Inhibited osteoclastic bone resorption through alendronate treatment in rats reduces severe osteoarthritis progression

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Inhibited osteoclastic bone resorption through alendronate treatment in rats reduces severe osteoarthritis progression

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ABSTRACT

Osteoarthritis (OA) is a non-rheumatoid joint disease characterized by progressive degeneration of extra-cellular cartilage matrix (ECM), enhanced subchondral bone remodeling, osteophyte formation and synovial thickening. Alendronate (ALN) is a potent inhibitor of osteoclastic bone resorption and results in reduced bone remodeling. This study investigated the effects of pre-emptive use of ALN on OA related osteoclastic subchondral bone resorption in an *in vivo* rat model for severe OA. Using multi-modality imaging we measured effects of ALN treatment within cartilage and synovium. Severe osteoarthritis was induced in left rat knees using papain injections in combination with a moderate running protocol. Twenty rats were treated with subcutaneous ALN injections and compared to twenty untreated controls. Animals were longitudinally monitored for 12 weeks with *in vivo* μCT to measure subchondral bone changes and SPECT/CT to determine synovial macrophage activation using a folate-based radiotracer. Articular cartilage was analyzed at 6 and 12 weeks with *ex vivo* contrast enhanced μCT and histology to measure sulfated-glycosaminoglycan (sGAG) content and cartilage thickness.
ALN treatment successfully inhibited subchondral bone remodeling. As a result we found less subchondral plate porosity and reduced osteophytosis. ALN treatment did not reduce subchondral sclerosis. However, after the OA induction phase, ALN treatment protected cartilage ECM from degradation and reduced synovial macrophage activation. Surprisingly, ALN treatment also improved sGAG content of tibia cartilage in healthy joints. Our data was consistent with the hypothesis that osteoclastic bone resorption might play an important role in OA and may be a driving force for progression of the disease. However, our study suggest that this effect might not solely be effects on osteoclastic activity, since ALN treatment also influenced macrophage functioning. Additionally, ALN treatment and physical activity exercised a positive effect in healthy control joints, which increased cartilage sGAG content. More research on this topic might lead to novel insights as to improve cartilage quality.

Introduction

Osteoarthritis (OA) is characterized by articular cartilage degradation and has long been seen as primarily a cartilage disorder. However, nowadays OA is considered as a 'whole joint disease' and it is thought that pathological changes in one joint tissue might compromise structure and function of other joint tissues. Changes within the subchondral bone have been known for a long time to play a role within OA development [1].

Within a healthy joint, the thin dome-like shaped subchondral plate is supported by vertical oriented trabeculae and plays an important role to evenly distribute forces from weight-bearing. Healthy subchondral bone protects cartilage from high peak stresses and possible matrix damage. Animal studies showed that during early OA there is a marked reduction in subchondral bone thickness [2,3] and there are increased numbers of subchondral pores [4,5]. On TRAP-stained histology sections, bone resorption and pore formation as a consequence of increased osteoclast activity [6], result in loss of integrity and plasticity at the osteochondral junction. This compromises its biomechanical function and could promote cartilage damage. Due to all the evidence that subchondral bone remodeling is involved in disease progression, bisphosphonates were suggested to be useful as an interesting intervention strategy to treat OA.

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Alendronate (ALN), risedronate and zoledronate are all nitrogen-containing bisphosphonates and potent inhibitors of osteoclastic resorption used clinically for the treatment of osteoporosis [7]. Both alendronate and zoledronate have demonstrated positive results when used as an OA modifying agent in preclinical animal studies [8–12]. It is suggested that osteoclast-mediated resorption of mineralized cartilage at the subchondral bone-cartilage interface is an early initiating event in OA pathobiology and that only early bisphosphonates use after OA induction will result in the observed positive effect on cartilage health [12]. If in fact osteoclast activation during OA is time-dependent and reduces with ongoing OA stages, this might explain the disappointing results from large clinical trials on the role of bisphosphonates as treatment for OA. These trials included a very heterogeneous patient population, in which a large portion of patients had already severely progressed OA. Therefore, it is less likely that these patients benefit from osteoclast inhibition through bisphosphonates [13–18].

Late or progressive OA shows a different type of subchondral bone remodeling. Several animal studies showed that an initial thinning of the subchondral bone plate [19,20] is followed by a recovery phase leading to subsequent thickening of the subchondral plate due to enhanced osteoblast activity [20–22]. During this un-physiological high bone turnover in OA joints, there is an altered phenotypic expression of osteoblasts, which results in the production of sclerotic bone together with cyst formation and osteophyte development [4,9,23]. It has been hypothesized that as a result of the functional coupling between osteoclasts and osteoblasts, increased osteoclastic bone resorption induces a rise in osteoblast activity leading to increased subchondral bone thickness and sclerosis [24]. If true, bisphosphonate intervention to inhibit osteoclastic bone resorption might intervene with eventual formation of subchondral sclerosis by osteoblasts.

Recently, we established a novel rat OA model using a combination of papain injections with a running protocol which induces severe knee joint articular cartilage degradation together with activation of synovial macrophages and prominent involvement of subchondral bone [25]. In this particular study we found that papain injection alone induced moderate OA features, like sGAG and slight cartilage matrix loss, enhanced loss of the subchondral cortical plate. As a result of OA induction through papain injections and running, there was a complete different response and rats develop a pronounced sclerotic bone phenotype within the lateral compartment of the proximal tibia plateau combined with severe loss of cartilage matrix. In the current study, we investigated whether pre-emptive inhibition of osteoclast function through bisphosphonate treatment could prevent the development bone sclerosis, and possibly could prevent or reduce the development of OA. We used longitudinal *in vivo* microCT scans to measure effects of ALN treatment on subchondral sclerosis development and *ex-vivo* microCT on cartilage samples to see if cartilage was protected against degradation. Besides marked changes of articular cartilage and subchondral bone in this model for OA, we know there is also abundant activation of synovial macrophages [25]. Therefore, we also measured whether ALN treatment had an effect on synovial macrophage activation using a folate-based radiotracer for *in vivo* SPECT/CT imaging [26].

Methods

Effect of systemic alendronate treatment on severe osteoarthritis progression

Forty 16-week-old male Wistar rats (Charles River Netherlands BV, Maastricht, the Netherlands) were housed in the animal facility of the Erasmus University Medical Centre, with a 12-h light–dark regimen, at 21 °C during the experimental period, and received standard food pellets and water *ad libitum*. Severe osteoarthritis was induced in all animals using intra-articular papain injections in their left knee joints combined with exposure to a moderate exercise protocol as described before [25]. In short, all animals received three intra-articular injections in their left knee joints with 30 μl papain/L-cystein solution [27]. Their right knee joint served as an internal healthy control. All rats were forced to run on a motorized rodent treadmill (LE-8700; Panlab Harvard Apparatus, Barcelona, Spain) 500 m/day during 5 days/week, for six weeks covering a distance of 15 km in total [25].

Animals were divided over two groups: twenty rats served as untreated controls and twenty rats were treated during the experiment with three times weekly subcutaneous ALN injections (2.4 μg/kg) (alendronate, Sigma-Aldrich, St. Louis, MO, USA) to inhibit osteoclast bone resorption, a dose previously reported to be comparable to the clinical dose of 10 mg/day prescribed for the treatment of postmenopausal osteoporosis [28] (Fig. 1). Sterile water was used as the vehicle for dissolving ALN. Untreated animals did not receive placebo injections.

During the study all animals were longitudinally monitored with microCT to measure subchondral bone changes. At six and twelve weeks, ten rats in both groups were selected for a full analysis sequence. This sequence consisted of a SPECT/CT using a folate-based radiotracer to quantify macrophage activation *in vivo* [28], and *ex vivo* EPIC-μCT and histology to measure cartilage quality [29]. For all procedures, the exact same procedures were followed as described earlier [25]. The animal ethic committee of the Erasmus University Medical Center, Rotterdam, the Netherlands, approved all conducted procedures. A detailed planning scheme of all groups and conducted tests is given in Fig. 1.

Subchondral bone measurements on μCT scans

Both knees of all animals were μCT scanned under isoflurane anesthesia, using a Skyscan 1176 *in vivo* μCT scanner (Skyscan, Kontich, Belgium). 10 min of scan time was required per knee at an isotropic voxel size of 18 μm, at a voltage of 65 kV, a current of 385 mA, field of view of 35 mm, using a 1.0 mm aluminum filter, over 198° with a 0.5° rotation step, and a 270 msec exposure time. All datasets were segmented with a local threshold algorithm [30]. Cortical and trabecular bone were automatically separated using in-house software [31]. Using Skyscan software, both subchondral plate thickness (Sb. Pl. Th. in μm) and subchondral plate porosity (Sb. Pl. Por. in mm^3) of the medial and lateral compartment of the tibial plateau were measured [24]. In the tibial epiphysis, the trabecular thickness (Tb. Th. in 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). We additionally quantified the amount of ectopic bone formation as a measure for osteophyte growth (mm^3) on longitudinal μCT scans.

Determination of activated macrophages by SPECT/CT using ^{111}In-EC0800

Activated macrophages express the folate receptor-β allowing monitoring macrophages *in vivo* using folate-based radiotracers [32–34]. Phosphate saline-buffered (PBS, pH 6.5) DOTA-Bz-folate (EC0800, kindly provided by Endocyte Inc., West Lafayette, USA) [35] was labeled with $^{111}InCl_3$ (Covidien, Petten, The Netherlands) as described previously [25]. Quality control was performed with ITLC-SG and revealed a radiochemical yield of >95% at a specific activity of 50 MBq/μg. ^{111}In-EC0800 (55 MBq) was administered *via* the tail vein 20 h prior to scanning. SPECT/CT scans were performed with a 4-head multiplex multi-pinhole small-animal SPECT/CT camera (NanoSPECT/CT™, Bioscan Inc., Washington DC, USA). All knee joints were scanned with both helical μCT (acquisition time 5 min) and SPECT (acquisition time 30 min). All scans were analyzed using InVivoScope post-processing software (Bioscan Inc.). To reduce inter-individual variation, the absolute difference in measured radioactivity (kBq/mm^3) of the OA knee joint compared to the contralateral control joint was calculated. This absolute difference was used when comparing mean values of untreated animals with ALN treated animals.

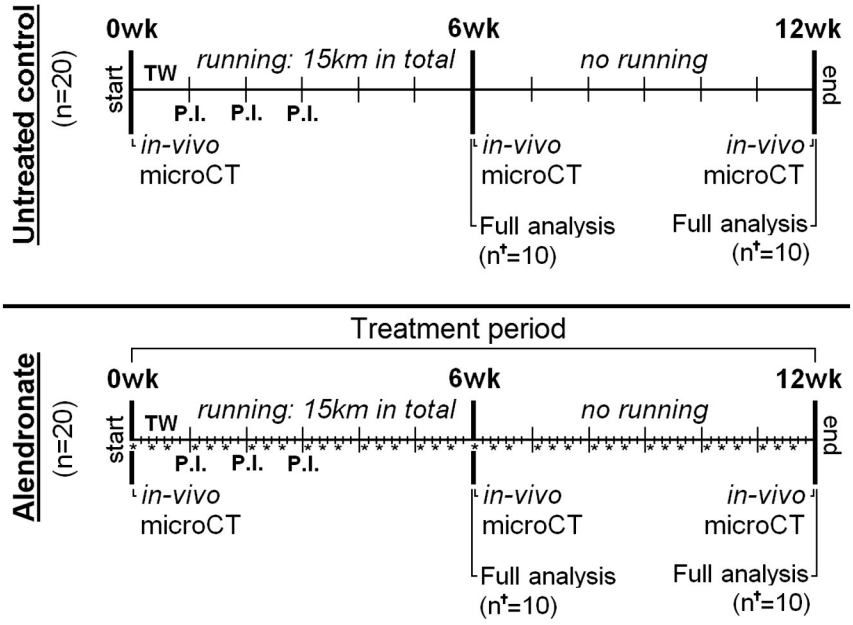

Fig. 1. Experiment design indicating analytical time points and methods for each experimental group. Forty 16-week-old male Wistar rats were injected with three papain intra-articular injections (P.I.) and forced to run 15 km on a motorized treadmill. Animals were divided over two different groups: an untreated group (n = 20) and a group treated with alendronate (n = 20). Treated animals received subcutaneous alendronate injections (2.4 μg/kg), indicated with * in the scheme. During the experiment three longitudinal microCT scans were made to measure subchondral bone changes. At six and twelve weeks a full analysis sequence was done in ten animals per group (n†), consisting of *in-vivo*: determination of activated macrophages using SPECT/CT; and *ex-vivo*: cartilage analysis with equilibrium partitioning of an ionic contrast agent using (EPIC-)microCT and histology.

Cartilage evaluation with contrast enhanced μCT and histology

Equilibrium partitioning of a contrast agent using microCT (EPIC-μCT) has a strong correlation with cartilage sulfated-glycosaminoglycan (sGAG) content [29]. In EPIC-μCT an equilibrium-state exists between sGAG and contrast agent after a 24 hour incubation period. Resulting cartilage X-ray attenuation in these scans is inversely related to sGAG content and thereby represent cartilage quality. This technique is suited for quantitative analysis of cartilage degradation for preclinical evaluation of OA [36].

Animals were euthanized directly after the last SPECT/CT scan and both knee joints were harvested for EPIC-μCT analysis. All specimens were incubated in 40% solution of ioxaglate for 24 h at room temperature [37]. EPIC-μCT was performed on the same μCT scanner, using the following scan settings: isotropic voxel size of 18 μm, a voltage of 65 kV, a current of 385 mA, field of view 35 mm, a 0.5 mm aluminum filter, 198° with a 0.5° rotation step, and a 235 ms exposure time. In all EPIC-μCT datasets, X-ray attenuation (arbitrary gray values related to sGAG content) and cartilage thickness (μm) was calculated for cartilage of the medial and lateral plateau of the tibia [25].

After EPIC-μCT, the separated parts of the knee joints were fixed in paraformaldehyde, decalcified with formic acid and embedded in paraffin. Sagittal sections were made at 300 μm intervals and stained with Safranin-O to image the amount and distribution of the GAGs. Sections were stained all at once, to minimize protocol differences between different samples.

Statistical analysis

All measurements were consistent with a normal distribution according to D'Agostino and Pearson omnibus normality tests. Differences between means of OA induced and healthy knee joints within the same animal were tested using paired *t*-tests at each time point for all outcome parameters (GraphPad Software, San Diego, California, USA). When comparing differences between means of untreated OA animals and ALN treated OA animals, an unpaired *t*-test was used at each time point for all outcome parameters (GraphPad Prism Software). When osteophytes and subchondral pores do not develop, this was scored as zero. Therefore, we used a one-sample *t*-test and tested whether the outcome of OA induced joints differed from zero (GraphPad Software). Longitudinal data from *in vivo* μCT were additionally analyzed using generalized estimating equations (GEE) (SPSS Inc., Chicago, USA). For all tests, p values ≤ 0.05 were considered significant.

Results

Effects of systemic alendronate treatment

All untreated (non-alendronate) rats did not increase in weight from 416.4 g (411.3–421.5 g) to 408.3 g (398.2–418.3 g) after six weeks of treadmill running. During subsequent six weeks of rest, all untreated rats increased in bodyweight to 485.5 g (473.0–498.0 g). ALN treated animals showed the same patterns, with no increase in weight during the first six weeks, from 419.4 g at the start of the experiment (413.8–425.0 g) to 416.9 g (408.4–425.4 g), and an increase in weight to 500.2 g at twelve weeks (483.9–516.5 g) (Fig. 2).

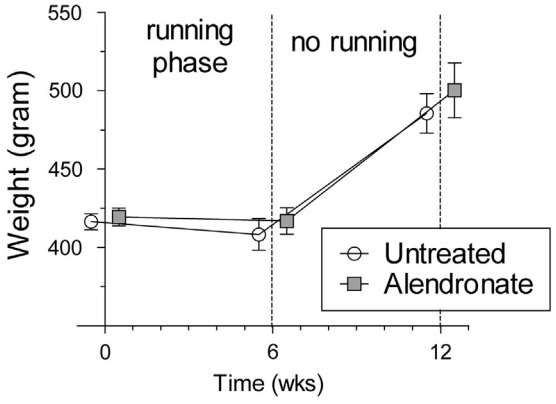

Fig. 2. Increase of bodyweight (gram) of untreated control rats (white circles) and alendronate treated rats (gray squares).

Subchondral bone changes

During the experiment, healthy knee joints of untreated animals showed increased subchondral trabecular thickness ($p < 0.001$) and decreased BV/TV ($p < 0.001$) (Figs. 3A–B). Due to OA induction there was a reduction in BV/TV ($p < 0.001$) compared to the contralateral healthy knee joint in untreated animals, while trabecular thickness was not different from the healthy control ($p = 0.29$). Both healthy and OA joints of ALN treated animals showed a reduced increase of trabecular thickness during the 6 weeks running and subsequent period to 12 weeks ($p < 0.001$) and higher BV/TV ($p < 0.001$) compared to untreated animals at 12 weeks but not at 6 weeks (Figs. 3A–B).

GEE analysis of medial subchondral plate thickness of untreated animals showed that the subchondral bone plate of OA joints increased less in thickness compared to healthy joints ($p = 0.008$), where in ALN treated animals there was no difference between healthy and OA joints ($p = 0.26$) (Fig. 3C). However, there was no significant difference in medial subchondral plate thickness of OA joints between untreated and ALN treated animals ($p = 0.12$). There was also no increased porosity of the medial subchondral bone plate for untreated and ALN treated rats.

At the lateral side after six weeks of running subchondral sclerosis developed in the OA joints of untreated animals compared to its contralateral healthy knee joint ($p < 0.0001$), which persisted after six weeks of rest ($p < 0.0001$) (Fig. 3E). In ALN treated animals sclerosis did also develop in OA induced joints. GEE analysis between OA joints of untreated and ALN treated animals did not find a significant difference in the development of subchondral sclerosis ($p = 0.12$) (Fig. 3E).

ALN treated animals totally lacked subchondral plate porosity at 6 weeks ($p = 0.02$) (Fig. 3F). At twelve weeks, this effect was however not significant anymore ($p = 0.24$). This was predominantly due to the rather large variation in subchondral porosity in untreated animals. This variation resulted from the reduction in number of animals at 12 weeks and some of them not showing any porosity.

Osteoarthritic changes of articular cartilage

OA induction in untreated animals induced severe sGAG depletion from both medial and lateral cartilage compartments of the tibia plateau. This sGAG depleted state persisted throughout the experiment (Fig. 4A). Although there was significant sGAG loss in OA joints of ALN treated rats compared to their healthy joints at both six ($p < 0.0001$) and twelve weeks ($p < 0.0001$), ALN treated animals had more sGAG in cartilage of the medial plateau at twelve weeks compared to OA joints of untreated rats ($p = 0.02$) (Fig. 4A). After the running protocol at six weeks, cartilage of the medial compartment was reduced in thickness compared to healthy knee joints in untreated rats ($p = 0.007$) and ALN treated rats ($p = 0.003$) (Fig. 4C). Compared to healthy joints this matrix degradation persisted during the following six weeks of rest in untreated rats ($p = 0.0005$). However, medial cartilage thickness of OA joints in ALN treated animals was thicker compared to OA joints of untreated animals ($p = 0.003$) (Fig. 4C). Lateral cartilage thickness was degraded (Fig. 4D) and resulted in almost completely denuded subchondral bone (Fig. 4E) in both untreated an ALN treated animals. Representative medial and lateral cartilage images from safranin-O stained histology from untreated OA controls at six and twelve weeks are shown in Fig. 5.

Interestingly, after the six week running phase in both medial ($p = 0.03$) and lateral ($p = 0.01$) cartilage of healthy joints in ALN treated animals, there was a reduced sGAG content compared to healthy cartilage of untreated animals (Figs. 4A–B). During the next six weeks of rest, untreated animals showed a ~3% improvement in sGAG content of medial tibia cartilage and 0.1% of the lateral tibia cartilage. Interestingly, after these six weeks of rest, the medial tibia cartilage improved ~13% and the lateral tibia cartilage ~7% in ALN treated animals and was significantly higher in both the medial ($p = 0.01$) and lateral cartilages ($p = 0.005$) compared to untreated animals (Figs. 4A–B).

In Fig. 4E representative images from EPIC-μCT scans are depicted. These figures clearly show the loss of sGAG from the articular cartilage due to the OA induction, as well as the loss of cartilage matrix on the lateral tibia plateau. At twelve weeks, ALN treated animals show less irregularity of medial tibia plateau cartilage matrix, which is also thicker compared to untreated animals.

Effect of systemic alendronate treatment on synovial macrophage activation

At both 6 weeks and 12 weeks each animal received 55 ± 5 MBq of ^{111}In-EC0800 with no significant differences of injected activity between experimental groups. At six weeks, untreated rats (~40%)

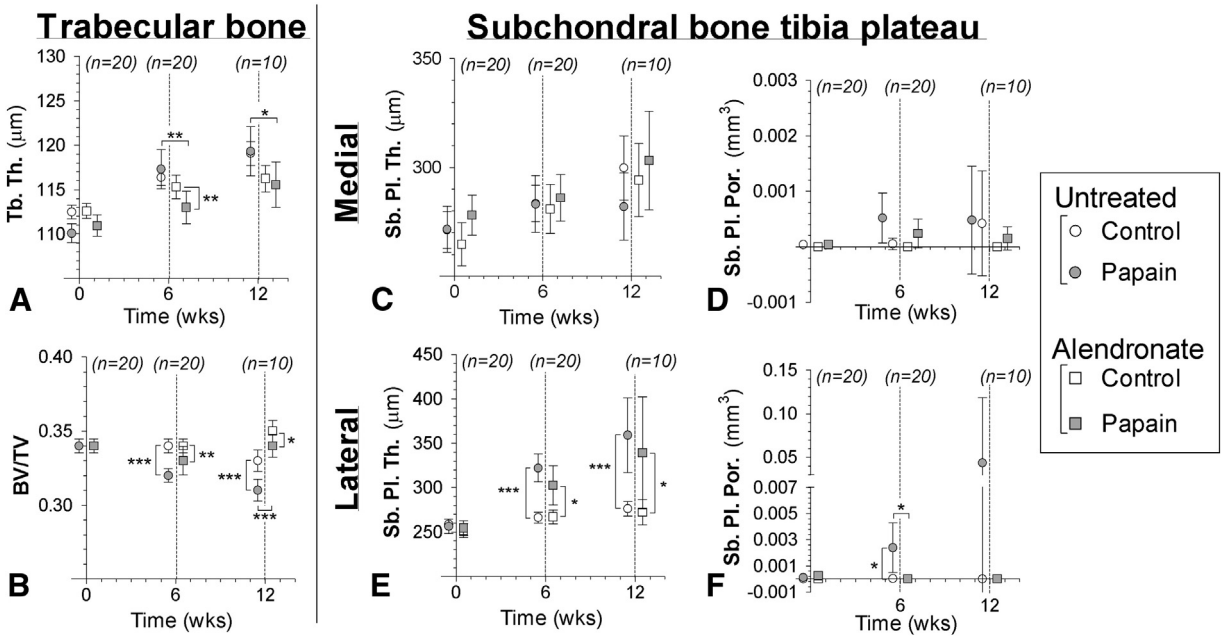

Fig. 3. Subchondral bone changes analyzed with longitudinal *in vivo* μCT in untreated animals (circles) and alendronate treated animals (squares). Changes in trabecular thickness (Tb. Th.; A) and trabecular bone volume fraction (BV/TV; B) were measured in tibial epiphysis bone marrow. Subchondral plate thickness (Sb. Pl. Th.; C, E) and porosity (Sb. Pl. Por.; D, F) were measured in the medial (C, D) and lateral (E, F) compartment of the tibial epiphysis. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, error bars indicate 95% confidence intervals.

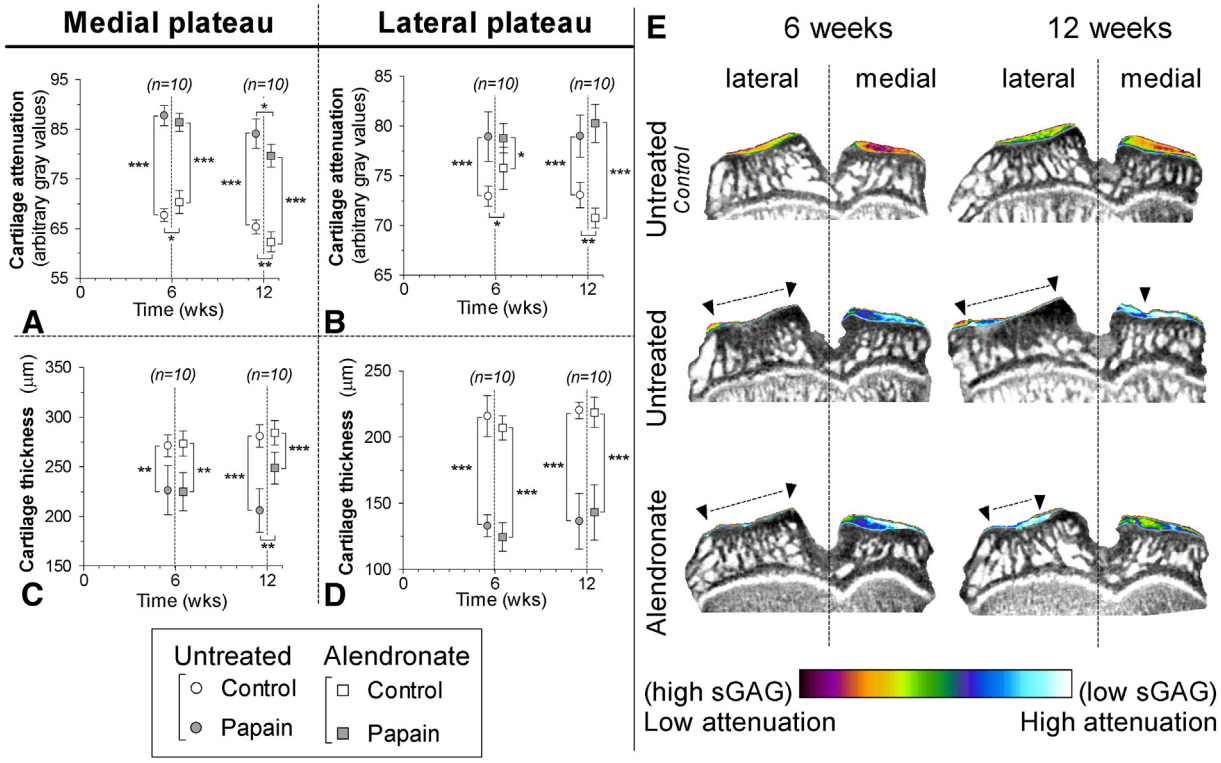

Fig. 4. Cartilage quality and quantity was determined from samples of untreated (circles) and alendronate treated (squares) rats with equilibrium partitioning of a ionic contrast agent using (EPIC-)µCT (A–D). The amount of sulfated-glycosaminoglycans (sGAG) (arbitrary gray values; A, B) and cartilage thickness (µm; C, D) were measured of medial (A, C) and lateral (B, D) cartilage compartments of the tibia plateau harvested from healthy joints (blank boxes) and OA induced joints (gray boxes). Attenuation values from EPIC-µCT scans are inversely related to the sGAG content, meaning that a high attenuation corresponds to low sGAG content. (E) Coronal images from representative EPIC-µCT scans of the tibia plateau show the amount of cartilage (erosions indicated with ▼ and dashed lines) and sGAG content (displayed in color). *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, error bars indicate 95% confidence intervals.

and ALN treated rats (~28%) had increased radioactive uptake in their OA-induced knee joints compared to their contralateral healthy control joints (Figs. 6A,C). However, a comparison between both groups showed no significant difference in radioactive uptake. During this period of moderate running, ALN treated animals formed less mineralized osteophyte formation compared to untreated animals, but this effect was not significantly different ($p = 0.07$) (Figs. 6B,C).

After six subsequent weeks of rest untreated animals still had ~23% increased radioactivity uptake in their OA-induced knee joints. In ALN treated animals this amount dropped to an ~8% increase, however it was still significantly more compared to their healthy control joint ($p = 0.02$). The absolute difference in radioactive uptake between OA induced and healthy control joints in ALN treated animals was lower compared to the absolute differences measured in untreated controls ($p = 0.003$) (Figs. 6A,C). At twelve weeks there was again a tendency of reduced osteophyte formation in ALN treated animals as compared to untreated controls, but again these data were not significantly different ($p = 0.09$) (Figs. 6B,C). However, GEE analysis showed that during the entire experiment, ALN treated animals developed significantly less ectopic bone formation compared to untreated controls ($p = 0.008$).

Discussion

Inhibiting osteoclastic bone resorption through bisphosphonate treatment has shown beneficial effects in pre-clinical animal OA studies, but these reporting studies made use of OA models that are relatively mild in nature [9,11,12]. Osteoclast activation is suggested to be time-dependent and reduces with ongoing OA stages, and might explain the disappointing results from large clinical trials on the role of bisphosphonates as treatment for OA [13–18]. In this study we investigated whether a preemptive start of alendronate could reduce the severe OA progression known to develop after papain injections combined with moderate running exercise [25].

This study demonstrates that healthy knee joints of untreated (but running) animals showed ~5% subchondral bone loss while trabecular thickness increased ~5%. This has previously been described to occur during normal bone remodeling as a consequence of aging and increased physical activity [38]. OA induced knee joints of untreated animals showed an enhanced loss of BV/TV, which can be related to increased trabecular bone remodeling in OA joints of rodents [21]. In contrast to untreated animals, our results show that ALN treatment resulted in functional impaired bone remodeling in both healthy and OA knee joints, which can be related to inhibited osteoclast bone resorption [28]. It has been suggested that a functional coupling between osteoclasts and osteoblasts eventually induces subchondral sclerosis [24], but ALN treatment in our study did not reduce subchondral sclerosis formation (Fig. 3). This suggests that a direct influence of osteoclastic function on the formation of subchondral sclerosis is rather unlikely.

This sclerotic bone phenotype only developed at sites where there was a total loss of articular cartilage. Due to this loss of cartilage, force dissipation through the subchondral bone must have changed severely. We hypothesize that subsequent increased mechanical stimuli within the underlying subchondral bone, might have triggered the mechanosensory response of osteocytes [39] and subsequently induced sclerosis. In OA patients osteocytes become more elongated [40] and produce less sclerostin [41]. Sclerostin is known for its anti-anabolic effect on osteoblasts through an antagonist function on the Wnt signaling pathway. Normally, Wnt signaling induces osteoblast maturation and prevents osteoblast apoptosis, which subsequently stimulates bone formation [42]. When sclerostin production by osteocytes is reduced, Wnt is promoted and osteoblasts are stimulated to form bone, which in this case, might result in increased or sclerotic bone formation. No direct

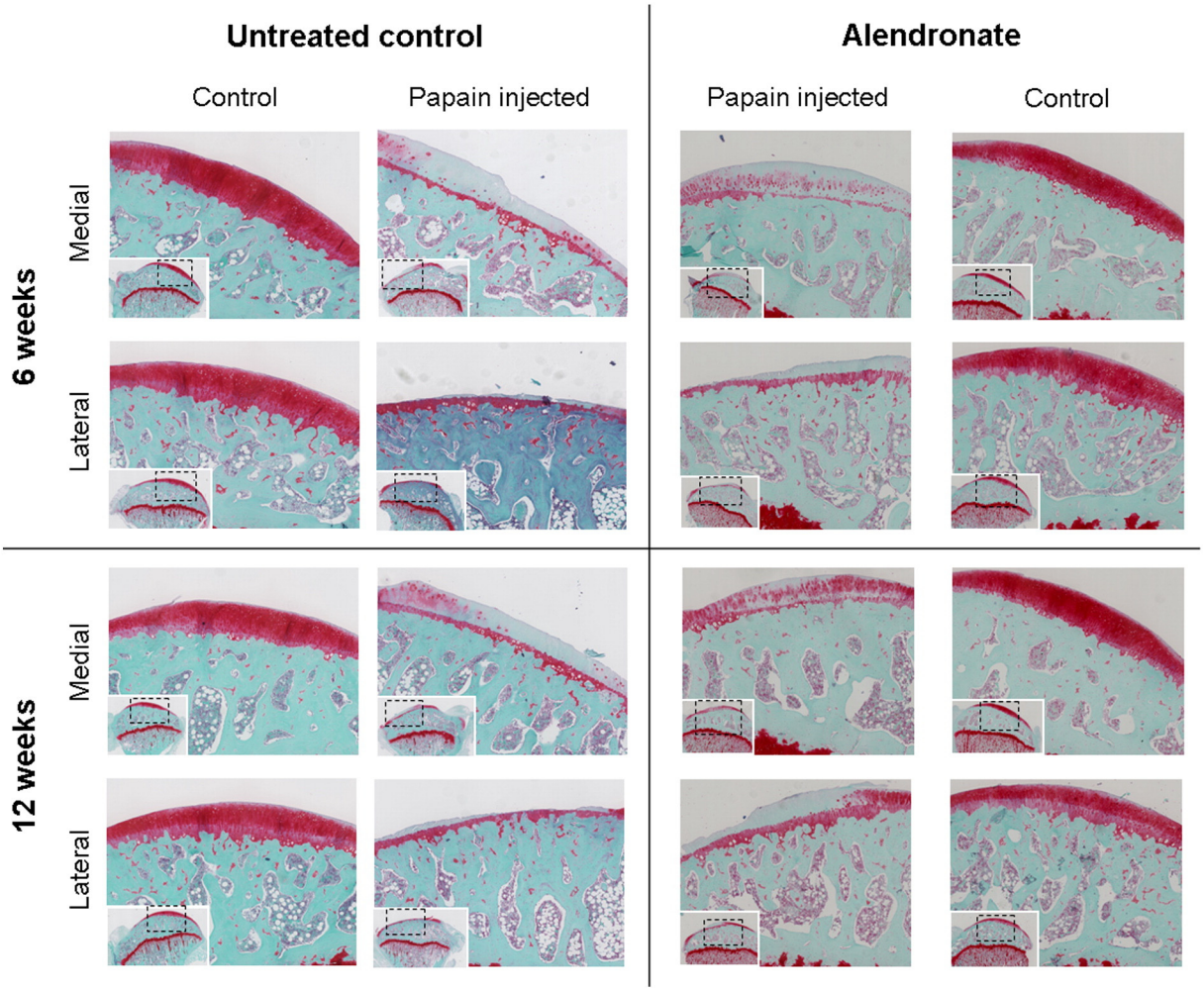

Fig. 5. Safranin-O stained histology sections of medial and lateral tibial plateau cartilage after six weeks and twelve weeks of follow up. Untreated animals show severely sulfated-glycosaminoglycan (sGAG) depleted medial tibia cartilage at six and twelve weeks. Cartilage extra-cellular matrix (ECM) was slightly degraded at six weeks, but progressive loss of cartilage ECM was found at twelve weeks. ALN treated animals showed a similar loss of sGAG and loss of ECM at six weeks, however, after twelve weeks more sGAG was present and the ECM was less degraded compared to untreated animals. In both untreated and ALN treated animals, lateral cartilage ECM was almost totally eroded with only the calcified cartilage layer that remained.

evidence for this specific relation was found in this study, and validation of such a theory would require more research.

ALN treatment did not prevent the deleterious erosion of lateral tibia cartilage. However, medial compartment cartilage was protected from further degradation of cartilage extra-cellular matrix due to ALN treatment. This suggests that in this model, osteoclastic activity somehow fuels an ongoing process of cartilage degradation. Besides this protective effect on cartilage matrix in OA induced joints, ALN treatment improved sGAG content in healthy joints of treated animals. Our results do not explain why ALN has this effect on cartilage. However, one hypothesis could be that through inhibition of osteoclast bone resorption by ALN, the supportive function of subchondral bone is not reduced and remains stiff during running exercise. As a consequence, chondrocytes are exposed to increased mechanical stress and produce less sGAG [43]. However when stress levels are relieved in the period between 6 and 12 weeks, chondrocytes recover and increase sGAG production. Possibly due to the stiffer subchondral cortical bone plate and higher stress levels, chondrocytes in ALN treated animals produced more sGAG to further enhance cartilage quality. The effects of training on cartilage are already well known in clinical patient care [44], possibly this effect might be enhanced with pre-emptive ALN treatment. However, more research is necessary to establish a relationship between osteoclast activity, chondrocyte sGAG production, and the role of biomechanical impact due to physical exercise.

Analysis of macrophage activation using folate receptor targeted SPECT/CT also showed interesting results. After six weeks of OA induction both groups showed no difference in macrophage activation, however, after 12 weeks macrophage activation was significantly reduced in ALN treated animals compared to untreated animals. Recently, bisphosphonates have been reported to significantly reduce pain in patients with clinical and radiographic knee osteoarthritis [45]. Synovitis and activation of synovial macrophages are related to patient complaints, like joint dysfunction and pain [46], and has been related to the progression of cartilage erosion [47,48]. Possibly, a loss of macrophage activation in ALN treated animals reflected the reduced amount of articular cartilage degradation. But, bisphosphonates are known to influence macrophage responses as well [49] and ALN treatment could directly have reduced macrophage activation. Then again, the finding that pre-emptive use of ALN did not reduce macrophage activation after six weeks does not support this explanation.

Although we found some promising results in this study, it is important to point out that using animal models for OA research does not allow for direct translation towards clinical care. There are simply too many factors related to the study design that might have a distinct influence on experimental outcome (for example, species, strain, age). Additionally, this study has two major limitations. First, we did not use a saline injection as a control in untreated animals. Hypothetically, handling of animals during subcutaneous injections might have caused

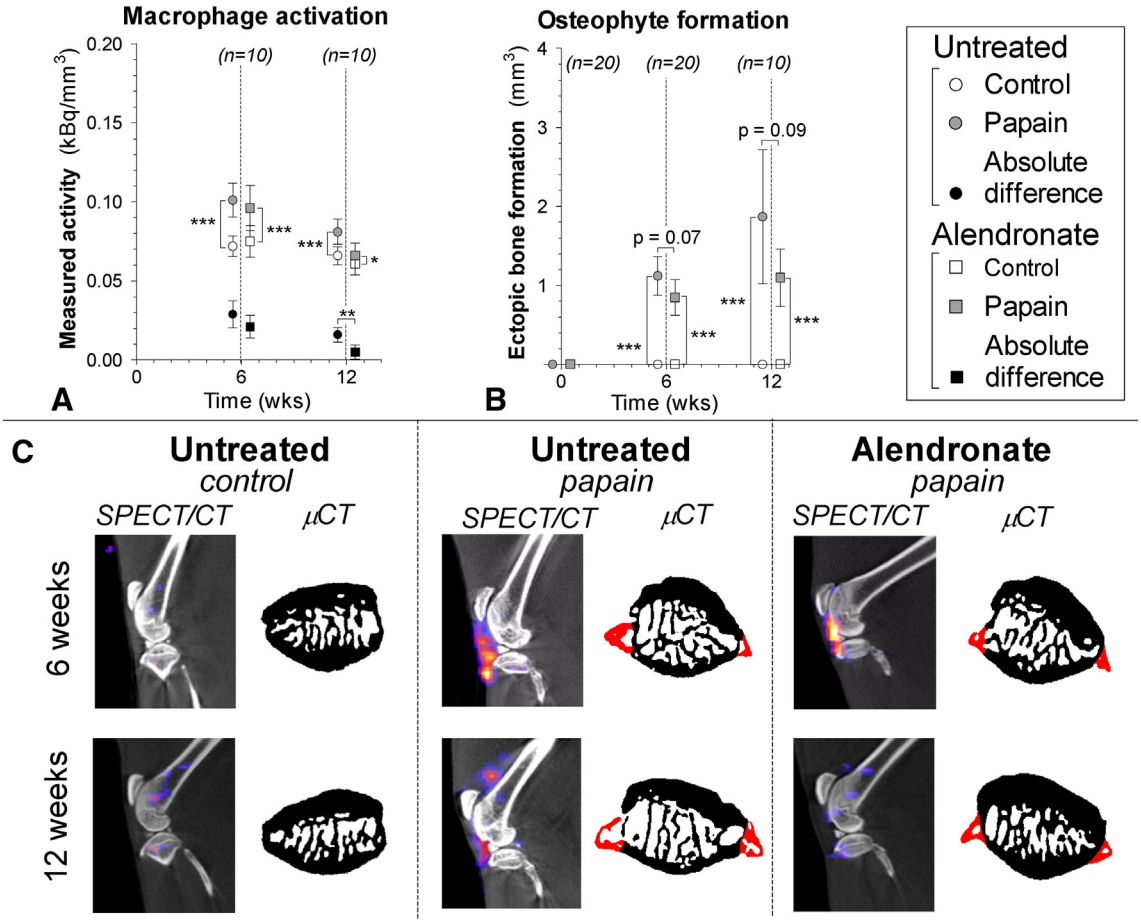

Fig. 6. Macrophage activation determined in untreated animals (circles) and alendronate treated animals (squares) after injection of ^{111}In-EC0800 using SPECT/CT. A: Quantitative outcome of measured radioactivity in the healthy joints (blank boxes) and OA joints (gray boxes) normalized to the size of the analyzed cylindrical region of interest (kBq/mm^3). Absolute differences per animal were calculated (kBq/mm^3) to correct for differences in biodistribution of ^{111}In-EC0800 (black boxes). A high radioactivity is related to more macrophage activation. B: Ectopic bone formation (mm^3) as a measure for osteophyte development was quantified on longitudinal bone µCT scans. C: Sagittal SPECT/CT images of knee joints from representative animals. CT images shown in black and white were used for anatomical reference, the SPECT images are shown in color. Micro-SPECT images show radioactivity accumulation, one fixed threshold used for all images. Transaxial images from patellar bone extracted from binary µCT images show ectopic bone formation (red color). *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, error bars indicate 95% confidence intervals.

some form of stress that might have caused a bias in our study. And second, we lacked a pure control without exercise and without OA induction, this may cause a bias in our study. From a biological point of view, it is known that skeletal growth in rats is related to changing cartilage matrix biology and phenotypic characteristics of chondrocytes [50,51]. And from a biomechanical point of view, the way rats run can never be compared to the way humans do. In light of biomechanics, pain is also an important aspect that is likely to have influenced our outcome. Rats that suffer pain from OA induction are known to change their weight-bearing behavior [52]. Unfortunately, we were not able to record their discomfort using an incapacitance test or pain measurement. Therefore, we are unable to discuss to what extent pain might have influenced our outcome.

Conclusion

Reduced subchondral bone loss and reduced osteophyte formation was found after OA induction in ALN treated compared to non-ALN treated rats. ALN treatment also reduced cartilage degradation and suggests that osteoclastic activity is a driving force behind ongoing OA articular cartilage degradation. However, this effect might not be solely due to osteoclastic activity, since the results of our study showed clear interaction of ALN treatment in macrophage activation. Furthermore, ALN treatment during moderate exercise influenced sGAG production in healthy cartilage and after a period of rest, resulted in increased cartilage sGAG content. More studies on the mechanisms of ALN treatment in healthy joints together with physical exercise training could provide more insight and potentially lead to new treatment strategies that can improve cartilage quality.

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