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Metabolic Dysregulation Accelerates Injury-Induced Joint Degeneration, Driven by Local Inflammation; An In Vivo Rat Study

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ABSTRACT: Evidence is growing for the existence of an obesity-related phenotype of osteoarthritis in which low-grade inflammation and a disturbed metabolic profile play a role. The contribution of an obesity-induced metabolic dysbalance to the progression of the features of osteoarthritis upon mechanically induced cartilage damage was studied in a rat in vivo model. Forty Wistar rats were randomly allocated 1:1 to a standard diet or a high-fat diet. After 12 weeks, in 14 out of 20 rats in each group, cartilage was mechanically damaged in the right knee joint. The remaining six animals in each group served as controls. After a subsequent 12 weeks, serum was collected for metabolic state, subchondral bone changes assessed by μCT imaging, osteoarthritis severity determined by histology, and macrophage presence assessed by CD68 staining. The high-fat diet increased statistically all relevant metabolic parameters, resulting in a dysmetabolic state and subsequent synovial inflammation, whereas cartilage degeneration was hardly influenced. The high-fat condition in combination with mechanical cartilage damage resulted in a clear statistically significant progression of the osteoarthritic features, with increased synovitis and multiple large osteophytes. Both the synovium and osteophytes contained numerous CD68 positive cells. It is concluded that a metabolic dysbalance due to a high-fat diet increases joint inflammation without cartilage degeneration. The dysmetabolic state clearly accelerates progression of osteoarthritis upon surgically induced cartilage damage supported by inflammatory responses as demonstrated by histology and increased CD68 expressing cells localized on the synovial membrane and osteophytes. © 2017 Orthopaedic Research Society. Published by Wiley Periodicals, Inc. J Orthop Res 36:881–890, 2018.

Keywords: osteoarthritis; metabolic syndrome; inflammation; animal model; synovitis

Osteoarthritis (OA) is the most prevalent joint disorder in the adult population with an estimated life-time risk of 13.8% and is an important contributor to the global burden of disease.[1,2] This prevalence is increasing as it is driven by ageing as well as trends of increasing obesity and physical inactivity in many countries.[2] Both overweight (Body Mass Index (BMI) $25 \leq 30$) and obesity (BMI ≥ 30) are well-known and important independent risk factors for OA.[3–5]

For a long time obesity was considered to be causative in OA because of the increased mechanical stress in weight-bearing joints.[6,7] However, increasing evidence is found that obesity is also a risk factor for OA of non-weight-bearing joints, like those of the hand.[8,9] This implies that this type of degeneration cannot be caused solely by increased mechanical stress. Obesity is highly associated with the metabolic syndrome (MetS).[10] MetS is defined as the simultaneous occurrence of the following factors: Obesity, insulin resistance, abnormal blood lipid levels, and hypertension.[11,12] Also MetS is an important world health problem with an increasing prevalence of 20% in the general population, contributing to higher disability and mortality.[13]

A relation between OA and MetS has been suggested. A metabolic dysregulation-related phenotype of OA, "metabolic OA" might be considered, with the second highest prevalence after aging.[10] Typically the patient population in this subset is middle-aged (between 45 and 65 years).[14] Cohort studies show faster development and progression of OA and an association with increased pain compared to OA patients without metabolic dysregulation.[15] To evaluate the effects of metabolic factors on joint degeneration, focus has been given to hand OA as hands are not influenced by the increase in loading due to obesity.[16] Establishing metabolic factors involved in OA development and progression, independently from overweight induced increased mechanical loading, is a challenge but considered essential in understanding "metabolic OA" in weight bearing joints. The relative contribution of the biomechanical factors versus metabolic dysfunction in the obese population have not yet been resolved.[17] Growing evidence is presented that both OA and MetS can be seen as low-grade inflammatory conditions with elevation in systemic inflammatory markers.[10,18]

Diet-induced obesity (DIO) is commonly used in animal models to induce characteristics of MetS and a corresponding elevation of inflammatory mediators.[19] Previous studies have shown that high-fat (HF) diet-induced animal models resulted in an approximately twofold increase in the incidence of knee OA.[17,20] Up to now, the exact underlying mechanisms of the systemic alterations due to a DIO in OA are not yet fully understood. Besides, it is still subject of discussion whether an early stage of MetS induced by a HF

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diet on its own leads to the onset of OA or only accelerates OA progression in case of an additional mechanical trigger.[21–24] Therefore, in the present study we hypothesize, that an early stage of MetS, and consequently a chronic low-grade systemic inflammatory state on its own is insufficient to induce clear OA, and that an additional trigger is needed to really give rise to progressive OA. We demonstrated earlier that mechanically induced cartilage damage, applied according to the Groove model in rats, leads on its own to degenerative features under the state of minimal joint inflammation, consistent with joint degeneration seen in early degenerative human disease.[25] The aim of this study was to determine the contribution of metabolic dysregulation, induced by a HF diet, on the progression of OA in this model and study the contribution of inflammatory macrophage activity in this model.

METHODS

Study Design

Forty, 12-week-old male Wistar rats (Charles-River, Sulzfeld, Germany) were housed two per cage in a 12:12 light-dark cycle. All rats were randomly divided into two groups of 20 rats. Twenty rats were fed a HF diet whereof 60% of the kcal contained fat (D12492i, 5.2 kcal/g, Research Diets Inc., NJ) and 20 rats were fed a standard diet whereof 9% of the kcal contained fat (8,01,730, 3.7 kcal/g, SDS, Essex, UK). All animals had access to food pallets and tap water ad libitum. The study was approved by the Utrecht University Medical Ethical Committee for animal studies (DEC 2013.III.12.086) and ARRIVE guidelines were fully complied.

Twelve weeks after randomization, in 14 rats of each diet group, groove surgery[25,26] was performed (see Fig. 1). In short, under general anesthesia, the articular cartilage of both femoral condyles of the right knee joint was damaged without damaging the underlying subchondral bone. In total, five longitudinal grooves at the weight-bearing surface of the medial and lateral femoral condyle, in addition to three grooves on the non-weight-bearing surface of the femoral trochlea were applied. The contralateral knee joint was not surgically damaged and served as internal control. Analgesia (Buprenorphine) was provided until 24 h after surgery and all animals were immediately allowed to move freely. The remaining six animals of each diet group served as control without surgery. To reach a relevant effect size of 50% increase in degeneration for the HF diet rats with groove surgery compared to the standard diet fed rats, power size calculations indicated that these 14 animals with mechanical induced cartilage damage are sufficient to provide enough statistical power (80%) with an α of 0.05. And six animals per group are sufficient to detect differences between a HF and a standard diet without groove surgery.

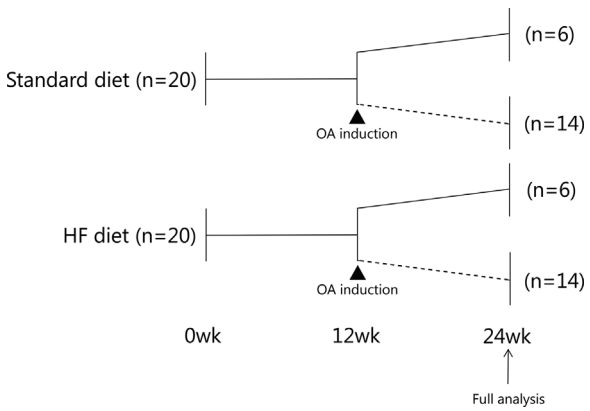

Figure 1. Experimental design of the study. Randomization was performed and 20 rats receive either a high fat (HF) diet or a standard diet. In 14 animals of each diet group, OA induction (groove surgery) was performed 12 weeks after the start of the diet. Full analysis at final time point, 24 weeks after randomization and 12-weeks post-surgery, included serum, and fasted plasma collection, ex vivo bone μ−CT and histological analysis.

Metabolic Parameters

To determine the effects of the HF diet on obesity, body weights were recorded weekly. At endpoint (24 weeks) in all rats, blood was sampled from the lateral tail vein. Prior to sampling all animals were fasted for a period of 6 h. In total, 0.8 ml fasted blood was collected in anticoagulant tubes for plasma, and 0.8 ml blood for serum was collected in a MiniCollect (Greiner bio-one, Alphen aan de Rijn, The Netherlands). Samples were immediately put on ice. One hour after collection, the whole blood was separated by centrifugation for a period of 15 min at 3,000 RCF. All samples were stored in aliquots at −80°C upon analysis. Plasma glucose levels were determined by the University Veterinary Diagnostic Laboratory of the Utrecht University and serum cholesterol levels were determined by an enzymatic colorimetric test (CHOD-PAP, Roche Diagnostics, Almere, The Netherlands). Levels of fasted plasma insulin (EZRMI-13K, Millipore, Amsterdam, The Netherlands), serum leptin (KRC2281, Invitrogen, CA), and serum resistin (RD391016200R, BioVendor, Brno, Czech Republic) were determined by sandwich ELISA specifically for the rat and were analyzed as recommended by the manufacturer. To determine insulin sensitivity, the homeostasis model assessment of insulin resistance (HOMA-IR) was used, which is calculated from a single measurement of fasting insulin and glucose in small laboratory animals.[27]

Subchondral Bone Evaluation With μ-CT

At endpoint, 24 weeks after randomization all rats were euthanized and transferred into a holder in supine position with the hind legs fixated in extension. To evaluate subchondral bone changes on the tibia compartment, a micro-computed tomography (μ-CT) scan, using a Quantum FX μ-CT scanner (PerkinElmar, MA), of both knee joints was performed as described before.[25] The μ-CT scans were made using a 3-min scan per knee joint at an isotropic voxel size of 42 μm, at a voltage of 90 kV, a current of 180 μA, field of view of 21 mm. Bone was segmented from the μ-CT datasets with a local threshold algorithm (Bernsen, radius 5).[28] Using ImageJ software (ImageJ) the regions of interest were manually drawn from coronal orientation for the subchondral plate and trabecular bone of the tibial epiphysis. Starting in the back of the knee joint from the point where the medial and lateral compartments of the tibial epiphysis unite to the front of the knee joint for a total of 90 slides onwards. In all μ-CT datasets, the mean subchondral plate thickness (μm), the mean trabecular bone thickness (μm), and trabecular bone volume fraction (BV/TV, representing the ratio of trabecular bone volume (BV, in mm^3) to endocortical tissue

volume (TV, in mm^3)) were calculated and the data for the medial and lateral side were averaged. Ectopic bone formation (μm^3) on the tibia compartment was quantified as a measure of osteophyte growth.

Histopathological Examination of the Knee Joint

The joint degeneration was evaluated using the OARSI histopathology score for rats.[29] Histological preparations were performed according to the guidelines. Coronal plane sections of 5 μm thickness were made at 100-μm intervals and stained with Hematoxylin & Eosin to visualize characteristics of inflammation and inflammatory cells and Safranin-O to envision the amount and distribution of the glycosaminoglycans. Assessment of joint degeneration and inflammation was performed in random order from sections originating from the middle of the joint, independently by two observers unaware of the source of the samples. The surgical applied grooves were not taken into account, but only the direct adjacent cartilage. The total OARSI score is based upon the sum of the following sub sections: Cartilage matrix loss width (0–2), cartilage degeneration (0–5), cartilage degeneration width (0–4), osteophytes (0–4), calcified cartilage and subchondral bone damage (0–5), and synovial membrane inflammation (0–4), and presented as a total score together with the individual subscores.

Immunohistochemistry for CD68 was performed to visualize cells in the monocyte/macrophage lineage. All sections were blocked for non-specific binding with endogenous enzyme block (DAKO S2003) following antigen retrieval. CD68 was retrieved by incubation with pepsin 0.1% at 37° for 30 min. Next, sections were incubated with primary antibody for CD68 (ab31630, Abcam, Cambridge, UK) at 4°C overnight. Subsequently, the antibody was visualized with Envision HRP anti-mouse (DAKO) for 30 min at room temperature following a 5-minute conversion of DAB. All sections were subsequently counterstained with Mayer's haematoxylin. Isotype control staining with IgG mouse antibody or by incubation without primary antibody was carried out.

Statistical Analysis

Systemic metabolic alterations from the blood samples are presented as mean values with 95% confidence interval of the mean of 20 animals in each group. Comparisons between the two dietary groups were performed by the independent samples *t*-test. Histological data is presented as mean values with 95% confidence interval of the mean for all different treatment groups and was not normally distributed. To analyze differences between experimental knee joints in the different groups the Mann–Whitney *U* test was used and to compare the experimental and their contralateral non-operated knee joint within a group the Wilcoxon signed ranks test was used. In addition, to correct for body weight and the metabolic parameters a linear regression analysis was performed. All subchondral bone changes, originated from the tibia by μ-CT imaging, are presented as mean change with 95% confidence interval and were normally distribute. To compare the experimental and contralateral non-operated joint within each animal, the paired samples *t*-test was used. And to determine if there are differences between the different study groups by μ-CT imaging, the independent samples *t*-test was used (SPSS statistics 21, SPSS inc., IL).

RESULTS

Systemic Metabolic Alterations of a HF Diet

After 24 weeks, rats on a HF diet had a statistically significant higher body weight compared to the standard diet-fed rats (Table 1). Moreover, HF feeding resulted in a statistically significant increase for total cholesterol and fasting insulin levels compared to the standard diet-fed rats. This is in line with previously described studies using this strain of rats.[30] No differences were detected in fasting blood glucose levels. However, the HOMA-IR was already increased compared to the standard diet-fed rats, confirming impaired insulin sensitivity. In addition, a statistically significant increase in the adipokines levels for leptin and resistin were determined in the HF diet-fed rats compared to the standard diet-fed rats (Table 1). All selected metabolic parameters, between groove surgery non-operated animals within both dietary groups were comparable.

Histopathological Joint Degeneration

Histology revealed a small, but statistically significant, increase in joint degeneration in rats with 24 weeks of HF diet feeding compared to the lean non-operated animals ($p = 0.002$; Fig. 2A). In addition, when HF feeding was combined with the surgical model of applied local cartilage damage, OA severity roughly doubled 12-weeks post-surgery compared to both the experimental group on a standard diet (+2.8 points;

Table 1. Metabolic Parameters

	Standard Diet	HF Diet	*p*-value
Weight (g)	734 (704–765)	861 (791–931)	0.001
Change Weight (g/day)	1.9 (1,7–2,1)	2.7 (2.3–3.0)	<0.001
Cholesterol (mg/dl)	113 (87–140)	150 (132–169)	0.003
Fasting Insulin (mU/l)	3.2 (1.9–4.5)	4.7 (3.5–6.0)	0.013
Fasting Glucose (mmol/l)	7.8 (7.2–8.4)	7.9 (7.5–8.4)	0.498
HOMA-IR	1.8 (1.0–2.6)	2.6 (1.9–3.3)	0.027
Leptin (ng/ml)	4.2 (3.3–5.2)	5.7 (4.5–6.9)	0.007
Resistin (ng/ml)	35.0 (30.3–39.6)	40.8 (38.1–43.4)	0.022

Metabolic parameters from blood sampling for the standard diet-fed ($n = 20$) and high fat (HF) diet-fed ($n = 20$) animals determined at endpoint (after 24 weeks of follow-up). Mean values are presented with 95% confidence interval and given *p*-values for differences between both groups were determined by the independent samples *t*-test.

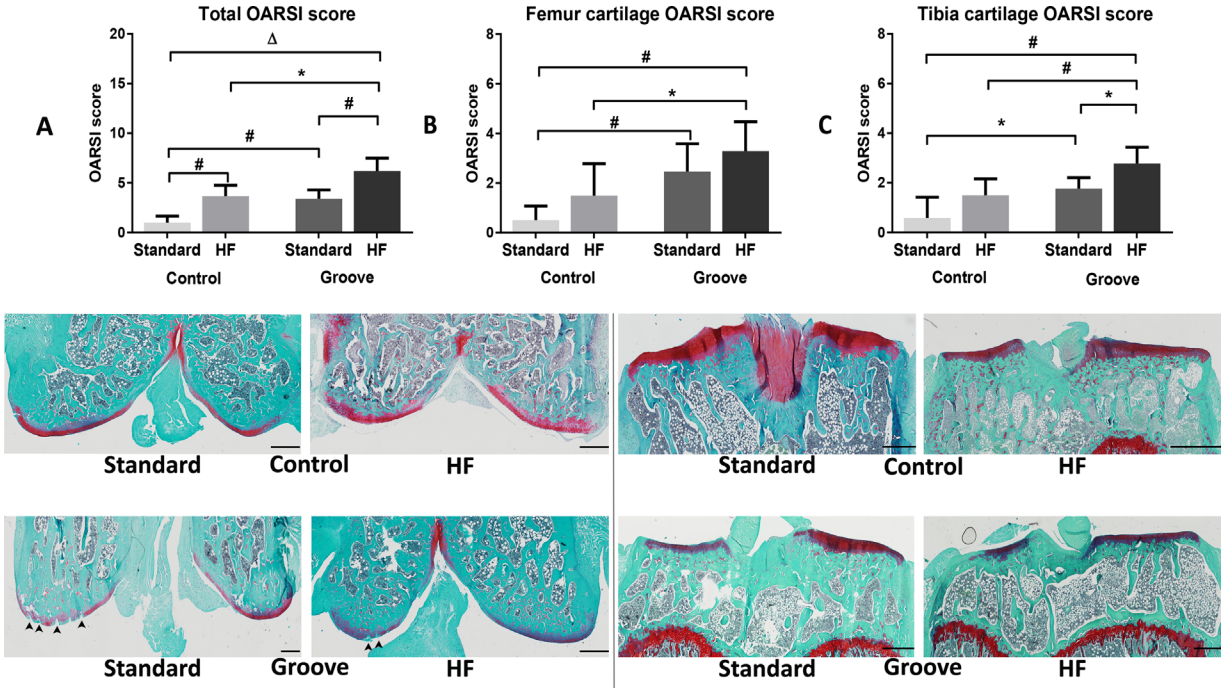

Figure 2. Histological change in joint degeneration as a result of a high fat (HF) diet and/or experimentally induced joint damage on the femoral condyles in the rat knee joint with groove surgery, values are presented for the total OARSI score of the knee joints (combination of cartilage matrix loss width, cartilage degeneration, cartilage degeneration width, osteophytes, synovial membrane inflammation and calcified cartilage, and subchondral bone damage; (A) as no differences were observed between the contralateral non-grooved knee joints and the control animals for each diet, only the experimental knee joints are presented. Histological changes for articular cartilage degeneration, an individual component of the total OARSI score, of joint location specific femoral condyles (B), and tibia plateau (C) for high fat (HF) diet and/or grooved rat knee joint 24 weeks after the start of the diet and 12-weeks post-surgery. Data is presented as mean value and 95% confidence intervals of the mean by the Mann–Whitney U test. $^{*}p < 0.05$, $\#p < 0.01$, $\Delta p < 0.001$. Representative images from safranine-O stained histological sections from the femoral condyles (left) and tibia plateau cartilage (right). Arrowheads indicate the locations of the surgically placed grooves (femoral condyle). Scale bar is 200 μm.

$p = 0.001$) and non-operated HF diet rats (+2.5 points; $p = 0.015$, Fig. 2A). Looking specifically at the articular cartilage, an individual component of the total OARSI score, HF feeding alone did not lead to statistically significant induction of articular cartilage degeneration on either femur or tibia compartment (Fig. 2B and Fig. 2C). Groove surgery with a standard diet on the other hand resulted in statistically significant higher degeneration of the articular cartilage on both the femoral condyles and the tibial compartment at 12-weeks post-surgery ($p = 0.003$; Fig. 2B and $p = 0.013$; Fig. 2C). When the HF diet was combined with groove surgery, again an approximate doubling of the articular cartilage degeneration was observed at the same sites, compared to the non-surgical controls ($p = 0.003$; Fig. 2B and $p = 0.013$; Fig. 2C). Moreover, when compared to the experimental group on a standard diet, an increase in articular cartilage degeneration was observed on the non-grooved tibial compartment, but not on the surgically damaged femoral compartment ($p = 0.010$; Fig. 2C). When the data was corrected for body weight and other metabolic parameters by linear regression analysis, the previous described differences remained statistically significant. Besides, of all selected metabolic parameters, not body weight, but leptin contributed most to the OA-score followed by cholesterol (Table 2).

Subchondral Bone Changes by μ-CT

At 24 weeks after the start of the diet, subchondral bone parameters originating from the tibia compartment of all knee joints were measured by μ-CT imaging. No differences could be demonstrated in the non-operated HF diet-fed rats compared to the non-operated standard diet-fed rats for mean subchondral plate thickness (243 μm (214–272) vs. 263 (250–276); $p = 0.107$), mean trabecular bone thickness (218 μm (165–271) vs. 230 (179–281); $p = 0.625$) and volume fraction (0.52 (0.48–0.57) BV/TV vs. 0.56 (0.51–0.61); $p = 0.134$). In the experimental knee joints on a standard diet, all subchondral bone parameters were increased compared to their contralateral knee joints. However, adding a HF diet to groove surgery did not result in statistically significant increase in any of the tibial subchondral bone parameters measured by μ-CT compared to the rats with groove surgery on a standard diet (Fig. 3).

Inflammatory Parameters

After 24 weeks of HF diet feeding, a mild increase in histological inflammation of the synovial membrane compared to the standard diet-fed rats was observed (Fig. 4A; averages and Fig. 4D; representative images). Groove surgery alone did not result in an increased inflammation of the synovial membrane,

Table 2. Linear Regression of Metabolic Parameters

Groups	B	Weight	Cholesterol	Insulin	Glucose	Leptin	Resistin
SD – SD + GR	2,358	0,085	−0,06	0,108	−0,019	−0,243	0,01
SD – HFD	2,667	−0,204	0,357	−0,183	−0,223	−0,643	0,031
SD – HFD + GR	5,214	−0,696	0,513	−0,571	0,022	0,991	0,697
SD + GR – HFD	0,282	−0,216	-0,85	−0,039	−0,052	0,121	−0,024
SD + GR – HFD + GR	2,83	−0,696	−0,999	−0,768	0,029	0,905	0,726
HFD – HFD + GR	2,548	−0,25	−0,18	−0,229	0,2	−0,177	0,548

SD, standard diet; SD + GR, Standard diet + groove surgery; HFD, High-fat diet; HFD + GR, High-fat diet + groove surgery.
Results of a linear regression analysis with correction for all metabolic parameters between the different study groups are presented. The data is presented as the regression equation for predicting the OARSI-score from the independent metabolic variables in relation to the unstandardized regression coefficient.

confirming earlier findings with the groove model.[25,31] Notably, the combination of the HF diet with the groove model aggravated the synovitis compared to the group with local cartilage damage on the standard diet. More interestingly, when compared to the contralateral knee joints of the HF diet only group, the surgical trigger did not result in an increased synovial membrane inflammation 12-weeks post-surgery (Fig. 4A; HF + Groove (R) vs. HF + Groove (L) and HF control).

By histology, osteophyte formation, was observed in a limited number of non-operated rats with a HF diet (Fig. 4B). In contrast, no osteophytes were observed in the experimental group on a standard diet up to 12-weeks post-surgery. However, when the groove surgery was added to a HF diet an increase in osteophyte formation is observed compared to both the non-operated HF diet and experimental rats on a standard diet. The presence of the osteophytes was supported by the μ-CT data in which the volume of the osteophytes was quantified (Fig. 4C). The observed osteophytes in the non-operated standard and HF diet group were limited and did not differ in size. On the other hand, a clear increase in osteophyte volume was observed in the experimental group with a HF diet compared to the rats with either a HF diet only or groove surgery on a standard diet (Fig. 4C; averages, 4E; representatives). This indicates that the additional surgical trigger, not leading to osteophytes by itself, on top of the HF diet is necessary to induce significant osteophyte formation.

Immunostaining for CD68 on paraffin embedded sections was performed to identify surface markers in the monocyte/macrophage and monocyte/osteoclast lineage. A small increase in CD68 expression was observed in the synovial membrane and subchondral bone in the experimental group on a standard diet and non-groove HF diet rats (Fig. 5). A further increased expression of CD68 positive cells was observed in the synovial lining, the newly formed osteophytes as well as in the bone marrow of the subchondral bone in the experimental group on a HF diet (Fig. 5). This increase in CD68 positive cells was not only observed in the experimental knee joints, but also on the subchondral bone of the contralateral knee joints were no cartilage damage was induced.

DISCUSSION

The present study shows that a systemic dysmetabolic state, induced by a HF diet, induces minor features of joint degeneration but clearly accelerates the progression of knee joint degeneration in the rat groove model of local articular cartilage damage. This can be explained by the increased inflammatory state of the

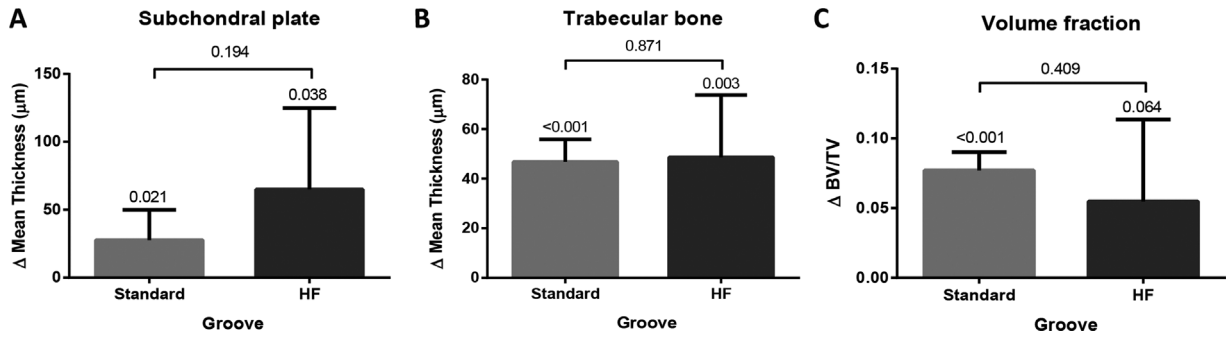

Figure 3. Subchondral bone changes of the surgically untouched tibia compartment as a result of the surgically induced joint damage with and without a high-fat (HF) diet as analyzed by μ-CT imaging. The average delta change between the experimental knee joint and its contralateral control knee joint, for the subchondral plate thickness (A), trabecular bone thickness (B), and bone volume fraction (C) are presented. Bars represent mean change and 95% confidence interval of the mean. *p*-value indicates statistical significant difference compared to their surgically untouched contralateral knee joint, as determined by the paired samples *t*-test. The differences between the grooved knee joints on top of a HF diet and groove surgery alone were determined by the independent samples *t*-test.

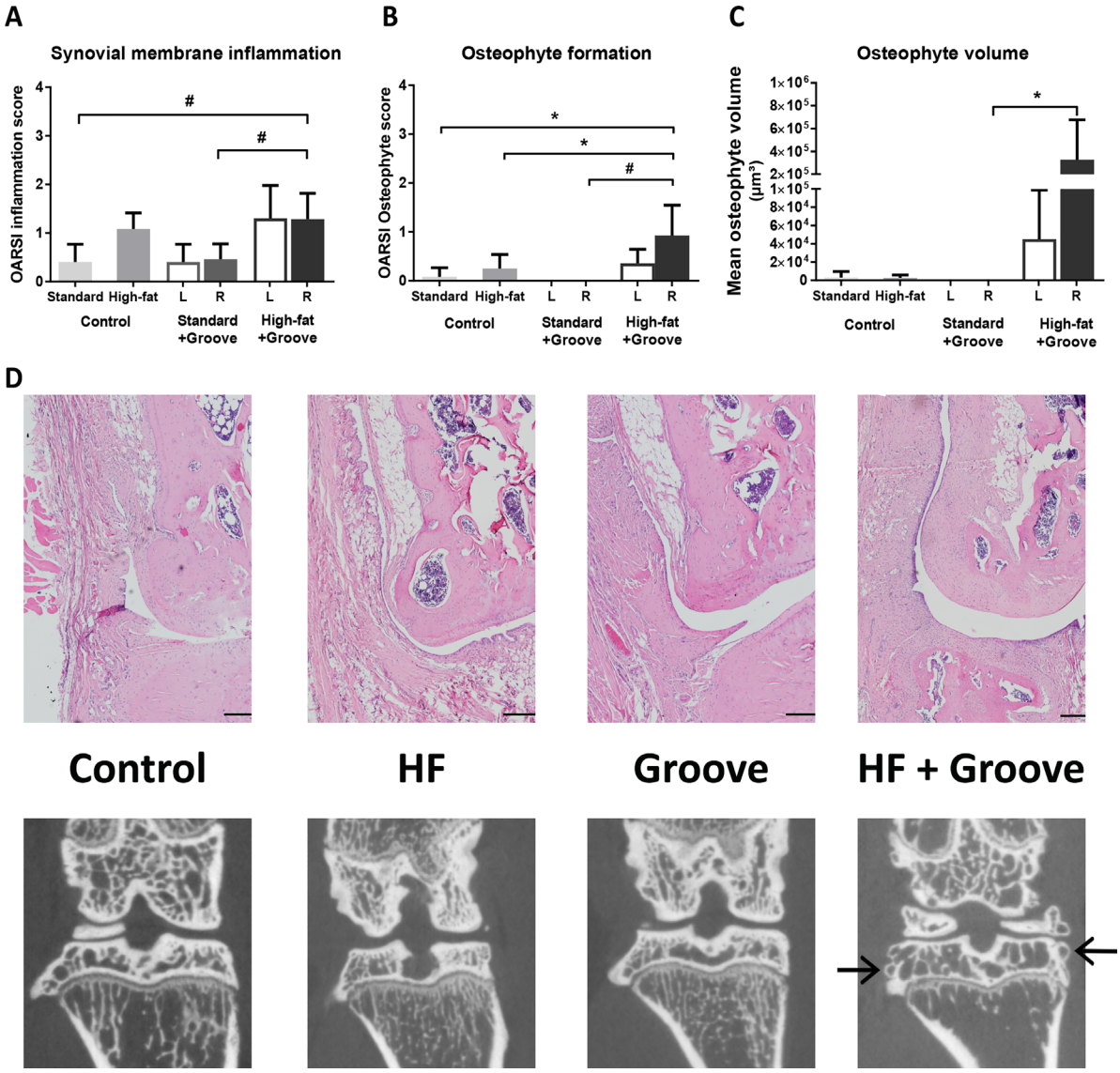

Figure 4. Overview of inflammatory parameters as a result of a high-fat (HF) diet and/or experimentally induced joint damage on the femoral condyles in the rat knee joint with groove surgery. Values are presented for synovial membrane inflammation (0–k4; A), osteophyte formation on histology (0–4; B) and osteophyte volume by μ-CT (C) 24 weeks after the start of the diet and 12-weeks post-surgery in all study groups. The study groups undergoing groove surgery were split for contralateral non-grooved knee joints (L) and experimental grooved knee joints (R). Data is presented as mean value and 95% confidence intervals of the mean. To compare the different experimental groups the Mann–Whitney *U* test was used. The Wilcoxon sign ranks test was used to compare the experimental and the contralateral knee joint. $^{*}p < 0.05$, $\#p < 0.01$. Representative images from H&E stained histological sections from the synovial membrane (D) and μ-CT unsegmented reconstructions (E). Scale bar is 100 μm on H&E stained histological sections.

knee joints illustrated by macrophage presence in bone and synovial tissue and highly elevated osteophytosis. The exact role of HF diet-induced systemic processes in OA of weight-bearing joints is debated on and difficult to identify, since in obese individuals increased mechanical stress and systemic metabolic effects frequently occur together.[4] The most evident change, induced by the HF diet, is the increase in body weight. Both overweight and obesity are highly associated with low-grade inflammatory MetS. Therefore, the prevalence of overweight and obesity is higher compared to MetS.[12,32] To represent the human situation of a dysmetabolic state not yet having full MetS, animal studies are frequently performed. Here, we selected a rat model to study the development of a dysmetabolic state, as rats are not as responsive to a HF diet as other animal models, for example, mice.[30] A specific metabolic syndrome component where this applies is high blood pressure that has been associated with OA, suggesting that hypertension might be related to symptomatic OA.[33] In Wistar rats however, feeding a HF diet does not increase the blood pressure.[30,34] In the present study, the HF diet-fed rats represent the human overweight and/or obese population with a tendency of MetS. Unexpectedly and in contrast to previous studies, the standard diet-fed rats gained more weight than anticipated. However, the state of the standard diet-fed rats is still

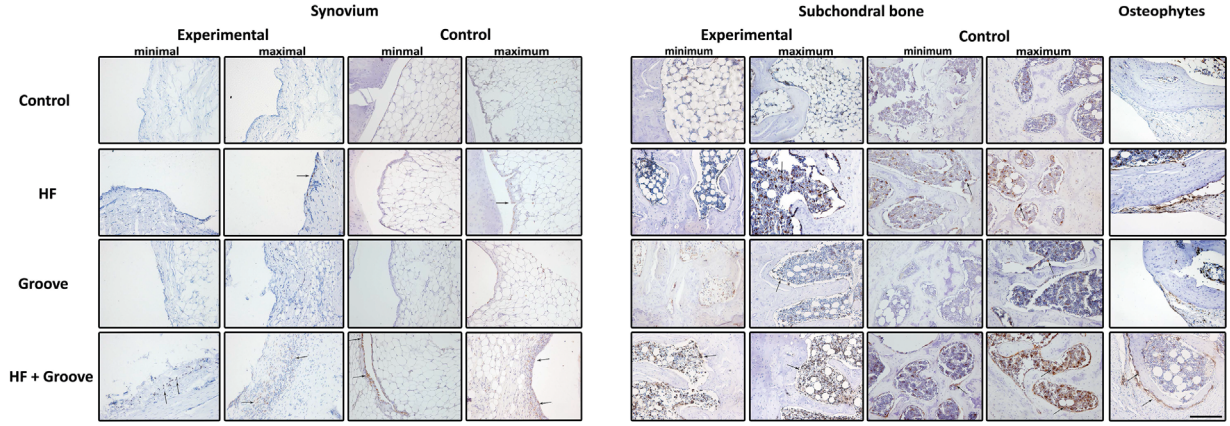

Figure 5. Immunostaining for CD68 on paraffin embedded slides from the synovial membrane and subchondral bone tibia of the experimental right (grooved) and contralateral control left knee joint for the different study groups. In addition to the CD68 positive cells direct adjacent to the newly formed osteophytes which were only present when HF diet was combined with groove surgery. Representative immunostaining images depict the least (minimum) and most (maximum) expression of CD68 positive cells in the synovium and subchondral bone per study group. Negative control staining did not show any a-specific binding. Arrowheads indicate positive cells and scale bar is 200 μm.

representative for the current western population, where overweight and/or obesity without MetS increases.[32] Although the difference in body weight was approximately 15%, the increased mechanical stress in the HF rats is probably less decisive in current findings of joint related phenomena. The increased mechanical stress, as a consequence of the increased body weight, can affect the different joint tissues and their biomechanics, including the subchondral bone turnover, subsequently leading to degenerative changes.[35] In our model, no differences were observed for subchondral bone changes in the HF diet-fed compared to standard diet-fed rats by μ-CT imaging, suggesting that the increased mechanical stress did not result in joint-related bone changes. Also when the OARSI-score was corrected for body weight the observed differences remained significant. In addition, the body weight in the HF diet group did not correlate to the metabolic parameters, as the metabolic parameters increased independent of the body weight in the HF diet group. More likely, the observed differences are a consequence of the systemic processes, in combination with increased mechanical stress, representing the human situation with overweight or obesity with a tendency of MetS, rather than solely increased mechanical stress. Besides, it remains unclear whether weight alone, in the absence of abnormal joint biomechanics, leads to OA development.[36] Conflicting evidence is presented that a DIO by itself is associated with increased OA progression.[20,23,37,38] Therefore, we included an additional surgical induced injury with the placements of grooves on the articular cartilage on top of the HF diet at the moment when the metabolic dysregulation was expectedly induced.[34,39] The rat groove model was used as an additional surgical induced injury in which local damage on the articular cartilage leads to early degenerative joint changes with slow onset without inflammatory OA related phenomena such as increased synovial activity or clear osteophytosis.[25] Therefore, this mechanical model mimics a mild human degenerative OA pathogenesis. Abnormal joint biomechanics cannot completely be ruled out in the groove model, but if present it would be less aberrant to the normal joint biomechanics than the surgical models creating a permanent joint instability or severe chemical joint destruction. Limitation of the present study is the lack of sham surgery in the HF diet fed control animals. Sham surgery was not performed in the contralateral knee joint, as previous work demonstrated no difference in synovial inflammation or cartilage degeneration 12 weeks after sham surgery compared to non-operated control rat knee joints in rats of the same strain and age on a standard diet.[26] In the HF diet sham surgery could control for the potential induced synovitis or altered load bearing. From another study we know that in HF diet fed rats sham surgery does not had an effect on the amount of synovitis (0.8 sham vs. 1.1 non-sham) or the load on the contralateral leg but rather a shift towards the front paws (data not shown).

In this study, the metabolic alterations induced by a HF diet alone for a period of 24 weeks do not induce articular cartilage changes on itself (Fig. 2B and C) but initially results in a more inflammatory state of the knee joint with thickening of the synovium (Fig. 4A). When the groove model is combined with HF diet feeding, the observed joint degeneration is still mild and clearly did not reach end-stage OA in the given time frame. However, the presence of synovitis in addition to multiple and extensive osteophytes and a more sclerotic structure of the subchondral bone is indicative of early degenerative changes considered the first signs of slowly progressive OA. As such, the presence of the increased metabolic alterations, by HF diet feeding is essential to develop increased degeneration of articular cartilage, synovial membrane inflammation, and osteophyte formation in the presence of

the additional surgical induced injury by the placements of grooves. This together result in elevated joint degeneration OARSI score as observed by histology. Nevertheless, a better insight into the mechanisms of early OA is key to understand and develop more targeted regenerative therapies at this early stage of the disease.[40]

We observed increased synovial membrane inflammation in all HF diet-fed rats, regardless of the additional groove surgery. Besides, no difference in synovitis between the grooved right knee and the non-grooved left knee joint was detected in the HF diet fed rats. This suggests that the observed increase in synovitis is a result of the systemic metabolic alterations by the HF diet and was not surgically driven. In previous animal studies looking at the effect of a DIO, the local inflammatory state of the knee joint, has often not been taken into account as a separate outcome parameter.[21,22,41] It was previously reported that osteophyte formation is a result of the presence of synovitis in the OA knee joint, suggesting that the formation of osteophytes is mainly driven by inflammation instead of mechanical stress.[42,43] Especially transforming growth factor-β and bone morphogenetic proteins have been implicated in the increased osteophyte formation.[44] In our model the increased inflammatory state induced by the HF diet alone did not lead to osteophyte formation by itself. The additional surgical induced injury on top of the HF diet was necessary to develop increased osteophytosis as seen on both histology and μ-CT (Fig. 4). Interestingly, a large number of CD68 positive cells were present direct adjacent to the newly formed bone of the osteophytes (Fig. 5), supporting that inflammation drives osteophyte formation. This positive staining likely shows monocytes because osteoclast (TRAP) staining was not positive (data not shown). These cells might later turn into osteoclasts and drive remodeling of the osteophytes. It remains unclear whether this osteophyte formation itself can cause further joint degeneration and cartilage loss or is the consequence. After HF diet feeding systemic metabolic alterations are known to increase the fat content, alter adipocytes size and stimulate their pro-inflammatory cytokine production such as tumor necrosis factor-α, interleukin-1β, and interleukin-6.[45–47] These cytokines are not only produced systemically but also locally by different tissues in OA knee joints including the infrapatellar fat pad and the synovial membrane.[6,18,48] In most tissues macrophages are a significant source of inflammatory cytokines, were both BMI and adipocyte size were strong predictors of the percentage of CD68-expressing macrophages.[49] Activation of adipose tissue macrophages within fat depots is also associated with the increase of an obesity-induced pro-inflammatory state.[49] This increase in macrophage activation, with the capacity to influence normal cell turnover and tissue remodeling, might play a key role in the increase of pro-inflammatory cytokines as seen in OA.[50] We observed that CD68 expression was a systemically induced and upregulated in the joint when combining groove surgery with HF diet f eeding, resulting in an increased obesity-induced pro-inflammatory state of the joint. This expression of CD68 positive cells was not limited to the synovial lining, as a clear increase was also observed in the bone marrow of the subchondral bone. Not only in the experimental joint, but surprisingly also in the contralateral control knee joint (Fig. 5). Besides the presence of macrophages and monocytes, literature suggests that the observed activity could also be fibroblasts and stromal cells.[51] Why the subchondral bone of the contralateral joint also shows increased CD68 expression is not clear at this point. Either there is an additional systemic effect as a consequence of the groove insult, or an altered loading in the contralateral joint that drives monocyte osteoclast precursors in the bone marrow.[52] However, the exact role of local monocyte/macrophage activity, as a consequence of systemic metabolic alterations, as well as the effect of an anti-inflammatory strategy has to be further elucidated.

In summary, these data support the role of systemic metabolic alterations contributing to the elevated joint degeneration upon a mechanical injury, most likely by the increased monocyte/macrophage formation and subsequent inflammatory factors. We showed in this study that in the background of a HF diet, local cartilage damage accelerates OA progression. Moreover, our findings indicate that mild systemic metabolic and subsequent inflammatory factors need an additional surgical induced injury to induce clear features of OA. Local cartilage damage by placement of grooves further accelerates the inflammatory process by macrophage activity resulting in increased osteophyte formation and synovial membrane inflammation.

AUTHORS' CONTRIBUTION

All authors have substantially contributed to the work presented in this paper. All authors contributed to the design, acquisition of data, or analysis and interpretation of data. Finally, all authors have read and approved the final version of the manuscript.

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