice were fed a cholesterol-supplemented Western type diet. After 3 weeks, half of the mice received intensive cholesterol-lowering treatment consisting of atorvastatin and the anti-PCSK9 antibody alirocumab. Three weeks after the start of the treatment, OA was induced via intra-articular injections of collagenase. Serum levels of cholesterol and triglycerides were monitored throughout the study. Knee joints were analyzed for synovial inflammation, cartilage degeneration, subchondral bone sclerosis and ectopic bone formation using histology. Inflammatory cytokines were determined in serum and synovial washouts.

Results

Cholesterol-lowering treatment strongly reduced serum cholesterol and triglyceride levels. Mice receiving cholesterol-lowering treatment showed a significant reduction in synovial inflammation (P = 0.008, WTD: 95% CI: 1.4 to 2.3; WTD + AA: 95% CI: 0.8 to 1.5) and synovial lining thickness (WTD: 95% CI: 3.0 to 4.6, WTD + AA: 95% CI: 2.1 to 3.2) during early-stage collagenase-induced OA. Serum levels of S100A8/A9, MCP-1 and KC were significantly reduced after cholesterol-lowering treatment (P = 0.0005, 95% CI: -46.0 to -12.0; P = 2.8x10^-10, 95% CI: -398.3 to -152.1; P=2.1x10^-9, -66.8 to -30.4, respectively). However, this reduction did not reduce OA pathology, determined by ectopic bone formation, subchondral bone sclerosis and cartilage damage at end-stage disease.

Conclusion

This study shows that intensive cholesterol-lowering treatment reduces joint inflammation after induction of collagenase-induced OA, but this did not reduce end stage pathology in female mice.

INTRODUCTION

Osteoarthritis is the most common joint disease worldwide and patients suffer from joint pain and stiffness, leading to disability. OA is a disease of the entire joint that affects various tissues including cartilage, synovium, subchondral bone and ligaments[1]. Currently, no disease-modifying treatments are available and treatment options are focused on prevention of the disease and reducing symptoms. OA is a complex and heterogeneous disease and many risk factors, including ageing, obesity and metabolic syndrome (MetS), have been associated with disease development[2, 40]. MetS comprises a cluster of metabolic conditions including obesity, hypertension and high blood sugar and insulin resistance and dyslipidemia. OA patients show an increased incidence of MetS[22] compared to the non-OA population and several studies have demonstrated that MetS is connected to disease development and progression[3, 41, 42].

Dyslipidemia refers to an imbalance of lipids in the blood such as decreased levels of high-density lipoprotein cholesterol (HDL-C), increased levels of low-density lipoprotein cholesterol (LDL-C) and increased triglycerides (TG) and has been defined as a separate risk factor for OA development. High cholesterol levels were associated with OA development in several clinical studies[40 41]. However, others have reported inconsistent findings with no association of dyslipidemia and OA development[57]. Statins are a class of drugs that are commonly prescribed to reduce systemic cholesterol levels. Several clinical studies have shown a protective effect of statin use on OA development[76, 80], while these findings could not be replicated by others[82]. Recently, monoclonal antibodies against proprotein convertase subtilisin/kexin 9 (PCSK9) were developed, which are highly effective in lowering systemic cholesterol levels in both mice and humans[100, 101]. Consistent with clinical studies, the use of cholesterol-lowering therapies have shown divergent effects in animal models. Gierman *et al*. have shown that atorvastatin as treatment reduced spontaneous OA pathology induced by a cholesterol-supplemented Western type diet (WTD) in ApoE3*Leiden.CETP (E3L.CETP) mice[47], a well-established mouse model for hyperlipidemia as they respond to lipid-lowering therapies in a human-like manner[132, 137-139]. In a recent study, however, we were not able to demonstrate these beneficial effects of novel cholesterol-lowering treatment on spontaneous OA development in E3L.CETP mice[140]. These inconsistent results imply that, next to high cholesterol levels, other mechanisms are involved in diet-induced OA pathology.

Over the last decades, the role of joint inflammation in the progression of OA has become increasingly recognized. The transformation of LDL into oxLDL by reactive oxygen species (ROS) that are produced in the joint under the influence of inflammatory factors, could be a mechanism associated with cholesterol-associated OA pathology. Similar to macrophages in atherosclerotic plaques, macrophages residing in the synovium can internalize and accumulate oxLDL, leading to an increased production of cytokines and matrix-degrading enzymes. Next to systemic lipid disturbance, local lipid dysregulation in the synovium has been shown to contribute to OA development[141].

In the recent study, we did not observe beneficial effects of intensive cholesterol-lowering treatments on spontaneous OA development in E3L.CETP mice in which joint inflammation was only minor[140]. We postulated that in the presence of local inflammation caused by a joint lesion cholesterol-lowering therapies may ameliorate development of OA pathology. To study that we used the collagenase-induced OA (CiOA) model, which is an injury-induced OA model with a strong local inflammatory response within the joint. In this study we determined whether high-intensive cholesterol-lowering treatment using a combination of atorvastatin and the PCSK9 inhibitor alirocumab can ameliorate OA development in WTD-fed E3L.CETP mice during CiOA.

MATERIALS AND METHODS

Animals and induction of collagenase-induced OA

Female E3L.CETP mice were obtained from the in-house breeding of TNO Leiden. E3L.CETP mice are an acknowledged model for dyslipidemia and show human-like responses to cholesterol-lowering therapies compared to other mouse strains and mice in a WT background[132, 137-139]. Female E3L.CETP mice were used since they are more susceptible to cholesterol-supplemented diets and develop more pronounced atherosclerosis due to higher systemic cholesterol and triglyceride levels[142]. Group sizes were calculated to be able to detect differences between groups with a power of 0.8 and a level of significance of 0.05 using Russ Lenth's sample size calculator (version 1.76) for the primary readout measure cartilage damage tested with a t-test, considering a change of 35% biologically relevant (detectable change of 0.35) with an expected SD of 0.31. This resulted in a total number of 14 mice per group. In separate groups of mice synovium was collected to study the local concentration of cytokines. Using a power of 0.8 and a level of significance of 0.05, with an expected SD of 0.2 and a decrease of 30% resulted in 8 mice per group for cytokine measurements. At the start of the study, mice were randomly assigned to an experimental group using an online randomizer using their individual tattooed number. Mice were housed with 6 animals in regular cages and received food and water *ad libitum*. 12-14 week old mice (n=14 mice per group) were switched to a cholesterol-supplemented Western-type Diet (15%w/w cacao butter, 40.5%w/w sucrose, + 0.3% w/w cholesterol). Mice were weighed regularly to monitor the response to the diet and cholesterol-lowering treatment. For measurements of systemic cholesterol and triglyceride levels, blood was collected via tail vein punction (weeks: 0, 3, 6, 7, 9, 12). Three weeks after the start of the diet, half of the mice received cholesterol-lowering treatment consisting of atorvastatin (0.008% mixed in the diet, about 7 mg/kg/d) and weekly subcutaneous injection of the anti-PCSK9 antibody alirocumab (10 mg/kg/week), which were shown to be effective concentrations in previous studies (**Figure 1A**)[102, 140]. Subcutaneous injections of saline were used as a control for the anti-PCSK9 treatment. Three weeks after the start of the treatment, collagenase-induced OA (CiOA) was induced via two intra-articular injections of bacterial collagenase (1 unit) into the right knee joint on day 0 and day 2 of the experiment. Mice were sacrificed on day 7 and 42 of collagenase-induced OA to study both early and late effects during CiOA. Knee joints were collected either for histological analysis

or collection of synovial RNA and washouts. Serum samples were collected for cytokine measurements. All animal studies were approved by the local ethics committees (Nijmegen, the Netherlands) and were performed according to the related codes of practice (CCD project number: 2018-0002).

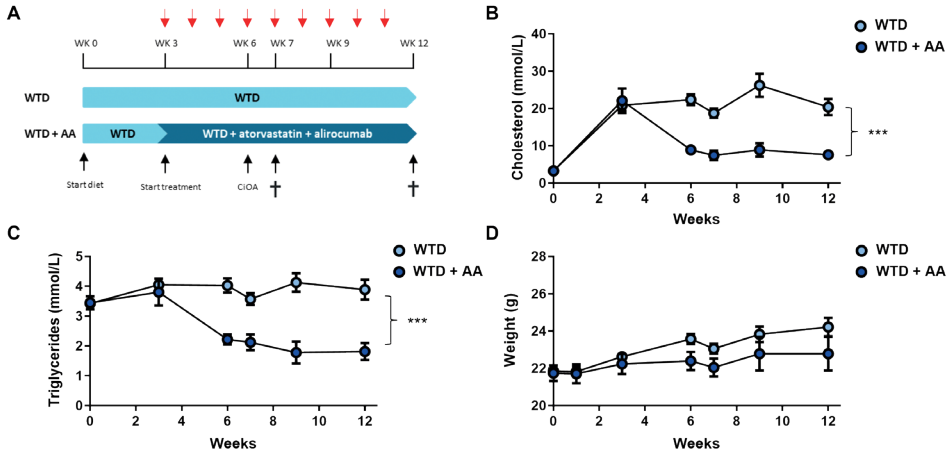

Figure 1. Cholesterol-lowering treatments strongly reduce diet-induced dyslipidemia during experimental OA.

Female E3L.CETP mice were fed a cholesterol-supplemented WTD for 3 weeks, after which half of the mice received cholesterol-lowering treatment consisting of atorvastatin and alirocumab. 3 weeks after the start of the treatments, CiOA was induced. Mice were sacrificed 7 and 42 day after the induction of CiOA. Serum cholesterol, triglycerides and weight of the mice was monitored throughout the study and blood was collected via tail vein punction (weeks: 0, 3, 6, 7, 9, 12). **(A)** Schematic overview of the experimental set-up of the experiment. **(B)** Cholesterol-lowering therapies strongly reduced systemic cholesterol levels compared to mice fed a cholesterol-supplemented WTD alone. **(C)** Cholesterol-lowering treatment reduced serum triglyceride levels compared to mice fed a WTD. **(D)** Mice that received cholesterol-lowering treatments showed a non-significantly reduced weight gain compared to mice fed a cholesterol-supplemented WTD alone. *, $P < 0.05$, ****, $P < 0.0001$. *N= 36 mice per group until week 7, N= 14 mice per group from week 7 to end point. Red arrows indicate weekly injections with alirocumab. AA = atorvastatin + alirocumab. Statistical are derived from post-treatment time points (from week six) and. Figures show data expressed as mean ± 95% confidence intervals.*

Determination of serum cholesterol and triglyceride levels

Serum cholesterol and triglyceride levels were monitored throughout the study. Peripheral blood was collected via tail vein punction at several time points throughout the study. Total cholesterol (TC) and triglyceride levels were determined at several time points throughout the study (**Figure 1B-C**) using a colorimetric enzymatic assay (Roche Diagnostics, Basel, Switzerland) according to manufacturer's instructions.

Histological processing and analysis

Murine knee joints were fixed in 4% formaldehyde and decalcified using 5% formic acid for 7 days. Subsequently, joints were embedded in paraffin and cut in 7µm coronal sections. Sections were stained using Safranin-O/fast green (SafO) or Haematoxylin/Eosin (H&E) for histological analysis. Mice with dislocations were excluded from histological analysis (day 7: 4, day 42; 5). Synovial inflammation was scored arbitrarily using H&E stained sections and averaged for three sections per joint with a scoring range from 0-3 (0 = no inflammation, 1 = mild inflammation, 2 = moderate inflammation; 3 = severe inflammation). Cell layers of the synovial lining were counted on both the lateral and medial side of the joint and were averaged per three H&E stained sections. Cartilage damage was quantified in SafO stained sections using a more detailed version of the OARSI scoring system adapted for mice, as described previously (0 = no damage, 30 = maximal damage)[118, 143]. Five sections at different depths in the knee joint were scored and averaged. Several locations throughout the whole joint were scored for presence of ectopic bone and the maturation stage on both the medial and lateral side of the joint[120]. Ectopic bone margins were manually traced by a researcher using the Leica Application suite image analysis software (Leica Microsystems, Rijswijk, the Netherlands) in three sections per joint and the surface area was averaged[120]. Subchondral bone scores (subchondral bone plate thickening, increased bone mass) were determined in SafO stained section using a scoring system ranging from 0-3 (0 is normal, 1 = mild, 2 = moderate, and 3 = severe)[144, 145]. Five sections were scored and averaged per joint. For all histological analyses, sections were scored in a blinded fashion.

Immunohistochemical analysis

For immunohistochemical analysis, knee joint sections were deparaffinized and endogenous peroxidase was blocked with H_2O_2 in methanol. Antigen retrieval was performed in 10 mM citrate buffer pH 6.0. Sections were stained with polyclonal antibodies against S100A9[146] or non-relevant rabbit IgG control (R&D Systems, Minneapolis, USA). Biotinylated anti-rabbit IgG was used as a secondary antibody. Subsequently, sections were stained with avidin-streptavidin-peroxidase (Elite kit, Vector Laboratories, Burlingame, USA) and diaminobenzidine (Sigma-Aldrich, St. Louis, USA) was used for visualization of peroxidase staining. Counterstaining was performed using hematoxylin (Merck, Kenilworth, USA).

Synovial wash-outs and cytokine measurements

Synovium was collected in a standardized manner using synovial punches from left and right knee joints seven days after the first injection of collagenase. Synovium was placed in 200 µl RPMI medium supplemented with penicillin-streptomycin and 0.1% bovine serum albumin (BSA) for 2 hours at room temperature and medium was collected to quantify protein levels of inflammatory cytokines. The levels of cytokines produced were corrected for the weight of the synovial punches. S100A8/A9 complexes were measured in the wash-outs or serum of mice using sandwich enzyme-linked immunosorbent assay (ELISA) as described previously[121]. KC and MCP-1 levels were measured in washouts and serum with Luminex technology using magnetic milliplex beads (Bio-Rad, Veenendaal, the Netherlands)

according to the manufacturer's protocol. Protein levels of IL-1β, IL-6 and IL-10 were below the detection limit. Concentrations of secreted cytokines were corrected for the weight of the synovial explant.

Statistical analysis

Statistical analysis was performed using GraphPad Prism version 9.0 and SPSS version 27. Normality was visualized using histograms and Q-Q plots using SPSS. Differences between groups were analyzed using a t-test. For the protein levels in washouts and serum of S100A8/A9, MCP-1 and KC, the nonparametric Mann-Whitney U was used for comparisons of the control group with the treatment group. To determine the relation between cholesterol, triglycerides and weight with the treatment and time, we performed multivariate generalized linear model analysis using SPSS from the start of the treatment (week 6). Systemic cholesterol and triglyceride levels and weight were included as dependent variables, and the treatment and time (treatment duration) were included as covariate including an intercept to account for clustering of measurements. Significance levels represent the interaction of the treatment effect over time. We performed multivariate generalized linear model analysis using SPSS to analyze ectopic bone formation. Ectopic bone formation quantified at the several locations were included as the dependent variable, and the treatment was included as covariate including an intercept to account for clustering of measurements. *P*-values below 0.05 were considered significant. Results are expressed as individual data points with mean ± 95% confidence intervals.

RESULTS

Cholesterol-lowering treatment attenuates dyslipidemia in E3L.CETP mice fed a cholesterol-supplemented WTD

E3L.CETP mice were fed a cholesterol-supplemented WTD. After 3 weeks of WTD-feeding, half of the mice received cholesterol-lowering treatment, consisting of a combination of atorvastatin and alirocumab. Three weeks after starting the cholesterol-lowering treatment, CiOA was induced in the right knee joints of the mice (**Figure 1A**). To determine the efficacy of the cholesterol-lowering treatment, serum levels of systemic cholesterol and triglyceride levels were monitored throughout the study. Three weeks of WTD feeding strongly increased systemic cholesterol levels (18.2 mmol/L increase) (**Figure 1B**). Cholesterol-lowering treatment strongly attenuated diet-induced dyslipidemia, demonstrated by a significant reduction of systemic cholesterol (on average by 13.1 mmol/L) and triglyceride (on average 1.7 mmol/L) levels over the course of the study (**Figure 1B, C**). To determine the relation of the cholesterol-lowering treatment with systemic cholesterol and triglyceride levels and weight, we performed a multivariate linear model analysis. The results showed a significant reduction in cholesterol and triglyceride levels, which was dependent on the cholesterol-lowering treatment (TC: $P = 1.21E-72$, 95% CI: -13.9 to -12.1; TG: $P = 1.33E-26$, 95% CI: -2.1to -1.5). The treatment resulted in a reduction in weight over the course of the study ($P = 1.1E-5$, 95% CI: -1.5 to -0.6 g) (**Figure 1D**).

Reduction of systemic cholesterol levels reduces early stage joint inflammation in dyslipidemic E3L.CETP mice

To assess whether cholesterol-lowering therapy could ameliorate local joint inflammation, we determined the inflammatory state of the synovium 7 days after the induction of CiOA. We observed that cholesterol-lowering treatment resulted in a significant reduction of synovial inflammation compared to mice fed a WTD alone ($P = 0.008$, WTD: 1.88 (95% CI: 1.4 to 2.3); WTD + AA: 1.2 (95% CI: 0.8 to 1.5) (**Figure 2A**). Quantification of cell layers in the synovial lining showed that cholesterol-lowering treatment significantly reduced lining thickness compared to mice fed a cholesterol-supplemented WTD alone, indicating reduced cellularity in the synovial lining ($P = 0.009$, WTD: 3.79 (95% CI: 3.0 to 4.6), WTD + AA: 2.6 (95% CI: 2.1 to 3.2) (**Figure 2B, C**). To examine the inflammatory state of the synovium in more detail, we determined gene expression and measured protein levels of several inflammatory cytokines (S100A8, IL-1β, IL-6, IL-10) and chemokines (KC, MCP-1) which are produced by the synovium in washouts of synovial explants. Gene expression levels in synovial tissue showed no significant differences between both groups (**Supplementary Figure 1A-F**). S100A8/A9, MCP-1 and KC levels were determined in washouts of synovial explants. We observed no significant differences in protein levels between both groups (**Figure 2D-F**). Protein levels of IL-1β, IL-6 and IL-10 were below the detection limit. Immunohistochemical staining for S100A9, an alarmin produced by activated macrophages, showed a strong staining in both groups in the synovial tissue (**Figure 2G**). We additionally quantified systemic protein levels of S100A8/A9, MCP-1 and KC in serum, where a strong reduction was observed in mice that received cholesterol-lowering treatment compared to mice fed a cholesterol-supplemented WTD only (S100A8/A9: -33.0 ng/ml (95% CI of difference: -46.0 to -12.0), U = 97.5, $P = 0.0005$; MCP-1: -242.4 pg/ml (95% CI of difference: -398.3 to -152.1), U = 12.5, $P = 2.8\text{x}10^{\wedge -10}$; KC: -48.1 pg/ml (95% CI of difference: -66.8 to -30.4), U = 19.5, $P = 2.1\text{x}10^{\wedge -9}$, (**Supplementary Figure 2A-C**). Protein levels of IL-1β, IL-6 and IL-10 were below the detection limit.

Cholesterol-lowering treatment does not reduce other early stage OA pathology in WTD-fed mice

Next, we determined whether the cholesterol-lowering treatment reduced OA pathology during early-stage CiOA that is characterized by superficial cartilage degeneration and ectopic bone formation along the joint margins. Cartilage damage was not decreased by a reduction of systemic cholesterol levels 7 days after the induction of CiOA (**Figure 3A, B**). We additionally determined the subchondral bone sclerosis scores. Similar to cartilage damage, subchondral bone sclerosis scores were comparable between both groups during early stage OA (**Figure 3C**). Early stage ectopic bone formation was mostly observed at the medial side of the joint. We investigated if the size of early ectopic bone formation was affected by the cholesterol-lowering treatment at several locations. The size of ectopic bone formation was not reduced by cholesterol-lowering treatment compared to mice fed a WTD alone (**Figure 3D-F**).

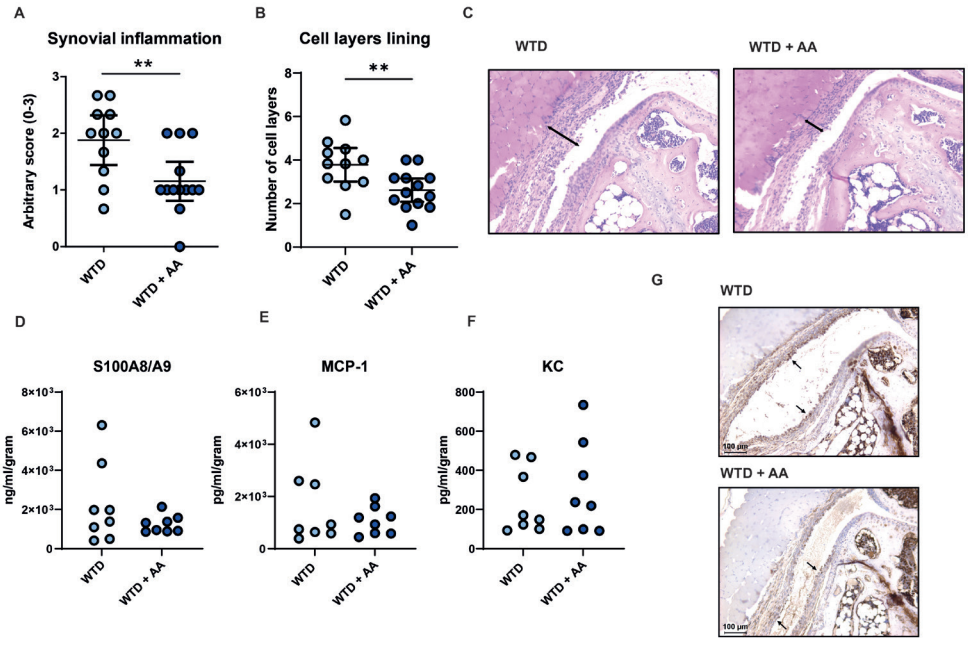

Figure 2. Cholesterol-lowering reduces synovial inflammation during early-stage OA in WTD-fed mice

Female E3L.CETP mice were fed a cholesterol-supplemented WTD for 3 weeks, after which half of the mice received cholesterol-lowering treatment. Mice were sacrificed 7 days after the induction of CiOA. **(A)** Scoring of synovial inflammation showed a significant reduction after cholesterol-lowering treatment. **(B)** Quantification of the cell layers in the synovial lining showed a significant reduction in lining thickness in mice that received cholesterol-lowering treatment (*P*=0.009). **(C)** Representative pictures of synovial inflammation. Protein levels of S100A8/A9, MCP-1 and KC were measured in synovial washouts and corrected for weight of the synovial explants (n=8). No significant differences were observed in protein levels of **(D)** S100A8/A9, **(E)** MCP-1 and **(F)** KC in mice that received cholesterol-lowering treatment compared to WTD-fed mice. Protein levels of IL-1β, IL-6 and IL-10 were below the detection limit. **(G)** Representative pictures of S100A9 staining, arrows indicate positive staining area. ***, $p < 0.01$, AA = atorvastatin + alirocumab. N=11-13 mice per group. Results are expressed as mean ± 95% confidence intervals.*

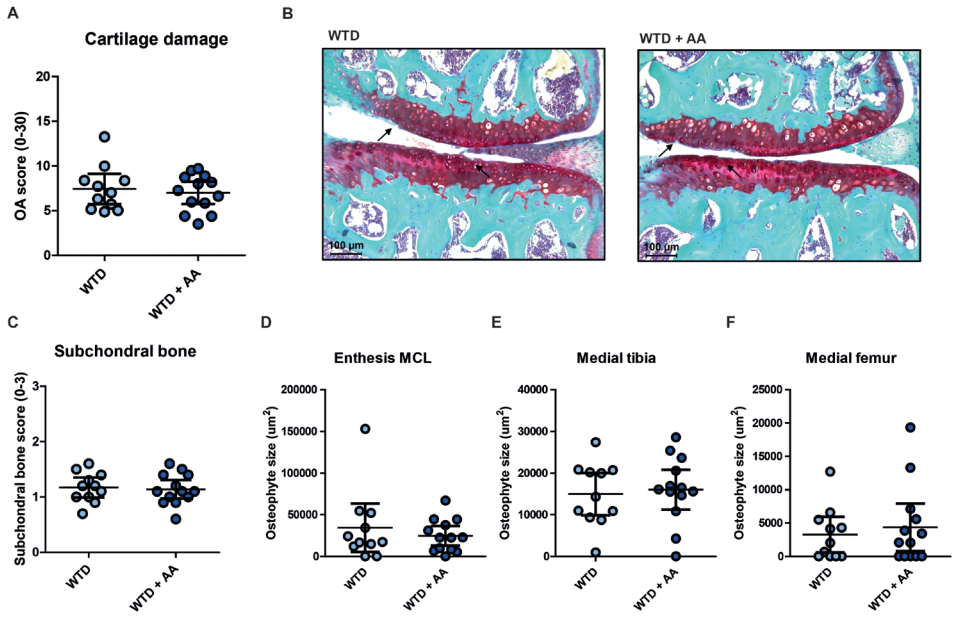

Figure 3. Cholesterol-lowering treatment does not reduce early stage OA pathology

Female E3L.CETP mice were fed a cholesterol-supplemented WTD for 3 weeks, after which half of the mice received cholesterol-lowering treatment. 3 weeks after the start of the treatments, CiOA was induced. Mice were sacrificed 7 days after the induction of CiOA **(A)** Cartilage damage was quantified on SafO stained sections with a score ranging from 0-30. 7 days after the induction of CiOA, no differences were observed in cartilage damage between both groups. **(B)** Representative pictures of cartilage damage, arrows indicate damages areas. **(C)** Subchondral bone sclerosis was scored using a graded scoring system ranging from 0-3. No differences were observed after cholesterol-lowering treatment compared to mice fed a cholesterol-supplemented WTD alone. **(D-F)** The size of ectopic bone formation was determined at the medial side of the joint in SafO stained sections. No differences were observed in ectopic bone size after cholesterol-lowering treatment. *AA = atorvastatin + alirocumab. N=11-13 mice per group. Results are expressed as mean ± 95% confidence intervals.*

Lowering of systemic cholesterol levels does not reduce end stage OA pathology in mice fed a cholesterol-supplemented WTD

To investigate if cholesterol-lowering therapies reduced end-stage OA pathology, we determined synovial inflammation, cartilage damage, subchondral bone sclerosis score and ectopic bone formation 42 days after the induction of CiOA. In contrast to day 7, the observed reduction on synovial inflammation was no longer significant after cholesterol-lowering treatment at end stage (**Figure 4A, B**). In addition, no reduction in both cartilage damage ($P = 0.09$, WTD: 17.5 (95% C: 15.5 to 19.5), WTD + AA: 14.9 (95% CI: 12.2 to 17.6) (**Figure 5A, C**) and subchondral bone sclerosis scores (WTD: 2.6 (95% CI: 2.2 to 2.9), WTD + AA: 2.1 (95% CI: 1.5 to 2.8) (**Figure 5B**) was found in mice that received cholesterol-lowering treatment compared to mice fed a WTD alone. We next determined whether cholesterol-lowering treatment could

reduce ectopic bone formation, multiple sites on the lateral and the medial side of the joint were scored for ectopic bone formation[120]. Ectopic bone was mainly observed at the joint margins and collateral ligaments (**Figure 5D**). We did not observe a reduction in ectopic bone size after cholesterol-lowering treatment at the quantified locations in the joint (MT: $P = 0.74$, (95% CI: -47832.2 to 66602.7); MF: $P = 0.78$, (95% CI: -38056.8 to 28122.1); enthesis: $P = 0.21$, (95% CI: --195498.5 to 44441.8); MCL: $P = 0.31$, (95% CI: --317240.9 to 105867.9) (**Figure 5E-H**). The total number of osteophytes (**Supplementary Figure 3A**) and their maturation stage remained similar between both groups (**Supplementary Figure 3B-F**).

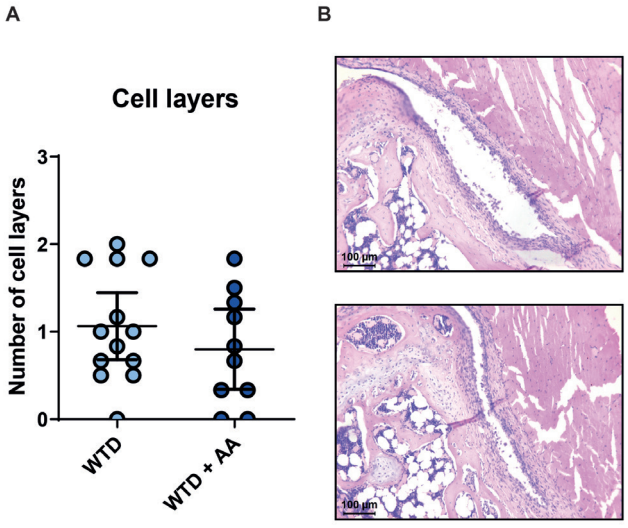

Figure 4. Cholesterol-lowering treatment does not reduce synovial inflammation at end stage collagenase-induced OA

Female E3L.CETP mice were fed a cholesterol-supplemented WTD for 3 weeks, after which half of the mice received cholesterol-lowering treatment. 3 weeks after the start of the treatments, CiOA was induced. Mice were sacrificed 42 days after the induction of CiOA. **(A)** Synovial inflammation was determined on both the medial and the lateral side of the joint. No significant differences were observed between both groups. **(B)** Representative pictures of synovial inflammation. *AA = atorvastatin + alirocumab. 10-13 mice per group. Results are expressed as mean ± 95% confidence intervals.*

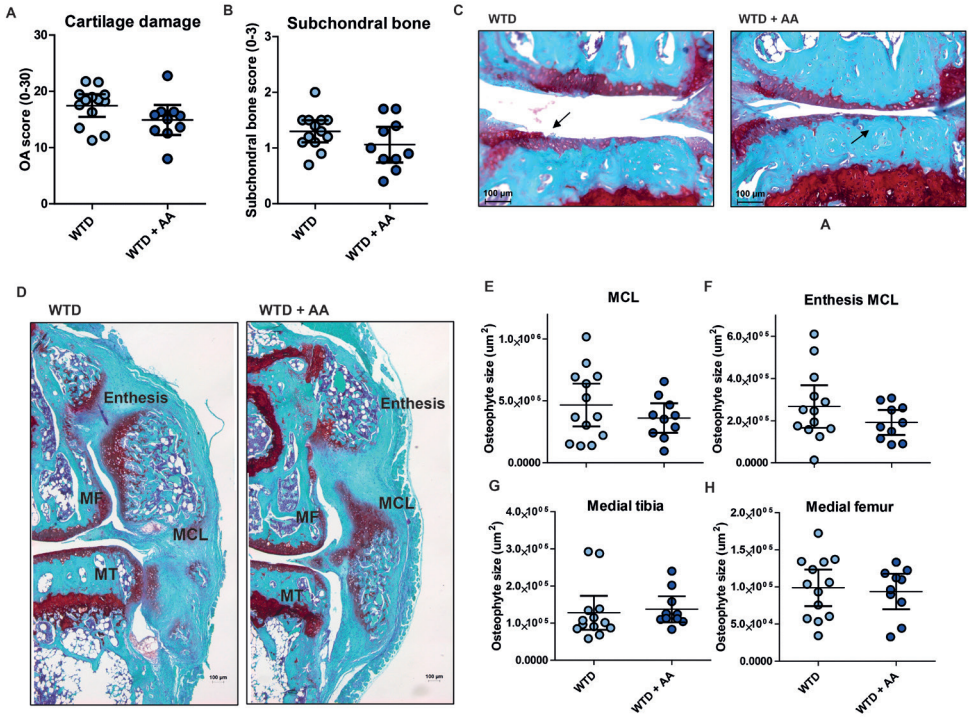

Figure 5. Cholesterol-lowering treatment does not reduce OA pathology at end stage OA

Female E3L.CETP mice were fed a cholesterol-supplemented WTD for 3 weeks, after which half of the mice received cholesterol-lowering treatment. 3 weeks after the start of the treatments, CiOA was induced. Mice were sacrificed 42 days after the induction of CiOA. **(A)** Cartilage damage was quantified on SafO stained sections with a score ranging from 0-30. 42 days after the induction of CiOA, no significant differences were observed in cartilage damage between both groups. **(B)** Subchondral bone sclerosis was scored using a graded scoring system ranging from 0-3. No differences were observed after cholesterol-lowering treatment compared to mice fed a cholesterol-supplemented WTD alone. **(C)** Representative pictures showing cartilage damage in SafO stained sections, arrows indicate sites of cartilage damages. **(D)** Representative pictures of ectopic bone formation and the locations scored. **(E-F)** The size of ectopic bone formation was manually traced using the Leica Application suite image analysis software at several sites of the joint in SafO stained sections. The size of ectopic bone formation was similar between both groups. *MT = medial tibia, MF = medial femur, MCL = medial collateral ligament. AA = atorvastatin + alirocumab. 10-13 mice per group. Results are expressed as mean ± 95% confidence intervals.*

DISCUSSION

The last decade, the association between MetS and OA development has become increasingly recognized. However, the exact role of cholesterol and cholesterol-lowering therapies in OA development has remained elusive. In this study, we used intensive cholesterol-lowering treatment consisting of atorvastatin and the novel anti-PCSK9 antibody alirocumab in E3L.CETP mice . We show that cholesterol-lowering treatment significantly reduces systemic levels of pro-inflammatory cytokines and synovial inflammation and lining thickness during early stage collagenase-induced OA, but this is not sufficient to ameliorate end stage pathology.

Clinical studies have reported contradictory findings regarding statin use and OA development[76, 80-83]. A recent meta-analysis by Wang *et al*. even showed no association between the use of statins and a reduced risk of OA incidence or progression[147]. In our study, we used a combination of atorvastatin and the anti-PCSK9 antibody alirocumab to strongly reduce cholesterol levels. In atherosclerosis, this combination treatment reduces the residual risk observed in cardiovascular patients that receive statin therapy only[104]. Even though we used high-intensive cholesterol-lowering therapies, we did not observe reduced OA pathology at end-stage CiOA. The contradictory findings regarding the effects of cholesterol-lowering treatment on OA pathology indicate that additional mechanisms are likely involved in cholesterol-associated OA pathology.

Previously, in a spontaneous OA model where similar cholesterol-lowering therapies were used, we did not observe a reduction in OA pathology while the development of atherosclerosis was strongly reduced[102, 140]. Previous studies in our lab showed that a cholesterol-supplemented diet increased synovial activation and ectopic bone formation, but not cartilage degeneration in a CiOA model[36, 49]. Others have shown that a high fat diet alone did not lead to joint pathology, but a combination with a secondary trigger such as DMM surgery[51] or groove surgery[50] was needed to induce cartilage degeneration. Therefore, we hypothesized that cholesterol-lowering therapies would be of benefit in an OA model with a substantial joint inflammation such as CiOA. Treatment was started before the induction of CiOA to ensure that systemic cholesterol levels were reduced before the induction of joint inflammation. Even though we observed a decrease in synovial inflammation and lining thickness and in systemic levels of inflammatory mediators, no significant differences were observed for ectopic bone formation and cartilage degeneration at end-stage OA. In several animal studies, a high-fat diet resulted in increased cartilage damage[47, 48, 133]. However, some studies have shown that a WTD increased macrophage infiltration[52] and inflammation in the synovium, while no effects on cartilage damage were observed[50, 52]. These results may suggest that high cholesterol mainly exacerbates early stage changes of inflammation which is insufficient to reduce end stage pathology. The latter could explain why we mainly observed anti-inflammatory effects of the cholesterol-lowering treatment during the early phases of our study.

There are several mechanisms that could explain why cholesterol-lowering alone was insufficient to significantly reduce end stage pathology. Firstly, it has been shown that lipoproteins, such as LDL and oxLDL, can induce trained immunity by metabolic and epigenetic reprogramming of monocytes and their myeloid progenitor cells in the bone marrow[148, 149]. Trained immunity increases the inflammatory response of monocytes and macrophages to secondary stimuli, such as Toll-like receptor (TLR) ligands like LPS or S100A8/A9[150]. In addition, Christ *et al*. have shown that immune training in monocytes was dependent on the NLRP3 inflammasome/IL-1β pathway[150]. In addition, Bekkering *et al*. have shown that trained immunity cannot be reversed by statin therapy in patients with familiar hypercholesterolemia[151]. The authors hypothesized that these results could explain why a residual risk is observed in cardiovascular patients even after successful reduction of cholesterol levels after statin treatment[151]. In a recent study, we showed that cholesterol-lowering treatment combined with inhibition of IL-1β could reduce synovial thickening and cartilage degeneration in dyslipidemic E3L mice (unpublished data). Possibly, cholesterol-lowering therapies should be supplemented with an anti-inflammatory treatment, such as inhibition of IL-1β, to successfully ameliorate diet-induced OA pathology. An overview of possible therapeutic strategies to target innate immune training has been published by Mulder *et al*[152].

A further explanation may be the contribution of glucose which levels are often increased when mice are fed a WTD which contain high amounts of fat and sugars. Similar to (ox)LDL, glucose is able to induce the production of inflammatory mediators in macrophages[153]. Moreover, it has been shown that induction of trained immunity in monocytes after oxLDL stimulation upregulates glycolytic metabolism[154]. OxLDL-induced trained immunity was increased in a high glucose environment, indicating that high glucose availability amplifies the pro-inflammatory effects of oxLDL-induced trained immunity[154]. In addition, these authors showed that trained immunity could be prevented by pharmacological inhibition of glycolysis[154]. It would be of interest to investigate if a combination of cholesterol- and glucose-lowering therapies can successfully reduce diet-induced OA pathology.

A limitation of our study is the absence of a chow control group. Even though we were able to investigate the effects of intensive cholesterol-lowering treatment on the development of OA, we were not able to determine the effect of the cholesterol-supplemented WTD alone on OA pathology in this study. However, previous studies have shown that a cholesterol-supplemented WTD contributes to the development of cartilage pathology in E3L.CETP mice[47, 155]. Another limitation is the use of only females in this study. Although in general male mice develop more severe OA pathology than female mice, we chose to use female mice in the current study as female E3L mice are more responsive to cholesterol containing diets by having higher cholesterol and TG levels compared to male mice.

Taken together, our study shows that intensive cholesterol-lowering treatment using a combination of atorvastatin and anti-PCSK9 antibody alirocumab reduces early stage synovial inflammation but this is insufficient to significantly reduce end stage pathology.

AUTHOR CONTRIBUTIONS

YG, ABB, EJP, HMGP, PLEML and MHJB have designed the study. YG, IDC, BW, MH, and AS have carried out experimental procedures and acquired the data. YG has been the primary person responsible for writing the manuscript. ABB, PLEML, PMK, NNLK, IDC, TV, JR, EJP, HMGP and MHJB were involved in drafting the work or revising it critically for important intellectual content. All authors approved the final version to be published.

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ROLE OF THE FUNDING SOURCE

The funding sources had no role in study design, in collection, analysis or interpretation of data, or in writing the manuscript and decision to submit the manuscript.

CONFLICTS OF INTEREST

The authors declare no competing interests.

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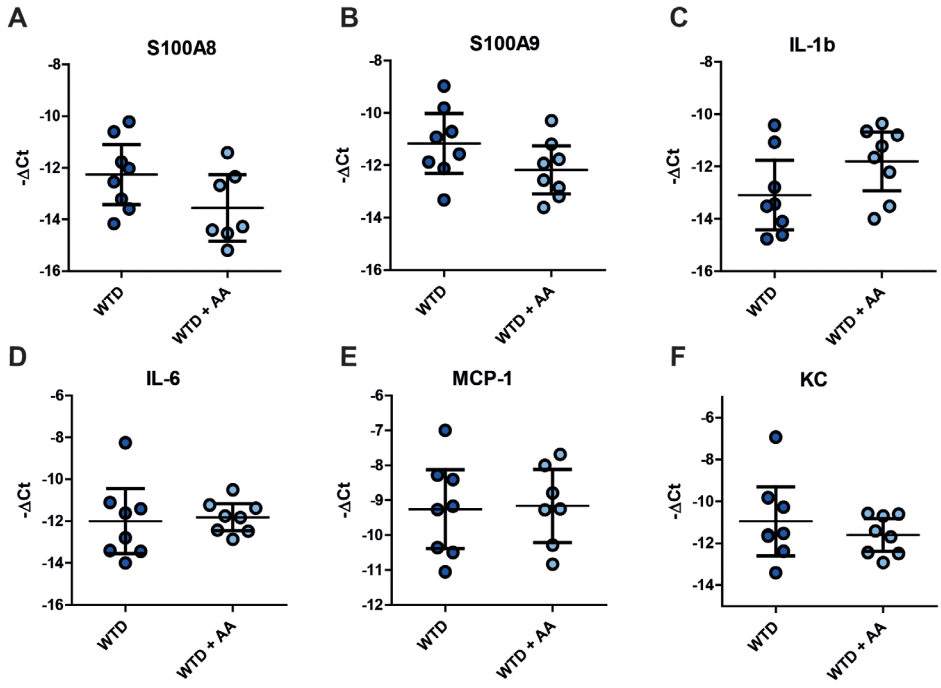
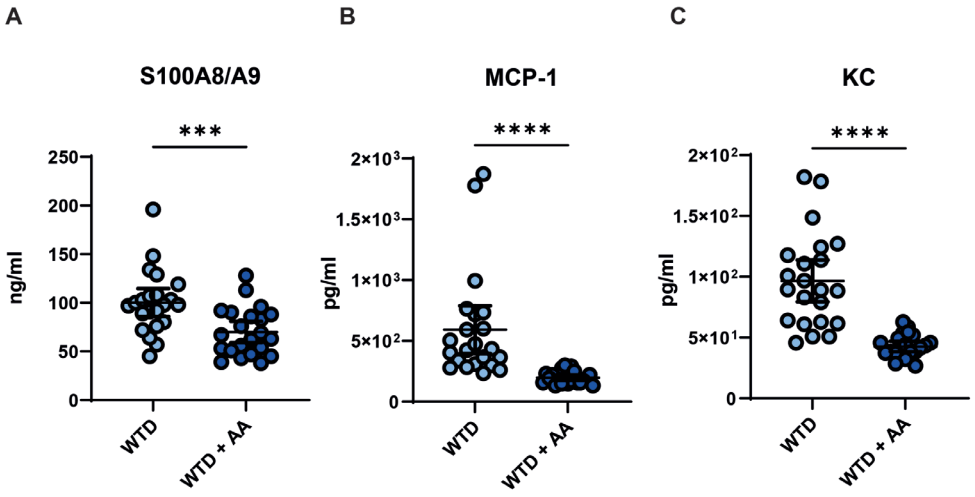
SUPPLEMENTARY MATERIAL



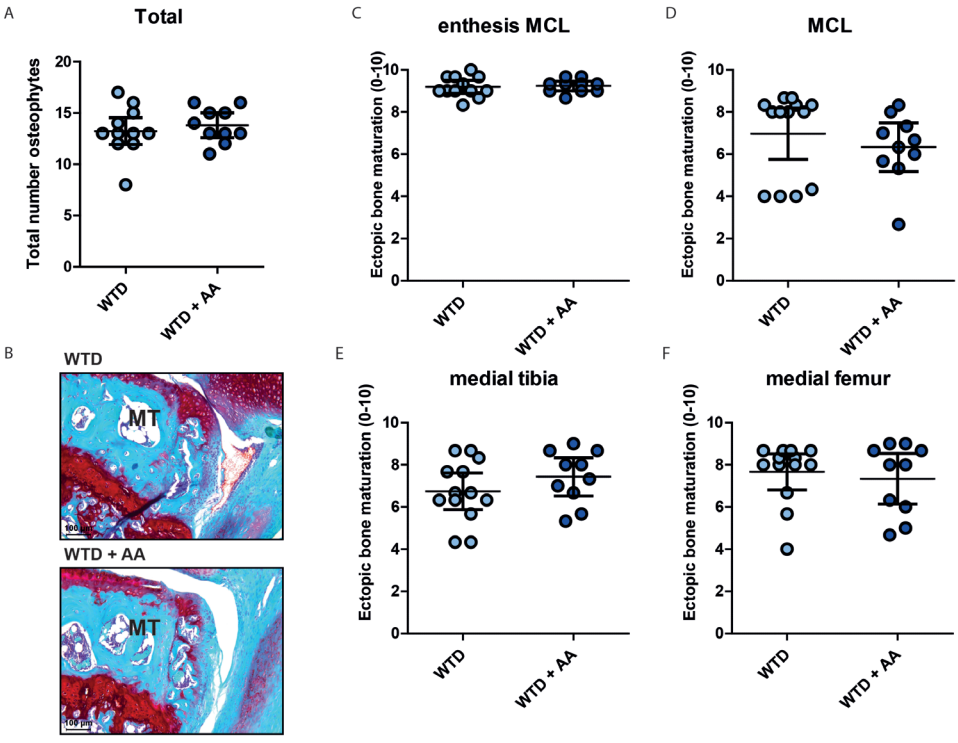
Supplementary Figure 1. Cholesterol-lowering treatment does not reduce gene expression of several pro-inflammatory cytokines in synovial tissue

Female E3L.CETP mice were fed a cholesterol-supplemented WTD for 3 weeks, after which half of the mice received cholesterol-lowering treatment. 3 weeks after the start of the treatments, CiOA was induced via two injections of collagenase into the right knee joint of the mice. Synovial punches (Ø 3 mm) were collected 7 days after the induction of CiOA. RNA was isolated using the RNeasy kit (Qiagen). Gene expression levels were determined using qPCR with specific primers and the SYBR Green Master Mix using the StepOnePlus RT-PCR System (Thermo Fisher Scientific). Expression levels are presented as –ΔCt, which is calculated by correcting for the household gene GAPDH. Primer sequences are provided in **Supplementary Table 1**. **(A-F)** Gene expression of S100A8, S100A9, IL-1b, IL6, MCP-1 and KC were not different between treatment groups. *AA = atorvastatin + alirocumab. Results are expressed as mean ± 95% confidence intervals.*



Supplementary Figure 2. Cholesterol-lowering treatment reduces serum levels of S100A8/A9, MCP-1 and KC in mice fed a cholesterol-supplemented WTD

Female E3L.CETP mice were fed a cholesterol-supplemented WTD for 3 weeks, after which half of the mice received cholesterol-lowering treatment. Protein levels of S100A8/A9, MCP-1 and KC were determined in serum 7 days after the induction of CiOA using Luminex. Protein levels of **(A)** S100A8/A9, **(B)** MCP-1 and **(C)** KC were significantly reduced in mice that received cholesterol-lowering treatment compared to mice fed a cholesterol-supplemented WTD alone.. Serum levels of IL-1β, IL-6 and IL-10 were below the detection limit. ***, $P < 0.001$, ****, $P < 0.0001$. *AA = atorvastatin + alirocumab. Results are expressed as mean ± 95% confidence intervals.*



Supplementary Figure 3. Cholesterol-lowering treatment does not reduce the number of osteophytes or their maturation stage during CiOA

Female E3L.CETP mice were fed a cholesterol-supplemented WTD for 3 weeks, after which half of the mice received cholesterol-lowering treatment. 3 weeks after the start of the treatments, CiOA was induced via two injections of collagenase into the right knee joint of the mice. **(A)** The total number of osteophytes were quantified at both the lateral and the medial side of the joint. No difference in the number of osteophytes was observed. **(B-F)** The maturation stage of several sites of ectopic bone formation was determined, ranging from chondrogenesis to the formation of mature bone (0 to 10). No differences in the maturation of osteophytes was observed after cholesterol-lowering treatment. *AA = atorvastatin + alirocumab. 10-13 mice per group. Results are expressed as mean ± 95% confidence intervals.*

Supplementary table 1

Gene	Forward primer 5' → 3'	Reverse primer 3' → 5'
S100A8	TGTCCTCAGTTTGTGCAGAATATAAAT	TTTATCACCATCGCAAGGAACTC
S100A9	GGCAAAGGCTGTGGGAAGT	CCATTGAGTAAGCCATTCCCTTTA
IL-1β	GGACAGAATATCAACCAACAAGTGATA	GTGTGCCGTCTTTCATTACACAG
IL-6	CAAGTCGGAGGCTTAATTACACATG	ATTGCCATTGCACAACTCTTTTCT
MCP-1	TTGGCTCAGCCAGATGCA	CCTACTCATTGGGATCATCTTGCT
KC	TGGCTGGGATTCACCTCAA	GAGTGTGGCTATGACTTCGGTTT

CHAPTER 5

A single dose of anti-IL-1β antibodies prevents Western diet-induced immune activation during early stage collagenase-induced osteoarthritis, but does not ameliorate end-stage pathology.

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ABSTRACT

Objective

Metabolic dysfunction can cause IL-1β mediated activation of the innate immune system, which could have important implications for the therapeutic efficacy of IL-1β neutralizing drugs as treatment for OA in the context of metabolic syndrome (MetS). In the present study, we investigated whether early treatment with a single dose of IL-1β blocking antibodies could prevent Western diet (WD) induced changes to systemic monocyte populations and their cytokine secretion profile and herewith modulate collagenase induced osteoarthritis (CiOA) pathology.

Methods

CiOA was induced in female C57Bl/6 mice fed either a standard diet (SD) or WD and treated with a single dose of either polyclonal anti-IL-1β antibodies or control. Monocyte subsets and granulocytes in bone marrow and blood were analyzed with flow cytometry, and cytokine expression by bone marrow cells was analyzed using qPCR. Synovial cellularity, cartilage damage and osteophyte formation were assessed on histology.

Results

WD feeding of C57Bl/6 mice led to increased serum levels of low-density lipoprotein (LDL) and innate immune activation in the form of an increased number of $Ly6C^{high}$ cells in bone marrow and blood and increased cytokine expression of especially IL-6 and TNF-α by bone marrow cells. The increase in monocyte number and activity was ameliorated by anti-IL-1β treatment. However, anti-IL-1β treatment did not significantly affect synovial lining thickness, cartilage damage and osteophyte formation during WD feeding.

Conclusions

Single-dose systemic anti-IL-1β treatment prevented WD-induced innate immune activation during early stage CiOA in C57Bl/6 mice, but did not ameliorate joint pathology.

Keywords

Metabolic syndrome, Western Diet, LDL, inflammation, CiOA, anti-IL-1β

INTRODUCTION

Osteoarthritis (OA) is a painful degenerative joint disease causing a substantial decrease in quality of life of those affected. Current treatment options are limited to lifestyle interventions, analgesics and joint replacement, urging for novel therapeutic options. This urge is substantiated by an ever increasing prevalence of the metabolic syndrome (MetS), a cluster of metabolic risk factors strongly associated with OA[1-4]. MetS often results from an unhealthy lifestyle, including a diet high in saturated fats and sugar. Symptoms associated to MetS like dyslipidemia, hyperglycemia and low-grade systemic inflammation are thought to regulate OA pathology by fueling synovitis[3, 5]. Despite attracting great scientific interest in recent years, the exact molecular drivers of MetS-associated OA remain unclear, thereby making targeted treatment difficult.

Inflammation of the synovium, characterized by infiltration of immune cells and high cytokine expression, is present in more than 50% of OA patients and has been linked to joint pain and disease progression[6, 7]. IL-1β, secreted by OA synovia, contributes to pain transmission and stimulates catabolic activity of chondrocytes *in vitro*[8, 9]. Despite these findings suggesting a central role for IL-1β in OA pathology, clinical trials in which IL-1β was targeted to treat OA have produced mainly disappointing results. It has to be noted that patients included in most of these trials were not stratified based on disease phenotype and suffered from advanced OA, at which point cartilage damage could be irreversible[10-13]. It was therefore suggested that the strategy of OA therapy targeting inflammation should be focused on treating at an early disease stage, and that treatment should be tailored to fit the specific disease phenotype of each patient[14].

One of the major challenges is to identify early OA, since it generally develops over a long period stretching multiple decades, while major symptoms often only present themselves when disease progression is already at an advanced stage. Interestingly however, 50-80% of patients suffering ligamentous injury of the knee develop OA over time, which is almost always accompanied by inflammation of the joint at early stages[15]. Since timing of injury and therefore onset of disease processes is known in these post-traumatic OA patients, they might benefit from anti-inflammatory therapy during the period shortly after joint reconstruction surgery to suppress early OA processes. Moreover, anti-inflammatory drugs might be especially suitable for OA phenotypes with a central inflammatory component like MetS-associated OA. Underlining this, it was shown in the CANTOS trial – a large clinical trial involving 10.061 patients with previous myocardial infarction and high systemic inflammation – that treatment with monoclonal antibody against IL-1β canakinumab lowered the rate of total knee and hip replacements in these patients.

In murine models, a metabolic syndrome-like phenotype is often simulated either by feeding of a calorie-rich diet like a Western diet (WD) - a variation of a high fat diet (HFD) that aims to reproduce human high caloric fast food feeding - or by using mice with genetic deficiencies like *Ldlr*$^{-/-}$ and *Apoe*$^{-/-}$

mice, often combined with a HFD[16, 17]. These knockout mice develop extreme hypercholesterolemia and are commonly used as models for atherosclerosis of which pathology is driven by low-density lipoprotein (LDL) and monocyte-derived macrophages. In numerous surgically-induced OA models, HFD feeding worsened OA progression and was accompanied by changes in IL-1β levels[18]. Moreover, studies performed in our lab showed that WD feeding of wild type (WT), *Ldlr*$^{-/-}$ and *Apoe*$^{-/-}$ mice increased OA disease parameters, presumably via local formation of oxidized LDL (oxLDL)[19, 20]. In addition, A. Christ and colleagues showed that WD feeding of *Ldlr*$^{-/-}$ mice trained myeloid cells to become hyperresponsive to TLR4 ligands in a mechanism that was shown to be dependent on NLRP3 signaling, suggesting an IL-1β mediated process[21].

Monocytes and macrophages are the most predominant immune cells in OA synovium and contribute to pathology by producing pro-inflammatory and catabolic factors like IL-1β, TNF-α, S100A8/A9 and metalloproteinases (MMPs). Whereas locally proliferating resident macrophages are thought to have a more stable anti-inflammatory phenotype[22, 23], monocyte-derived macrophages have a more flexible and dynamic pro- or anti-inflammatory phenotype[24]. This suggests an important role for especially the monocyte-derived macrophages in disturbing the balance of synovial macrophage activity. In mice, two functionally distinct monocyte subsets are identified; patrolling Ly6C^{low} and pro-inflammatory Ly6C^{high} monocytes, the latter being the monocyte that preferentially infiltrates OA synovium[25].

Even though we previously showed that IL-1β is not important for the development of CiOA pathology[26], this might be different in the context of metabolic syndrome. WD-trained monocytes and monocyte-derived macrophages could worsen OA pathology when they encounter TLR4 ligands like S100A8/A9 in the synovium and react with production of radical oxygen species (ROS) and cytokines. In the present study, we therefore investigated whether early treatment with IL-1β blocking antibodies could prevent WD mediated aggravation of CiOA pathology and coupled this to systemic and local changes in monocyte phenotype and activation status.

METHODS

Collecting blood and OA conditioned medium of OA patients

Serum was collected from patients planned to undergo total knee replacement and LDL levels were determined by our diagnostics lab (n=16). After surgery, synovial explants of approximately 1x1x½ cm (2-8 per patient) were dissected and incubated at 37˚C for 24 hours in DMEM (+0.1% BSA). Levels of IL-1β, TNF-α, IL-10 and MCP-1 secreted in the conditioned medium were measured using Luminex (Bio-Rad). Levels of S100A8/A9 were measured using ELISA. Concentrations of secreted factors were normalized for wet tissue weight. All donors provided informed consent under institutional ethics committee approved protocols (2018-4876).

Animals

Female wild type (WT) C57BL6/J mice were obtained from Janvier. Mice were 12 weeks old at the time of induction of the disease model, were housed in open cages and received food and water *ad libitum*. Cages (5 mice/cage) were randomly allocated to timepoint day 7 or day 42 and were fed either standard diet (SD) or WD (15% cacao butter, 1% cholesterol, 0.05% NaCholate; 43% metabolized energy from fat, 42% from carbohydrates, 15% from protein (Sniff Spezialdiäten GmbH)), starting after 1 week of acclimatization,4 weeks before induction of the disease model and continuing until the end of the experiment. Mice were randomly allocated to control or anti-IL-1β treatment groups (split 2 to 3 or vice versa in each cage). Randomization of cage/mice-to-group and cage-to-location allocation was performed using Microsoft Excel. Animal studies were approved by the Institutional Review Board and were performed according to the related codes of practice (CCD project number: 2018-0002).

Collagenase-induced osteoarthritis

Collagenase-induced OA (CiOA) was induced by two time intra-articular injection of 1U bacterial collagenase type VII (Sigma-Aldrich) into the right knee cavity, on day 0 and 2 of CiOA. One day before the first collagenase injection, mice were given a single intraperitoneal injection of either normal rabbit serum (control) or rabbit anti-IL-1β serum (anti-IL-1β) according to the previously described protocol[27]. Investigators were aware of diet groups due to food coloring, but blinded for treatment groups from day 0 onwards. On day 7 and day 42 of CiOA, mice were weighed and sacrificed, after which right knee joints were isolated and processed for histological analysis. Total femurs from mice undergoing the same treatment protocol were isolated for flow cytometry analysis at day 7 of CiOA. Serum samples were obtained from all mice to determine lipoprotein and cytokine levels. The nuclear factor Kappa B (NF-κB) luciferase reporter assay was used to test the IL-1β neutralizing capacity of the rabbit anti-IL-1β serum the mice were treated with, and that of sera of treated mice.

Histological analysis of human synovium and murine knee joints

Human synovial explants were fixed in 4% buffered formalin and embedded in paraffin. Cellular infiltration and synovial lining hyperplasia were arbitrarily scored on hematoxylin & eosin (H&E) stained

sections (3 sections per tissue sample). Isolated murine knee joints were fixed in 4% buffered formalin and subsequently decalcified in formic acid and embedded in paraffin. Synovial lining thickness was scored on H&E stained sections using an arbitrary scoring system ranging from 0-3 (0 being no infiltration and 3 being the highest level of infiltration in this specific set of samples) (3 sections per knee joint). Cartilage damage in the tibiofemoral joint was scored using a modified OARSI score, in which the grade (severity of erosion) is multiplied by the stage (affected area), resulting in a score of 0-30 (five sections per knee joint)[28]. Osteophytes, enthesophytes and bone marrow was manually traced by an investigator, after which the surface area and percentage of white area was calculated by Leica-software (3 sections per knee joint). All histological analysis was performed blinded for the experimental condition. Both tibiofemoral and patellofemoral dislocations were *a priori* excluded from analysis.

Flow cytometry

Femur-derived bone marrow and blood was analyzed for monocyte subpopulations and granulocytes using the Gallios flow cytometer (Beckman Coulter). The antibodies and fluorophores that were used are summarized in **supplementary table 1**. First, doublets (based on FS-A/SS-W) and dead cells (positive for eFluor780 viability dye) were excluded. Monocytes were identified as $CD11b^{high}$ (B220/CD3/CD49b/Ter119)^{neg} $Ly6G^{low}$ SS^{int} $Ly6C^{pos}$ and further subdivided into $Ly6C^{high}$ and $Ly6C^{low}$ monocytes, whereas granulocytes were identified as $CD11b^{high}$ (B220/CD3/CD49b/Ter119)^{neg} $Ly6G^{high}$ SS^{high} $Ly6C^{int}$.

Quantitative PCR

Bone marrow cells were isolated from the murine femur by flushing with plain Roswell Park Memorial Institute (RPMI) medium using a needle and syringe and subsequently plated and stimulated for 6 hours. Supernatant was collected and cells were lysed in Trizol (MilliporeSigma) reagent for RNA isolation and quantitative PCR (qPCR) analysis. Gene expression was determined using qPCR with specific primers (primer sequences provided in **supplementary table S2**) and the Sybr Green Master Mix using the StepOnePlus RT-PCR System (Thermo Fisher Scientific). Between-group differences in expression levels are calculated based on -ΔCt (Ct(GAPDH) - Ct(gene of interest), and plotted as ΔΔCt (Average -ΔCt of control group (Gene of interest)) - -ΔCt(Gene of interest), with the control group being: standard diet, control treatment, unstimulated *in vitro*).

Statistical analysis

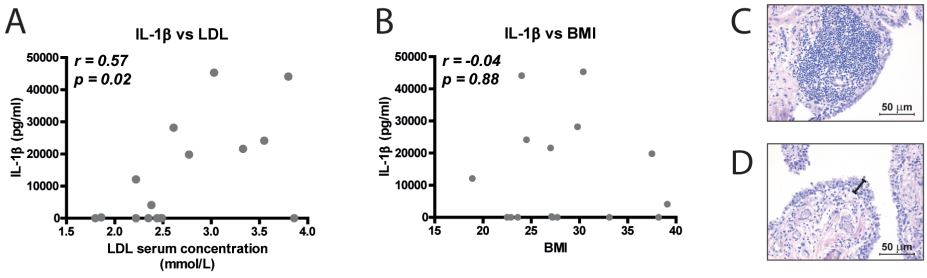
Group sizes were calculated to be able to detect differences between groups with a power of 0.8 and a level of significance of 0.05. We based our sample size calculation on the hypothesis that there would be a significant interaction between the independent variables diet (SD or WD) and treatment (control or anti-IL-1β) in determining the level of the dependent variable cartilage damage. Group sizes were calculated using Russ Lenth's sample size calculator (version 1.76) for the primary readout measure cartilage damage tested with a two-way ANOVA, considering a difference smaller than 3.2 as not biologically relevant. Assuming a mean of 10 in the control group (female C57Bl/6 mice, standard diet, untreated) at day 42 of CiOA, an SD of 2 and a detectable contrast of 3.2, this resulted in a total

required number of 10 mice per group. Two-way-ANOVA was used to test for interaction between diet and treatment (4 groups) using R studio (v4.0.3) for all histological outcome parameters, flow cytometry and qPCR, separately on both timepoints. In case of no significant interaction, two-way-ANOVA was repeated without testing for interaction to determine the significance of effects of diet and treatment. Tukey/HSD was used to determine between-group differences and 95% Confidence Intervals (CI's). All other statistical analyses were performed using Graphpad Prism v5.01. Pearson's test was used to test for correlations, after D'Agostino and Pearson omnibus was used to test for normality. *P*-values lower than 0.05 were considered significant. Results are expressed as mean values ± 95% CI's.

RESULTS

IL-1β secretion by human OA synovium is correlated to serum LDL levels, but not to synovial cell infiltration and lining hyperplasia.

To investigate if systemic LDL levels are associated with the inflammatory status of human OA synovium, we studied the correlation between serum LDL levels and concentrations of IL-1β, TNF-α, MCP-1, IL-10 and S100A8/A9 in the OA conditioned media. Interestingly, secretion levels of only IL-1β positively correlated with serum levels of LDL but did not significantly correlate with BMI, whereas MCP-1 on the other hand did not show a correlation with LDL while showing a strong correlation with BMI (Fig. 1A-B, E). No correlations between LDL or IL-1β with patient age or sex were found (patient characteristics shown in **supplementary figure 1**). None of the measured cytokines showed correlation with cellular infiltration and synovial hyperplasia (Fig. 1C-E). Serum IL-1β levels were too low for a reliable readout.



E

	LDL		BMI		Infiltration		Hyperplasia	
Protein	r	(95% CI)	r	(95% CI)	r	(95% CI)	r	(95% CI)
IL-1β	**0.57**	**(0.08 , 0.8)**	-0.04	(-0.6 , 0.4)	0.11	(-0.4 , 0.6)	-0.20	(-0.6 , 0.4)
TNF-α	0.37	(-0.2 , 0.7)	0.12	(-0.4 , 0.6)	-0.09	(-0.6 , 0.4)	-0.22	(-0.7 , 0.3)
MCP-1	-0.05	(-0.5 , 0.4)	**0.68**	**(0.3 , 0.9)**	0.27	(-0.3 , 0.7)	-0.33	(-0.7 , 0.2)
S100A8/A9	-0.23	(-0.7 , 0.3)	0.50	(-0.05 , 0.8)	0.27	(-0.3 , 0.7)	-0.41	(-0.8 , 0.1)
IL-10	0.40	(-0.1 , 0.8)	-0.25	(-0.7 , 0.3)	-0.04	(-0.5 , 0.5)	-0.38	(-0.7 , 0.2)

Figure 1. IL-1β secretion by human OA synovium is correlated to serum LDL levels.

IL-1β concentrations measured in OA synovium conditioned media correlated positively with LDL serum concentrations **(A)**, but not with BMI **(B)** (2-8 samples/patient, plots show averages). **C-E)** Cellular infiltration was scored on histology using an arbitrary score of 0-3 (2-8 samples/patient), with 0 being no infiltration and 3 being the highest level of infiltration in this specific set of samples (**C**, showing a score of 3. 400x magnification). Lining hyperplasia was scored on histology based on the average lining thickness in number of cells based on histology (**D**, 400x magnification). Cellular infiltration and lining hyperplasia did not correlate with concentrations of IL-1β, MCP-1, S100A8/A9, TNF-α and IL-10 measured in OA synovium conditioned media (**E**). Significant correlations are presented bold (P<0.05) (n=16).

Western diet feeding increased LDL serum levels without increasing weight.

Five weeks after start of the diet, at day 7 of CiOA (Fig. 2A), total serum cholesterol was increased in WD fed mice compared to mice fed SD (1.8 to 3.5 mmol/L) mainly due to an increase in LDL cholesterol at day 7 of CiOA (Fig. 2B), and this was unchanged at day 42 of CiOA (Fig. 2C). WD feeding did not lead to significant weight gain over the entire disease duration (Fig. 2D), excluding weight as a factor mediating any observed effect. Interestingly, WD-feeding seemed to increase the volume of lipids in the bone marrow especially in mice treated with anti-IL-1β (Fig. 2E).

To validate that IL-β was functionally neutralized during the early stage of CiOA, we pre-incubated mouse sera obtained from mice of all 4 treatment groups with recombinant mouse IL-1β (rmIL-1β) and analyzed the NF-κB-inducing capacities of the samples using a NF-κB-luc 3T3 reporter assay. Indeed, sera from anti-IL-1β-treated mice showed a strong reduction in rmIL-1β-induced NF-κB signal compared to control mice despite being pre-incubated with an identical concentration (d(anti-IL-1β – Ctr) = -2.79x10^5, $CI_{95\%}$ = [-3.03x10^5, -2.55x10^5]) (Fig. 2F). However, this difference was gone when using sera of mice from day 42

of CiOA (*d*(anti-IL-1β – Ctr) = -2.26x10^{3}, CI$_{95\%}$ = -3.07x10^{4}, 2.62x10^{4}]) (Fig. 2G), suggesting the antibodies were already cleared at this timepoint. A proof of concept experiment confirmed that pre-incubation with anti-mIL-1β polyclonal antibodies could completely block the rm-IL-1β induced NF-κB-luc signal (Fig. 2H).

Western diet-induced alteration of monocyte subsets in bone marrow and blood is affected by anti-IL-1β treatment.

We next determined the effect of WD feeding of mice on monocyte subsets in bone marrow and blood, and whether IL-1β blocking interfered in this process. WD and anti-IL-1β treatment did not significantly alter the total number of nucleated bone marrow cells in the femur, despite a decreasing trend in anti-IL-1β treated mice (Fig. 3A, D). Flow cytometry analysis revealed that WD feeding increased the absolute number of Ly6C^{high} monocytes in the bone marrow, which was not affected by anti-IL-1β treatment (Fig. 3B, D). The same effect was found in the percentages of Ly6C^{high} monocytes in the blood (Fig. 3C, D). Here, the effect of WD seems to be lower in mice treated with anti-IL-1β, although no significant interaction or treatment effect was observed. In addition, WD feeding increased the percentage of Ly6C^{low} monocytes in blood while showing an interaction with treatment (Fig. 3C, D). Anti-IL-1β treatment furthermore decreased the number of granulocytes in the bone marrow, which was not reflected in blood (Fig. 3B-D).

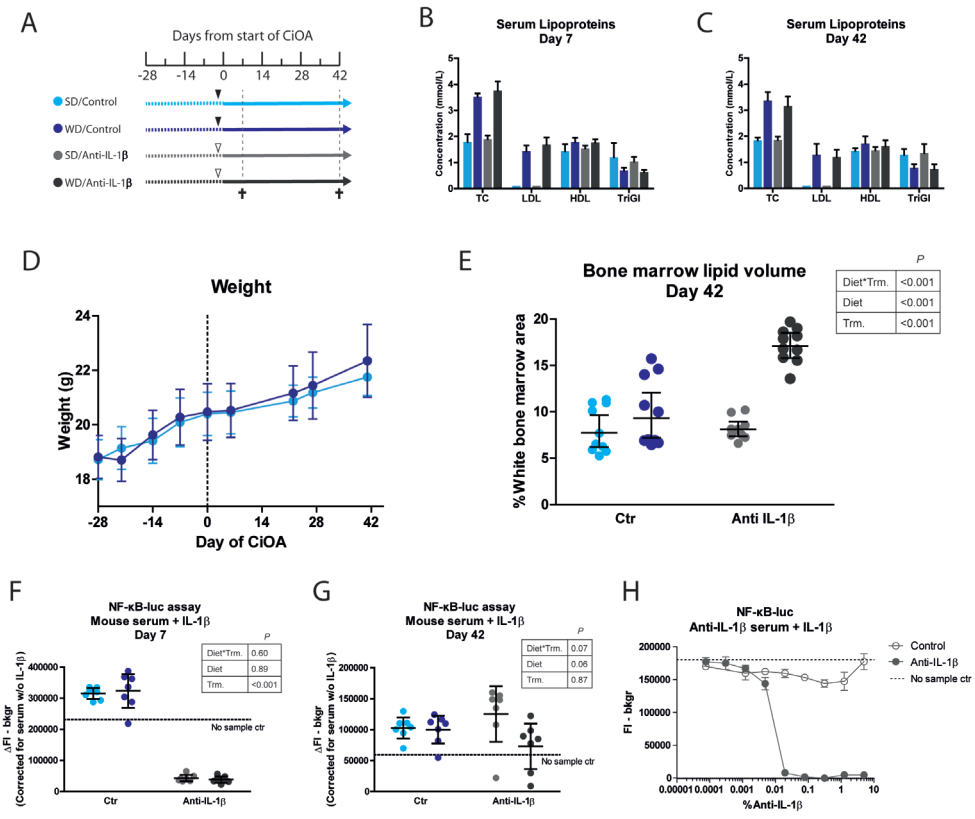
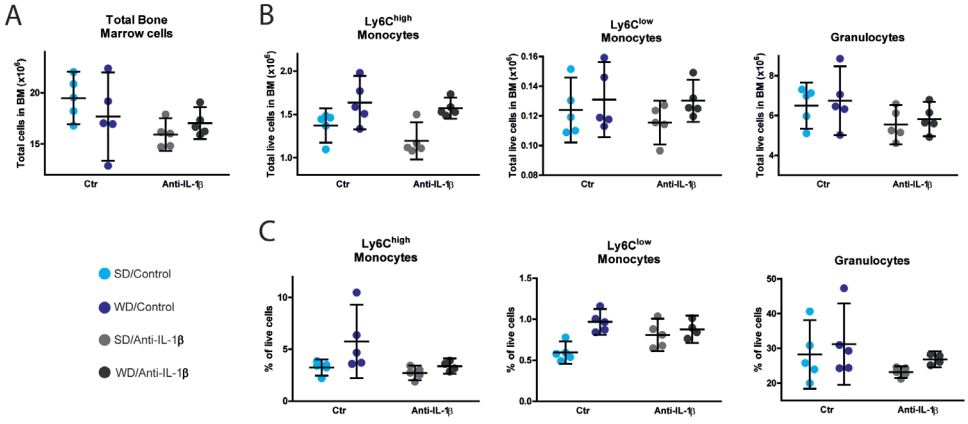


Figure 2. Western diet feeding increased LDL serum levels and systemic inflammation without increasing weight.

A) Schematic of dietary and therapeutic interventions. Female C57Bl/6 were fed either a standard diet (SD) or western diet (WD), and treated with the control (Ctr) or polyclonal anti-IL-1β antibodies (anti-IL-1β). Mice were sacrificed on day 7 and 42 of CiOA. n=10 for histology (day 7 and 42) and n=5 for flow cytometry (day 7), resulting in a total number of 100 mice. **B,C)** Serum concentrations of total cholesterol (TC), low-density lipoprotein (LDL), high-density lipoprotein (HDL) and triglycerides (TriGl) were determined at day 7 and 42 of CiOA. **C)** Body weight during the course of the experiment. **D)**Percentage of white area in the bone marrow as an indication of relative bone marrow lipid volume. **E,F)** NF-κB-luc assay of culture medium (DMEM supplemented with 5% FCS, pyruvate, penicillin/streptomycin) supplemented with 5% sample (mouse serum of day 7 (**E**) and day 42(**F**)) spiked with rmIL-1β (100pg/mL). Fluorescence intensity (FI) is shown. All values are corrected for background (only culture medium) and non-rmIL-1β spiked samples (n=7 (from 10 obtained samples)). **G)** NF-κB-luc of culture medium supplemented with control or anti-IL-1β antibodies (serial dilution) spiked with rmIL-1β (100 pg/mL). Trm.=Treatment. Mean and 95% CI (**E-G**) or SD (**B-D, H**) are shown.



D

Bone marrow		*P*-value			Difference in number of live cells x 10^5	
		Diet*Trt	Diet	Trt	WD - SD (95% CI)	Anti-IL-1β - Ctr (95% CI)
Total	Total BM cells	0.16	0.74	0.06	0.34 (-2.5 , 1.8)	-2.10 (-4.3 , 0.06)
Cell type	Monocytes (Ly6C^{high})	0.49	**<0.001**	0.14	**0.32 (0.2 , 0.5)**	-0.12 (-0.3 , 0.05)
	Monocytes (Ly6C^{low})	0.59	0.13	0.51	0.01 (-0.004 , 0.03)	0.00 (-0.02 , 0.01)
	Granulocytes	0.97	0.55	**0.04**	0.26 (-0.6 , 1.2)	**-0.94 (-1.8 , -0.03)**

Blood		*P*-value			Difference in % of live cells	
		Diet*Trt	Diet	Trt	WD - SD (95% CI)	Anti-IL-1β - Ctr (95% CI)
Cell type	Monocytes (Ly6C^{high})	0.21	**0.04**	0.06	**1.65 (0.1 , 3.2)**	-1.49 (-3.0 , 0.05)
	Monocytes (Ly6C^{low})	**0.02**	**<0.001**	0.34	**0.23 (0.08 , 0.4)**	0.06 (-0.09 , 0.2)
	Granulocytes	0.91	0.26	0.10	3.29 (-2.8 , 9.4)	-4.93 (-11.0 , 1.1)

Figure 3. Western diet-induced alteration of monocyte subsets in bone marrow and blood is affected by anti-IL-1β treatment.

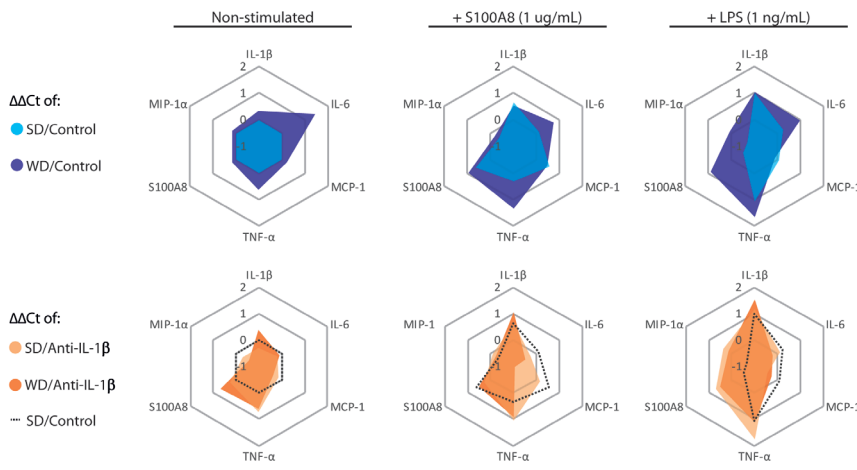
A) Complete femurs of the left hind legs were isolated, cleaned crushed and strained, after which the total numbers of bone marrow cells were determined. **B)** Absolute numbers of Ly6C^{high} monocytes, Ly6C^{low} monocytes and granulocytes (Ly6G^{high}) in the bone marrow of the femur calculated using the total number of bone marrow cells and the percentage of total cells as determined by flow cytometry. **C)** Percentages of Ly6C^{high} monocytes, Ly6C^{low} monocytes and granulocytes (Ly6G^{high}) in the blood as determined by flow cytometry. Mean and 95% CI's are plotted. **D)** Table showing *P*-values resulting from 2-way-ANOVA and between-group differences and 95% CI's resulting from Tukey/HSD (n=5, 1 blood sample was lost during processing). Trm.=Treatment.

Bone marrow cells of WD fed mice express increased cytokine levels, and this effect is dampened by anti-IL-1β treatment.

To investigate whether WD feeding in addition to changing the numbers of circulating monocytes also functionally altered the monocyte/macrophage precursor population, we isolated femoral bone marrow cells at day 7 of CiOA and directly stimulated them with TLR4 ligands S100A8 and LPS for 6 hours.

qPCR analysis confirmed that WD feeding caused the bone marrow cells to express a more inflated cytokine expression pattern, exemplified by increased IL-6 and TNF-α in particular, while this effect was nearly absent in mice treated with anti-IL-1β (Fig. 4A, B). This effect of WD was significant for IL-6 in absence of TLR4 ligands, and for TNF-α only in presence of S100A8 or LPS (Fig. 4B). For IL-6, a significant interaction between diet and treatment was observed in absence and presence of S100A8 or LPS (Fig. 4B).

A



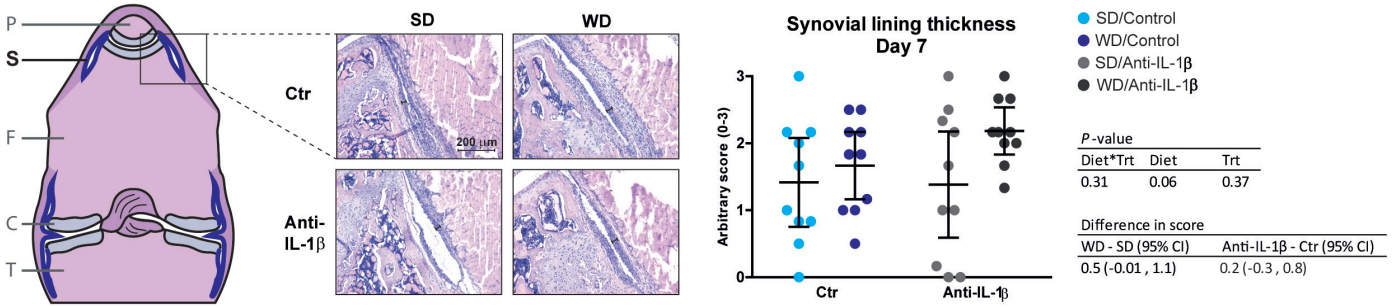
B

P -value	NS			S100A8			LPS		
	Diet*Trm.	Diet	Trm.	Diet*Trm.	Diet	Trm.	Diet*Trm.	Diet	Trm.
IL-1β	0.20	0.41	0.72	0.27	0.81	0.99	0.36	0.23	0.08
IL-6	**0.01**	***0.008***	***0.003***	0.13	0.46	**0.02**	0.09	0.51	**0.048**
MCP-1	0.59	0.99	0.32	0.44	0.36	0.17	0.58	0.99	0.42
TNF-α	0.14	0.31	0.19	**0.02**	0.07	0.53	***0.002***	0.82	0.60
S100A8	0.14	0.31	0.19	0.91	0.47	0.32	**0.03**	0.08	0.25
MIP-1α	0.35	0.80	**0.03**	0.56	0.93	0.63	0.09	0.33	0.07

Difference in -ΔCt	NS		S100A8		LPS	
	WD - SD (95% CI)	αIL1β - Ctr (95% CI)	WD - SD (95% CI)	αIL1β - Ctr (95% CI)	WD - SD (95% CI)	αIL1β - Ctr (95% CI)
IL-1β	0.26 (-0.4 , 0.9)	0.11 (-0.5 , 0.8)	-0.11 (-1.0 , 0.8)	0.00 (-1.0 , 1.0)	0.20 (-0.1 , 0.5)	0.31 (-0.0 , 0.7)
IL-6	***0.76 (0.1 , 1.4)***	***-0.89 (-1.5 , -0.3)***	0.22 (-0.4 , 0.8)	**-0.78 (-1.4 , -0.2)**	0.20 (-0.4 , 0.8)	**-0.64 (-1.3 , -0.006)**
MCP-1	0.00 (-0.8 , 0.8)	0.37 (-1.1 , 0.4)	-0.59 (-1.9 , 0.7)	-0.90 (-2.2 , 0.4)	0.00 (-0.8 , 0.8)	-0.31 (-1.1 , 0.5)
TNF-α	0.26 (-0.3 , 0.8)	0.34 (-0.2 , 0.9)	0.45 (-0.1 , 1.0)	0.15 (-0.4 , 0.7)	-0.04 (-0.5 , 0.4)	0.09 (-0.4 , 0.6)
S100A8	0.26 (-0.3 , 0.8)	0.35 (-0.2 , 0.9)	0.30 (-0.6 , 1.2)	-0.41 (-1.3 , 0.4)	0.61 (-0.2 , 1.4)	0.40 (-0.4 , 1.2)
MIP-1α	-0.06 (-0.5 , 0.4)	**-0.52 (-1.0 , -0.1)**	0.02 (-0.5 , 0.6)	0.13 (-0.4 , 0.7)	0.28 (-0.3 , 0.9)	0.54 (-0.0 , 1.1)

Figure 4. Bone marrow cells of WD fed mice express increased cytokine levels, and this effect is dampened by anti-IL-1β treatment.

A) Bone marrow cells isolated at day 7 of CiOA following dietary and therapeutic intervention, stimulated *ex vivo* with TLR4 ligands S100A8 (1 µg/mL) or LPS (1 ng/mL), or left unstimulated for 6 hours. Cytokine expression as determined by qPCR, represented in radar plots as ΔΔCt values corrected for the reference gene GAPDH (-ΔCt) and calculated relative to the mean -ΔCt of the control group (standard diet, control treatment, unstimulated). **B)** Table showing *P*-values resulting from 2-way-ANOVA and between-group differences and 95% CI's in -ΔCt resulting from Tukey/HSD (n=5). Values are presented bold when $P<0.05$ and cursive when $P<0.0027$ (0.05/18 comparisons). Trm.=treatment.



P-value

Diet*Trt	Diet	Trt
0.31	0.06	0.37

Difference in score

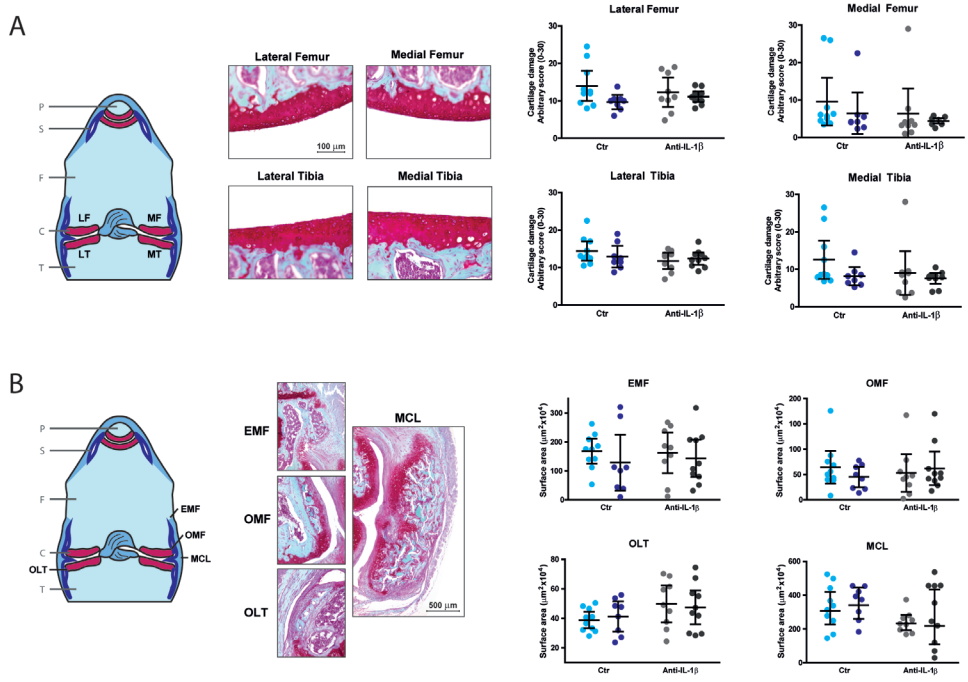
WD - SD (95% CI)	Anti-IL-1β - Ctr (95% CI)
0.5 (-0.01 , 1.1)	0.2 (-0.3 , 0.8)

Figure 5. Synovial lining thickness was not significantly affected by anti-IL-1β treatment during WD feeding at day 7 of CiOA.

Illustration shows a schematic overview of a frontal section of an H&E stained knee joint. P=patella, S=synovium, F=femur, C=cartilage, T=tibia. Pictures are representative of the mean (100x magnification). Synovial lining thickness at day 7 of CiOA was scored on H&E stained histological sections using an arbitrary score of 0-3 (where 0 = no thickening and 3 = most observed thickening). Three sections of different standardized depths were scored per knee joint. Trm.=Treatment. Mean and 95% CI's are plotted. Table shows *P*-values resulting from 2-way-ANOVA and between-group differences and 95% CI's resulting from Tukey/HSD (n=10). No mice were excluded from analysis.

CiOA pathology was not significantly affected by anti-IL-1β treatment during WD feeding

Next, we investigated whether the observed increase in monocyte reactivity by WD feeding was reflected in the inflammatory status of the synovium. However, histological analysis revealed no significant difference in synovial lining thickness on day 7 of CiOA, despite a trend towards a significant effect of diet (Fig. 5). Finally, we studied whether WD induced systemic innate immune activation worsens CiOA pathology by scoring cartilage damage and ectopic bone formation by histology on day 42 of CiOA. No significant effects of WD feeding and anti-IL-1β treatment on cartilage degeneration were observed (Fig. 6A, C). In addition, osteophyte and ectopic bone size was scored at four sites in the joint, but also here no significant differences were observed (Fig. 6B, C).



C

Cartilage damage		*P*-value			Difference in score			
		Diet*Trt	Diet	Trt	WD - SD (95% CI)		Anti-IL-1β - Ctr (95% CI)	
Location	Lateral Femur	0.26	0.06	0.77	-2.7	(-5.5 , 0.1)	-0.4	(-3.2 , 2.4)
	Medial Femur	0.81	0.28	0.22	-2.5	(-7.1 , 2.1)	-2.8	(-7.4 , 1.8)
	Lateral Tibia	0.30	0.69	0.11	-0.4	(-2.4 , 1.7)	-1.6	(-3.7 , 0.4)
	Medial Tibia	0.42	0.13	0.20	-2.9	(-6.6 , 0.8)	-2.4	(-6.1 , 1.3)
Average		0.38	0.12	0.18	-2.1	(-4.8 , 0.6)	-1.8	(-4.5 , 0.8)

Osteophytes and ectopic bone		*P*-value			Difference in µm²x 10⁴			
		Diet*Trt	Diet	Trt	WD - SD (95% CI)		Anti-IL-1β - Ctr (95% CI)	
Location	EMF	0.73	0.33	0.95	-2.9	(-8.8 , 3.0)	0.2	(-5.7 , 6.1)
	OMF	0.32	0.74	0.89	-0.5	(-3.3 , 2.4)	0.2	(-2.7 , 3.1)
	OLT	0.60	0.98	0.06	-0.0	(-0.9 , 0.9)	0.9	(-0.03 , 1.8)
	MCL	0.71	0.37	0.09	3.8	(-4.7 , 11.9)	-7.3	(-15.8 , 1.2)

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Figure 6. Cartilage destruction and ectopic bone formation was not significantly affected by anti-IL-1β treatment during WD feeding at day 42 of CiOA.

A+B) Illustrations each show a schematic overview of a frontal section of a SafO stained knee joint. P=patella, S=synovium, F=femur, C=cartilage, T=tibia. **A)** Sites where cartilage was scored is presented as LF (lateral femur), MF (medial femur), LT (lateral tibia) and LF (lateral femur). Cartilage damage in the tibial-femoral joint was scored using a modified OARSI score, in which the grade (severity of erosion) is multiplied by the stage (affected area), resulting in a score of 0-30 (0 = no damage, 30 = maximal damage). Five sections were scored per knee joint. Pictures are representative of the mean across groups (200x magnification). **B)** Sites where ectopic bone formation was scored is presented as EMF (enthesophyte medial femur), OMF (osteophyte medial femur), OLT (osteophyte lateral femur) and MCL (medial collateral ligament). Osteophytes and enthesophytes were manually circled by an investigator blinded for the experimental condition, after which the surface areas were calculated. The mean cross-sectional surface area in three sections per knee joint was determined. Pictures are representative of the mean across groups (50x magnification). Trm.=Treatment. Mean and 95% CI's are plotted. **C)** Table showing *P*-values resulting from 2-way-ANOVA and between-group differences and 95% CI's resulting from Tukey/HSD (n=10). Tibiofemoral and patellofemoral dislocations were excluded from analysis (**A+B,** 2 in SD/Ctr, 0 in WD/Ctr, 1 in SD/anti-IL-1β and 0 in WD/anti-IL-1β).

DISCUSSION

One of the biggest challenges in finding a treatment for OA is to identify specific OA phenotypes to enable tailored treatment. OA phenotyping research is still in its early stage, but so far MetS associated OA has been heavily suggested as one of the disease subtypes covering a distinct molecular endotype[29-31]. Anti-inflammatory therapy might be especially suitable for MetS associated OA because of its systemic inflammatory component. Even though anti-IL-1β therapies have so far proven unsuccessful in clinical trials and preclinical studies, there are clues that IL-1β plays a more central role in OA development in the context of metabolic imbalances. Firstly, it was shown in the CANTOS trial that treatment with monoclonal antibody against IL-1β canakinumab lowered the rate of total knee and hip replacements in these patients, although structural joint outcomes were not the focus of this study and therefore not collected[32]. Secondly, IL-1β is known to play an important role in mouse models of atherosclerosis, which are LDL and macrophage driven, and IL-1β targeting treatment in these mice showed therapeutic effects[33-35]. Whether this beneficial effect of IL-1β blocking also applies to metabolically associated OA development remains to be seen, but the overlapping risk factors of atherosclerosis and OA indicate common biochemical pathways. Here, we showed that IL-1β secretion by human OA synovium is positively correlated to serum LDL levels but not to BMI. Studies with larger cohorts and more information on confounding factors should confirm these findings. Dyslipidemia is one of many biochemical branches of the metabolic syndrome that could eventually lead to worsening of OA development while also affecting IL-1β, and this might run via immune programming of innate immune cells which is shown to be dependent on IL-1β signaling.

In the here presented *in vivo* study, we show that WD feeding of C57Bl/6 mice results in systemic activation of the innate immune system within a relatively short time frame of 5 weeks. This innate immune activation was exemplified by an increase of Ly6C^{high} monocytes in bone marrow and blood at day 7 of CiOA. We observed similar changes in the Ly6C^{low} compartment of monocytes, although we believe that especially the changes in Ly6C^{high} monocytes are relevant for OA development since these cells preferentially infiltrate OA synovium[25].
In addition to a systemic increase in monocyte number, WD feeding caused an increase of cytokine secretion by bone marrow cells, both in absence and presence of TLR4 ligands S100A8 or LPS. The cytokines most notably increased by WD were IL-6 and TNF-α, which have both been implicated in OA pathology as stimulators of cartilage degenerating processes[36, 37]. An increased activity of these factors in the joint could in theory worsen OA related processes like synovitis, cartilage degradation and pain. Surprisingly, we did not observe a WD-induced increase in IL-1β, although this needs to be confirmed on protein level. Moreover, IL-1β may not be required to be increased by WD to mediate its pro-inflammatory effect as is indicated by the effectiveness of anti-IL-1β to ameliorate WD-induced IL-6 expression.

The systemic increase in number and activity of innate immune cells did not seem to cause increased synovial cellularity based on histological H&E stained knee sections, on which no significant effects of WD or anti-IL-1β were observed. Whether this negative result was due to an absence of effect or due to the large variation within groups could not be evaluated with this sample size, but we could observe a trend towards an increasing effect of WD. We were unfortunately unable to collect a reliable readout for monocyte/macrophage phenotype and activation status to assess if the systemically observed effects of WD and anti-IL-1β were reflected in the synovium. If they indeed were, however, these local alterations were seemingly unable to significantly influence the severity of end stage disease. Possibly, the local immune environment in the synovium simply remains unaffected by the WD-induced systemic immune changes, hence the unaltered end stage pathology. As explained in the study by Culemann and colleagues, synovial lining macrophages have an anti-inflammatory phenotype which persists even in a strongly inflamed environment[22]. To this end, the physiological function of the lining macrophages might have to be compromised first to then allow the WD-activated immune cells to infiltrate the synovium and locally skew macrophage phenotypes, consequently worsening OA pathology. This would be in line with results previously found in our lab, which show that intra-articular injection of oxLDL in naïve mouse knee joints induces chemokine production and immune cell infiltration, but only after depletion of synovial macrophages using clodronate liposomes.

The disease-worsening effects of WD were either absent or imperceptible in this study. In addition to the statistical insignificance of the diet effect, the between-group differences do not meet the value of 3.2 to be considered biologically relevant, and hint more towards a disease-relieving effect of WD rather than a disease-aggravating one. This is in contrast to studies previously performed in our lab where WD feeding of C57Bl/6 mice did indeed result in increased pathology in the form of increased

ectopic bone formation[19, 20]. There are several differences between the experimental designs that might explain the different outcomes. First of all, the WD used in this study is supplemented with cholic acid (0.05%), whereas previously used diets weren't. Supplementation of this bile acid makes WD especially effective in enhancing the dyslipidemia aspect of metabolic syndrome, and causes mild atherosclerosis when fed to C57Bl/6 mice for a period of 14 weeks[38, 39]. However, it might have additional effects which unintentionally counteract the OA aggravating effects of WD, for example via its ability to locally suppress innate immunity[40]. Secondly, the duration of WD-feeding before induction of CiOA differs between experiments. In this study, WD-feeding was started 4 weeks before CiOA induction, whereas in previous studies this was 12 weeks and 2 weeks[19, 20]. Nonetheless, disease-aggravating effects of WD were observed also with only 2 weeks of WD preceding CiOA induction, making it less likely that diet duration is the prime cause of the differences in outcome. Alternatively, the WD-induced immune activation has to persist longer to be able to affect local pathology. This particular model of metabolic syndrome, being wild type mice fed WD, realistically reflects a physiological metabolic phenotype and proved suitable to show short term effects on the innate immune system, but is possibly not suitable to simulate the longevity of exposure to this altered immune system that possibly takes place in the human situation. More exaggerated metabolic syndrome models may simulate this better, given the relatively short duration of the experimental OA models compared to human OA development.

The absence of effect of anti-IL-1β on pathology suggests that in this setting, WD does not alter OA development to become more dependent on IL-1β activity. Although the effect of anti-IL-1β treatment on cartilage damage shows consistently negative values, they are statistically insignificant and additionally do not meet the value of 3.2 to be considered biologically relevant. This is in line with a study of Collins and colleagues in which the authors show that lipodystrophy protects against cartilage damage in a DMM model of OA despite strongly increased serum IL-1β levels[41], indicating that they are independent processes. Further evaluation of the effect of anti-IL-1β on the WD-aggravated part of OA pathology requires additional experiments that reproduce the disease-worsening effects of WD previously found.

Potentially, IL-1β signaling possibly needs to be blocked during the entire disease duration to affect CiOA pathology. A single early treatment time point was chosen to block IL-1β especially in the early disease stage when inflammation and synovial IL-1β expression is highest[42], but a potential role for IL-1β in later disease stages cannot be excluded. A similar experiment with continued anti-IL-1β delivery should still be performed to conclude that IL-1β does not play a role also in later stages of CiOA. Interestingly, we found that WD-induced bone marrow lipid accumulation was strongly increased in anti-IL-1β treated mice, suggesting a suppressive role for IL-1β in bone marrow lipid formation. Increased bone marrow lipid volume has previously been linked to formation of bone marrow lesions which form a risk factor for OA[43, 44].

Even though it needs to be further investigated whether the here observed WD-induced and IL-1β dependent systemic immune alterations have the potential to alter OA pathology, one can speculate

on the underlying mechanisms which could provide valuable insight for future studies on this topic. Our findings in C57Bl/6 mice are in line with the study from A. Christ and colleagues, who show that WD induces an increased immune response of granulocyte-monocyte progenitor cells in *Ldlr*$^{-/-}$ mice through long lasting epigenetic reprogramming[21]. In both studies, the WD-mediated effects were shown to rely on the IL-1 pathway. OxLDL was suggested by the authors as a potential inducer of these effects, which was supported by the finding that oxLDL-induced trained immunity of human monocytes is ameliorated by IL-1 receptor antagonist (IL-1ra). However, oxLDL represents just one of many biological factors which potentially mediate WD-induced immune training. Alternatively, WD affects the innate immune system via microbiome alterations or hyperglycemia. Huang and colleagues showed that mice that received fecal bacteria from OA patients with metabolic syndrome developed more severe cartilage degradation and synovitis during DMM as compared to mice who received fecal bacteria from OA patients without metabolic syndrome or individuals without OA, and this was associated with changes in IL-1β[45]. Furthermore, hyperglycemia is known to cause systemic macrophage activation and increased IL-1β production[46], and the glucose-lowering drug metformin was shown to ameliorate DMM with both therapeutic and prophylactic strategies[47].

To conclude, we report here that WD feeding of C57Bl/6 mice systemically activates the innate immune system in an IL-1β dependent manner, but that these changes were unable to significantly modulate CiOA pathology. This result is in line with previous studies that show no beneficial effects of IL-1β neutralizing drugs and suggests this does not change in the context of WD-feeding.

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AUTHOR CONTRIBUTIONS

Conception and design of study: NK, MK, FvdL, PvdK, AB, MvdB, PvL
Acquisition of data: NK, YvG, BW, MH, AS, JR, TV, AB, MvdB
Analysis and interpretation of data: NK, AB, MvdB, PvL
Drafting the article: NK, AB, MvdB, PvL
Revising the article critically: NK, YvG, MK, FvdL, JR, TV, PvdK, AB, MvdB, PvL
Final approval of the submitted manuscript: NK, YvG, BW, MH, AS, MK, FvdL, JR, TV, PvdK, AB, MvdB, PvL

ROLE OF THE FUNDING SOURCE

The funding sources had no role in study design, collection, analysis or interpretation of data, or in writing the manuscript and decision to submit the manuscript.

CONFLICT OF INTEREST STATEMENT

None of the authors had financial or personal relationships with people or organizations that could inappropriately influence the bias of the presented work.

ETHICS APPROVAL

All animal studies were according to the Dutch law and were approved by the local Animal Experimentation Committee (CCD number: 2018-0002).

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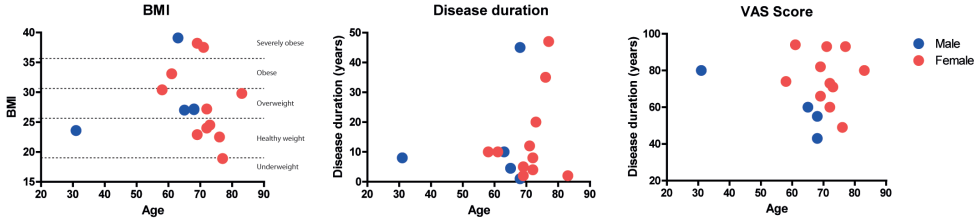
SUPPLEMENTARY MATERIAL



Supplementary figure 1. Patient characteristics

Supplementary table 1. Flow cytometry antibodies-fluorophores

Marker	Marker for	Fluorophore	Concentration	Manufacturer	Clone	Catalogue #
B220	B-cell eclusion	FITC	1:200	Biolegend	RA3-6B2	103206
CD3	T-cell exclusion	FITC	1:200	Biolegend	145-2C11	100306
CD49b	NK-cell exclusion	FITC	1:100	Biolegend	DX5	108906
Ter119	Ery-exclusion	FITC	1:400	Biolegend	TER-119	116206
CD45	Leukocytes	PE	1:200	Biolegend	30-F11	103106
CD11b	Myeloid cells	APC	1:800	Biolegend	M1/70	101212
Ly6G	Granulocytes	Alexa Fluor 700	1:400	Biolegend	1A8	127622
Ly6C	Monocytes	Brilliant Violet 421	1:200	Biolegend	HK1.4	128032
Fixable viability Dye	Live/death staining	efluor 780	1:10.000	eBioscience	NA	65-0865-18

Supplementary table 2. Primer sequences

Gene	Forward primer 5' → 3'	Reverse primer 3' → 5'
GAPDH	GGCAAATTCAACGGCACA	GTTAGTGGGGTCTCGCTCCTG
IL-1β	GGACAGAATATCAACCAACAAGTGATA	GTGTGCCGTCTTTCATTACACAG
IL-6	CAAGTCGGAGGCTTAATTACACATG	ATTGCCATTGCACAACTCTTTTCT
MCP-1	TTGGCTCAGCCAGATGCA	CCTACTCATTGGGATCATCTTGCT
TNF-α	CAGACCCTCACACTCAGATCATCT	CCTCCACTTGGTGGTTTGCTA
S100A8	TGTCCTCAGTTTGTGCAGAATATAAAT	TTTATCACCATCGCAAGGAACTC
MIP-1α	CAAGTCTTCTCAGCGCCATATG	TCTTCCGGCTGTAGGAGAAGC

CHAPTER 6

IL-1β inhibition combined with cholesterol-lowering therapies decreases synovial lining thickness and spontaneous cartilage degeneration in a humanized dyslipidemia mouse model

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Osteoarthritis Cartilage. 2023 Mar;31(3):340-350

ABSTRACT

Introduction

Both systemic inflammation and dyslipidemia contribute to osteoarthritis (OA) development and have been suggested as a possible link between metabolic disease and OA development. Recently, the CANTOS trial showed a reduction in knee and hip replacements after inhibition of IL-1β in patients with a history of cardiovascular disease and high inflammatory risk. In this light, we investigated whether inhibition of IL-1β combined with cholesterol-lowering therapies can reduce OA development in dyslipidemic APOE*3Leiden mice under pro-inflammatory dietary conditions.

Materials and methods

Female ApoE3*Leiden mice were fed a cholesterol-supplemented Western-Type diet (WTD) for 38 weeks. After 14 weeks, cholesterol-lowering and anti-inflammatory treatments were started. Treatments included atorvastatin alone or with an anti-IL1β antibody, and atorvastatin combined with proprotein convertase subtilisin-kexin type 9 (PCSK9) inhibitor alirocumab without or with the anti-IL1β antibody. Knee joints were analyzed for cartilage degradation, synovial inflammation and ectopic bone formation using histology at end point.

Results

Cholesterol-lowering treatment successfully decreased systemic inflammation in dyslipidemic mice, which was not further affected by inhibition of IL-1β. Synovial thickening and cartilage degeneration were significantly decreased in mice that received cholesterol-lowering treatment combined with inhibition of IL-1β ($P < 0.01$, $P < 0.05$, respectively) compared to mice fed a WTD alone. Ectopic bone formation was comparable between all groups.

Conclusion

These results indicate that inhibition of IL-1β combined with cholesterol-lowering therapy diminishes synovial thickening and cartilage degeneration in mice and may imply that this combination therapy could be beneficial in patients with metabolic inflammation.

INTRODUCTION

Osteoarthritis (OA) is the leading cause of disability worldwide and is characterized by joint pain and stiffness. Treatment options are currently limited to lifestyle interventions and analgesics, and patients ultimately need whole joint replacement at end-stage disease. OA is a complex and heterogeneous disease that affects all joint structures such as cartilage, synovium, subchondral bone and ligaments[1]. Many risk factors, including aging, obesity, trauma and metabolic syndrome (MetS) have been associated with the development of OA[2, 3]. MetS is characterized by a cluster of metabolic conditions (visceral obesity, diabetes and insulin resistance, dyslipidemia and hypertension) and its prevalence has increased over the last decades. An increased prevalence of MetS in the OA population was observed compared to the non-OA population[22] and MetS has been associated with the progression and development of OA[2, 3].

Dyslipidemia is frequently described as an increase of circulating lipid levels, such as cholesterol and triglycerides. Several clinical studies showed an association between high cholesterol levels and the development of OA[41, 42, 204], while others did not observe this association[57]. Previously, we found that mice fed a cholesterol-supplemented diet spontaneously developed OA compared to chow-fed controls[205]. Additionally, we and others have shown that dietary cholesterol also exacerbates disease development in both the collagenase-induced OA (CiOA) model and a surgically induced OA model[36, 49, 206, 207]. Although cholesterol-lowering therapies successfully reduce the risk of cardiovascular disease , the exact role of high cholesterol and cholesterol-lowering therapies on OA progression remains unclear. Results from clinical studies using statins are variable, showing beneficial or no effects on the incidence or progression of OA[76, 80, 82]. These inconsistent results imply that additional mechanisms, combined with high cholesterol levels, are likely involved in diet-induced OA development.

Low-grade systemic inflammation, associated with metabolic disease, is thought to contribute to OA development and has been suggested as a connection between MetS and OA[17]. Metabolic inflammation is mainly caused by metabolic factors that induce oxidative stress and inflammation, such as lipids, glucose and adipokines[18, 19]. Increased levels of circulating inflammatory markers and adipokines are observed in OA patients and have been associated with increased joint inflammation[20, 21]. The role of joint inflammation in the progression of OA is increasingly recognised[134, 135], and OA patients frequently present with various degrees of synovial inflammation. Although inflammation is believed to be involved in the pathogenesis of OA, clinical trials targeting inflammatory factors proved disappointing in ameliorating disease progression. Interleukin-1β (IL-1β) is an important driver of inflammation and is thought to be involved in OA pathology. Despite the fact that IL-1β was thought to play a critical role in the pathogenesis of OA, several pre-clinical and clinical studies were not able to demonstrate any disease-modifying effects after inhibition of IL-1β[64-67]. Recently, a secondary analysis of the Canakinumab Anti-inflammatory Thrombosis Outcome Study (CANTOS) trial showed that systemic inhibition of IL-1β resulted in a decreased incidence of total knee and hip replacements in patients with CVD[69]. Interestingly, this IL-1β effect was found on top of cholesterol-lowering treatment, since over 93% of

patients enrolled in the CANTOS study received cholesterol-lowering therapy at the time the anti-IL1-β treatment started. This study indicates that inhibition of IL-1β could be beneficial in a subset of patients with high inflammatory risk or metabolic disease.

In a previous study we have shown that therapeutic cholesterol-lowering treatment in hypercholesterolemic mice successfully attenuated atherosclerosis progression[102] but proved insufficient in reducing spontaneous OA development[130]. In this study, we investigated the effects of IL-1β inhibition on top of high-intensive cholesterol-lowering treatments on spontaneous OA development in dyslipidemic APOE*3Leiden mice with elevated systemic inflammation.

MATERIALS AND METHODS

Animals

ApoE*3Leiden (E3L) and ApoE*3Leiden.CETP (E3L.CETP) transgenic mice fed fat- and cholesterol-containing diets have high translatability for lipoprotein metabolism and are well-established models to study dyslipidemia, atherosclerosis and metabolic disease[132, 137, 142, 208, 209]. E3L and E3L.CETP mice show human-like responses to cholesterol-lowering therapies[132, 138, 139]. The study was carried out in female E3L and E3L.CETP transgenic mice on a C57BL/6 background (8-12 weeks of age), obtained from the in-house breeding colony (TNO Metabolic Health Research, Leiden, the Netherlands). Female mice were used since they are more susceptible to cholesterol-supplemented diets and develop more pronounced atherosclerosis due to higher systemic cholesterol and triglyceride levels compared to male mice[142]. All groups had 15 mice per group at the beginning of the study. 2 animals of the WTD + atorvastatin group were excluded from the study, one mouse was found dead in cage and one mouse was taken out of the study because of human endpoints. The study was approved by the Governmental Central Committee on animal experiments (AVD5010020172064) and the Institutional Animal Care and Use Committee of TNO (TNO-400) and was in compliance with European Community specifications regarding the use of laboratory animals.

Diet and treatments

8-12 week old E3L and E3L.CETP mice were fed a cholesterol-supplemented WTD for 38 weeks. After 14 weeks of WTD feeding, mice were matched into several treatment groups based on age, body weight, plasma total cholesterol (TC), plasma total triglycerides (TG) and cholesterol exposure (mmol/L*weeks). Treatments included atorvastatin (10mg/kg bw/d) (A), atorvastatin combined with PCSK9 inhibitor alirocumab (10 mg/kg bw/wk) (AA) or with a mouse IL-1β blocking monoclonal antibody (10 mg/kg bw/wk) (AI), or triple treatment (AAI). For more details about the diet and treatments, see the online supplementary methods.

Determination of systemic cholesterol and inflammatory factors

Plasma cholesterol levels were monitored throughout the study. Peripheral blood was collected via tail vein incision using EDTA-coated tubes (Sarstedt) after 4h of food deprivation or by heart puncture at sacrifice. Total cholesterol levels were determined throughout the study using an enzymatic assay (Roche Diagnostics) according to manufacturer's instructions. Plasma levels of Serum amyloid A (SAA), E-selectin and Monocyte chemoattractant protein-1 (MCP-1) were determined in individual plasma samples at end point using ELISA kits from R&D (Minneapolis, MA, USA), and SAA with an ELISA kit from Tridelta Development Limited (Maynooth, County Kildare, Ireland). All assays were performed according to the manufacturer's instructions.

Histology and histological analysis

Murine knee joints were fixed in formalin and decalcified using formic acid. Joints were subsequently embedded in paraffin and cut in 7µm sections. Sections were stained using Safranin-O/Fast Green and Hematoxylin/Eosin for histological analysis. Cartilage damage in the joint was quantified using a more detailed version of the OARSI scoring system, as described previously[143, 210] (0 = no damage, 30 = maximal damage). The cartilage damage score represents the assessment of the grade (progression of damage into the cartilage; 0-6) multiplied by the grade (percentage of the damaged cartilage surface; 0-5). Five sections from various depths, representing the entire knee joint, were scored and averaged per joint after blinding. Osteophyte formation and maturation were determined using an arbitrary scoring system as described previously[120]. Different locations throughout the whole joint were scored for osteophyte formation and maturation on both the medial and lateral side[120]. Synovial thickness was scored using a scoring range from 0-3 in a blinded fashion (0 = no thickening, 1 = low thickening, 2= mild thickening, 3 = moderate thickening). Three sections per joint were scored and averaged using a fixed position in the joint.

Immunohistochemistry

For immunohistochemical analysis, knee joint sections were deparaffinized and endogenous peroxidase was blocked with H_2O_2 in methanol. Antigen retrieval was performed in citrate buffer pH 6.0. Sections were stained with polyclonal antibodies against S100A9 (kindly provided by Thomas Vogl, Institute of Immunology, University of Muenster, Germany) and MMP13 (ab219620) or non-relevant rabbit IgG control (R&D systems). Biotinylated anti-rabbit IgG was used as a secondary antibody. Subsequently, sections were stained with avidin-streptavidin-peroxidase (Elite kit, Vector Laboratories) and diaminobenzidine (Sigma-Aldrich) was used for visualization of peroxidase staining. Counterstaining was performed using hematoxylin (Merck).

Statistical analysis

We determined the statistical power of our study based on the main readout parameter, cartilage degradation. The mean differences between groups detectable with a power of 0.8 for each analysis was determined using 15 mice per group, a two sided *t*-test, a 95% confidence interval and the observed

SD, which is 1.7 for our main read-out parameter cartilage degeneration. This resulted in a detectable difference of 1.8. Statistical analysis was performed using GraphPad Prism version 9. Normality was assessed using a Shapiro-Wilk test. A nonparametric Kruskal-Wallis test followed by a Dunn's Multiple Comparison Test was used for comparisons of the control group with different treatment groups. *P*-values below 0.05 were considered significant. For cholesterol exposure, a parametric One-Way ANOVA followed by a Bonferroni post hoc test to was used. Results are expressed as individual data points with mean ± 95% confidence intervals.

RESULTS

Dyslipidemic E3L mice show an increased inflammatory profile compared to E3L.CETP mice

We first validated the most optimal translational mouse model with diet-induced systemic inflammation to study cholesterol-lowering treatment combined with inhibition of IL-1β in mice. Therefore, we compared systemic cholesterol levels and the inflammatory profile in plasma of E3L and E3L.CETP mice fed a pro-atherogenic hypercholesterolemic diet. After 14 weeks of WTD feeding, plasma cholesterol levels were comparable between E3L and E3L.CETP mice (25.7 mmol/L and 27.2 mmol/L, respectively). E3L mice fed a cholesterol-supplemented WTD showed higher systemic levels of SAA and E-selectin when compared to E3L.CETP mice, while MCP-1 levels were comparable between both strains (SAA: 3-fold difference; P<0.001, E-selectin: 1.3-fold difference; p<0.001; **Figure 1A-C**). Hence, we selected the more pro-inflammatory E3L model for further analysis of the treatments and analyzed spontaneous OA development in their knee joints.

Cholesterol-lowering treatments successfully reduce systemic cholesterol levels and inflammatory markers, which was not further affected by inhibition of IL-1β

We first determined the efficacy of the cholesterol-lowering treatments throughout the study. Mice were fed a cholesterol-supplemented WTD for 38 weeks and treatments were started after 14 weeks (**Figure 2A**). Atorvastatin treatment successfully reduced systemic cholesterol levels and cholesterol exposure (A: -34% compared to WTD control; P<0.0001, AI: -28%; P<0.0001, AA: -55%; P<0.0001, AAI: -54%; P<0.0001), which was further reduced when combined with inhibition of PCSK9 (P<0.0001 (**Figure 2B, C**). In addition, we checked whether the anti-IL-1β antibodies lowered IL-1β levels properly. Since IL-1β levels in the circulation are very low and at the limit of detection, we measured its levels in liver homogenates. Treatment with the anti-IL-1β antibodies significantly reduced hepatic IL-1β levels at endpoint compared to the WTD control (AI: -83%; p<0.0001, AAI: -82%; p<0.0001) and to mice that received cholesterol-lowering therapy alone (A-AI:-82.2%; p<0.001), AA-AAI: -67.1%; p<0.05) (**Figure 2D**). We next determined whether the treatment combinations reduced circulating inflammatory markers by quantification of SAA, E-selectin and MCP-1 at end point. Atorvastatin treatment significantly reduced levels of all inflammatory markers compared to the WTD group, and there were no additional effects of more intensive cholesterol lowering by PCSK9 inhibition and by inhibition of IL-1β (SAA: A:

-51%; p<0.0001, AI: -48%; p<0.0001, AA: -33%, AAI: -39%; E-selectin: A: -30%; p=0.006, AI: -27%; p=0.01, AA: -23%; p=0.05, AAI: -15%; MCP-1: A: -31%; p=0.03, AI: -39%; p-0.004, AA: -46%; p=0.0002, AAI: -40%; p=0.003; **Figure 2E-G**). We observed a significant correlation with cholesterol exposure and plasma MCP-1 levels, but no significant correlation was observed for SAA or E-selectin (**Figure 2H-J**).

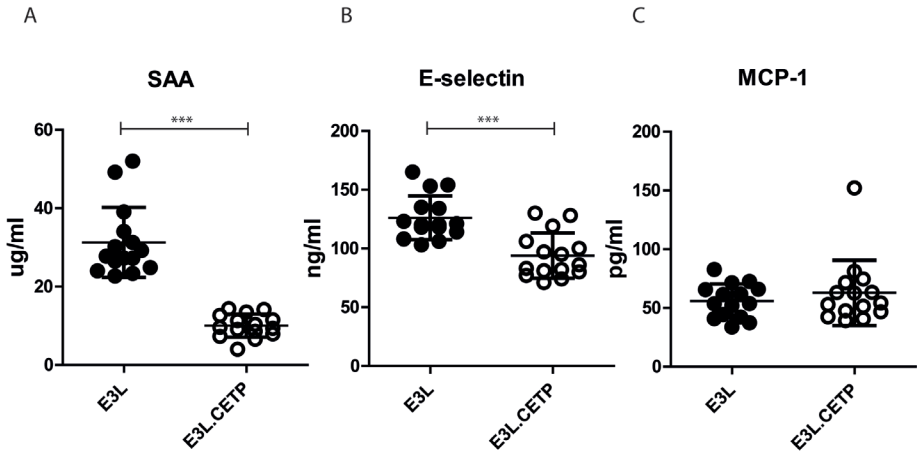

Figure 1. E3L mice show higher levels of inflammatory markers when fed a cholesterol-supplemented WTD compared to E3L.CETP mice

E3L and E3L.CETP mice were fed a cholesterol-supplemented WTD for 14 weeks. Plasma levels of SAA, E-selectin and MCP-1 were determined after sacrifice. **(A)** SAA levels were significantly higher in E3L mice compared to E3L.CETP mice (p<0.001). **(B)** E-selectin were significantly higher in E3L mice compared to E3L.CETP mice (p<0.001). **(C)** MCP-1 levels were comparable between both mouse strains. *Results are expressed as individual data points with mean ± 95% confidence intervals.*

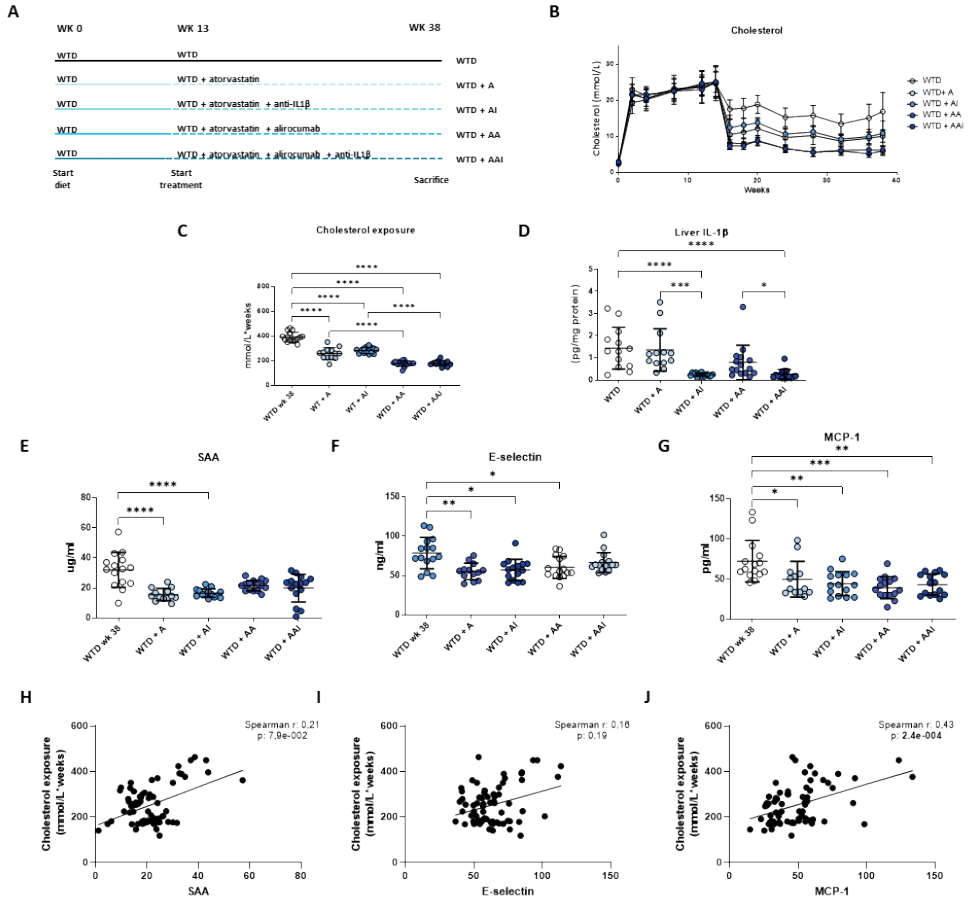

Figure 2. Cholesterol-lowering treatment attenuates dyslipidemia and reduces systemic cytokine levels

E3L mice were fed a cholesterol-supplemented WTD for 38 weeks, treatments were started after 14 weeks. **(A)** Schematic overview of the study design and the different treatment groups. **(B)** Plasma cholesterol levels are successfully reduced after cholesterol-lowering treatments, the combination treatments being most effective. **(C)** Cholesterol exposure based on systemic cholesterol levels in time was reduced upon cholesterol-lowering treatment (A: P<0.001, AI: P<0.001, AL: P<0.001, AAI: P<0.001). **(D)** IL-1β levels were quantified in liver homogenates. Treatment with the anti-IL-1β antibodies significantly reduced hepatic IL-1β levels at endpoint compared to the WTD control (AI: p<0.001, AAI: p<0.001) **(E-G)** SAA, E-selectin and MCP-1 plasma levels were determined at end point. Cholesterol-lowering treatment successfully reduced inflammatory mediators, which was not further affected by inhibition of IL-1β (SAA: A: -51%; p<0.0001, AI: -48%; p<0.0001, AA: -33%, AAI: -39%; E-selectin: A: -30%; p=0.006, AI: -27%; p=0.01, AA: -23%; p=0.05, AAI: -15%; MCP-1: A: -31%; p=0.03, AI: -39%; p-0.004, AA: -46%; p=0.0002, AAI: -40%; p=0.003) **(H-J)** Correlation of cholesterol exposure with systemic cytokines showed a mild correlation with MCP-1 (Spearman R:0.43).* = p <0.05, ** = p <0.01, *** = p <0.001. *A= atorvastatin, AI= atorvastatin + anti-IL-1β, AA= atorvastatin + alirocumab, AAI= atorvastatin + alirocumab + anti-IL1β. n=13-15 mice per group. Results are expressed as individual data points with mean ± 95% confidence intervals.*

Intensive cholesterol-lowering combined with inhibition of IL-1β results in a decrease of synovial thickness in knee joints of dyslipidemic mice

Next we determined whether these treatments were able to ameliorate OA pathology in the knee joints of these mice. As inflammatory processes in the joint contribute to the development of structural OA pathology, we first determined whether the treatments affected synovial thickness. Although we observed only mild synovial thickening, intensive cholesterol-lowering with atorvastatin and alirocumab significantly reduced synovial thickness compared to mice fed a cholesterol-supplemented WTD alone, which was not further decreased by inhibition of IL-1β. (AA: -52%; $p<0.05$, AAI: -62%; $p<0.01$, **Figure 3A,B**). To investigate the inflammatory state of the synovium in more detail, we performed immunohistochemical analysis for S100A9 as a marker for activated monocytes and macrophages. We observed only minor expression of S100A9 in the synovium, supporting the observation that local activation of myeloid cells was minor in this model (**Figure 3C**).

Ectopic bone formation and maturation is not affected by cholesterol-lowering treatment and inhibition of IL-1β in dyslipidemic mice

We next determined whether the different treatments affected ectopic bone formation by determining the total amount of osteophytes and their maturation stage at end point. Ectopic bone formation was determined at several sites throughout the joint and their maturation stage was determined ranging from chondrogenesis to the formation of mature bone. The treatment regimens did not show an effect on ectopic bone formation regarding the number of osteophytes, or on their maturation stage compared to the WTD control group at end point (Figure 4A,B).

Inhibition of IL-1β combined with intensive cholesterol-lowering treatment lowers spontaneous cartilage degeneration in dyslipidemic mice

Finally, we determined whether inhibition of IL-1β on top of cholesterol-lowering treatments could ameliorate cartilage pathology in dyslipidemic mice. Similar to previous observations in a diet-induced model of spontaneous OA[9,130], cartilage damage was mild and most cartilage damage occurred at the lateral site of the joint at end point (**Figure 5A, B, C**). A significant correlation between synovial thickness and cartilage damage was observed (Spearman $r=0.44$, $p=0.0001$, **Supplementary Figure 1**). Cholesterol-lowering treatments alone did not ameliorate spontaneous cartilage damage compared to the WTD control group. Interestingly, inhibition of IL-1β on top of atorvastatin and alirocumab treatment resulted in a significant decrease in cartilage damage when compared to mice fed a WTD alone (AAI: -47.% $p<0.05$,) (**Figure 5A,C**). To investigate enzymatic activity in the articular cartilage in more detail, we performed an MMP13 staining. MMP13 expression was absent in the articular cartilage in all groups (**Figure 5D**).

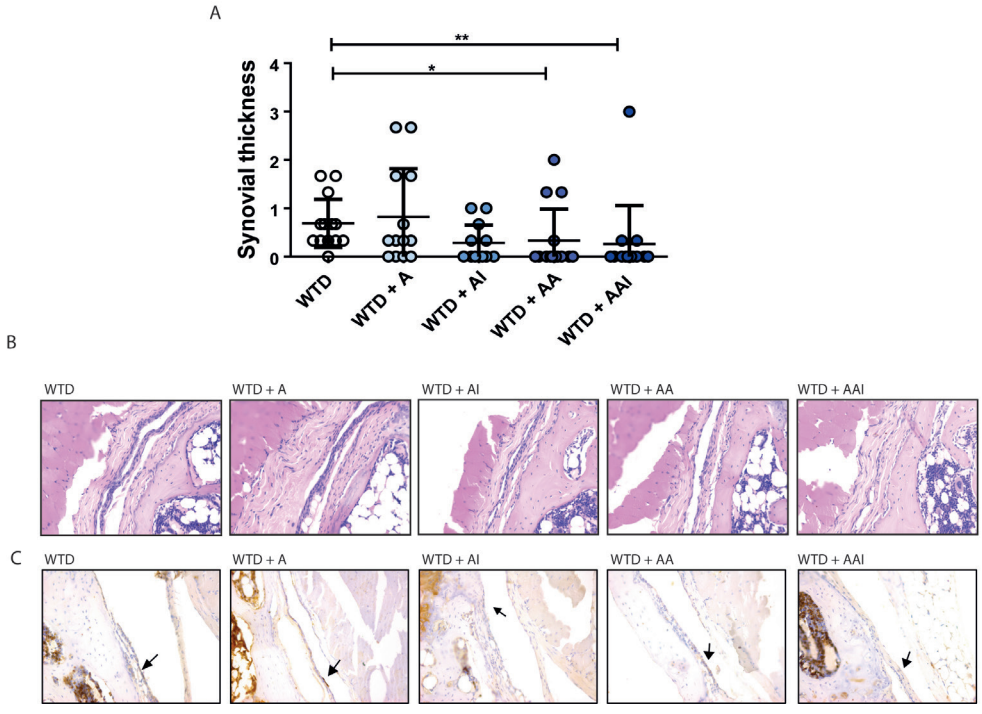

Figure 3. Synovial thickness is reduced in mice receiving intensive cholesterol-lowering treatment combined with inhibition of IL-1β

E3L mice were fed a cholesterol-supplemented WTD for 38 weeks, treatments were started after 14 weeks. Synovial thickness was determined at end point using H&E stained sections. **(A)** Combination treatments reduced synovial thickness compared to mice fed a WTD alone (AA p <0.05, AAI p <0.01). **(B)** Representative pictures of synovial thickness. **(C)** S100A9 staining in the knee joints showed only minor expression in the synovium. * = p <0.05, ** = p <0.01. *A= atorvastatin, AI= atorvastatin + anti-IL-1β, AA= atorvastatin + alirocumab, AAI= atorvastatin + alirocumab + anti-IL1β. n=13-15 mice per group. Results are expressed as individual data points with mean ± 95% confidence intervals.*

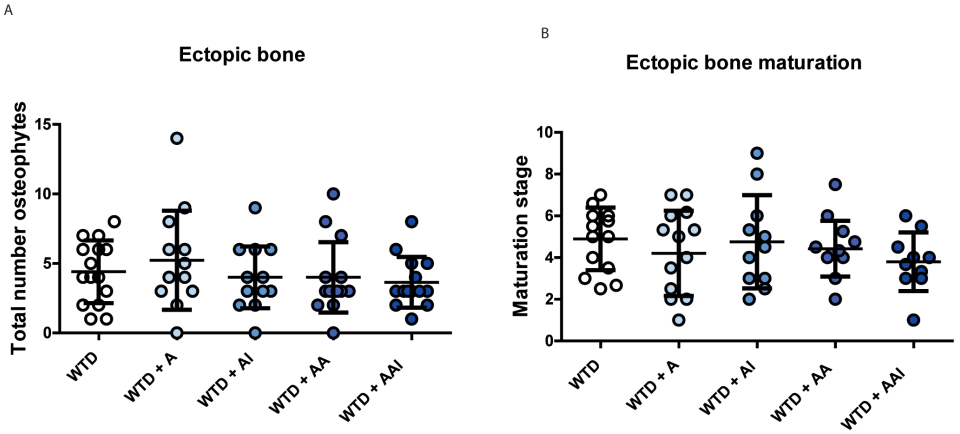

Figure 4. Ectopic bone formation is not affected by cholesterol-lowering treatment and inhibition of IL-1β in dyslipidemic mice.

E3L mice were fed a cholesterol-supplemented WTD for 38 weeks, treatments were started after 14 weeks. Ectopic bone formation was determined throughout the joint using SafO/FG stained sections. (A) The total number of osteophytes throughout the joint was comparable between all treatment groups. (B) Maturation of ectopic bone (0=chondrogenic, 10=mature bone) was similar between all groups and not affected by cholesterol-lowering treatment or inhibition of IL-1β. *A= atorvastatin, AI= atorvastatin + anti-IL-1β, AA= atorvastatin + alirocumab, AAI= atorvastatin + alirocumab + anti-IL1β. n=13-15 mice per group. Results are expressed as individual data points with mean ± 95% confidence intervals.*

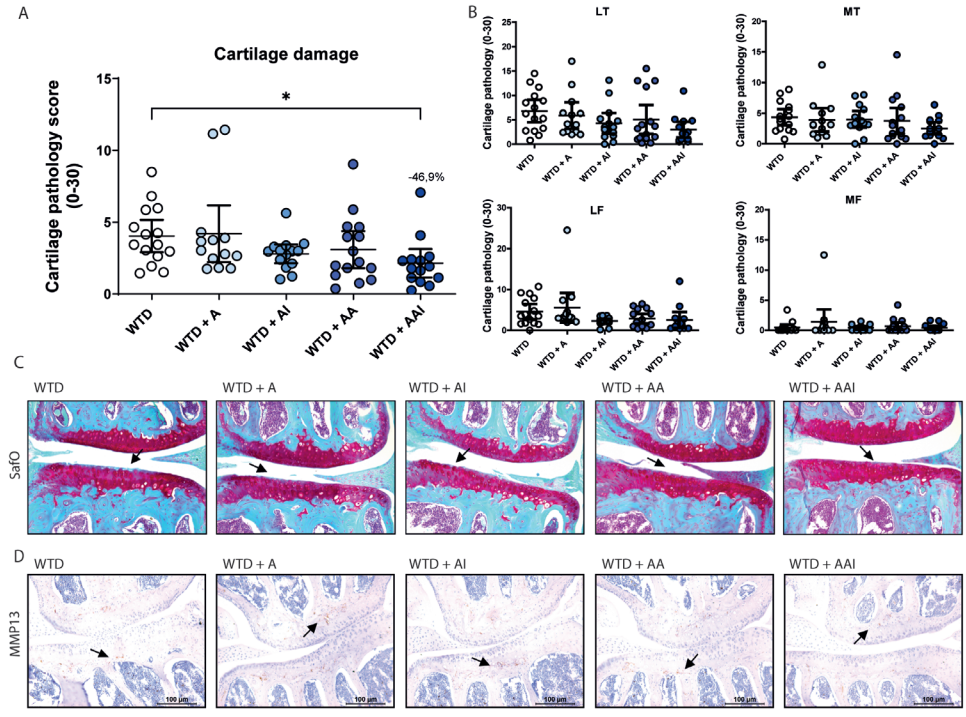

Figure 5. Cartilage damage is reduced in dyslipidemic E3L mice after intensive cholesterol-lowering treatment combined with inhibition of IL-1β

E3L mice were fed a cholesterol-supplemented WTD for 38 weeks, treatments were started after 14 weeks. Cartilage damage was determined at end point using an adapted OARSI scoring system (0-30). **(A)** OA scores were mild and cholesterol-lowering alone did not reduce cartilage damage compared to the WTD control. Triple treatment with atorvastatin, alirocumab and an IL-1β antibody significantly reduced cartilage damage compared to WTD controls (AAI: $p<0.05$). Cartilage damage score represents an average of the four individual compartments that were analyzed. **(B)** Most damage was observed at the lateral side of the joint. **(C)** Representative pictures of cartilage damage. **(D)** MMP13 staining was absent in articular cartilage * = $p<0.05$. *A= atorvastatin, AI= atorvastatin + anti-IL-1β, AA= atorvastatin + alirocumab, AAI= atorvastatin + alirocumab + anti-IL1β. n=13-15 mice per group. Results are expressed as individual data points with mean ± 95% confidence intervals.*

DISCUSSION

The association of MetS and OA has become increasingly recognized and metabolic inflammation has been suggested as a potential link between both diseases. In this study in mimicking the CANTOS Study, we showed that inhibition of IL-1β combined with intensive cholesterol-lowering treatment resulted in a reduction of both synovial thickness and spontaneous cartilage degeneration in knee joints of diet-induced dyslipidemic E3L mice. Lowering of cholesterol levels resulted in a decrease in systemic levels of inflammatory mediators SAA, E-selectin and MCP-1 levels. These findings may imply that inhibition

of IL-1β combined with cholesterol-lowering treatment could be beneficial for a subset of OA patients with high inflammatory risk.

This is the first study to show the effects of inhibition of IL-1β combined with cholesterol-lowering interventions on spontaneous OA development in dyslipidemic mice. IL-1β is produced by inflammasomes and is an important driver of inflammation[211]. IL-1β inhibits proteoglycan synthesis and is a strong inducer of catabolic enzymes by chondrocytes[212, 213] leading to the degradation of the extracellular matrix. Additionally, IL-1β has been linked to increased joint pain in OA patients[214]. Metabolic inflammation can increase activation of the NLR family pyrin domain containing 3 (NLRP3) inflammasome[33, 68], making IL-1β a target of interest in diseases associated with MetS, such as cardiovascular disease and OA. Although IL-1β is thought to play a critical role in the pathogenesis of OA, several clinical studies were not able to demonstrate disease-modifying effects after inhibition of IL-1β[65, 67, 215]. In contrast, a secondary analysis of the CANTOS trial showed that systemic inhibition of IL-1β resulted in a 40-47% decreased incidence rate of total knee and hip replacements compared to the placebo group[69]. Additionally, OA-related adverse events (new onset, worsening of symptoms) were reduced by 23%[69]. Of note, 93% of patients included in the CANTOS trial received lipid-lowering therapy at the onset of the study and cholesterol levels were generally well managed. This study suggested that inhibition of IL-1β could reduce OA development in OA patients with systemic inflammation associated with metabolic disease. As the CANTOS trial was designed to study the effects on cardiovascular events, data on structural outcomes or radiographic evidence were not collected. In the current study, we observed that inhibition of IL-1β combined with cholesterol-lowering treatment resulted in a reduction of structural joint parameters such as synovial thickness and cartilage damage in mice fed a pro-atherogenic WTD. These results substantiate the findings of the CANTOS trial that inhibition of IL-1β together with cholesterol-lowering therapy could reduce OA progression in a subset of patients with systemic inflammation. As structural joint parameters were not assessed during the CANTOS trial, it would be of interest to investigate if inhibition of IL-1β in OA patients with high inflammatory risk reduces structural pathology such as cartilage damage or joint space narrowing. Moreover, IL-1β is believed to play an important role in joint pain via the upregulation of nociceptive mediators and it has been hypothesized that the beneficial effects observed in the CANTOS trial could be attributed to a reduction in joint pain. In future studies where induced OA models such as CiOA or DMM are used, it would be of value to include pain measurements.

Low-grade systemic inflammation is associated with dyslipidemia and is thought to contribute to OA development. Several clinical studies showed an association between high cholesterol levels and the development of OA[41, 42, 204]. The inconsistent effects of cholesterol-lowering therapies on OA development indicate that additional mechanisms, next to high cholesterol levels, are involved[76, 80, 82, 155]. Recently, we have shown that high-intensive cholesterol-lowering therapy alone does not ameliorate the development of spontaneous OA pathology in dyslipidemic E3L.CETP mice with low inflammatory involvement[130]. Therefore, we aimed to determine the effects of cholesterol-lowering combined with inhibition of IL-1β in a more pro-inflammatory environment in the current study. We focused on the

analysis of OA pathology in the E3L model as these mice developed higher levels of inflammatory cytokines on a hypercholesterolemic diet compared to the E3L.CETP mice. Systemic levels of SAA, MCP-1 and E-selectin were significantly reduced by the cholesterol-lowering treatments. While they were not reduced any further upon inhibition of IL-1β, we observed a local reduction of synovial lining thickness and cartilage damage in the knee joints of mice that received high-intensive cholesterol-lowering treatment combined with inhibition of IL-1β. We found a mild significant correlation of hepatic IL-1β levels with synovial thickening but not with cartilage pathology or ectopic bone formation (**Supplementary Figure 2**), which indicates a positive association of systemic IL-1β levels with the inflammatory state of the synovium. To investigate the contribution of systemic inflammation in more detail, we determined OA development in the less inflammatory E3L.CETP model (**Supplementary Figure 3**). In the latter model, the treatments did not reduce cartilage damage or synovial lining thickness, which suggests that the treatment is more beneficial in mice with a more pronounced pro-inflammatory profile. Metabolic inflammation can induce activation of the NLRP3 inflammasome which could explain why inhibition of IL-1β proved more beneficial in the E3L model. Although both strains develop metabolic and cardiovascular disease when fed a atherogenic WTD and show human-like responses to cholesterol-lowering therapies, we only observed effects of the treatments in the E3L mice with increased inflammation. This is similar to observations in the CANTOS trial, where patients with high CRP levels showed the most benefit from the IL-1β inhibition. These results suggest that using translatable models, such as the E3L and E3L.CETP mouse models, can increase the predictive value of pre-clinical studies. As all patients in the CANTOS trial had a history of cardiovascular disease, the presence of metabolic inflammation could explain why beneficial effects of IL-1β were observed in this, but not in previous OA trials in which IL-1β was inhibited[65, 67, 215]. Since these parameters are usually not used as inclusion criteria of OA patients in clinical trials, these results could point towards a subgroup of OA patients that would respond to anti-inflammatory therapy.

Inhibition of IL-1β could reduce OA development via several mechanisms. Possibly, systemic inhibition of IL-1β could act locally, via a reduction of inflammatory mediators or reduced production of cartilage degrading enzymes like matrix metalloproteinases (MMPs) in the knee joint. Moreover, IL-1β inhibits proteoglycan synthesis by chondrocytes[212, 213], thereby hampering repair of the cartilage matrix. In addition, IL-1β stimulates cartilage degeneration via an increased production of several MMPs by chondrocytes, which are released in a latent form within the cartilage matrix and become activated in an inflammatory environment. This may explain why the beneficial effects of IL-1β inhibition were observed in the more inflammatory E3L model. To investigate enzymatic activity in articular cartilage in more detail, we determined expression of MMP13 and in articular cartilage. MMP13 staining in articular cartilage was absent, which can be explained by the mild cartilage damage and the spontaneous model used in this study.

Although inhibition of IL-1β on top of cholesterol-lowering treatment ameliorated synovial thickening and cartilage damage, no effect was found on ectopic bone formation. We observed a correlation with

systemic cholesterol exposure and synovial lining thickness and cartilage damage, but not with ectopic bone formation in this study (**Supplementary Figure 4**). We additionally found a significant correlation with cholesterol exposure and plasma MCP-1 levels, but not with systemic levels of SAA or E-selectin. This indicates that more intensive cholesterol-lowering treatment affects systemic inflammation and OA pathology. However, as cholesterol-lowering treatments alone were insufficient in ameliorating spontaneous OA development, these results suggest that high cholesterol drives other processes involved in OA development such as inflammation or innate immune training. We and others showed that induced post-traumatic OA models show more severe pathology in hypercholesterolemic mice[36, 49, 51]. In an inflammatory environment, low-density lipoprotein cholesterol (LDL-C) is transformed into oxidized LDL (oxLDL) due to the presence of inflammatory mediators like reactive oxygen species (ROS). In previous studies, we have shown that mainly oxLDL, and not LDL, is responsible for the OA pathology associated with high cholesterol in mice[206]. Furthermore, it was shown that lipoproteins like oxLDL can induce reprogramming of monocytes and their myeloid progenitor cells, which is referred to as trained immunity. Trained immunity increases the inflammatory response of monocytes and macrophages to secondary stimuli, like Toll-like receptor (TLR) ligands. Recently, it was shown by Christ *et al*. that a WTD can induce trained immunity in pro-atherogenic *Ldlr*$^{-/-}$ mice, which was dependent on the NLRP3 inflammasome/IL-1β pathway[150]. In OA, these (ox)LDL-primed monocytes could enter the joint during inflammation and instigate an aggravated local inflammatory response. As we observed a reduction of synovial lining thickness and cartilage damage in mice treated with a combination of atorvastatin, alirocumab and inhibition of IL-1β, it is of interest to explore the efficacy of these combination treatments in induced post-traumatic OA models with more pronounced local inflammation, such as destabilization of the medial meniscus (DMM) or CiOA.

Some limitations of this study are the absence of a chow control group and a group in which only IL-1β was inhibited. Firstly, as this study focused on the effects of different treatments in dyslipidemic mice, a regular chow control group was not taken along so we were unable to study the effect of the WTD only on development of spontaneous OA. However, previous studies showed that a cholesterol-supplemented WTD contributes to OA development in E3L and E3L.CETP mice[155, 205]. Secondly, we did not include a group in which only IL-1β was inhibited. We find that cholesterol-lowering therapies combined with inhibition of IL-1β reduces cartilage damage in dyslipidemic mice. It would be of interest to investigate whether anti-IL1β alone is also effective under high cholesterol conditions without the use of cholesterol-lowering therapies. Further studies using these treatments in mice that were fed a regular chow diet should proof whether this protective effect is specific for high cholesterol conditions. Another potential limitation if the use of only females in this study. Although in general male mice develop more severe OA pathology that female mice, we chose to use female mice in the current study as female E3L mice are more responsive to cholesterol containing diets by having higher cholesterol and triglyceride levels compared to male mice. However, for the arthroplasty outcomes in the CANTOS trial, similar effects of IL-1β inhibition were observed after the analyses were stratified by sex[69].

Taken together, this study supports the hypothesis that systemic inflammation associated with metabolic dysregulation could contribute to OA development. We have shown that inhibition of IL-1β combined with intensive cholesterol-lowering treatment reduces OA pathology in dyslipidemic mice with systemic inflammation. Our results may indicate that targeting IL-1β in combination with cholesterol-lowering therapies could be beneficial for a subset of OA patients with metabolic dysfunction and high inflammatory risk.

AUTHOR CONTRIBUTIONS

HMGP and EJP have designed the study. EJP and YG have carried out experimental procedures and acquired the data. YG has been the primary person responsible for writing the manuscript. NNLK, MHJB, ABB, PvdK, EJP, HMGP and PLEML were involved in drafting the work or revising it critically for important intellectual content. All authors approved the final version to be published.

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ROLE OF THE FUNDING SOURCE

The funding sources had no role in study design, in collection, analysis or interpretation of data, or in writing the manuscript and decision to submit the manuscript.

CONFLICTS OF INTEREST

The authors declare no competing interests.

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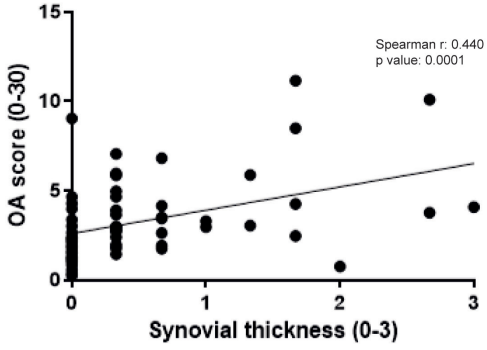
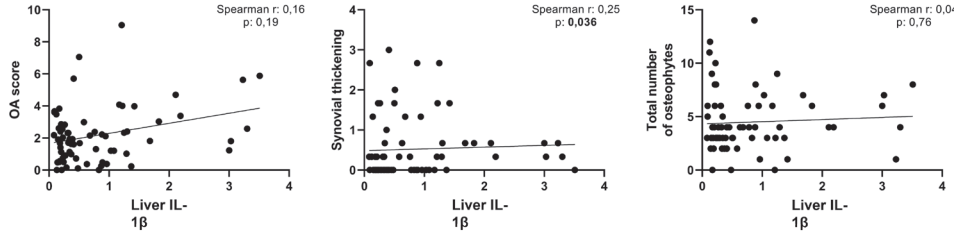
SUPPLEMENTARY MATERIAL



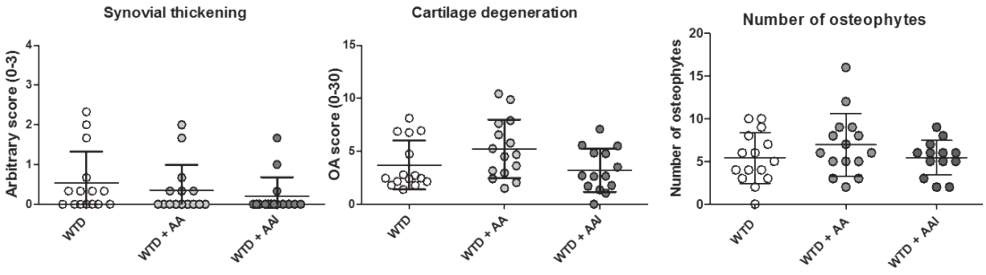
Supplementary Figure 1. Synovial thickness correlated to cartilage damage in knee joints of E3L mice fed a cholesterol-supplemented WTD

E3L mice were fed a cholesterol-supplemented WTD for 38 weeks, treatments were started after 14 weeks. Synovial lining thickness correlated to the observed pathology in the knee joints. (*p = 0.0001, Spearman r = 0.440)*



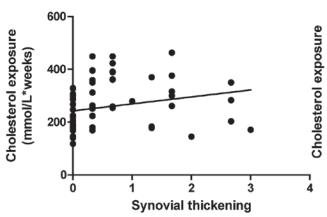
Supplementary Figure 2: IL-1β correlated with synovial thickening, but not with cartilage damage or ectopic bone formation

E3L mice were fed a cholesterol-supplemented WTD for 38 weeks, treatments were started after 14 weeks. IL-1b produced by the liver showed a mild correlation with synovial thickening. (*p = 0.036, Spearman r = 0.25),* but not with cartilage damage or ectopic bone formation.

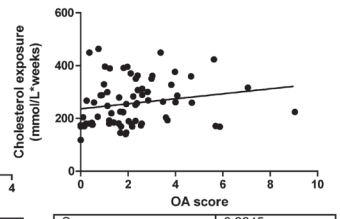


Supplementary Figure 3. Cholesterol-lowering treatment combined with inhibition of IL-1β does not reduce OA pathology in E3L.CETP mice fed a cholesterol-supplemented WTD

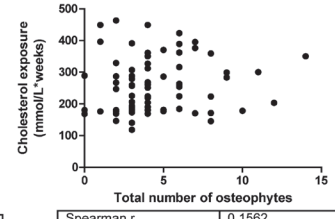
E3L.CETP mice were fed a cholesterol-supplemented WTD for 38 weeks, treatments were started after 14 weeks. **(A)** Synovial thickening was determined using an arbitrary score from 0-3. **(B)** Cartilage damage was determined at end point using an adapted OARSI scoring system (0-30). Cartilage damage was not affected by the treatment groups compared to mice fed a WTD alone. **(C)** Ectopic bone formation was determined throughout the joint using SafO/FG stained sections. The total number of osteophytes throughout the joint was comparable between all treatment groups. *A= atorvastatin, AA= atorvastatin + alirocumab, AAI= atorvastatin + alirocumab + anti-IL1β. Results are expressed as individual data points with mean ± 95% confidence intervals.*



Spearman r	0.4040
95% confidence interval	0.1833 to 0.5859
P value (two-tailed)	0.0004
P value summary	***



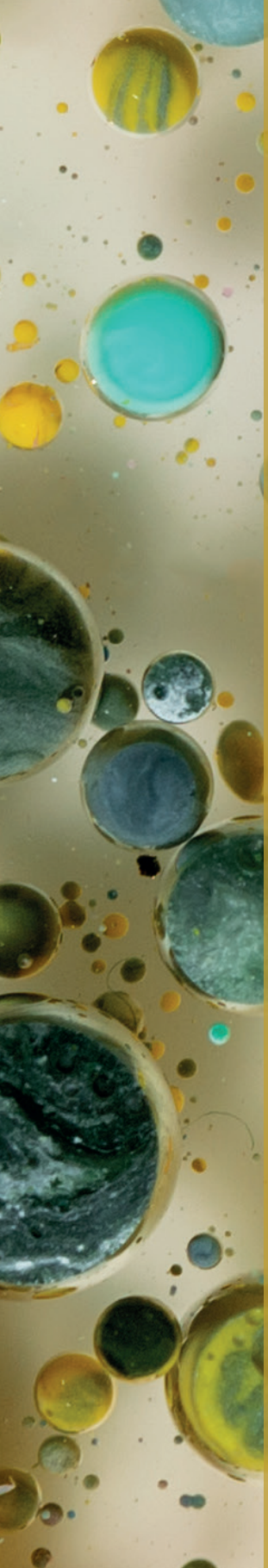
Spearman r	0.2845
95% confidence interval	0.05129 to 0.4883
P value (two-tailed)	0.0147
P value summary	*



Spearman r	0.1562
95% confidence interval	-0.08360 to 0.3788
P value (two-tailed)	0.1871
P value summary	ns

Supplementary Figure 4. Cholesterol exposure correlated to synovial thickness and cartilage damage in dyslipidemic E3L mice

Cholesterol exposure was correlated to synovial lining thickness, cartilage degeneration and the total number of osteophytes at end point. **(A+B)** Cholesterol exposure correlates to synovial lining thickness ($p = 0.0004$, Spearman r = 0.4*)* and cartilage damage ($p = 0.01$, Spearman r = 0.28) observed after 38 weeks of WTD feeding. **(C)** No correlation was observed between systemic cholesterol exposure and the number of osteophytes.



CHAPTER 7

Summary, discussion and final considerations

Currently, there are no curative or disease-modifying treatments available for OA and symptoms are generally managed by lifestyle interventions and analgesics. Although it has become clear that OA is a highly multifactorial and heterogenous disease, the underlying mechanisms that drive OA pathology remain unclear. One identified risk factor for OA development is the metabolic syndrome (MetS). MetS comprises a cluster of multiple metabolic conditions including visceral obesity, high glucose levels and insulin resistance, hypertension and dyslipidemia. Previously, it was thought that MetS mainly increased OA development via loading on the joint due to obesity. However, the observation that metabolic syndrome also increased the risk of developing OA in non-weight bearing joints, such as the hands, indicated that systemic mechanisms are most likely involved[1-4]. High systemic cholesterol is a component of MetS and has been suggested as a contributor to osteoarthritis development. Several studies have indicated that hypercholesterolemia leads to the progression of OA. The use of statins have shown variable results concerning the development or progression of OA, which is discussed in detail in **Chapter 1**. Statins are the most commonly used group of cholesterol-lowering drugs and are highly effective for the treatment of cardiovascular disease and atherosclerosis. However, the observed effects regarding statin use and OA progression have been highly variable, where some studies showed a protective effect of statins while others did not observe these protective effects. There are several potential mechanisms that can account for the conflicting results regarding statin therapy and the progression of OA. Statin intolerance is estimated to occur in 20-30% of patients and often leads to discontinuation of statin treatment[5]. Moreover, the presence of statin-associated muscle pain could interfere with the beneficial effects on OA symptoms such as pain, and confound the results. In addition, the limited availability of information regarding the dosage and the duration of statin use could contribute to the high heterogeneity observed amongst studies. In this thesis I studied the potency of more intensive cholesterol-lowering therapies, by combinational treatment of statins and newly-developed cholesterol-lowering drugs like anti-PCSK9 and anti-ANGPTL-3 antibodies, to reduce the development of joint pathology during both spontaneous and induced models of OA. These novel cholesterol-lowering drugs have been successfully used in cardiovascular disease and are able to even further reduce cholesterol levels on top of statin therapy. As high systemic cholesterol levels are associated with systemic inflammation[6, 7], more optimal cholesterol-lowering therapies could be of additional benefit for OA patients. It was shown that more optimal cholesterol-lowering treatment could further reduce cardiovascular events in patients that already received statin therapy. Moreover, statin therapy combined with PCSK9 inhibition resulted in stabilization and regression of atherosclerotic plaques in both humans and mice[8, 9]. Since cardiovascular disease and OA share common pathophysiological risk factors, these could be promising new treatment options for MetS-associated OA.

PCSK9 DEFICIENCY DOES NOT AFFECT OA PATHOLOGY IN DYSLIPIDEMIC MICE

In **Chapter 2** we investigated the role of PCSK9 in OA development in more detail. *Pcsk9*$^{-/-}$ mice have reduced systemic cholesterol levels and therefore can be used to study the effects of diet and cholesterol on disease development[10]. In our study, PCSK9 deficiency resulted in reduced systemic cholesterol levels in mice fed a regular chow or cholesterol-supplemented WTD, although less than expected. Possibly, other pathways that regulate LDLR expression, such as the inducible degrader of LDLR (IDOL)[11], or pathways involved in cholesterol efflux could become more active and act as a compensatory mechanism in mice lacking the PCSK9 gene. In this study we showed that synovial inflammation was reduced in *Pcsk9*$^{-/-}$ mice fed a cholesterol-supplemented WTD compared to WT mice. In contrast, *Pcsk9*$^{-/-}$ mice fed a regular chow diet had higher levels of synovial inflammation. These observed changes in synovial inflammation did not result in differences in end stage pathology such as cartilage damage or ectopic bone formation. In contrast to previous experiments performed in dyslipidemic *Apoe*$^{-/-}$ *and Ldlr*$^{-/-}$ mice, a cholesterol-supplemented WTD did not increase OA pathology during experimental collagenase-induced OA in WT or *Pcsk9*$^{-/-}$ mice[12, 13]. *Apoe*$^{-/-}$ *and Ldlr*$^{-/-}$ mice have much higher systemic cholesterol levels compared to WT mice when fed a (cholesterol-supplemented) WTD. Although in these previous studies mouse models that develop supraphysiological cholesterol levels were used, increased OA pathology such as ectopic bone formation and synovial activation was also observed in dyslipidemic WT mice that show a more modest increase in cholesterol levels upon WTD feeding[12, 13]. Difference in the duration or the composition of the diet possibly explain these discrepancies between studies.

In contrast to humans, mice with a WT background predominantly carry their cholesterol in HDL particles, while their (V)LDL levels are lower, because they lack the CETP gene which transports cholesterol esters from HDL to VLDL and LDL particles in exchange for triglycerides. Therefore, using mice with a more human-like lipoprotein profile, like the profile present in transgenic E3L.CETP mice, increases the translatability of cholesterol-associated research on OA development[14]. Interestingly, the use PCSK9-inhibitor alirocumab was previously shown to effectively reduce circulating cholesterol levels in E3L.CETP mice and could therefore be a more translatable approach to investigate the use of PCKS9 inhibition in OA[9, 14].

TARGETING OF CHOLESTEROL DURING SPONTANEOUS OA DEVELOPMENT

In **Chapter 3** we determined the effects of various intensities of cholesterol-lowering treatment on spontaneous OA development in E3L.CETP mice that were fed a cholesterol-supplemented WTD, using a combination of atorvastatin therapy with the novel PCSK9 inhibitor alirocumab and ANGPLT3-inhibitor evinacumab. Contrary to experiments performed in *Pcsk9*$^{-/-}$ mice, we observed a robust reduction in systemic cholesterol levels in this study. However, these decreased systemic cholesterol levels did not result in decreased cartilage degeneration, synovial inflammation or ectopic bone formation as

compared to the non-treated control mice. A possible explanation could be that in contrast to our previous experiments where induced OA models were used that show profound synovial inflammation, the joint inflammation in the spontaneous model used in this study was only minor. In an inflammatory environment, there is an increased production of reactive oxygen species (ROS) in the joint. ROS induce the transformation of LDL into the more pro-inflammatory oxLDL. Similar to macrophages in atherosclerotic plaques, macrophages residing in the synovium can internalize and accumulate oxLDL, leading to an increased production of cytokines and matrix-degrading enzymes. Therefore, we hypothesized that inflammation is an important driver of cholesterol-associated OA pathology and that cholesterol-lowering treatments could be more effective when there is more substantial joint inflammation.

TARGETING OF CHOLESTEROL DURING INJURY-INDUCED OA DEVELOPMENT

As we have previously shown that intensive cholesterol lowering treatment did not reduce spontaneous OA development[15], in **Chapter 4** we determined if cholesterol-lowering treatment could reduce OA development in WTD-fed E3L.CETP mice in which the CiOA model was induced. We observed that the combination of atorvastatin with PCSK9-inhibitor alirocumab reduced both synovial inflammation and systemic inflammatory markers in serum early after induction of the CiOA model. However, this reduction in inflammation did not result in a decrease in cartilage pathology or ectopic bone formation at end-stage disease. The observed pathology in this study was severe, demonstrated by substantial ectopic bone formation and cartilage damage. Possibly, more subtle effects of the cholesterol-lowering treatment could be lost, as the observed pathology leads to more mechanically induced damage. In several animal studies, a high-fat diet resulted in increased cartilage damage[16-18]. However, some studies have shown that a WTD increased macrophage infiltration[19] and inflammation in the synovium, while no effects on cartilage damage were observed[19, 20]. These results suggest that high cholesterol primarily exacerbates early stage changes of inflammation, which could explain why we mainly observed anti-inflammatory effects of the cholesterol-lowering treatment during the early phases of our study.

TARGETING OF IL-1B IN DYSLIPIDEMIC MICE

Metabolic inflammation is thought to contribute to OA development and has been suggested as a possible link between MetS and OA[21]. Metabolic inflammation is mainly caused by metabolic factors that induce metabolic stress and inflammation, such as lipids and glucose[6, 7]. Interleukin-1β (IL-1β) is an important driver of inflammation and is considered by many to be involved in OA pathology. Metabolic inflammation can increase activation of the NLRP3 inflammasome[22-24], which mediates the secretion of IL-1β. Therefore, IL-1β is a target of interest in diseases associated with MetS, such as cardiovascular disease and OA. Several in vitro studies showed that IL-1β inhibits proteoglycan synthesis and is a

strong inducer of cartilage-degrading enzymes by chondrocytes[25, 26] leading to the degradation of the extracellular matrix. Additionally, IL-1β has been linked to increased joint pain in OA patients[27]. Despite the fact that IL-1β is thought to play a critical role in the pathogenesis of OA, several pre-clinical and clinical studies were not able to demonstrate any disease-modifying effects after inhibition of IL-1β[28-31]. Interestingly, a recent secondary analysis of the Canakinumab Anti-inflammatory Thrombosis Outcome Study (CANTOS) trial showed that systemic inhibition of IL-1β resulted in a 40-47% decreased incidence of total knee and hip replacements in patients with CVD[32]. Additionally, OA-related adverse events (new onset, worsening of symptoms) were reduced by 23%[32]. As the CANTOS trial was designed to study the effects on cardiovascular events, data on structural outcomes or radiographic evidence were not collected and more detail on structural parameters could provide insight for future studies. Hereto, in **Chapter 5** we showed that a single dose of anti-IL-1β serum could prevent diet-induced activation of innate immune cells in WT mice fed a cholesterol-supplemented WTD during early-stage CiOA. However, we did not observe a reduction in OA pathology after inhibition of IL-1β in mice fed a cholesterol-supplemented WTD. These data indicate that inhibition of IL-1β alone, also in an environment with high cholesterol levels, is not sufficient to reduce OA pathology.

As all patients in the CANTOS trial had a history of cardiovascular disease, the presence of metabolic inflammation could explain why beneficial effects of IL-1β were observed in this, but not in previous OA trials in which IL-1β was inhibited[29, 31, 33]. Interestingly, this IL-1β effect was found on top of cholesterol-lowering treatment, since over 93% of patients enrolled in the CANTOS study received cholesterol-lowering therapy at the time the anti-IL1-β treatment started. Moreover, patients with increased systemic inflammation, shown by the highest CRP levels, were most responsive to the treatment. Therefore, in **Chapter 6** we investigated if inhibition of IL-1β combined with cholesterol-lowering therapy could decrease spontaneous OA pathology in E3L and E3L.CETP mice, which show different degrees of systemic inflammation when fed a cholesterol-supplemented WTD. Cholesterol levels were strongly reduced in mice receiving cholesterol-lowering treatment. E3L mice exhibited increased systemic inflammation compared to the E3L.CETP strain. We showed that the combined treatment resulted in a decrease in synovial thickening and cartilage degeneration in E3L mice compared to mice fed a WTD only. These results strengthen the findings of the CANTOS trial and indicate that the use of IL-1β inhibitors combined with cholesterol-lowering treatment could be beneficial in patients with metabolic inflammation. Interestingly, we observed that OA pathology was reduced in the more inflammatory E3L mice and not in the E3L.CETP mice which is in agreement with the CANTOS trial. It would be of interest to investigate the efficacy of cholesterol-lowering treatment combined with IL-1β inhibitors in a CiOA model.

FUTURE PERSPECTIVES

Literature already showed a strong link between metabolic syndrome and the development of osteoarthritis and high cholesterol levels seem to drive the progression and development of OA. Although cholesterol-lowering treatment is very effective in the treatment of cardiovascular disease, their effects on OA development are not fully elucidated yet and the use of statins showed variable results on OA development.

The use of novel cholesterol-lowering therapies could, similar to observations made for cardiovascular disease, be more efficient to ameliorate OA development than the use of statin therapy only. In our studies we observed that the use of intensive cholesterol-lowering treatment such as anti-PCSK9 antibodies resulted in a robust reduction of systemic cholesterol. However, this reduction of systemic cholesterol levels did not result in changes in end-stage OA pathology such as cartilage degeneration or ectopic bone formation. Interestingly, we did show that cholesterol-lowering therapies mainly reduce the inflammatory effects of WTD feeding that are observed in dyslipidemic mice, shown by a decrease in both systemic and synovial inflammation. In addition, the results described in this thesis indicate that cholesterol-lowering by itself might not be enough to ameliorate OA development, but that a combination of cholesterol-lowering therapies with anti-inflammatory therapies is needed to reduce the development of cholesterol-associated OA development. Moreover, our studies show that the use of cholesterol-lowering therapies might be most effective in dyslipidemic patients with increased levels of metabolic inflammation.

Together, our research highlights the importance of concentrating on specific subgroups of OA patients. Investigating the use of cholesterol-lowering therapies in OA patients with (metabolic) inflammation or patients with co-morbidities such as CVD could point towards specific subgroups of patients for whom these therapies could be of benefit. Moreover, the use of anti-inflammatory therapies combined with cholesterol-lowering therapies could be beneficial for a subset of OA patients with metabolic dysfunction and high inflammatory risk and calls for further investigation.

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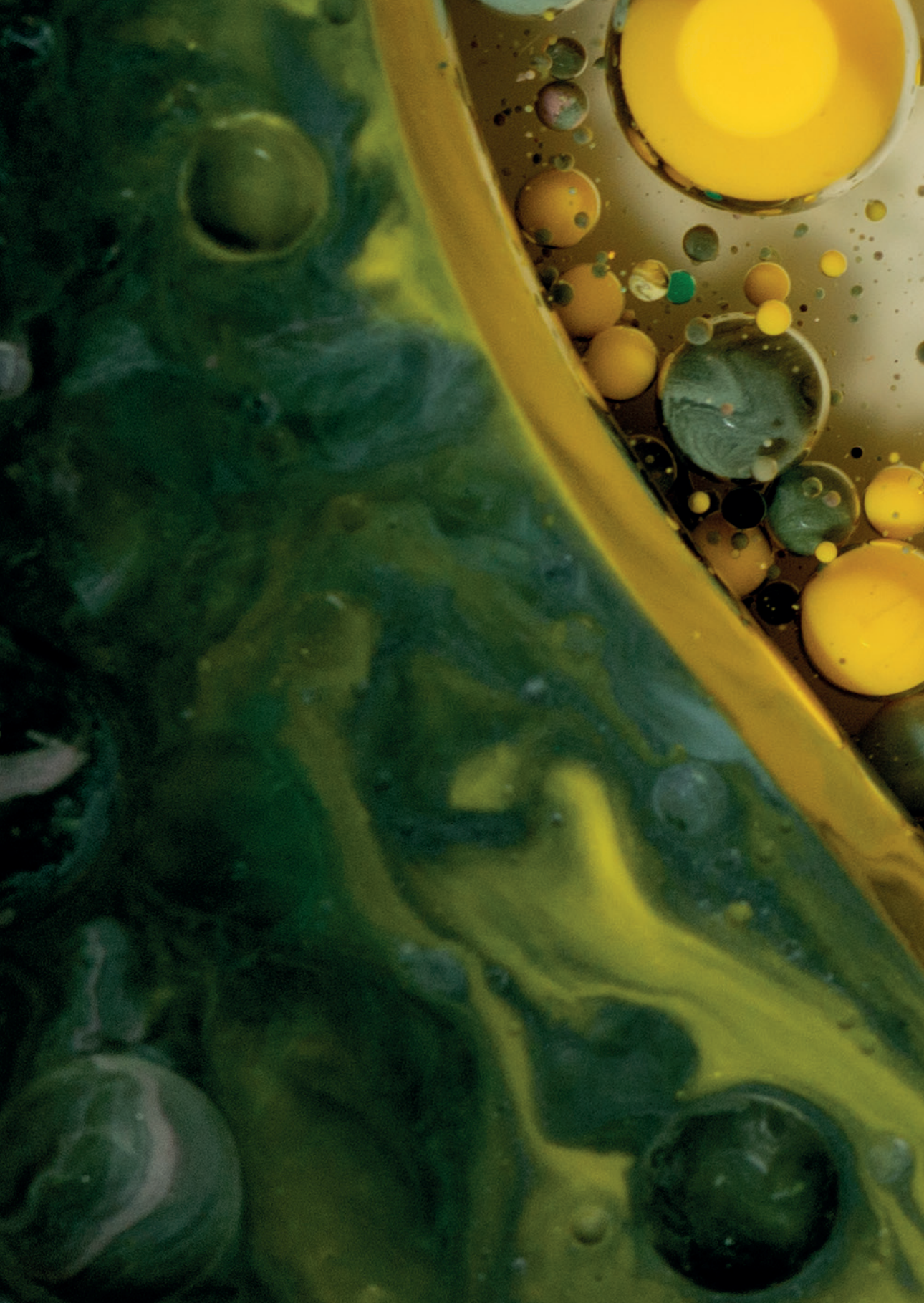

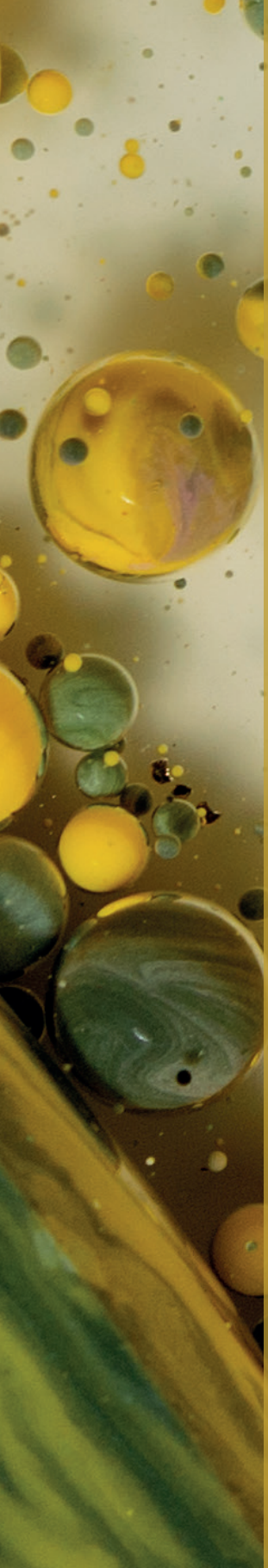

APPENDICES

Nederlandse samenvatting

Curriculum Vitae

List of Publications

Phd Portfolio

Research data management

Dankwoord

ARTROSE

Artrose is de meest voorkomende aandoening van het bewegingsapparaat. In 2019 hadden ongeveer 1,5 miljoen mensen in Nederland artrose. Er wordt verwacht dat dit aantal aanzienlijk zal toenemen, onder andere door de toenemende vergrijzing en overgewicht. Er zijn verschillende factoren die van invloed kunnen zijn op het ontstaan van artrose. Enkele van deze factoren zijn leeftijd, erfelijkheid, het vrouwelijke geslacht, overgewicht en het metabool syndroom. Het metabool syndroom omvat een verzameling van meerdere problemen rond de stofwisseling zoals overgewicht , een hoge bloeddruk, hoge bloedsuikerspiegels en een verstoorde vetzuurbalans (dyslipidemie). Artrose kan voorkomen in één gewricht, zoals de knie, of de heup, maar ook in meerdere gewrichten tegelijkertijd. Artrose is een chronische ziekte waarbij afbraak van het gewrichtskraakbeen een van de belangrijkste kenmerken is. Gewrichtskraakbeen is het weefsel dat de uiteinden van botten bekleedt, op de plek waar deze botten scharnieren. Gezond kraakbeen heeft een schokdempende functie en zorgt ervoor dat de botuiteinden binnen het gewricht soepel over elkaar heen kunnen bewegen. In gezond kraakbeen is er een evenwicht tussen opbouwende en afbrekende processen. Tijdens artrose is dit evenwicht ontregeld en verschuift de balans richting de afbrekende processen, waarbij onder andere een verhoogde hoeveelheid kraakbeen afbrekende enzymen wordt geproduceerd, zoals MMPs en ADAMTSs. Bij langdurige afbraak van kraakbeen zal er uiteindelijk bot op bot contact ontstaan, wat leidt tot pijn en functieverlies van het gewricht. Naast de schade aan het kraakbeen spelen ook andere weefsels in het gewricht een belangrijke rol bij artrose. Er kunnen bijvoorbeeld abnormale botknobbels ontstaan aan de rand van de gewrichtsholte. Ook de binnenbekleding van het gewricht, het synovium, kan ontstoken raken en bijdragen aan het ziekteproces van artrose. Huidige therapieën richten zich voornamelijk op symptoombestrijding, zoals het verminderen van pijn, en het geven van levensstijl adviezen zoals meer beweging en gewichtsverlies. Bij vergevorderde artrose kan het pijnlijke gewricht worden vervangen door een kunstgewricht. Tot op heden zijn er nog geen behandelingen beschikbaar die de ontwikkeling van artrose kunnen voorkomen of afremmen, daarom is er een grote behoefte aan de ontwikkeling van nieuwe therapieën die het ziekteproces kunnen remmen.

HOOG CHOLESTEROL EN ARTROSE

Hoewel het duidelijk is dat artrose van veel actoren afhankelijk is, en ook veel verschillende oorzaken kan hebben is, blijven de onderliggende mechanismen achter artrose-pathologie slecht begrepen. Zoals eerder genoemd is een van de risicofactoren voor het ontwikkeling van artrose het metabool syndroom. Het metabool syndroom is zeer levensstijl afhankelijk en staat ook bekend als een welvaartsziekte. Het metabool syndroom komt dan ook vaak voor bij de Westerse bevolking en hangt samen met overconsumptie van vetten en suikers. Eerder werd gedacht dat metabool syndroom gekoppeld was aan de ontwikkeling van artrose als gevolg van overgewicht en de daardoor toegenomen belasting op bijvoorbeeld knieën of heupen. Echter is gebleken dat metabool syndroom ook samenhangt met de

ontwikkeling van artrose in gewrichten die geen lichaamsgewicht hoeven dragen, zoals die in de hand. Dit geeft aan dat er hoogstwaarschijnlijk ook andere factoren dan alleen het gewicht betrokken zijn bij het ontwikkelen van artrose. Bij mensen met het metabool syndroom is er vaak sprake van dyslipidemie. Mensen met een verstoorde vetzuurbalans kunnen bijvoorbeeld een verstoorde balans hebben tussen het 'slechte' LDL en 'goede' HDL cholesterol. LDL staat bekend als het 'slechte' cholesterol en transporteert voornamelijk cholesterol van de lever naar de perifere organen, terwijl HDL bekend staat als 'goed' cholesterol omdat het cholesteroldeeltjes van de periferie terug naar de lever transporteert. In de lever kan het cholesterol vervolgens worden omgezet in galzuren, waarna het in de darmen terechtkomt en uiteindelijk wordt uitgescheiden. Een verhoogde inname van vetten en cholesterol via de voeding leidt tot een overbelasting van dit systeem, wat resulteert in een verhoogde productie van cholesterol in de lever en een toename van de hoeveelheid cholesterol in het bloed. Er zijn verschillende onderzoeken die hebben laten zien dat het hebben van hoog cholesterol geassocieerd is met de ontwikkeling van artrose.

CHOLESTEROLVERLAGING EN ARTROSE

Cholesterol lijkt een rol te spelen bij de ontwikkeling van artrose. Er zijn dan ook onderzoeken gedaan waarin is gekeken of het gebruik van statines, een van de meest voorkomende cholesterolverlagende therapieën, het ontstaan van artrose kan verminderen of voorkomen. Tot op heden laten deze onderzoeken sterk wisselende resultaten zien, waarbij sommige studies een beschermend effect van statines aantoonden, terwijl andere onderzoeken concludeerden dat het gebruik van statines geen effect had op het ontwikkelen van artrose. Er zijn verschillende redenen die de uiteenlopende resultaten van statinegebruik zouden kunnen verklaren. Het komt bijvoorbeeld met regelmaat voor dat het gebruik van statines niet leidt tot de beoogde verlaging van het cholesterol, wat ook deels verklaard kan worden door de slechte therapietrouw bij statines. Daarnaast is een vaak voorkomende bijwerking van statinegebruik spierpijn, wat de gunstige effecten op symptomen zoals pijn zou kunnen maskeren. Cholesterolverlagende therapieën zoals statines worden vaak met succes gebruikt bij de behandeling van hart- en vaatziekten zoals aderverkalking. Er zijn veel overeenkomsten tussen de risicofactoren voor het ontwikkelen artrose en hart- en vaatziekten. De ontwikkeling van nieuwe cholesterolverlagende zoals anti-PCSK9- of anti-ANGPTL-3-antistoffen zouden potentiële nieuwe strategieën kunnen bieden voor cholesterolverlagende behandelingen voor artrose patiënten. Er is namelijk aangetoond dat het gebruik van statines in combinatie met het remmen van PCSK9 zorgt voor een sterkere vermindering van het cholesterol en van aderverkalking dan wanneer er alleen statines worden gebruikt. Het gebruik van deze nieuwe cholesterolverlagende therapieën zou, vergelijkbaar met de behandeling van hart- en vaatziekten, ook efficiënter kunnen zijn om de ontwikkeling van artrose te remmen dan het gebruik van alleen statines.

DOEL VAN DIT PROEFSCHRIFT

In de experimenten die in dit proefschrift staan beschreven hebben we onderzocht of intensieve cholesterolverlaging door middel van het combineren van cholesterolverlagende therapieën de ontwikkeling van artrose in muizen met een verstoorde vetzuurbalans kon verminderen. Daarnaast hebben we onderzocht of het gebruik van ontstekingsremmende therapieën in combinatie met cholesterolverlaging de artrose pathologie kan verminderen. Hiervoor hebben we gebruik gemaakt van verschillende cholesterolverlagende therapieën, zoals statines en anti-PCSK9 antilichamen.

DELETIE VAN HET PCSK9 GEN HEEFT GEEN EFFECT OP ARTROSE PATHOLOGIE IN DYSLIPIDEMISCHE MUIZEN

In **Hoofdstuk 2** hebben we in meer detail onderzocht welke rol het PCSK9 eiwit speelt in de ontwikkeling van artrose. PCSK9 is een van de belangrijkste eiwitten die het cholesterol metabolisme in ons lichaam regelt. Daarnaast is gesuggereerd dat PCSK9 een directe rol speelt bij ontstekingen. PCSK9-deficiente muizen hebben lagere cholesterol waarden en kunnen worden gebruikt om de effecten van voeding en cholesterol op de ontwikkeling van ziekten, zoals artrose, te bestuderen. Wij hebben bestudeerd of PCSK9-deficientie de artrose pathologie kon verminderen in muizen met verhoogde cholesterol waardes. Dit hebben wij onderzocht in een experimenteel model voor artrose (het collagenase-geïnduceerd artrose model (CiOA)), waarin gewrichtsontsteking een belangrijke rol speelt. Wij zagen dat muizen zonder PCSK9 op een normaal dieet een verlaging van cholesterol in het bloed hadden. Er was echter een minder efficiënte verlaging in muizen die een cholesterol-rijk dieet hadden gekregen. In PCSK9-deficiente muizen die een cholesterol-rijk dieet kregen was er een vermindering in ontsteking van het gewrichtskapsel, terwijl muizen die een normaal dieet kregen juist meer ontsteking hadden. Deze veranderingen in gewrichtsontsteking hebben niet geleid tot vermindering van kraakbeenbeschadiging of abnormale botvorming.

HET VERLAGEN VAN CHOLESTEROL IN EEN MODEL WAAR SPONTAAN ARTROSE ONTSTAAT

In **Hoofdstuk** 3 hebben wij onderzocht wat het effect is van het gebruik van cholesterolverlagende behandelingen met een toenemende intensiteit op de spontane ontwikkeling van artrose in E3L.CETP muizen. In E3L.CETP muizen zijn een deel van de genen die het cholesterolmetabolisme aansturen vervangen door stukjes menselijk DNA, waardoor ze een cholesterolmetabolisme hebben dat meer op dat van de mens lijkt. Deze muizen hebben een dieet gekregen met hoog cholesterol. We gebruikten een combinatie van atorvastatine met de PCSK9-remmer alirocumab en ANGPLT3-remmer evinacumab om de hoeveelheid cholesterol te verlagen. In tegenstelling tot het experimenten in PCSK9-deficiente muizen, zagen we in deze studie een sterke verlaging van het systemische cholesterolgehalte. We zagen

echter geen vermindering van artrose pathologie zoals kraakbeen afbraak of synoviale ontsteking. In tegenstelling tot eerdere experimenten waarbij modellen voor geïnduceerde OA werden gebruikt, is gewrichtsontsteking in dit spontane model gering. In een gewricht met een ontstekingsomgeving is er namelijk een verhoogde productie van reactieve zuurstofsoorten (ROS) in het gewricht. ROS leiden tot de omzetting van LDL in het meer ontstekings-stimulerende oxLDL. Macrofagen die zich in het synovium bevinden kunnen dit oxLDL opnemen, wat leidt tot een verhoogde productie van ontstekingsstoffen en kraakbeen afbrekende enzymen. Hierdoor veronderstelden we dat ontsteking een belangrijke rol speelt in de ontwikkeling van artrose pathologie die geassocieerd is met hoog cholesterol.

HET VERLAGEN VAN CHOLESTEROL IN GEÏNDUCEERDE ARTROSE MODELLEN MET ONTSTEKING

Eerder hebben we aangetoond dat intensieve cholesterolverlagende behandelingen geen effect hadden op de spontane ontwikkeling van artrose. Daarom hebben we in **Hoofdstuk 4** onderzocht of ontsteking een belangrijke rol speelt bij artrose ontwikkeling in muizen met hoog cholesterol. We hebben gekeken of de PCSK9-remmer alirocumab in combinatie met atorvastatine therapie de ontwikkeling van artrose zou kunnen verminderen. Dit hebben we onderzocht in E3L.CETP muizen waarin door middel van het CiOA model artrose is opgewekt die een dieet kregen met hoog cholesterol. Hier zagen we dat de combinatie van atorvastatine met PCSK9-remmer alirocumab synoviale ontsteking en systemische ontstekingsmarkers in serum verminderde tijdens een vroeg stadium van de artrose. Deze vermindering van ontsteking resulteerde echter niet in een afname van kraakbeenschade of botvorming op plekken waar dit niet hoort in het eindstadium van de ziekte. Verschillende onderzoeken hebben eerder laten zien dat een dieet met hoog cholesterol leidt tot een toename in de afbraak van het kraakbeen. Andere onderzoeken hebben echter aangetoond dat een cholesterol-rijk dieet het aantal ontstekingscellen in het synovium verhoogde, terwijl er geen effecten op kraakbeenschade werden waargenomen. Deze resultaten suggereren dat hoog cholesterol voornamelijk zorgt voor een toename van ontsteking in de vroege stadia van artrose, wat zou kunnen verklaren waarom we voornamelijk ontstekingsremmende effecten van de cholesterolverlagende behandeling tijdens de vroege fasen van onze studie zagen.

VERLAGEN VAN DE ONTSTEKINGSFACTOR IL-1B IN DYSLIPIDEMISCHE MUIZEN

Er wordt gedacht dat systemische ontsteking die voortkomt uit problemen met de stofwisseling, ook wel metabole ontsteking genoemd, een mogelijke link kan zijn tussen het metabool syndroom en artrose. Metabole ontsteking wordt voornamelijk veroorzaakt door factoren zoals glucose en vetten zoals cholesterol. Interleukine-1β (IL-1β) is een belangrijke aanjager van ontsteking en er wordt gedacht dat deze ontstekingsstof een belangrijke bijdrage levert aan het ontstaan van artrose. Metabole ontsteking leidt vaak tot activering van de IL-1β productie, waardoor IL-1β een interessant doelwit is

voor ziekten die gerelateerd zijn aan het metabool syndroom, zoals hart- en vaatziekten en artrose. Verschillende onderzoeken toonden al aan dat IL-1β de opbouw van kraakbeen remt en daarnaast zorgt voor de aanmaak van kraakbeen afbrekende enzymen. Daarnaast is IL-1β ook in verband gebracht met verhoogde gewrichtspijn bij artrosepatiënten. Ondanks het feit dat IL-1β een cruciale rol lijkt te spelen in de pathogenese van artrose, lieten verschillende preklinische en klinische onderzoeken geen effecten zien op de ontwikkeling van artrose na remming van IL-1β.

Een recente secundaire analyse van de Canakinumab Anti-inflammatory Thrombosis Outcome Study (CANTOS) studie, waarin een IL-1β remmer (canakinumab) werd gegeven aan mensen met hart- en vaatziekten, toonde aan dat remming van IL-1β resulteerde in een verminderd aantal totale knie- en heupvervangingen bij deze patiënten. Het is interessant dat dit effect van IL-1β remming werd gevonden in deze groep patiënten, aangezien meer dan 93% van de patiënten die deelnamen aan de CANTOS studie ook een cholesterolverlagende therapie kreeg. Dit zou kunnen verklaren waarom gunstige effecten van IL-1β remming werden waargenomen in deze, maar niet in eerdere artrose-onderzoeken waarin IL-1β werd geremd. Daarnaast zijn de grootte van de patiëntengroepen en de relatief lange volgduur van de deelnemers anders dan bij standaard klinische onderzoeken voor artrose. Aangezien de CANTOS studie was ontworpen om cardiovasculaire gebeurtenissen te bestuderen, werden er geen gedetailleerde gegevens over de artrose pathologie verzameld. Om dit in meer detail te onderzoeken hebben we in **Hoofdstuk 5** onderzocht of de remming van IL-1β de ontwikkeling van artrose zou kunnen verminderen in een CiOA-model in dyslipidemische muizen. Hier hebben we laten zien dat een enkele dosis anti-IL-1β-serum activering van immuun cellen, veroorzaakt door hoog cholesterol, kon voorkomen. We hebben hier echter geen vermindering van artrose pathologie waargenomen.

In de CANTOS-studie reageerden patiënten met verhoogde systemische ontsteking het beste op de behandeling. Daarom hebben we in **Hoofdstuk 6** onderzocht of remming van IL-1β in combinatie met cholesterolverlagende therapie, bestaande uit statinetherapie en de PCSK9-remmer alirocumab, spontane artrose-pathologie zou kunnen verminderen bij dyslipidemische E3L- en E3L.CETP-muizen, die verschillende gradaties van systemische ontsteking hebben. E3L-muizen hadden verhoogde systemische ontsteking in vergelijking met de E3L.CETP muizen wanneer ze een cholesterol-rijk dieet kregen. De cholesterolverlagende behandeling was zeer effectief in het verlagen van de systemische cholesterol waardes. In deze studie hebben wij aangetoond dat de cholesterolverlagende behandeling resulteerde in een afname van synoviale ontsteking en kraakbeenafbraak bij de E3L muizen. Zoals eerder benoemd reageerden patiënten met hoge niveaus van systemische ontsteking in de CANTOS-studie het meest op de behandeling. Wij zagen vergelijkbare resultaten in onze studie, aangezien de ontwikkeling van artrose alleen werd verminderd bij de E3L muizen met meer systemische ontsteking, en niet bij de E3L.CETP muizen met milde systemische ontsteking. Deze resultaten ondersteunen de bevindingen van de CANTOS-studie en geven aan dat het gebruik van IL-1β-remmers in combinatie met een cholesterolverlagende behandeling gunstig zou kunnen zijn bij de behandeling van artrose patiënten met metabool syndroom.

CONCLUSIE

De resultaten beschreven in dit proefschrift geven aan dat cholesterolverlaging alleen niet voldoende blijkt te zijn om de ontwikkeling van artrose te verminderen, maar dat een combinatie van cholesterolverlagende therapieën met ontstekingsremmende therapieën nodig is om de ontwikkeling van cholesterol-geassocieerde artrose te verminderen. Verder hebben we aangetoond dat cholesterolverlagende therapieën voornamelijk de inflammatoire effecten verminderen die worden geassocieerd met het metabool syndroom. Bovendien tonen de resultaten beschreven in dit proefschrift aan dat het gebruik van cholesterolverlagende therapieën mogelijk het meest effectief is bij patiënten met een verstoorde vetzuurbalans gecombineerd met een hoge mate van systemische ontsteking.

Ons onderzoek benadrukt het belang van het focussen op specifieke subgroepen van artrosepatiënten bij het zoeken naar een geschikte behandeling. Verder onderzoek naar patiënten met (metabole) ontsteking of patiënten met een co-morbiditeit zoals hart- en vaatziekten zou kunnen leiden naar specifieke subgroepen van artrosepatiënten voor wie cholesterolverlagende therapieën nuttig kunnen zijn. Bovendien zou het gebruik van ontstekingsremmende therapieën in combinatie met cholesterolverlagende therapieën gunstig kunnen zijn voor deze subgroepen van artrose patiënten. Daarnaast is het belangrijk dat er voldoende aandacht wordt besteed aan preventie om het ontwikkelen van metabool syndroom te voorkomen, zoals voldoende bewegen en een gezond dieet.

CURRICULUM VITEA

Yvonne van Gemert werd geboren op 5 November 1993 te Nijmegen. Na het behalen van haar VWO diploma aan het Pantarijn te Wageningen in 2011, ging ze (Medische) Biologie studeren aan de Radboud Universiteit. Ter afronding van haar bachelor opleiding in 2014 liep zij drie maanden stage op de afdeling Neuroinformatics aan de Radboud Universiteit. In 2015 is zij begonnen aan de master Medical Biology aan de Radboud Universiteit. Tijdens haar master heeft Yvonne verschillende onderzoeksstages gelopen. Tijdens haar eerste masterstage liep zij zeven maanden stage op de afdeling Laboratoriumgeneeskunde in het Radboudumc in de groep van Harry Dolstra, onder directe begeleiding van Willemijn Hobo en Janneke Hoogstad-van Evert. Haar tweede masterstage heeft ze gelopen op de afdeling Fysiologie in het Radboudumc in de groep van Peter Deen, onder directe begeleiding van Marjolein ter Laak.

Na het behalen van haar master in 2018 is Yvonne in Mei dat jaar begonnen aan haar promotieonderzoek op de afdeling experimentele reumatologie onder supervisie van Peter van der Kraan met dagelijkse begeleiding van Peter van Lent en Martijn van den Bosch. Hier werkte zij aan het PhD project genaamd "Inhibition of PCSK9 dampens the flames of synovitis and destruction in cholesterol fuelled osteoarthritis", waarin zij het effect van cholesterol verlaging op ontsteking en artroseontwikkeling onderzocht. Tijdens haar promotietraject heeft Yvonne de mogelijkheid gekregen om haar bevindingen te presenteren op internationale congressen, waaronder de European Workshop for Rheumatology (EWRR) en Annual meeting of the Osteoarthritis Research Society International (OARSI).

Sinds maart 2023 is Yvonne werkzaam als Informatiespecialist bij de Radboud Universiteit.

LIST OF PUBLICATIONS

1. **van Gemert Y**, Blom AB, Di Ceglie I, Walgreen B, Helsen M, Sloetjes A, Vogl T, Roth J, Kruisbergen NNL, Pieterman EJ, Princen HMG, van der Kraan PM, van Lent PLEM, van den Bosch MHJ. *Intensive cholesterol-lowering treatment reduces synovial inflammation during early collagenase-induced osteoarthritis, but not pathology at end-stage disease in female dyslipidemic E3L.CETP mice.* Osteoarthritis Cartilage. 2023 Jul;31(7):934-943. doi: 10.1016/j.joca.2023.01.577. PMID: 36898656.

2. **van Gemert Y**, Kruisbergen NNL, Blom AB, van den Bosch MHJ, van der Kraan PM, Pieterman EJ, Princen HMG, van Lent PLEM. *IL-1β inhibition combined with cholesterol-lowering therapies decreases synovial lining thickness and spontaneous cartilage degeneration in a humanized dyslipidemia mouse model.* Osteoarthritis Cartilage. 2023 Mar;31(3):340-350. doi: 10.1016/j.joca.2022.09.014. PMID: 36442605.

3. Kruisbergen NNL, **van Gemert Y**, Blom AB, van den Bosch MHJ, van Lent PLEM. *Activation of circulating monocytes by low-density lipoprotein-a risk factor for osteoarthritis?* Rheumatology (Oxford). 2022 Dec 23;62(1):42-51. doi: 10.1093/rheumatology/keac359. PMID: 35863051.

4. Kruisbergen NNL, Di Ceglie I, **van Gemert Y**, Walgreen B, Helsen MMA, Slöetjes AW, Koenders MI, van de Loo FAJ, Roth J, Vogl T, van der Kraan PM, Blom AB, van Lent PLEM, van den Bosch MHJ. *Nox2 Deficiency Reduces Cartilage Damage and Ectopic Bone Formation in an Experimental Model for Osteoarthritis.* Antioxidants (Basel). 2021 Oct 22;10(11):1660. doi: 10.3390/antiox10111660. PMID: 34829531**.**

5. Kruisbergen NNL, **van Gemert Y**, Walgreen B, Helsen MMA, Slöetjes AW, Koenders MI, van de Loo FAJ, Roth J, Vogl T, van der Kraan PM, Blom AB, van den Bosch MHJ, van Lent PLEM. *A single dose of anti-IL-1β antibodies prevents Western diet-induced immune activation during early stage collagenase-induced osteoarthritis, but does not ameliorate end-stage pathology.* Osteoarthritis Cartilage. 2021 Oct;29(10):1462-1473. doi: 10.1016/j.joca.2021.07.005. PMID: 34298196**.**

6. **van Gemert Y**, Kozijn AE, Pouwer MG, Kruisbergen NNL, van den Bosch MHJ, Blom AB, Pieterman EJ, Weinans H, Stoop R, Princen HMG, van Lent PLEM. *Novel high-intensive cholesterol-lowering therapies do not ameliorate knee OA development in humanized dyslipidemic mice.* Osteoarthritis Cartilage. 2021 Sep;29(9):1314-1323. doi: 10.1016/j.joca.2021.02.570. PMID: 33722697.

7. Olde Loohuis NF, Kole K, Glennon JC, Karel P, Van der Borg G, **Van Gemert Y**, Van den Bosch D, Meinhardt J, Kos A, Shahabipour F, Tiesinga P, van Bokhoven H, Martens GJ, Kaplan BB, Homberg JR, Aschrafi A. *Elevated microRNA-181c and microRNA-30d levels in the enlarged amygdala of the valproic acid rat model of autism.* Neurobiol Dis. 2015 Aug;80:42-53. doi: 10.1016/j.nbd.2015.05.006. PMID: 25986729.

PHD PORTFOLIO OF YVONNE VAN GEMERT

Department: **Experimental Rheumatology**
PhD period: **01-05-2018 – 30-04-2022**
PhD Supervisor(s): **Prof. dr. P.M. van der Kraan**
PhD Co-supervisor(s): **dr. P.L.E.M. van Lent, dr. M.H.J. van den Bosch**

Training activities	Hours
Courses	
- Introduction day radboudumc (2018)	14.00
- RIMLS - Introduction course "In the lead of my PhD" (2018)	15.00
- RIMLS PhD course (2018)	21.00
- Management voor Promovendi (2018)	56.00
- Presentation Skills (2019)	42.00
- RIMLS PhD course "Within Sight of my PhD" (2019)	21.00
- Mindfulness Based Stress Reduction (2019)	28.00
- Advanced Conversation (2020)	42.00
- Radboudumc - Scientific integrity (2021)	20.00
- Scientific Integrity for PhD candidates (2021)	28.00
- Design and Illustration (2021)	28.00
- Loopbaanmanagement voor Promovendi (2021)	28.00
Conferences	
- Sleutel tot actieve patientenparticipatie (STAP) meetings (2021)	28.00
- Weten en Eten Scholingsavond Reumatische Ziekten 2018/2019 (2021)	11.20
- Masterclass "Molecular cardiology and vascular biology in the picture" (2018)	5.60
- PhD retreat 2019 (poster presentation)	28.00
- EWRR congress (poster presentation) (2020)	42.00
- PhD retreat 2020 (poster presentation)	14.00
- OARSI congress 2021, Oral presentation (2021)	56.00
- PhD retreat 2021 (poster presentation)	14.00
- New frontiers Symposium (poster presentation) (2021)	28.00
Other	
- Reviewing scientific paper (2022)	6.00
Teaching activities	
Lecturing	
- Meet the PhD voor 1e jaars studenten biomedische wetenschappen 2019/2020 (2021)	11.20
- Wetenschap in de klas (2019)	14.00
- Meet the PhD 2021 (2021)	8.00
- Wetenschap in de klas 2022 (2021)	14.00
- Meet the PhD 2022 (2022)	14.00
- Klinische Immunologie rondleiding en werkgroepen (2022)	8.00
- STAP (2022)	18.00
- STAP (2022)	14.00
Supervision of internships / other	
- Supervision Kavita Lips, BSc, 4 months (2020)	56.00
- Supervision Aranka Gerritsen, Medical Biology, BSc, 3 months (2020)	42.00
- Supervision Rojina Duwal, MMD, MSc, 6 months (2020)	56.00
Total	**831.00**

RESEARCH DATA MANAGEMENT

Findable:

All data described in this thesis are stored and can be found at the department of Experimental Rheumatology of the Radboud university medical center in (Nijmegen, the Netherlands).

Accessible:

The data and protocols can be obtained on request from the department of Experimental Rheumatology of the Radboud university medical center (Nijmegen, the Netherlands).

Interoperable:

The data presented in this thesis are documented in English according to the FAIR principles and include qualified references to other data.

Reusable:

The data shown in this thesis are documented to be reusable for further research and analysis.

DANKWOORD

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