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

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Abstract

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

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

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

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

The apolipoprotein E*3Leiden.human Cholesteryl Ester Transfer Protein (APOE*3Leiden.CETP) transgenic mouse is a model which resembles human lipoprotein metabolism and is sensitive to lipid modulating therapies in contrary to wild type mice (9-11). Feeding a HFD to these mice leads to a profile of anomalies quite similar to those that are present in the majority of the patients having metabolic syndrome (12). In an unpublished study which was designed for lipid research purposes we found that APOE*3Leiden.CETP mice receiving a HFD and fructose in their drinking water developed severe features of OA. Moreover, we observed a strong inhibitory effect on OA development when mice were treated with fenofibrate (figure 1). Fibrates are agonists for the PPAR-α receptors and used in the clinic as drugs to reduce plasma triglycerides and cholesterol levels (13). Fibrates are, like statins, recognized for their pleiotropic effects (14). With respect to OA it has been shown in in vitro experiments that a PPAR-α agonist counteracts IL-1-induced proteoglycan degradation and increases MMP-activities in rabbit articular chondrocytes (15). Furthermore, Clockaerts et al. showed that a PPAR-α agonist inhibited inflammatory and destructive responses in human OA cartilage explants, while collagen type II or aggrecan mRNA expression remained unaffected (16). In addition, PPAR-α agonists have an inhibitory effect on inflammatory cytokines in infrapatellar fat pad explants and to a lesser extent in synovium explants (17).
Figure 1. OA grades of male APOE*3Leiden.CETP mice included in a study designed for lipid research purposes. Mice received high fat diet (HFD) for 32 weeks or HFD supplemented with 0.0212% w/w fenofibrate for 9 weeks. Fructose (10%) was added to the drinking water during the treatment period. Each point represents the value of an individual mouse. Line indicates mean ± SEM (n=8/group).

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

The pro-inflammatory cytokine interleukin (IL)1-β is suggested to have an important role in OA pathogenesis (24-26) as well as in obesity (27). IL-1β is synthesized as an inactive precursor and needs to be converted by the enzyme caspase-1 to become active. Caspase-1 itself requires activation via a molecular platform called the inflammasome (28). The activation of inflammasome-mediated caspase-1 plays a key role in the enhanced inflammatory state characteristic of obesity and has a central role in the pathogenesis of type 2 diabetes (27). Although recently it has been demonstrated that OA cartilage may be degraded independently
of any inflammasome activity (29), the role of inflammasome in relation to obesity-induced OA merits more investigation.

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

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

Methods

Mice
Male APOE*3Leiden mice, characterized by an enzyme-linked immunosorbent assay (ELISA) for human APOE, were crossbred with human CETP transgenic mice which express CETP under control of its natural flanking regions in our animal facility to obtain heterozygous APOE*3Leiden.CETP mice (10-12). Mice were housed in groups under standard conditions with a 12 h light-dark cycle and had free access to water and food. Body weight (BW) and food intake were monitored during the study. Mice
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received a standard lab chow (V1534 Ssniff Spezialdiäten GmbH, Germany) until the start of the study at the age of 10-16 weeks (t=0 weeks). Experiments were approved by the institutional Animal Care and Use Committee of TNO and were in compliance with European Community specifications regarding the use of laboratory animals.

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

Main study
APOE*3Leiden.CETP mice (n=150) were switched to a HFD at t=0 weeks. After a 12 weeks run-in period (t=12), mice were matched based on BW, plasma cholesterol, TG and insulin levels in 8 different groups (n=12/group) and low responders were removed from the study, except for a group of n=12 mice that continued on HFD (HFD Low Responders) to investigate whether the low response on above-mentioned parameters is indicative for less OA progression. All other groups continued on HFD with or without treatment (HFD), except for one group (HFD_Chow) which was switched back to standard lab chow after 22 weeks (t=22 weeks). Two groups received a HFD supplemented with 0.005% w/w rosuvastatin (Provisacor®, Astrazeneca, Zoetermeer, the Netherlands) in which the first group received rosuvastatin from 12 weeks onward (HFD + Rosu t=12) while the other received this drug from 22 weeks onward (HFD + Rosu t=22). The same treatment schedule was applied for 2 groups receiving HFD supplemented with 0.0212 % w/w fenofibrate (art no F6020, Sigma-Aldrich, St. Louis, USA) (HFD + Feno t=12 and HFD + Feno t=22). Furthermore, one group received a daily intra-peritoneal (i.p) injection with 10 mg/kg caspase-1 inhibitor (AC-YVAC-CMK art.no. N-1330, Bachem, Bubendorf, Switzerland) dissolved in PBS/0.6% DMSO from 14 weeks (t=14) onward (HFD + Casp.inh). A control group
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of n=12 mice received standard lab chow diet until the end of the study (Control). All mice were sacrificed after 32 weeks (t=32).

**Cholesterol, triglyceride and insulin analyses**

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

**Serum Amyloid A analysis**

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

**Histopathology**

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

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

**Results**

**Pilot study**

BW, plasma cholesterol and TG levels were assessed during the study. All parameters increased gradually (Table 1). Mice were sacrificed at t=12, 16, 20 and 24 weeks on HFD to investigate the development of OA in the knee joint over time. After 12 weeks on a HFD, the OA score was relatively homogeneous. The longer the mice received a HFD, the more variation in OA score was observed (Figure 2A). Based on the severity of OA score, it was decided to start the late treatments in the main study at t=22 weeks and to run the main study till 32 weeks. Plasma SAA levels at the start of the study were comparable between the different pilot groups with an average of 29.6 ± 37.3 µg/ml (Figure 2B).
Table 1. Body weight, plasma cholesterol and triglyceride levels of APOE*3Leiden.CETP mice in the pilot study.

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

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

Mice characteristics

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

To evaluate the effects of a HFD-intake plasma cholesterol and TG levels were determined every 4 weeks. Plasma cholesterol levels in the HFD group were significantly higher than in the control group (HFD vs Control p<0.001) (Figure 4A). No such significant effect was observed for the TG levels, which already reached substantial levels in the control group (Figure 4D). A switch back from a HFD to a control chow diet decreased cholesterol levels immediately, returning to almost the levels in the control group (HFD_Chow vs HFD p=0.002). This was accompanied by a reduction in TG levels. The HFD Low Responder group had significantly lower cholesterol (p<0.001) and TG (p<0.01) levels than the HFD group. Rosuvastatin, fenofibrate and caspase-1 inhibitor treatments did not significantly influence these parameters (Figure 4BCEF).
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Figure 3. Body weight (BW). Time course of BW of APOE*3Leiden.CETP mice in the A. chow (Control), high fat diet (HFD), HFD_Chow and HFD Low Responders groups, B. HFD, HFD treated with rosvastatin started from t=12 or t=22 onwards (HFD + Rosu t=12/22) and HFD treated with fenofibrate started from t=12 or t=22 weeks onwards (HFD + Feno t=12/22) groups and C. HFD and HFD treated with caspase-1 inhibitor (HFD Casp.inh.) groups. Each point represents the mean of the group (Control, HFD, HFD_Chow, HFD Low Responders, HFD + Rosu t=12/22, HFD + Feno t=12: n=12/group, HFD + Feno t>22 and HFD Casp.inh.: n=11/group). ***P<0.001 vs HFD, ###p<0.001 vs Control.
Figure 4. Plasma cholesterol and triglycerides (TG) levels. Time course of plasma cholesterol (mmol/L) and triglycerides (mmol/L) levels of APOE*3Leiden.CETP mice in the chow (Control), high fat diet (HFD), HFD_Chow and HFD Low Responders groups (A,D) or the HFD, HFD treated with rosvastatin started from t=12 or t=22 weeks onwards (HFD + Rosu t=12/22) and HFD treated with fenofibrate started from t=12 or t=22 weeks onwards (HFD + Feno t=12/22) groups (B,E) or HFD and HFD treated with caspase-1 inhibitor (HFD Casp.Inh.) groups (C,F). Each point represents the mean (Control, HFD, HFD_Chow, HFD Low Responders, HFD + Rosu t=12/22, HFD + Feno t=12: n=12/group, HFD + Feno t=22 and HFD Casp.Inh.: n=11/group). ***P <0.001, **p<0.01, *p<0.05 vs HFD and ###p<0.001 vs Control.

OA grades

The knee joints of the APOE*3Leiden.CETP were analysed for the effects of HFD as well as different interventions on the development of OA. Surprisingly, the control group tended to display a higher overall OA grade than the HFD group (Control vs HFD p=0.081). The HFD Low Responders group as well as the group which was switched from a HFD to a control diet halfway the study had no significantly different OA grades than the HFD group (Figure 5A). In line with this, no significant effect of the
various treatments on HFD-induced OA were observed (Figure 5BC). With respect to the individual components of the knee joint (femoral condyle and tibia plateau at the lateral and medial side, trochlear groove and the patella) no significant effects were observed either (data not shown).

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

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

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

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

The current study was based on an unpublished study designed for lipid research purposes. Herein we found promising results for the APOE*3Leiden.CETP mouse as a model for HFD-induced OA. Interestingly, in that experiment treatment for 9 weeks with fenofibrate significantly suppressed OA grades (HFD 13.4 ± 3.8 vs HFD fenofibrate 7.9 ± 5.7, p=0.04), thereby substantiating in vitro observations that fenofibrate has the potential to interfere with degenerative and inflammatory processes in OA. Unexpectedly, we could not validate these data in the current study. We found an overall OA score of 6.0 ± 2.9 in the HFD group which was substantially lower than the OA score of 13.4 ± 3.8 found in the HFD group of the lipid study. With respect to the in-life parameters BW, plasma cholesterol and TG levels no anomalies were observed during the study. Moreover the pilot study, which ran 5 weeks ahead of the main study, gave no indication which would explain the lack of effect of HFD on OA development. These unexpected results may potentially be explained by several observations. At first, although a comparable protocol with regard to strain, length of the study, HFD and gender was applied as in the lipid study, the addition of fructose to the drinking water was omitted. Addition of fructose to the drinking water results in a switch from HDL to ApoB containing lipoprotein (V)LDL in the lipoprotein profile of APO*3Leiden.CETP mice inducing a more human-like cholesterol distribution (12). Fructose consumption in human subjects has been linked to features of the metabolic syndrome and can induce hepatic TG overproduction and accumulation which leads
to the activation of classical inflammatory pathways such as nuclear factor kappa B (37). In a previous publication, OA development and progression was accelerated in the human C-reactive protein (hCRP) transgenic mice, a translational model to monitor inflammation, on HFD (8). As these mice have no VLDL fraction, these data suggest that VLDL particles are not contributing to the process of HFD-induced OA. The HFD-induced OA mouse model in combination with a fructose component has never been investigated (38). At this moment we cannot exclude that fructose water is required to induce OA in the APOE*3Leiden.CETP strain in combination with a HFD. If the hypothesis that fructose is essential for inducing OA is prospectively validated then a highly specific pathway for induction of OA emerges.

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

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

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

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References


