Osteoarthritis and Cartilage 29 (2021) 1314–1323

Osteoarthritis and Cartilage

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

Y. van Gemert [† a], A.E. Kozijn [‡ § ‖ a], M.G. Pouwer [‡ ¶], N.N.L. Kruisbergen [†], M.H.J. van den Bosch [†], A.B. Blom [†], E.J. Pieterman [‡], H. Weinans [§ #], R. Stoop [‡], H.M.G. Princen [‡ a], P.L.E.M. van Lent [† * a]

† *Experimental Rheumatology, Radboud University Medical Center, Nijmegen, the Netherlands*
‡ *Metabolic Health Research, TNO, Leiden, the Netherlands*
§ *Department of Orthopaedics, UMC Utrecht, Utrecht University, Utrecht, the Netherlands*
‖ *Department of Rheumatology & Clinical Immunology, UMC Utrecht, Utrecht University, Utrecht, the Netherlands*
¶ *Department of Cardiology, Leiden UMC, Leiden, the Netherlands*
# *Department of Biomechanical Engineering, Delft University of Technology, Delft, the Netherlands*

ARTICLE INFO

Article history:
Received 29 May 2020
Accepted 25 February 2021

Keywords:
Osteoarthritis (OA)
Cholesterol
Mouse model
Dyslipidemia
Western-type diet

SUMMARY

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 five 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.

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 LDL-C[5,6], the role of an impaired lipid metabolism in OA pathology remains unclear. Increased total cholesterol (TC) levels were recently associated with increased risk of generalized OA[1,7], while others found no association with LDL-C or TC[8] and even describe protective

* Address correspondence and reprint requests to: P L. van Lent, Radboud university medical center, Geert Grooteplein 28, Nijmegen, 6525 GA, the Netherlands. Tel: 31243610512; Fax: 31243540403.
E-mail address: peter.vanlent@radboudumc.nl (P.L.E.M. van Lent).
[a] These authors contributed equally to this work.

https://doi.org/10.1016/j.joca.2021.02.570

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].

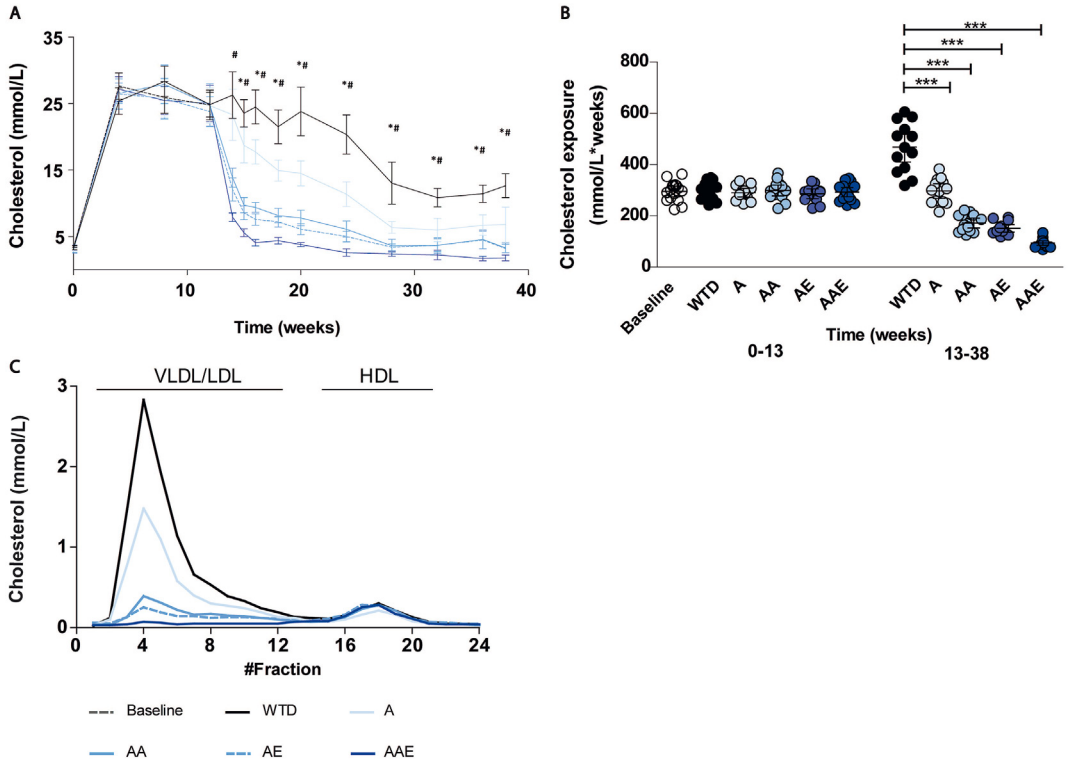


Fig. 1

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 Fig. 1(A)–(C) are given as background information and have been published previously 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. © Pouwer, M. G. ***et al.***[26] 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 vs WTD; * ***P*** < 0.001 WTD vs A, # ***P*** < 0.001 WTD vs AA, AE and AAE in Fig. 1(A).

Osteoarthritis and Cartilage

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 the Netherlands Organization for Applied Scientific Research (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 six groups based on age, body weight, plasma 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 five groups continued to receive WTD alone or with treatment for 25 weeks (Supplementary Fig. 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 Fig. 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. TC levels were determined throughout the study with an enzymatic assay (Roche Diagnostics) according to manufacturer's instructions and TC 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 7 μm 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

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.
*$P < 0.05$.
**$P < 0.01$.
***$P < 0.001$ vs WTD.

Table I Levels of inflammation markers in plasma

Osteoarthritis and Cartilage

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 to 2 (0 = no inflammation, 1 = mild inflammation, 2 = moderate inflammation; Supplementary Fig. 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% coincidence 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 [Fig. 1(A) and (B)]. The decrease in systemic cholesterol levels coincided with a reduced body weight gain in the treated groups compared to WTD controls (Supplementary Fig. 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 [Fig. 1(C)]. 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 I). E-selectin and MCP-1 levels were low and not affected by cholesterol-lowering treatment (Table I).

*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; Fig. 2(A)). The observed decline in systemic cholesterol levels did not attenuate progression of cartilage destruction in treatment groups as compared to WTD controls [Fig. 2(A)]. Cartilage degradation was mainly observed at the lateral tibia and femur (Supplementary Fig. 4(A)). 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 matrix metalloproteases (MMPs) and a marker of advanced cartilage degradation, was not observed in articular cartilage [Fig. 2(D)]. NITEGE staining, as marker of both early was significantly increased at 38 weeks in WTD-fed mice compared to baseline controls ($p < 0.01$; Fig. 2(B)). 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, $p < 0.01$; Fig. 2(B)) when compared to WTD controls. Representative pictures of cartilage degeneration and NITEGE and VDIPEN staining are shown [Fig. 2(C)–(E)]. The combined data indicate that increased cartilage degradation also induces an active repair process in articular cartilage.

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 [Fig. 3(A) and (B)]. The alarmin S100A8, a marker for activated macrophages, also showed only minor expression in the synovial lining and was comparable between all groups [Fig. 3(C)]. 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 [Fig. 4(A)]. Most osteophytes occurred at the anterior side of the medial femoral condyle [Fig. 4(B)].

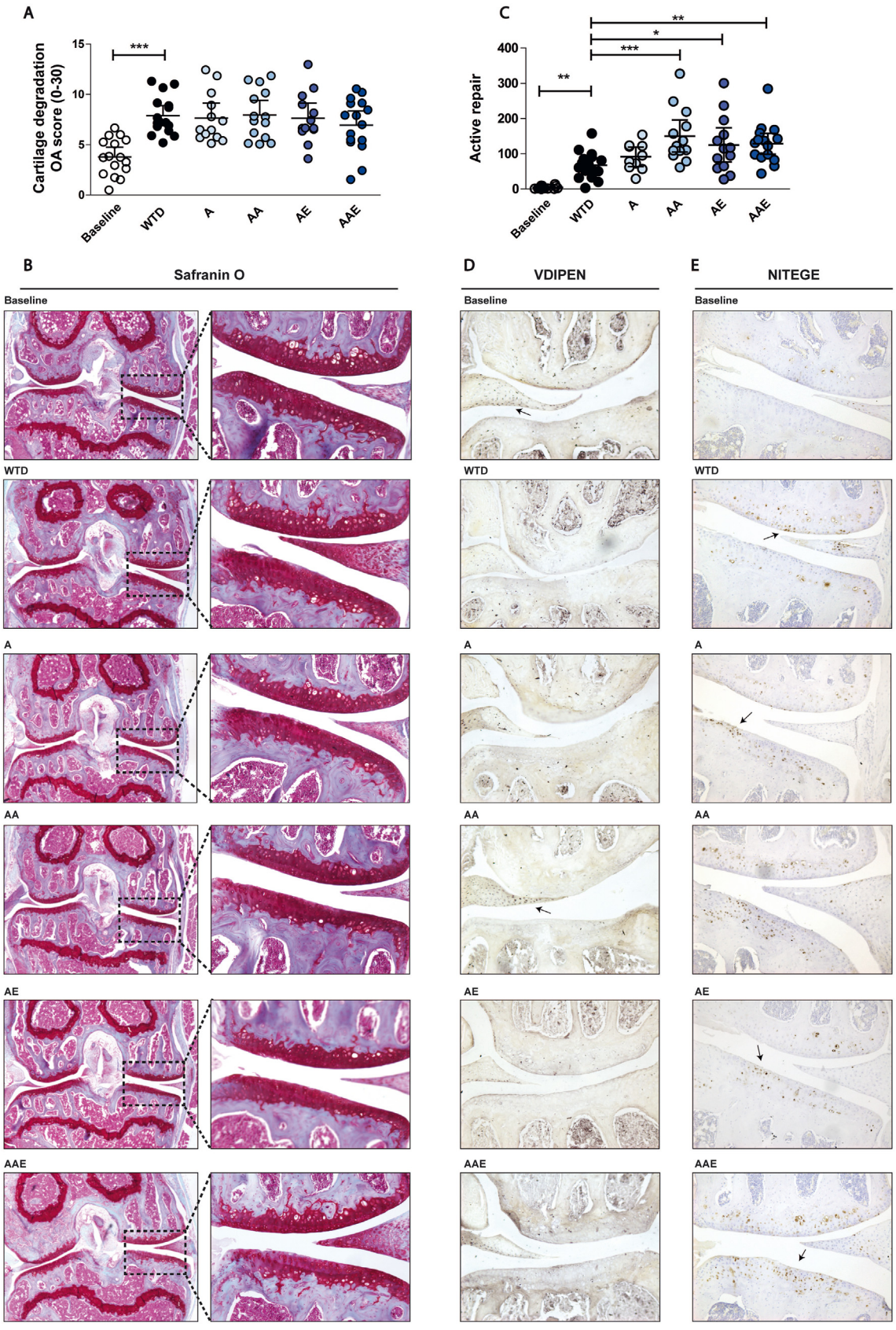


Fig. 2 **Cholesterol-lowering interventions do not ameliorate cartilage degradation in dyslipidemic mice.** APOE*3Leiden.CETP mice received a Western Type Diet for 38 weeks. **(A)** Cartilage degradation was determined using histological analysis and revealed a significant increase in cartilage degeneration after 38

Discussion

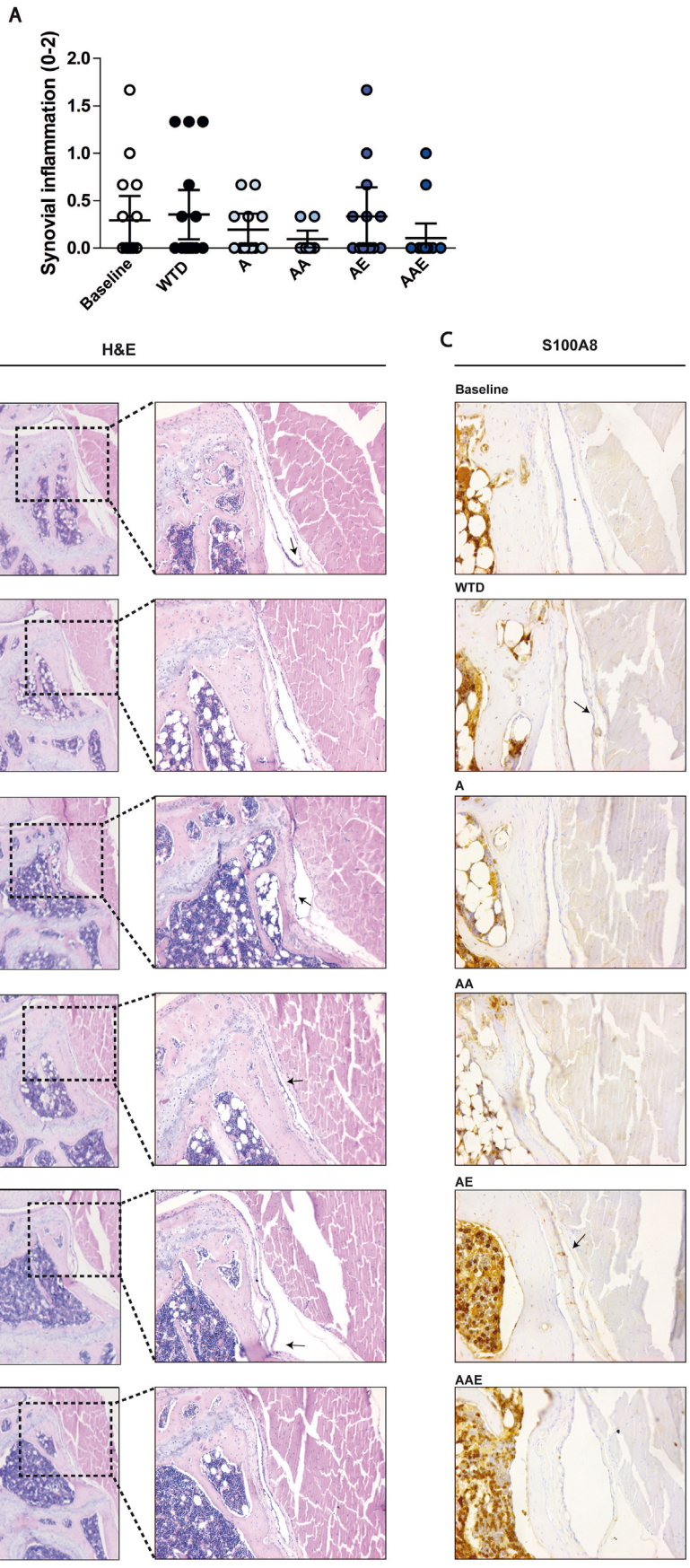
The association of CVD with OA has become increasingly recognized and understanding their interrelationship is imperative for improving therapeutic approaches. Cholesterol, with its crucial role in CVD, could be a potential link. Our study demonstrates that therapeutic cholesterol-lowering therapies 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 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

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 (20× magnification). Representative pictures of cartilage degradation and NITEGE staining are depicted in figures **(B)** and **(E)** (20× 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. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs WTD.


A
Synovial inflammation (0-2)
2.0
1.5
1.0
0.5
0.0
Baseline
WTD
A
AA
AE
AAE
B
H&E
C
S100A8
Baseline
WTD
A
AA
AE
AAE

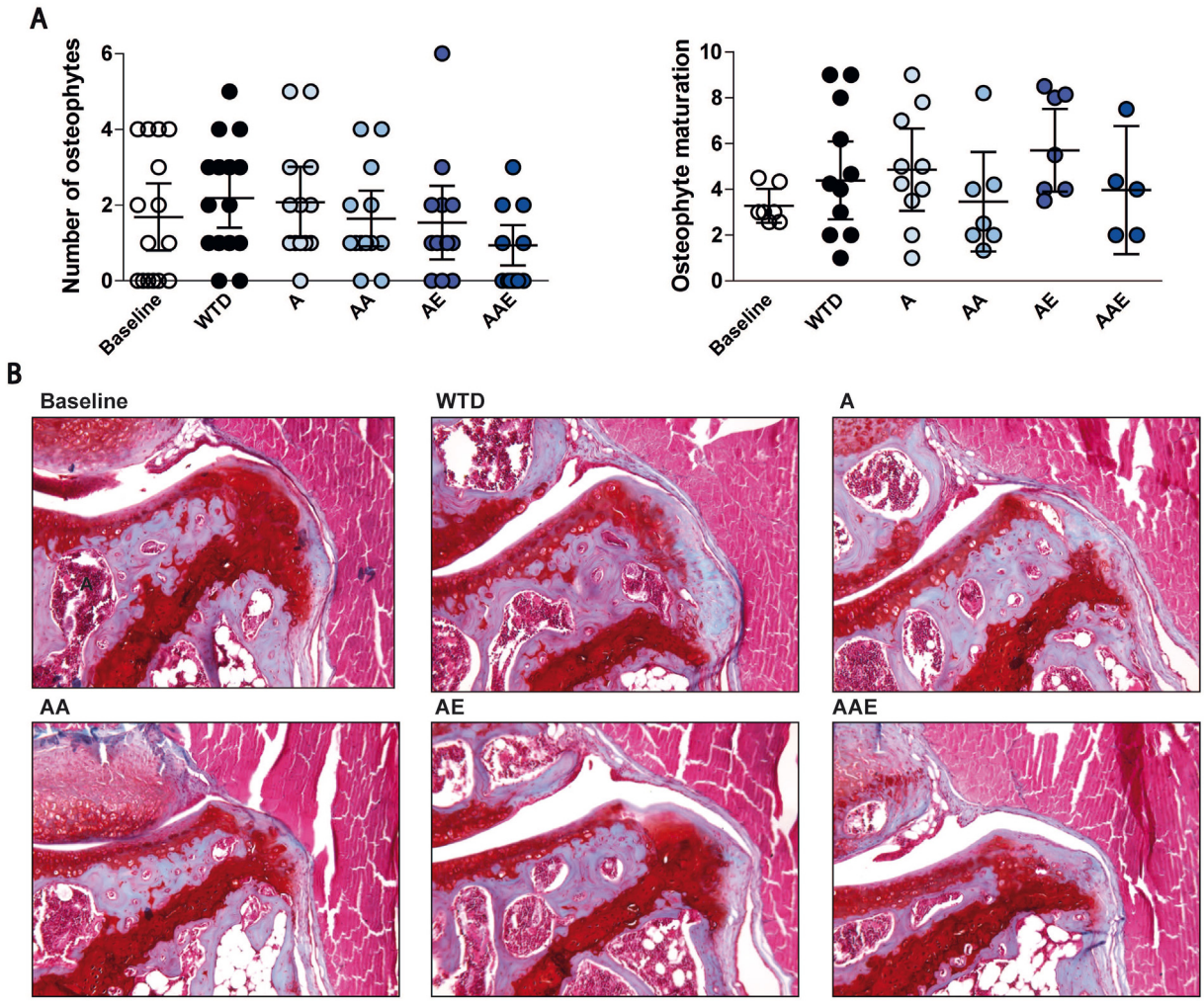


Fig. 4

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 (10× magnification). A = atorvastatin, AA = atorvastatin + alirocumab, AE = atorvastatin + evinacumab, AAE = atorvastatin + alirocumab + evinacumab.

Osteoarthritis and Cartilage

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

Fig. 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: 5× magnification, right 10× 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 (10× magnification). Arrows indicate positive staining. A = atorvastatin, AA = atorvastatin + alirocumab, AE = atorvastatin + evinacumab, AAE = atorvastatin + alirocumab + evinacumab.

Osteoarthritis and Cartilage

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.

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.

Conflict of interest

Alirocumab (Praluent®) and evinacumab (REGN1500) are developed by Regeneron Pharmaceuticals and evinacumab is currently in clinical trials. EJP, RS and HMGP are employees of TNO.

Sources of funding

This manuscript was supported by funding from the Dutch Arthritis Foundation (Grant number 17-1-404 and LLP-22) and Regeneron Pharmaceuticals, by an allowance for TKI-LSH from the Ministry of Economic Affairs in the Netherlands (reference number 060.23203), TNO research program “Preventive Health Technologies”, the Dutch Arthritis Foundation under grant agreement n◦17-1-404, and the European Union Seventh Framework Programme under grant agreement n◦305815 (EU FP7/2007–2013; HEALTH.2012.2.4.5–2; D-BOARD: Novel Diagnostics and Biomarkers for Early Identification of Chronic Inflammatory Joint Diseases).

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.

Acknowledgments and affiliations

The authors would like to thank Nanda Keijzer, Nicole Worms, Erik Offerman, Annet Sloetjes and Birgitte Walgreen for their excellent technical assistance.

Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.joca.2021.02.570.

References

1. Buchele G, Gunther KP, Brenner H, Puhl W, Sturmer T, Rothenbacher D, *et al*. Osteoarthritis-patterns, cardio-metabolic risk factors and risk of all-cause mortality: 20 years follow-up in patients after hip or knee replacement. Sci Rep 2018;8:5253.
2. Wang H, Bai J, He B, Hu X, Liu D. Osteoarthritis and the risk of cardiovascular disease: a meta-analysis of observational studies. Sci Rep 2016;6:39672.
3. Hall AJ, Stubbs B, Mamas MA, Myint PK, Smith TO. Association between osteoarthritis and cardiovascular disease: systematic review and meta-analysis. Eur J Prev Cardiol 2016;23:938–46.
4. Hoeven T, Leening M, Bindels P, Castaño-Betancourt M, van Meurs J, Franco O, *et al*. Disability and not osteoarthritis predicts cardiovascular disease: a prospective population-based cohort study. Ann Rheum Dis 2015;74:752–6.
5. Mishra R, Singh A, Chandra V, Negi MP, Tripathy BC, Prakash J, *et al*. A comparative analysis of serological parameters and oxidative stress in osteoarthritis and rheumatoid arthritis. Rheumatol Int 2012;32:2377–82.
6. Oliviero F, Nigro AL, Bernardi D, Giunco S, Baldo G, Scanu A, *et al*. A comparative study of serum and synovial fluid lipoprotein levels in patients with various arthritides. Clin Chim Acta 2012;413:303–7.
7. Tootsi K, Märtson A, Kals J, Paapstel K, Zilmer M. Metabolic factors and oxidative stress in osteoarthritis: a case–control study. Scand J Clin Lab Investig 2017;77:520–6.
8. Garcia-Gil M, Reyes C, Ramos R, Sanchez-Santos MT, Prieto-Alhambra D, Spector TD, *et al*. Serum lipid levels and risk of hand osteoarthritis: the chingford prospective cohort study. Sci Rep 2017;7:3147.
9. Clockaerts S, Van Osch G, Bastiaansen-Jenniskens Y, Verhaar J, Van Glabbeek F, Van Meurs J, *et al*. Statin use is associated with reduced incidence and progression of knee osteoarthritis in the Rotterdam study. Ann Rheum Dis 2012;71:642–7.
10. Riddle DL, Moxley G, Dumenci L. Associations between statin use and changes in pain, function and structural progression: a longitudinal study of persons with knee osteoarthritis. Ann Rheum Dis 2013;72:196–203.
11. Michaëlsson K, Lohmander L, Turkiewicz A, Wolk A, Nilsson P, Englund M. Association between statin use and consultation or surgery for osteoarthritis of the hip or knee: a pooled analysis of four cohort studies. Osteoarthr Cartil 2017;25:1804–13.
12. Burkard T, Hugle T, Layton JB, Glynn RJ, Bloechliger M, Frey N, *et al*. Risk of incident osteoarthritis of the hand in statin initiators: a sequential cohort study. Arthritis Care Res 2018;70:1795–805.
13. Robinson JG, Farnier M, Krempf M, Bergeron J, Luc G, Averna M, *et al*. Efficacy and safety of alirocumab in reducing lipids and cardiovascular events. N Engl J Med 2015;372:1489–99.
14. Dewey FE, Gusarova V, Dunbar RL, O'Dushlaine C, Schurmann C, Gottesman O, *et al*. Genetic and pharmacologic inactivation of ANGPTL3 and cardiovascular disease. N Engl J Med 2017;377:211–21.
15. Gaudet D, Gipe DA, Pordy R, Ahmad Z, Cuchel M, Shah PK, *et al*. ANGPTL3 inhibition in homozygous familial hypercholesterolemia. N Engl J Med 2017;377:296.
16. van den Hoek AM, van der Hoorn JWA, Maas A, van den Hoogen R, van Nieuwkoop A, Droog S, *et al*. APOE* 3 Leiden. CETP transgenic mice as model for pharmaceutical treatment of the metabolic syndrome. Diabetes Obes Metabol 2014;16:537–44.

17. Westerterp M, van der Hoogt CC, de Haan W, Offerman EH, Dallinga-Thie GM, Jukema JW, *et al*. Cholesteryl ester transfer protein decreases high-density lipoprotein and severely aggravates atherosclerosis in APOE* 3-Leiden mice. Arterioscler Thromb Vasc Biol 2006;26:2552–9.
18. Pouwer MG, Heinonen SE, Behrendt M, Andréasson A-C, van Koppen A, Menke AL, *et al*. The APOE3-leiden heterozygous glucokinase knockout mouse as novel translational disease model for type 2 diabetes, dyslipidemia, and diabetic atherosclerosis. J Diabetes Res 2019;2019.
19. Zadelaar S, Kleemann R, Verschuren L, de Vries-Van der Weij J, van der Hoorn J, Princen HM, *et al*. Mouse models for atherosclerosis and pharmaceutical modifiers. Arterioscler Thromb Vasc Biol 2007;27:1706–21.
20. Kühnast S, Fiocco M, van der Hoorn JW, Princen HM, Jukema JW. Innovative pharmaceutical interventions in cardiovascular disease: focusing on the contribution of non-HDL-C/LDL-C-lowering versus HDL-C-raisingA systematic review and meta-analysis of relevant preclinical studies and clinical trials. Eur J Pharmacol 2015;763:48–63.
21. Kühnast S, van der Hoorn JW, Pieterman EJ, van den Hoek AM, Sasiela WJ, Gusarova V, *et al*. Alirocumab inhibits atherosclerosis, improves the plaque morphology, and enhances the effects of a statin. J Lipid Res 2014;55:2103–12.
22. Gierman LM, Kuhnast S, Koudijs A, Pieterman EJ, Kloppenburg M, van Osch GJ, *et al*. Osteoarthritis development is induced by increased dietary cholesterol and can be inhibited by atorvastatin in APOE*3 Leiden.CETP mice–a translational model for atherosclerosis. Ann Rheum Dis 2014;73:921–7.
23. de Munter W, van den Bosch M, Slöetjes A, Croce K, Vogl T, Roth J, *et al*. High LDL levels lead to increased synovial inflammation and accelerated ectopic bone formation during experimental osteoarthritis. Osteoarthr Cartil 2016;24:844–55.
24. Farnaghi S, Prasadam I, Cai G, Friis T, Du Z, Crawford R, *et al*. Protective effects of mitochondria-targeted antioxidants and statins on cholesterol-induced osteoarthritis. Faseb J 2017;31:356–67.
25. Kozijn A, Gierman L, van der Ham F, Mulder P, Morrison M, Kühnast S, *et al*. Variable cartilage degradation in mice with diet-induced metabolic dysfunction: food for thought. Osteoarthr Cartil 2018;26:95–107.
26. Pouwer MG, Pieterman EJ, Worms N, Keijzer N, Jukema JW, Gromada J, *et al*. Alirocumab, evinacumab, and atorvastatin triple therapy regresses plaque lesions and improves lesion composition in mice. J Lipid Res 2020;61:365–75.
27. Van Vlijmen B, van't Hof HB, Mol M, van der Boom H, van der Zee A, Frants RR, *et al*. Modulation of very low density lipoprotein production and clearance contributes to age-and gender-dependent hyperlipoproteinemia in apolipoprotein E3-Leiden transgenic mice. J Clin Invest 1996;97:1184–92.
28. Van Der Hoorn J, Jukema J, Havekes L, Lundholm E, Camejo G, Rensen P, *et al*. The dual PPARα/γ agonist tesaglitazar blocks progression of pre-existing atherosclerosis in APOE* 3 Leiden. CETP transgenic mice. Br J Pharmacol 2009;156:1067–75.
29. Van Den Bosch M, Blom A, Kram V, Maeda A, Sikka S, Gabet Y, *et al*. WISP1/CCN4 aggravates cartilage degeneration in experimental osteoarthritis. Osteoarthr Cartil 2017;25:1900–11.
30. Pritzker KP, Gay S, Jimenez SA, Ostergaard K, Pelletier J-P, Revell P, *et al*. Osteoarthritis cartilage histopathology: grading and staging. Osteoarthr Cartil 2006;14:13–29.
31. Davidson EB, Vitters E, Van Beuningen H, Van de Loo F, Van den Berg W, Van der Kraan P. Resemblance of osteophytes in experimental osteoarthritis to transforming growth factor β–induced osteophytes: limited role of bone morphogenetic protein in early osteoarthritic osteophyte formation. Arthritis Rheum: Offic J American College Rheumatol 2007;56:4065–73.
32. van Meurs JB, van Lent PL, Holthuysen AE, Singer II , Bayne EK, van den Berg WB. Kinetics of aggrecanase- and metalloproteinase-induced neoepitopes in various stages of cartilage destruction in murine arthritis. Arthritis Rheum 1999;42:1128–39.
33. Veronese N, Koyanagi A, Stubbs B, Cooper C, Guglielmi G, Rizzoli R, *et al*. Statin use and knee osteoarthritis outcomes: a longitudinal cohort study. Arthritis Care Res 2019;71:1052–8.
34. de Munter W, Geven EJ, Blom AB, Walgreen B, Helsen MM, Joosten LA, *et al*. Synovial macrophages promote TGF-beta signaling and protect against influx of S100A8/S100A9-producing cells after intra-articular injections of oxidized low-density lipoproteins. Osteoarthr Cartil 2017;25:118–27.
35. Warmink K, Kozijn AE, Bobeldijk I, Stoop R, Weinans H, Korthagen NM. High-fat feeding primes the mouse knee joint to develop osteoarthritis and pathologic infrapatellar fat pad changes after surgically induced injury. Osteoarthr Cartil 2020;28:593–602.
36. Mooney RA, Sampson ER, Lerea J, Rosier RN, Zuscik MJ. High-fat diet accelerates progression of osteoarthritis after meniscal/ligamentous injury. Arthritis Res Ther 2011;13:1–10.