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Osteoarthritis and Cartilage

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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SUMMARY

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– 2.3; WTD + AA: 95% CI: 0.8– 1.5) and synovial lining thickness (WTD: 95% CI: 3.0–4.6, WTD + AA: 95% CI: 2.1–3.2) 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; $P = 2.8 \times 10^{-10}$, 95% CI: −398.3 to −152.1; $P = 2.1 \times 10^{-9}$, −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 (OA) 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

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is a complex and heterogeneous disease and many risk factors, including aging, obesity and metabolic syndrome (MetS), have been associated with disease development[2,3]. 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[4] compared to the non-OA population and several studies have demonstrated that MetS is connected to disease development and progression[5–7].

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[3,6]. However, others have reported inconsistent findings with no association of dyslipidemia and OA development[8]. 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[9,10], while these findings could not be replicated by others[11]. 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[17,18]. 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[12], a well-established mouse model for hyperlipidemia as they respond to lipid-lowering therapies in a human-like manner[13–16]. 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[19]. 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[20].

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[19]. 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 wild type (WT) background[13–16]. 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[21]. 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 eight 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 [Fig. 1(A)][19,22]. Subcutaneous injections of saline were used as a control for the anti-PCSK9 treatment. Three weeks after the start of the treatment, 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 CiOA to study both early and late effects during CiOA. Knee joints were collected either for histological analysis or collection of synovial ribonucleic acid (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).

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 [Fig. 1(B) and (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 7 μm 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 to 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

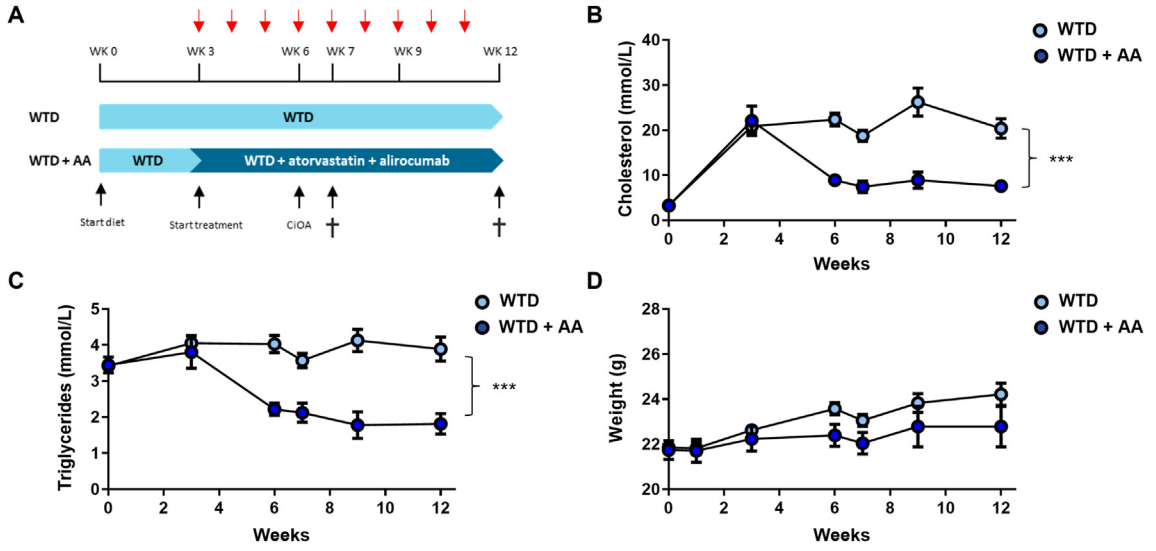

Fig. 1

Osteoarthritis and Cartilage

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.

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)[23,24]. 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[25]. 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[25]. Subchondral bone scores (subchondral bone plate thickening, increased bone mass) were determined in SafO stained section using a scoring system ranging from 0 to 3 (0 is normal, 1 = mild, 2 = moderate, and 3 = severe)[26,27]. 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[28] 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 7 days after the first injection of collagenase. Synovium was placed in 200 μl Roswell Park Memorial Institute (RPMI) medium supplemented with penicillin–streptomycin and 0.1% bovine serum albumin (BSA) for 2 h 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[29]. 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 [Fig. 1(A)]. 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) [Fig. 1(B)]. 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 [Fig. 1(B) and (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) [Fig. 1(D)].

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

aTo 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: 1.4–2.3); WTD + AA: 1.2 (95% CI: 0.8–1.5)) [Fig. 2(A)]. 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: 3.0–4.6), WTD + AA: 2.6 (95% CI: 2.1–3.2)) [Fig. 2(B) and (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 Fig. 1(A)–(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 [Fig. 2(D)–(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 [Fig. 2(G)]. 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, $P = 2.8 \times 10^{-10}$; KC: −48.1 pg/ml (95% CI of difference: −66.8 to −30.4), U = 19.5, $P = 2.1 \times 10^{-9}$ (Supplementary Fig. 2(A)–(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 [Fig. 3(A) and (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 [Fig. 3(C)]. 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 [Fig. 3(D)–(F)].

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 [Fig. 4(A) and (B)]. In addition, no reduction in both cartilage damage (P = 0.09, WTD: 17.5 (95% C: 15.5–19.5), WTD + AA: 14.9 (95% CI: 12.2–17.6) [Fig. 5(A), (C)] and subchondral bone sclerosis scores (WTD: 2.6 (95% CI: 2.2–2.9), WTD + AA: 2.1 (95% CI: 1.5–2.8) [Fig. 5(B)] 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[25]. Ectopic bone was mainly observed at the joint margins and collateral ligaments [Fig. 5(D)]. 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) [Fig. 5(E)–(H)]. The total number of osteophytes

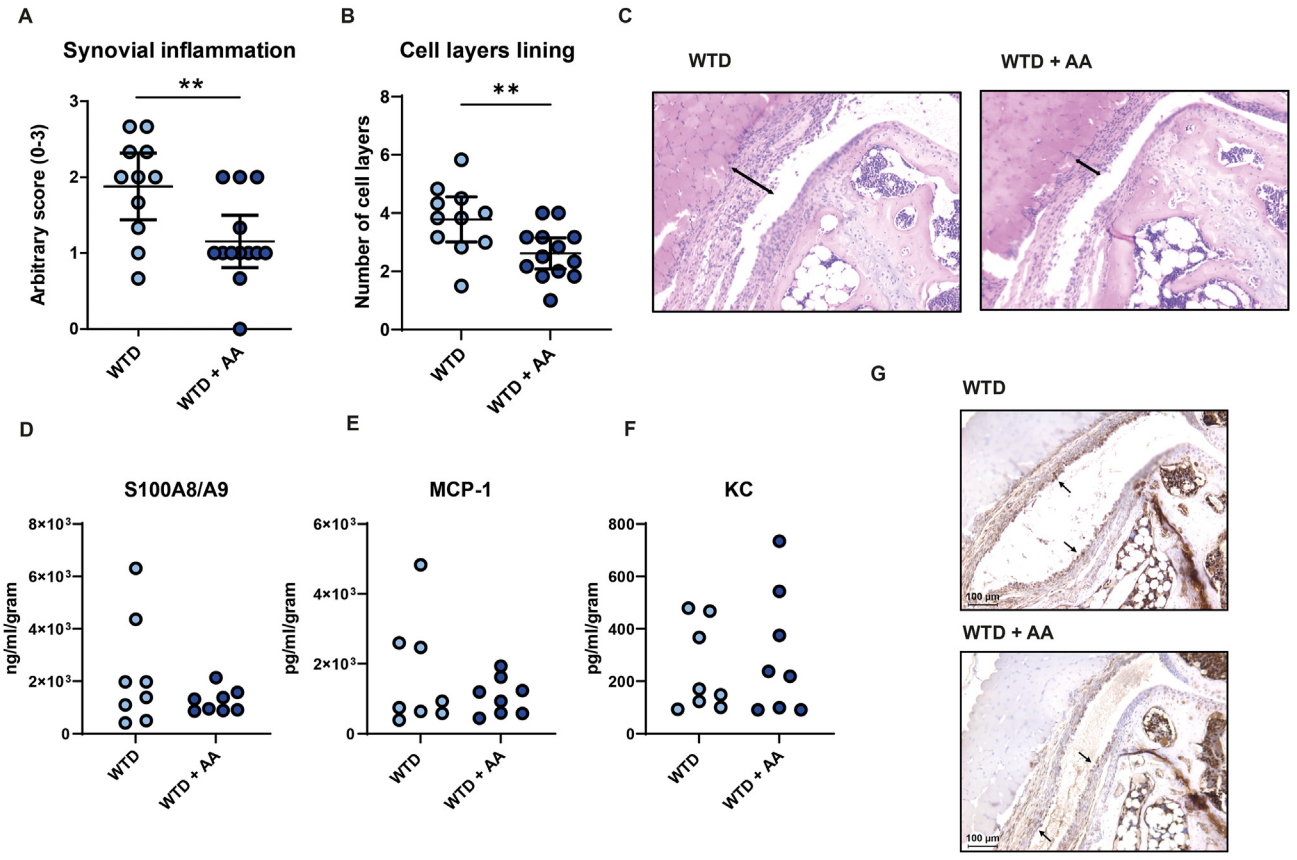

Fig. 2

Osteoarthritis and Cartilage

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.

(Supplementary Fig. 3(A)) and their maturation stage remained similar between both groups (Supplementary Fig. 3(B)–(F)).

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 CiOA, but this is not sufficient to ameliorate end stage pathology.

Clinical studies have reported contradictory findings regarding statin use and OA development[9–11,30,31]. 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[32]. 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[33]. 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[19,22]. Previous studies in our lab showed that a cholesterol-supplemented diet increased synovial activation and

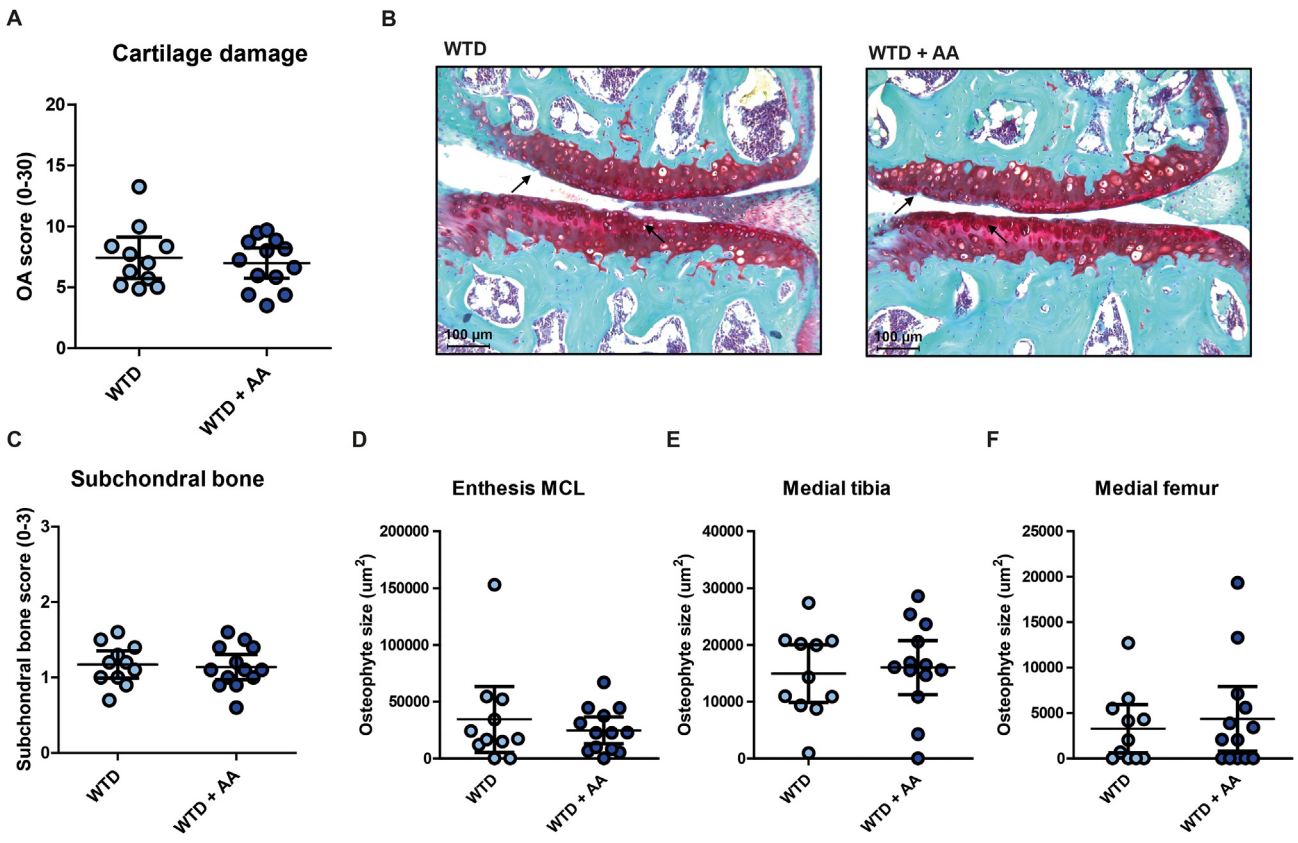

Fig. 3

Osteoarthritis and Cartilage

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 to 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 to 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.

ectopic bone formation, but not cartilage degeneration in a CiOA model[34,35]. Others have shown that a high fat diet alone did not lead to joint pathology, but a combination with a secondary trigger such as destabilization of the medial meniscus (DMM) surgery[36] or groove surgery[37] 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[12,38,39]. However, some studies have shown that a WTD increased macrophage infiltration[40] and inflammation in the synovium, while no effects on cartilage damage were observed[37,40]. 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[41,42]. Trained immunity increases the inflammatory response of monocytes and macrophages to secondary stimuli, such as Toll-like receptor (TLR) ligands like lipopolysaccharide (LPS) or S100A8/A9[43]. In addition, Christ *et al.*

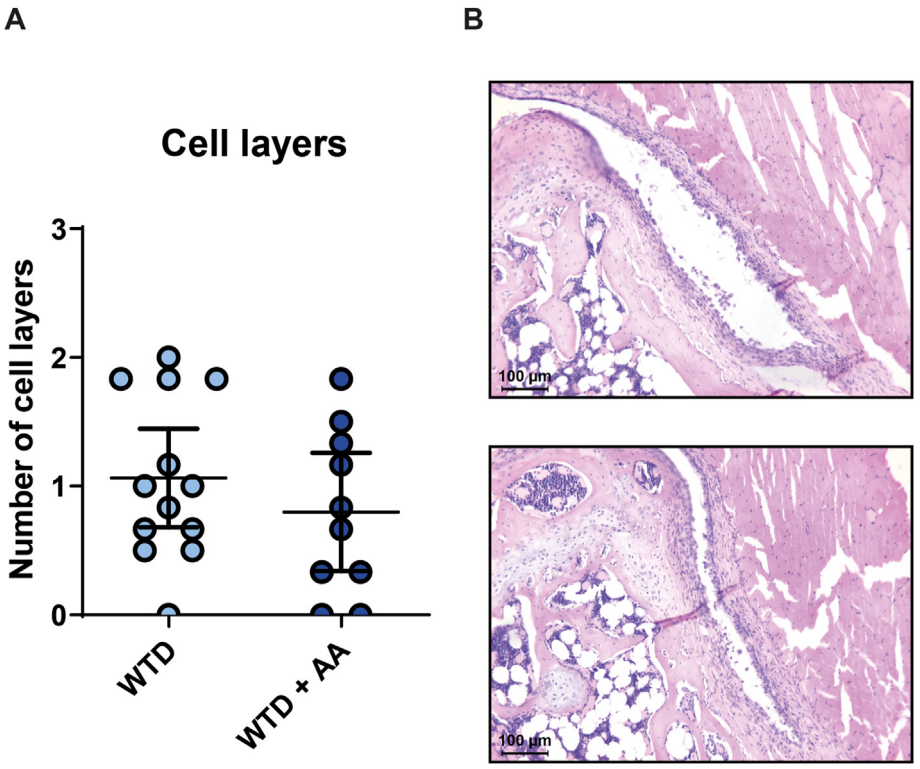

Fig. 4

Osteoarthritis and Cartilage

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.

have shown that immune training in monocytes was dependent on the NLRP3 inflammasome/IL-1β pathway[43]. In addition, Bekkering *et al.* have shown that trained immunity cannot be reversed by statin therapy in patients with familiar hypercholesterolemia[44]. 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[44]. 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.*[45].

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[46]. Moreover, it has been shown that induction of trained immunity in monocytes after oxLDL stimulation upregulates glycolytic metabolism[47]. 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[47]. In addition, these authors showed that trained immunity could be prevented by pharmacological inhibition of glycolysis[47]. 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[12,48]. 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.

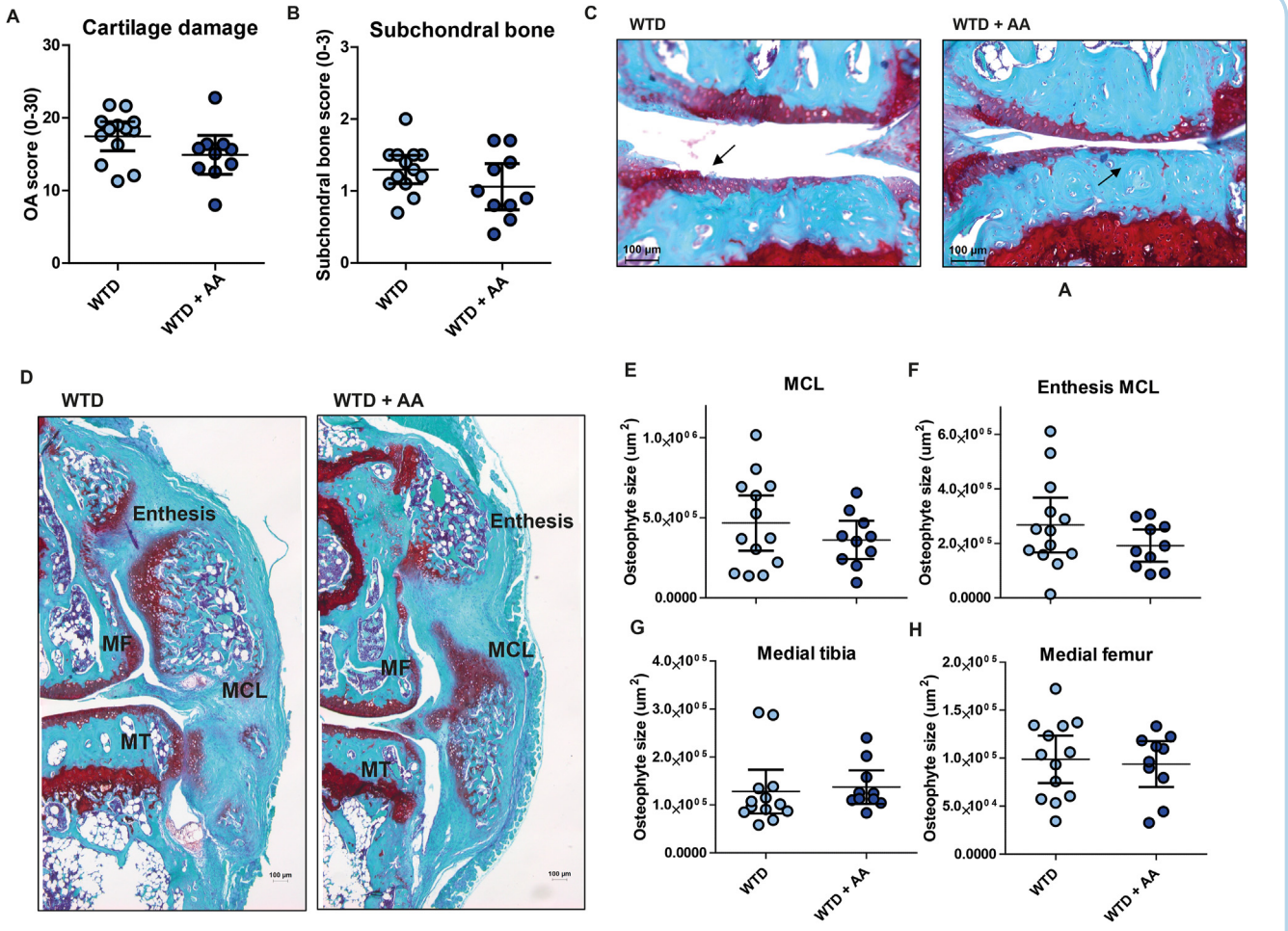

Fig. 5

Osteoarthritis and Cartilage

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 to 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 to 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.

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.

Declaration of competing interest

The authors declare no competing interests.

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Supplementary data

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