

# Early Signs of Bone and Cartilage Changes Induced by Treadmill Exercise in Rats

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## ABSTRACT

This study aims to investigate the earliest alterations of bone and cartilage tissues as a result of different exercise protocols in the knee joint of Wistar rats. We hypothesize that pretraining to a continuous intense running protocol would protect the animals from cartilage degeneration. Three groups of animals were used: (i) an adaptive (pretraining) running group that ran for 8 weeks with gradually increasing velocity and time of running followed by a constant running program (6 weeks of 1.12 km/hour running per day); (ii) a non-adaptive running (constant running) group that initially rested for 8 weeks followed by 6 weeks of constant running; and (iii) a non-running (control) group. At weeks 8, 14, and 20 bone and cartilage were analyzed. Both running groups developed mild symptoms of cartilage irregularities, such as chondrocyte hypertrophy and cell clustering in different cartilage zones, in particular after the adaptive running protocol. As a result of physical training in the adaptive running exercise a dynamic response of bone was detected at week 8, where bone growth was enhanced. Conversely, the thickness of epiphyseal trabecular and subchondral bone (at week 14) was reduced due to the constant running in the period between 8 and 14 weeks. Finally, the intermediate differences between the two running groups disappeared after both groups had a resting period (from 14 to 20 weeks). The adaptive running group showed an increase in aggrecan gene expression and reduction of MMP2 expression after the initial 8 weeks running. Thus, the running exercise models in this study showed mild bone and cartilage/chondrocyte alterations that can be considered as early-stage osteoarthritis. The pretraining adaptive protocol before constant intense running did not protect from mild cartilage degeneration. © 2017 The Authors. *JBMR Plus* is published by Wiley Periodicals, Inc. on behalf of American Society for Bone and Mineral Research.

**KEY WORDS:** ANIMAL MODELS; BONE MODELING AND REMODELING; EXERCISE; BIOMECHANICS; OSTEOARTHRITIS

## Introduction

Osteoarthritis (OA) is a progressive degenerative disease affecting the whole articulating joint with disturbed bone remodeling, degenerated extracellular matrix of articular cartilage, synovial inflammation, and changes in the normal functions of cells.<sup>(1)</sup> OA progresses from a mild form to advanced pathology characterized by debilitating pain.<sup>(2)</sup> Multiple studies have indicated that joint tissues respond to exercise, which could have both positive and negative effects on bone and cartilage depending on the frequency and amplitude of the load.<sup>(3–5)</sup> Finding changes of cartilage and subchondral bone as a consequence of exercise can be challenging, but is important for a better understanding of joint tissue adaptation to mechanical triggers. This could also elucidate both how the involved tissues improved after exercise,<sup>(6,7)</sup> and how they start to become damaged in cases where the exercise reaches a state of overloading.<sup>(2,8)</sup>

In the current study we aimed to study the earliest signs of a response to a biomechanically induced trigger or an anomaly in exercised joints in a rat model. Potential positive effects of physical activity may be attributed to the adjustment of the metabolism and viability of chondrocytes and osteocytes through which mechanical loading influences both cartilage matrix and bone architecture.<sup>(7)</sup> Roos and Dahlberg<sup>(9)</sup> tested the effects of a moderate training protocol in women at high risk of knee OA. They found that moderate exercise improved the glycosaminoglycan (GAG) content as measured by delayed gadolinium-enhanced magnetic resonance imaging (MRI) and concluded that exercise may be a good treatment to improve cartilage and joint function.<sup>(9)</sup> The negative effects of intensive physical activity, on the other hand, are likely due to the overload damages incurred in the involved tissues which exceed their regenerative capacity.<sup>(10)</sup> Chondrocytes respond to mechanical loading by changing the proteoglycan content of cartilage, while they do not make more collagen.<sup>(11)</sup> Because

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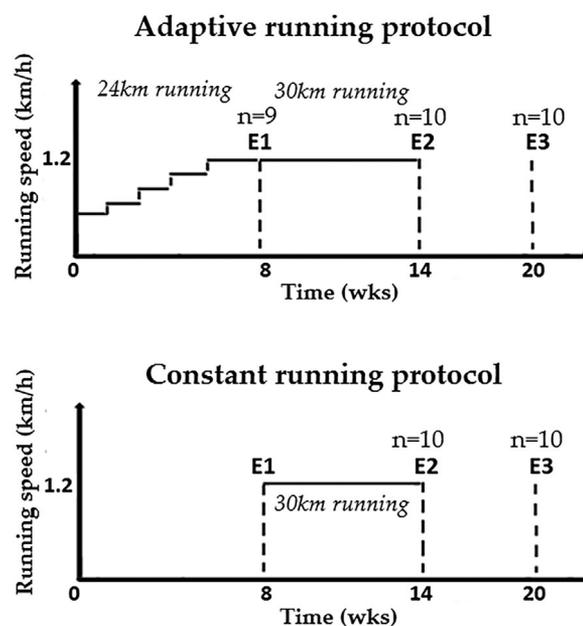
GAG level in cartilage, among other parameters, is dependent on the duration and magnitude of mechanical loading,<sup>(5,12)</sup> moderate levels of physical activity may be beneficial for cartilage health. Intensive mechanical loading may, nevertheless, result in collagen damage, which is considered irreversible. It is therefore quite difficult to distinguish the narrow margin between too little and too much physical activity.<sup>(5,13)</sup>

Many studies have focused on the effects of one type of treadmill running such as mild, moderate, or strenuous/excessive on the involved tissues.<sup>(2,7,8)</sup> However, here, we study the response of the knee joint to a combined approach of a stepwise gradually increasing training protocol followed by an intense running exercise to investigate the continuous cartilage and bone adaptation to the exercise protocol. To study the earliest alterations in the involved tissues, we started from a 6-week protocol with 1.12-km running in 1 hour each day in young rats that provoked mild GAG loss in earlier work.<sup>(14,15)</sup> We hypothesized that this running protocol provides adverse effects on the cartilage tissue because the rats have never been active and started to run without any previous pretraining protocol. Therefore we added an additional 8-week running protocol with gradually increasing intensity in a pretraining phase, which would (potentially) create an adaptation of the tissues that improves the cartilage and thereby protects it from the mild GAG loss in the next period of constant (intense) running. Both of these running groups were compared to a non-running control group. The effects on cartilage and bone as a consequence of the various running protocols were closely monitored at different time intervals using micro-computed tomography ( $\mu$ CT) (bone morphometry), histology, microindentation, and RT-PCR for gene expression.

## Materials and Methods

### Experimental animals and study design

Eighty male Wistar rats (8 weeks old, weight 280 to 350 g) obtained from Charles River (Sulzfeld, Germany) were used in this study. The total study period was 20 weeks. The animals were randomly divided into three groups based on the running protocol: (i) an adaptive running group that ran for 8 weeks with gradually increasing velocity and time of running followed by a constant running program which was 6 weeks of 1.12 km/hour running per day; (ii) a non-adaptive running group (constant running group) that initially rested for 8 weeks followed by 6 weeks of constant running; and (iii) a control group (no running) (Fig. 1). The rats in the constant running group were trained in the week prior to the start of the treadmill exercise program.<sup>(15)</sup> Running took place on a five-lane motorized rodent treadmill (LE-8700; Panlab Harvard Apparatus). The pace and duration of the constant running protocol is equal to about 50% of a total exhaustion protocol.<sup>(16)</sup> Animals were kept under controlled temperature with 12-hour light/dark cycle with *ad libitum* access to tap water and standard food pellets. Detailed information regarding the running regimes for each group and cumulative running distance are described in Supporting Table 1. As shown in Fig. 1, we had 8 subgroups, each comprising 10 rats with endpoints at 8 weeks (endpoint-1), 14 weeks (endpoint-2), and 20 weeks (endpoint-3). The constant running protocol was the same as the non-running protocol up to endpoint-1 so only one subgroup (n=10) was included for both protocols at this endpoint. One rat from the

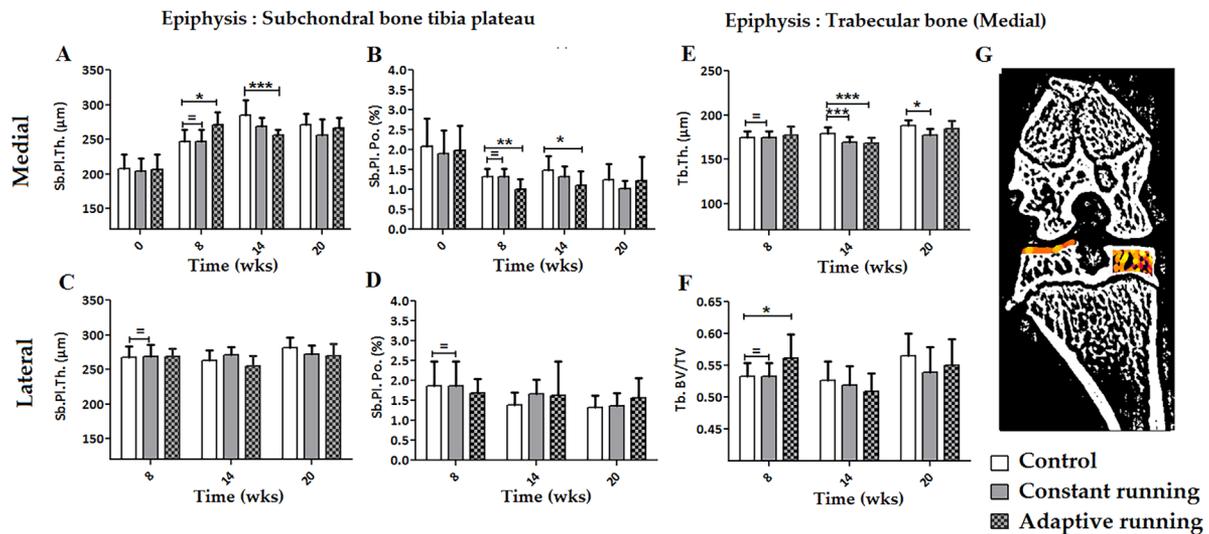


**Fig. 1.** Exercise protocols of different experimental groups, E1, E2, and E3, respectively, indicate various time points: endpoint-1 (after 8 weeks), endpoint-2 (after 14 weeks), and endpoint-3 (after 20 weeks). Male Wistar rats (8 weeks old) were divided into three groups: one adaptive running group ( $n = 29$ ), one constant running group ( $n = 20$ ), and one control group ( $n = 30$ ). Except the rats in control group, the rest were subjected to a constant running regime for a period of 6 weeks. The rats in adaptive running group started their exercise training with an adaptive running regime 8 weeks before the constant running period.

adaptive running group was removed from the experiment at endpoint-1, because it did not run on the treadmill. The animals were weighed at regular intervals. Before starting the experiment (week 0), we performed *in vivo*  $\mu$ CT to assess bone morphology-related parameters for each rat. At each follow-up time point, we analyzed the influence of running protocols on the cellular, molecular, and tissue level properties using  $\mu$ CT, histology, RT-PCR, and microscale indentation tests. The study was approved by the Utrecht Animal Ethics Committee (DEC, 2014.III.01.010).

### $\mu$ CT analysis to measure bone characteristics

$\mu$ CT (Quantum FX, Perkin Elmer, MA, USA) scans were made with the following parameters: time = 3 min, voxel size =  $42 \mu\text{m}^3$ , tube voltage = 90 kV, and tube current =  $180 \mu\text{A}$ . Serial 2D coronal images were obtained from 3D reconstructed  $\mu$ CT images using Analyze 11.0 (Perkin Elmer, USA). We performed local segmentation on the preprocessed images in ImageJ (1.47v) using Bernsen algorithm with a radius equal to 5. The bone parameters were measured in different regions of the tibia including epiphysis, metaphysis, and diaphysis (Fig. 2G, Fig. 3C) using BoneJ (a plug-in of ImageJ software; NIH, Bethesda, MD, USA; <https://imagej.nih.gov/ij/>) by manually selecting the proper regions of interest. In the tibial epiphysis, we calculated subchondral bone plateau thickness (Sb.PI.Th.) and its porosity (Sb.PI.Po.) as well as trabecular bone thickness (Tb.Th.) and its bone volume fraction (Tb.BV/TV). Furthermore, metaphyseal



**Fig. 2.**  $\mu$ CT result on tibial epiphysis. (A, B) Sb.PI.Th. and Sb.PI.Po. of the medial and (C, D) lateral plateau, as well as (E, F) medial Tb.Th. and medial Tb. BV/TV were measured. (G) Coronal view of reconstructed image of the rat knee joint and the selected ROIs in subchondral bone tibia plateau and trabecular bone. Control, constant running, and adaptive running groups are shown with white, gray, and gray/black, respectively. \* $p \leq 0.05$ , \*\* $p \leq 0.01$ , \*\*\* $p \leq 0.005$ . Sb.PI.Th. = subchondral bone thickness; Sb.PI.Po. = subchondral bone porosity; Tb.Th. = trabecular bone thickness; Tb.BV/TV = trabecular bone volume fraction; ROI = region of interest.

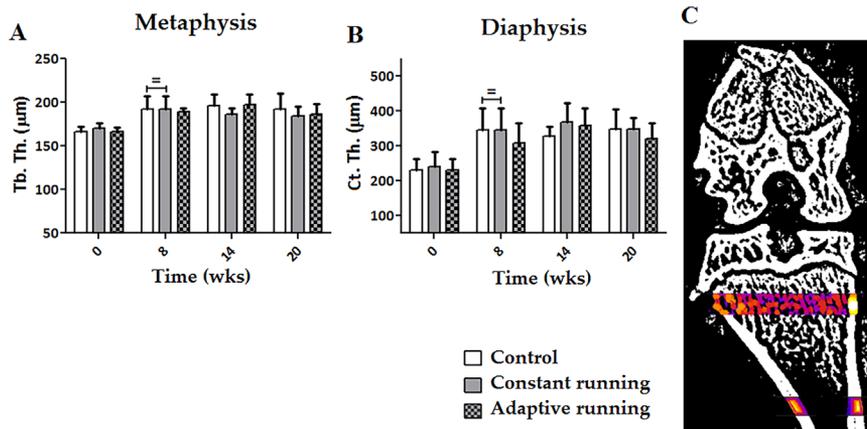
trabecular thickness (Tb.Th.) and diaphyseal cortical shell thickness (Ct.Th.) were computed.

#### Histopathological examination of the knee joint

After  $\mu$ CT scanning, animals were euthanized and left knees were harvested and fixed in 4% formaldehyde solution (Klinipath, The Netherlands) for 1 week. They were then decalcified by EDTA solution (12.5%; VWR, Leuven, Belgium) for 40 days and after serial dehydration in graded ethanol solutions (Brunschwig Chemie, Amsterdam, the Netherlands) were embedded in paraffin. Five-micromillimeter-thick (5- $\mu$ m-thick) sections at

300- $\mu$ m intervals were obtained using a microtome (Thermo Scientific Micron HM 340E, Germany).

Knee joint slides were examined by Safranin-O with a fast green to investigate the whole tissue structure, GAG content, and chondrocytes. Histological sections for both medial and lateral portions were semiquantitatively scored. The four grades were defined as follows: 0 = intact cartilage surface and normal chondrocytes morphology; 1 = slight GAGs loss (up to 10% of the cartilage area) in the superficial zone, cell proliferation and clustering (up to 30%) in the superficial and middle zones, and hypertrophic chondrocytes (up to 20%) in the deep zone



**Fig. 3.**  $\mu$ CT result on tibial metaphysis and diaphysis. (A) Tb.Th. and (B) Ct.Th. were measured. (C) Coronal view of reconstructed image of the rat knee joint and the selected ROIs in metaphysis and diaphysis. Control, constant running, and adaptive running groups are shown with white, gray, and gray/black, respectively. (=) shows that only one subgroup was considered for both control and constant running groups at endpoint-1. Tb.Th. = trabecular thickness; Ct.Th. = cortical shell thickness; ROI = region of interest.

and tidemark; 2 = moderate GAGs loss (up to 30%) in the superficial zone, cell proliferation and clustering (up to 60%) in the superficial and middle zones, and hypertrophic chondrocytes (up to 40%) in the deep zone and tidemark; and 3 = marked GAGs loss (greater than 30%) in the superficial zone, cell proliferation and clustering (greater than 60%) in the superficial and middle zones, and hypertrophic chondrocytes (greater than 40%) in the deep zone and tidemark.

### RNA isolation and quantitative RT-PCR

The effect of exercise on the response of chondrocytes was investigated using RT-PCR for the following cartilage relevant genes: type II collagen (*COL2A1*), aggrecan (*ACAN*), and matrix metalloproteinases (*MMP-2*, *MMP-9*, and *MMP-13*). Femoral condyles of the right knee joint were isolated. After immediate snap-freezing in liquid nitrogen, the samples were stored in a  $-80^{\circ}\text{C}$  freezer. A maximum cartilage thickness of 200  $\mu\text{m}$  from both medial and lateral femoral condyles was cut using a cryotome (Thermo Scientific CryoStar NX70, China). RNA was extracted from cartilage tissue using an RNA isolation kit (miRCURY<sup>TM</sup> cell and plant; Exiqon 300110, Vedbaek, Denmark) in accordance with the instructions provided by manufacturer. The RNA concentrations were quantified using nanodrop (DeNovix DS-11, Wilmington, USA) and Agilent bioanalyzer (RNA 6000 Pico Assay, Waldbronn, Germany). cDNA was synthesized from 30 ng total RNA in a total volume of 20  $\mu\text{L}$  using iScript<sup>TM</sup> cDNA Synthesis Kit (Bio-Rad Laboratories B.V., Veenendaal, The Netherlands). Quantitative PCR was performed in mono using a BioRad iCycler CFX384 Touch<sup>TM</sup> thermal cycler, and IQ SYBR Green Super mix (Bio-Rad Laboratories). First, the difference between cycle threshold (Ct) value of each target gene from the average Ct value of two housekeeping genes, namely glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) and  $\beta$ -actin (*ACTB1*) was calculated ( $\Delta\text{Ct}$ ). Then the normalized expression levels were determined.

The primers were purchased from Eurogentec (Maastricht, The Netherlands) (Table 1). The primers were designed and the sequences were confirmed according to a previously described method.<sup>(17)</sup>

### Nanoindentation for cartilage stiffness

The tibia component of the right knee joint was isolated and stored at  $-20^{\circ}\text{C}$ . To perform indentation experiments, the tibia plateau was defrosted and equilibrated in PBS supplemented with protease inhibitor (Complete; Roche, Mannheim, Germany). Indentations were performed on the central part of both medial and lateral components of the tibia plateau using a displacement-controlled nanoindenter instrument (Piuma, Amsterdam, the Netherlands) and a spherical probe (Optics, Netherlands) with a radius of 50  $\mu\text{m}$  and a cantilever stiffness of

$\sim 100\text{ N/m}$ . Indentation tests were conducted on the cartilage tibia plateaus with 81 indentations ( $9 \times 9$  matrix) at a 1  $\text{mm}^2$  square.<sup>(18)</sup> The effective elastic modulus was calculated based on the Oliver-Pharr theory from the initial portion of the unloading part of the load-displacement curve in order to detect the elastic response of the cartilage.<sup>(19)</sup> See Moshtagh and colleagues<sup>(18)</sup> for a more detailed description of this mechanical experiment.

### Statistical analysis

One-way ANOVA followed by Bonferroni correction was used to test the differences between the control group and two running groups at each time point. Differences in semiquantitative histology grades and gene expression between rats in three experimental groups were tested using Kruskal-Wallis one-way ANOVA. At endpoint-1, differences between the control group and adaptive running group were assessed using the Student's unpaired *t* test. The differences between the groups over time were compared using two-way ANOVA. A linear mixture model was used to compare the mechanical properties measured at different locations between the three groups at each time point (IBM SPSS, v22; IBM Corp., Armonk, NY, USA). Statistical significance threshold was set at  $p < 0.05$ .

## Result

### Body mass

The mean weight of all rats was ( $308.0 \pm 15\text{ g}$ , mean  $\pm$  SD) at the beginning (8 weeks old), and  $489.5 \pm 46\text{ g}$ ,  $522.9 \pm 57\text{ g}$ , and  $589.9 \pm 58\text{ g}$ , at first, second, and final (28 weeks old) endpoints of the experiment, respectively. At endpoint-2 the weight of both running groups was significantly lower than the sedentary controls ( $p < 0.0001$ , see Supporting Fig. 1), but the difference between the groups decreased in the remaining 6 weeks of non-running.

### Bone changes

#### *$\mu\text{CT}$ result on tibial epiphysis*

The bone morphometric parameters had a rather small variation between animals within the group (Fig. 2). The non-running control animals showed a clear increase of the subchondral bone plate in the first 14 weeks of the follow-up period (from  $207.9 \pm 21\ \mu\text{m}$  to  $284.8 \pm 22\ \mu\text{m}$ ), likely related to normal growth. Similarly the subchondral plate porosity decreased in the first 8 weeks when the rats aged from 8 weeks to 16 weeks. The time curves of the animals of the two running regimes were considerably different for some of

**Table 1.** Primer Sequences Used for Quantitative RT-PCR

Primer	Forward (5'-3')	Reverse (5'-3')
<i>COL2A1</i>	AAAGTCTTCTGCAACATGGAG	TAGCTGAAGTGGGAAGCCG
<i>ACAN</i>	CTTAGCAGGGATAACGGAC	ATGGTCTGGAACCTCTCTG
<i>MMP-2</i>	TCCCTGATAACCTGGATGC	CCAACCTCAGGTAATAAGCACC
<i>MMP-9</i>	CGCTTGGATAACGAGTTCTCTG	TCACACGCCAGAAGTATTTGTC
<i>MMP-13</i>	TAACCAGACTATGGACAAAGAC	CTGTATCAAACCTGTATGGGC
<i>GAPDH</i>	CAATGACAACCTTTGTGAAGC	CATGTAGGCCATGAGGTC
<i>ACTB1</i>	AAGGAGATTACTGCCCTGG	GCTGATCCACATCTGCTG

the bone morphometric parameters, clearly indicating an effect of the running protocols. Bone changes between groups were mainly observed on the medial side of the knee joint. After the initial 8 weeks of the adaptive running, the thickness of medial tibial subchondral bone plate increased (+9.7%,  $p=0.015$ ) and the porosity decreased relative to the non-running control group (-24.2%,  $p=0.008$ ) (Fig. 2A, B). The bone volume fraction of trabecular bone underlying the subchondral bone was also higher in the adaptive running animals at endpoint-1 ( $p=0.04$ ) (Fig. 2F).

After 14 weeks, at endpoint-2, the situation reversed; the medial subchondral bone plate thickness in both running groups ( $255.7 \pm 9 \mu\text{m}$  and  $268.2 \pm 13 \mu\text{m}$ ) was thinner than that of the non-running control group ( $284.8 \pm 22 \mu\text{m}$ ,  $p=0.0018$ ) (Fig. 2A). When compared to the starting point (delta value for each animal), the thickness of the subchondral bone tibia plateau similarly tended to be thinner at endpoint-2 ( $p=0.015$ ). This was coincident with significantly reduced epiphyseal trabecular bone thickness of both running groups ( $168.4 \pm 6 \mu\text{m}$  and  $169.4 \pm 6 \mu\text{m}$ ) in comparison with that of the control group ( $178.8 \pm 8 \mu\text{m}$ ,  $p=0.0047$ ) (Fig. 2E). However, the adaptive running group had lower subchondral plate porosity (-26.2% relative to the control,  $p=0.041$ ) (Fig. 2B).

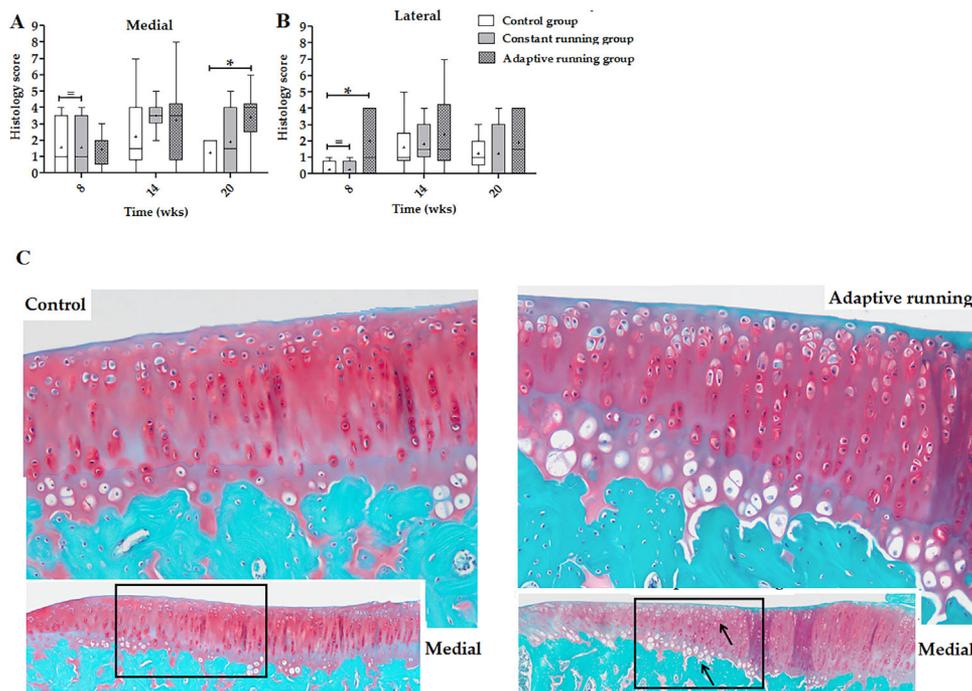
At the end of the experiment at week 20 these differences disappeared except for a slightly lower epiphyseal bone thickness for the constant running group at endpoint-3 (Fig. 2E).

#### $\mu\text{CT}$ result on tibial metaphysis and diaphysis

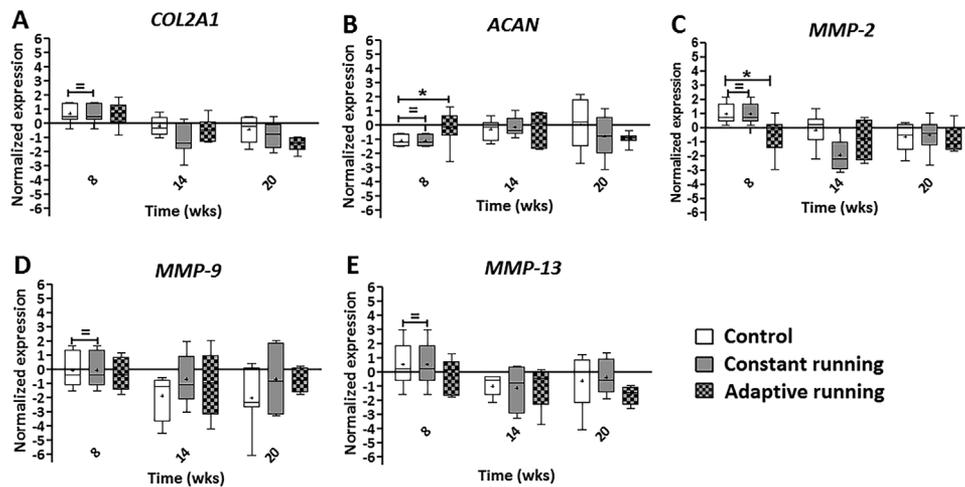
To verify if the differences found in the epiphyseal regions were related to potential altered (metabolic) activity of the joint itself and not a general bone adaptation to the loading only, we analyzed the metaphyseal and diaphyseal bone farther away from the joint. No significant differences were found in metaphyseal trabecular thickness and diaphyseal cortical shell thickness between the three groups (Fig. 3A, B). After the initial 8-week training exercise program, the adaptive running group showed thinner metaphysis and diaphysis, although not significant, compared with the two other experimental groups.

#### Histological changes of articular cartilage

The histological appearance was different between the running and control animals (Fig. 4). Both the running groups had higher scores at the medial side shortly after the constant running period (Fig. 4, 14 weeks). This continued after the 6-week resting period, in particular for the adaptive running group (Fig. 4, 20 weeks), which was characterized by a reduction in the proteoglycan content of the cartilage surface layer, hypercellularity and cell clustering in the superficial and mid-layers, and hypertrophic chondrocytes in the deep zone ( $p=0.035$ ) (Fig. 4C). At the lateral side, the adaptive running group showed more sensitivity in terms of chondrocyte morphological changes after 8 weeks as compared to the



**Fig. 4.** Changes in the chondrocyte population and morphology as well as GAGs loss determined by safranin-O histological staining on (A) medial and (B) lateral component of the rat knee joint for all three groups at different experimental time points. (C) Representative safranin-O-stained images of tibial cartilage of (left) control and (right) adaptive running groups at endpoint-3. Arrows indicate hypercellularity, cell cloning in the superficial and mid-zones, and abnormality in tidemark. In the box-whiskers plots, the lines show the lowest and highest values, and the boxes represent the 25th to 75th percentiles as well as the median. The average value for each box is shown with (+). Control, constant running, and adaptive running groups are respectively shown with white, gray, and black. \* $p \leq 0.05$ . (=) shows that only one subgroup was considered for both control and constant running groups at endpoint-1. wks = weeks.



**Fig. 5.** Normalized gene expression for (A) *COL2A1*, (B) *ACAN*, (C) *MMP-2*, (D) *MMP-9*, and (E) *MMP-13* in three experimental groups ( $n = 6-7$ ) after 8, 14, and 20 weeks of follow-up. The values are expressed as  $\log_2$ . Positive values depict upregulation and negative values depict downregulation of gene expression. *GAPDH* and *ACTB1* were used as reference genes. In the box-whiskers plots, the lines show the lowest and highest values, and the boxes represent the 25th to 75th percentiles as well as the median. The average value for each box is shown with (+). Control, constant running and adaptive running groups are respectively shown with white, gray, and gray/black. \* $p \leq 0.05$ . (=) shows that only one subgroup was considered for both control and constant running groups at endpoint-1. wks = weeks.

control group ( $p = 0.025$ ) (Fig. 4B). OA Research Society International (OARSI) histopathology initiative score,<sup>(20)</sup> which does not include specific chondrocyte morphology at different cartilage layers, was not different between the groups (Supporting Fig. 2).

### Gene expression

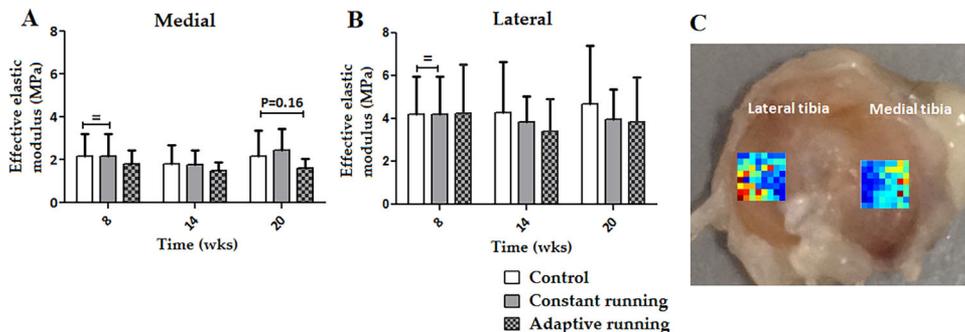
At endpoint-1, aggrecan was 1.55-fold upregulated while *MMP-2* was 2.38-fold downregulated in the adaptive group compared to control ( $p < 0.05$ ) (Fig. 5B, C). Gene expression was not significantly different for other genes (Fig. 5A, D, E). Furthermore, no significant changes were detected in the expression of either anabolic or catabolic factors at endpoint-2 or endpoint-3 (Fig. 5).

### Mechanical properties

There were no clear differences in cartilage stiffness between the running groups or between the running versus the control group. There appeared a small trend that the running led to a slightly lower stiffness compared to control. The lateral tibia exhibited approximately a twofold higher effective elastic modulus as compared to the medial side for all three groups (Fig. 6A, B, C).

### Discussion

In this study, we used two carefully designed running protocols to study both the cartilage and bone tissue responses using a multitude of assays at multiple points. The constant running



**Fig. 6.** The effective elastic modulus measured from a  $9 \times 9$  matrix of indentation on the center of both (A) medial and (B) lateral tibia plateaus of the rat knee joint. (C) The interpolated distribution of the effective elastic modulus obtained from a series of indentations in the  $900 \times 900 \mu\text{m}^2$  square frame in which the indentations were performed. Control, constant running and adaptive running groups are shown with white, gray, and gray/black, respectively. (=) shows that only one subgroup was considered for both control and constant running groups at endpoint-1. wks = weeks.

protocol applied in the current study was used before and induced mild cartilage degeneration in earlier studies with some GAG loss and small fissures as well as periarticular bone resorption.<sup>(14,21,22)</sup> The adaptive running protocol in the current study was introduced under the hypothesis that a more gradual introduction of the loading would allow for adaptation and protect the joint from further damage during the constant running protocol. On the whole, the involved tissues showed consistent and significant, albeit very mild changes. In fact, both running protocols induced subtle GAG loss at the surface, lower surface elasticity and cell clustering with hypertrophic cells in the deep zone; a joint status that can be considered unfavorable. However, this unfavorable joint status found after the running regimes was in the current study even milder than previously reported.<sup>(14,21,23)</sup>

The most important aspects regarding the degenerative effects in the cartilage are the histological findings with respect to the superficial loss of proteoglycans, the superficial and mid-zonal chondrocyte clustering and the hypertrophic chondrocytes in the deep zone found in the running animals. This was not repaired after the 6 weeks rest (Fig. 4A, C). We assume that the chondrocytes have been subjected to some kind of pathological stress and are experiencing abnormal conditions during the exercise.<sup>(24)</sup>

It seems reasonable to conclude that the applied running protocols have been successful in eliciting changes especially on bone in the joint region. Although the degradation on cartilage tissue is milder than the more severe cases shown in previous studies, in terms of cartilage matrix damage, cartilage surface fibrillation and degradation.<sup>(7,14,21)</sup> This can be due to various reasons: the rat strains have different origin, food suppliers are different, and they may experience different day/night cycles. Furthermore, the animals showed different weight gains than reported in the literature.<sup>(10,21)</sup> Therefore, comparing the running exercise in terms of the amount of load (body weight) on the joint induced by different protocols, could be useful. Miller and colleagues<sup>(25)</sup> measured the peak knee joint loads during walking and running with respect to the body weight using gait analysis. Vingard and colleagues<sup>(26)</sup> obtained an estimated relative risk of OA as a result of different physical activities. They have taken various factors including age, body mass index, and the amount of physical load into account.

Interestingly, gait analyses of rats have shown that these animals put more weight on their medial than their lateral tibia compartment.<sup>(10,27)</sup> This might be the reason that our data shows significant differences between medial and lateral responses in both bone and cartilage parameters (Fig. 2A, B vs. Fig. 2C, D). We also performed histological scoring in accordance with OARSI histopathology initiative 2010 for rat cartilage degeneration.<sup>(20)</sup> However, this grading system could not show the small changes caused by the applied running protocols (Supporting Fig. 2) because it does not represent the specific chondrocyte morphology and bone adaptations reported here. There are other studies that have also shown that OARSI scoring system may not adequately describe small initial changes.<sup>(28,29)</sup>

In normal situations, there is a natural balance between the anabolic and catabolic activity of cartilage tissue, which is controlled by chondrocytes.<sup>(30,31)</sup> Cartilage cells responded first (at week 8) to the mild adaptive running protocol by increased expression of aggrecan and decreased expression of MMP-2 (Fig. 5), which represents a strengthening of the cartilage matrix in response to exercise. However, at later time points neither

anabolic nor catabolic genes had altered expression in the running animals compared to controls. This might be because the running between week 0 and week 8 is performed in relatively young animals (8 weeks to 16 weeks old), whereas in the 6 weeks constant running protocol the animals are 8 weeks older and maybe less sensitive to the (over) loading.<sup>(5)</sup>

Despite the slightly increased aggrecan expression, no increase in cartilage stiffness was found between the different running programs. Counterintuitively, the articular cartilage in the adaptive running group showed the lowest stiffness (Fig. 6). Perhaps, the adaptive loading protocol in these young animals leads to biomechanically stressed chondrocytes, which then start proliferating and clustering. However, they are not capable of increasing the rate of synthesis of proteoglycans and ultimately less GAGs are produced,<sup>(10)</sup> causing less osmotic pressure and less pre-stress on the collagen network, which translates to a softer cartilage matrix.<sup>(32)</sup>

One of the major findings of the current study is the bone morphology changes in the joint region induced by the running protocols. Eight weeks of mild running (endpoint-1) seems to accelerate bone growth in the subchondral bone of the tibia plateau of the animals who had started running from a young age (8 weeks old) (Fig. 2A, B). This is also represented in the trabecular bone volume fraction in the epiphysis, which is increased after 8 weeks (Fig. 2F). After 14 weeks follow-up and 6 weeks of intensive running in both running groups (endpoint-2) this effect reversed and the trabecular and subchondral bones were thinner for both running groups (Fig. 2A, E, F). This observation is in line with the findings of previous studies according to which the constant exercise regime can cause thinning in the underlying subchondral bone through enhancing osteoclast activity.<sup>(33)</sup> However, the subchondral plate bone porosity was lower for the adaptive running group in comparison with control animals at endpoint-2 (Fig. 2B), which confirms the increased bone density during the 8 weeks of initial training before the start of 30 km of constant running. After 20 weeks (endpoint-3), the remarkable differences observed at 14 weeks had disappeared for the most part (Fig. 2). The thickness of trabecular bone in the constant running group was still lower than that of the sedentary control at week 20 (Fig. 2E), which indicates that trabecular bone is still suffering from the consequence of solely constant running exercise (without initial mild running program) in this group of animals. The small standard deviation of bone morphological parameters obtained from  $\mu$ CT makes these observations fairly precise.

Presumably, changes in bone morphology induced by the running protocols are specific for the joint region, as they are quite different from the adaptations further away from the joint (in metaphysis and diaphysis). Therefore, it can be assumed that the changes in the subchondral bone are not only a direct adaptation to the increased loading but reflect an interaction of subchondral bone with the cartilage as seen generally in OA joints.<sup>(34)</sup> Furthermore, the presences of hypertrophic and clustered chondrocytes in different layers of articular cartilage, together with reduced thickness of the underlying bone, are clear signs of early-stage OA. Chondrocytes with abnormally high proliferation rate synthesize more cytokines and mediators, which can raise the biomolecules' penetration within the cartilage matrix and toward the underlying bone, thereby adversely affecting the normal homeostasis of cartilage and bone.<sup>(10,24,34)</sup> Alternatively, bone alterations may precede the morphological changes of the chondrocytes, which further highlights the interaction between epiphyseal bone and

cartilage tissue in response to mechanical stimuli.<sup>(35)</sup> In addition, cartilage tissue did not seem to fully recover from the adaptive running exercise, while we clearly detected bone adaptation after the sedentary period (Figs. 2 and 4). More detailed investigation regarding bone biology in order to measure bone formation/resorption rates might discover useful information about the interaction between cartilage/bone interfaces, which is not included in the current study. Considering the growth effects in young rats used in the current study, dynamic histomorphometry can be useful to clearly distinguish the effect of growth disturbance. Further  $\mu$ CT analysis in order to measure the entire tibial length could also improve our understanding regarding the age effects.

The presented results cannot be used to draw a mechanistic picture of how subchondral bone adaptation relates to joint disruption, cellular response, and alterations in cartilage tissue. Considering the observed changes in terms of cartilage degradation were mild, it is difficult to definitively conclude whether the exercised animals are still within the limits of healthy exercise or whether they are entering an early stage OA. Unfortunately, because the histological scores for cartilage and chondrocyte morphology are not very precise and resulted in high standard deviations, they could not elucidate this question. Thus, the study overall is inconclusive and longer investigations are needed to determine whether the observed early changes would progress to OA. What can be concluded though is that a pretraining adaptive running protocol in young rats before an intense period of constant running does not provide any protection for mild cartilage degeneration. In addition, the study showed that bone turnover and mild cartilage degeneration are likely related phenomena that can result after (intense) running and might both be signs of early stage OA.

## Disclosures

All authors state that they have no conflicts of interest.

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