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Comparative effects of various running exercise modalities on femoral bone quality in rats

Authors: Rkia Wazzani ¹, Céline Bourzac ^{2,3}, Hanane Elhafci ², Philippe Germain ^{4,5}, Said Ahmaidi ¹, Stéphane Pallu ^{2,4}, Christelle Jaffré ¹ and Hugues Portier ^{2,4}

Affiliations:

- 1: Laboratory APERE, University de Picardie Jules Verne, Avenue Paul Claudel F-80000 Amiens- France; rkia.wazzani@etud.u.picardie.fr ,
- 2: B3OA Laboratory, CNRS 7052, INSERM 1271, University of Paris, 10 Avenue de Verdun, 75010 Paris, France;
- 3 : Plateforme de recherche biomédicale, Ecole Nationale Vétérinaire d'Alfort, F-94700 Maisons-Alfort, France
- 4: Research group Sport, Physical Activity, Rehabilitation and Movement for Performance and Health, University of Orleans, 45067 Orléans, France.
- 5 : Centre de Biophysique Moléculaire (CBM), UPR CNRS 4301, Université d'Orléans, Rue Charles Sandron, CS 80054, F-45071 Orléans, France;

Key words: intermittent exercise; continuous exercise; combined running; bone quality; femur; rat

Abstract:

Background It is now well established that physical exercise is an effective preventive method to reduce and treat certain chronic diseases, particularly musculoskeletal disorders. At the bone level, running exercise is well known for its positive effects on various parameters of bone quality. There is, however, no consensus regarding the effects of different running exercise modalities on bone quality.

Aim the objective of this study was to compare the effects of three treadmill running modalities: intermittent, moderate continuous, and a combination of both - on bone quality parameters in rats.

Methods Thirty-nine, 5-week-old, male Wistar rats were randomly divided in 4 groups: sedentary control (SED; n=10), intermittent running exercise (IE; n=10), continuous running exercise (CE; n=10) and combined running exercise (COME; n=9). Rats in running groups were exercised 45 min/day, 5 days/week, for 8 consecutive weeks. Femoral micro-architectural parameters were assessed by micro-CT; femoral osteocyte apoptosis, osteoclast resorption and bone histomorphometry were assessed by histology.

Results Femoral trabecular thickness in the combined running group was increased ($p < 0.0001$) compared to respective results in the other running groups (0.13 mm vs 0.11 mm). The cortical thickness, osteocyte lacunae occupancy rate in the whole femur, numbers of apoptotic osteocytes and osteoclastic resorption surfaces were not significantly different between groups. Statistical differences were occasionally noted depending on the femoral anatomical region.

Conclusion These results suggest that the femur should not be considered as the better bone to study the effects of running protocols.

Abbreviations:

- BV/TV: Trabecular bone fraction
Tb.Th: Trabecular thickness
Tb.N: Trabecular number
BMD: Bone mineral density
NTx: Cross-linked N-Telopeptide of type I collagen
BMI: Body mass index
SED: Sedentary control
IE: Intermittent running exercise
CE: Continuous running exercise
COME: Combined running exercise

MAS:	Maximal aerobic velocity
ALP:	Alkaline phosphatase
PTH:	Parathyroid hormone
AP:	Antero-posterior
LM:	Latero-medial
μ CT:	Micro-Computed Tomography
VOI:	Volumes of interest
ROI:	Region of interest
Tb.BMD:	Trabecular BMD
Tb.Sp:	Trabecular spacing
TRAP:	Tartrate-Resistant Acid Phosphatase activity
SD:	Standard deviation
SEM:	Standard error of the mean

Introduction:

Bone marrow stromal cells (BMSC), Bone is a dynamic living tissue, in which the modeling and re-modeling processes are affected by the mechanical stresses it undergoes (Wolff 1986). The bone sensitivity to mechanical stresses is achieved through the osteocytes, which represents 90-95% of all bone cells in adult bone tissue (Bonewald 2011). The activity of this cell associated with other bone cells, i.e. osteoblasts and osteoclasts, constitutes an important parameter of bone quality, with other well-defined multi-scale parameters (Fonseca et al. 2014). These latter include various bone properties, including overall bone geometry, microarchitecture and tissue properties based on cell density, balance of cellular activities, degree of mineralization and collagen stability (Fonseca et al. 2014). Bone quality depends on the level of stress and constraints received by the bone (Fonseca et al. 2014).

In the literature, physical exercise is considered as a real constraint applied to the bone (Rubin et Lanyon 1984; Vicente et al. 2013). Indeed, physical exercise provokes mechanical strains directly affecting the bone through the impact on the ground, and torsion or traction of tendons, for example (Rubin et Lanyon 1984). Also, according to Wolff's law (1886), physical exercise leads to modifications causing bone resorption or anabolism, thus modifying the internal architecture and external morphology of the bone (Wolff 1986). Numerous animal and human studies have demonstrated the importance of physical exercise with an optimal load level to maintain the integrity of the skeleton (Barlet et al. 1995).

Multiple exercise modalities have been studied in animal models. Among them, treadmill running is one of the most widely used protocol to modulate exercise in rats and study its effects on bone (Portier et al 2020).

According to a recent review, this treadmill running protocols have induced positive results on bone tissue in 67.7% of the studies analyzed (Portier et al. 2020). However, the parameters of this exercise protocol (i.e. intensity duration, etc.) differ from one study to another. It is therefore difficult to draw solid conclusions. Indeed, running exercise exists in the literature in different modalities with scattered and controversial effects on bone quality. Most of the protocols for this exercise have focused on running at moderate and continuous intensity, while few studies have focused on the so-called "intermittent" or "interval" running, in which a period of rest is inserted between the exercise periods (Nakajima et al. 2001; Boudenot et al. 2014; Boudenot et al. 2015).

Moderate continuous running induces adaptations in the biomechanical properties of bone (Vicente et al. 2013), in the

trabecular bone microarchitecture (increase in the trabecular bone fraction (BV/TV) (Iwamoto et al. 1999; Vicente et al. 2013), trabecular thickness (Tb.Th) and trabecular number (Tb.N) (Joo et al. 2003)), and in bone mineral density (BMD) compared to the sedentary condition (Joo et al. 2003; Hamann et al. 2012). Intermittent running also improves BMD (Nakajima et al. 2001; Chen, et al. 2004), the surface area of osteocyte lacunae and their occupancy rate in subchondral bone in osteoarthritis rat model (Boudenot et al. 2014) compared to the sedentary condition. However, only one study directly compared the two exercise running modalities, demonstrating that intermittent running can improve BMD by 20% compared to the moderate continuous running, even with a 30% shorter training period (Boudenot et al. 2015). Moderate continuous running is also known to stimulate bone formation and limit bone resorption to a certain extent. This is sustained by the increase in the levels of bone formation biomarkers, including osteocalcin, and the decrease in bone resorption biomarkers, such as Cross-linked N-Telopeptide of type I collagen (NTx), after training sessions (Maurel et al. 2013).

In spite of all these studies showing the positive effects of running on bone, there are other studies in which no effect (Chang et al. 2010; Maurel et al. 2011; Nordsletten et al. 1994; Hamann et al. 2012), or even a negative effect of running (Maurel et al. 2013; Bourrin et al. 1995) were reported.

To date, there is no consensus regarding the effects of intermittent and continuous running on bone quality because very few protocols investigating this question are comparable. In addition, to our knowledge, there are no studies that have investigated the effects of the combination of these 2 running modalities, i.e. alternating intermittent and continuous exercises during a training program, on the quality of bone tissue. Therefore, the objective of this study was to compare the effects of three treadmill running modalities: intermittent running, moderate continuous running and the combination of the two, on bone quality in rats.

Combined exercise is expected to have superior positive effects on various bone parameters by accumulating the benefits of both the intermittent and the continuous running modalities.

Materials and Methods:

Animals

Experiments were carried out on thirty-nine 5-week-old, male, Wistar rats. All the rats were housed in controlled facilities (4 rats per standard cage, maintained on a 12h light/dark cycle, at a

constant temperature of $21 \pm 2^\circ\text{C}$). A commercial standard diet (Genestil, Royaucourt, France) and tap water were provided ad libitum to all animals.

The experimental protocol was approved by the Ethics Committee on Animal Research of Lariboisiere/Villemin (Paris, France) and by the French Ministry of Agriculture (Paris, France, reference number APAFIS # 9505).

Body weight, body lengths (nose to anus), body mass index (BMI = body weight (g)/length² (cm²) (Novelli et al. 2007)) were recorded at the beginning (T0) and end (T8) of the experimental protocol.

Exercise protocol

Rats were randomly assigned to one of the four following groups: sedentary control (SED) (n=10), intermittent running exercise (IE) group (n=10), continuous running exercise (CE) group (n=10), and combined running exercise (COME) group (n=9). SED rats had only normal movement in their cage as a daily activity. After one week of acclimation, treadmill running learning (25 minutes per day, at different speeds) was carried out during 4 consecutive days before any testing.

Rats in the exercise groups performed treadmill exercise 5 days per week, for 8 consecutive weeks.

At the end of the protocol, after induction of general anesthesia with ~5% isoflurane in air, rats were sacrificed by cardiac puncture and exsanguination.

Maximal aerobic speed test

At the end of the protocol, after induction To determine running speeds, each rat underwent a maximal aerobic velocity (MAS) test on day 5 of running acclimation, following the progressive running test protocol (Bourzac et al. 2020). The test began with a warm-up session at an incline of 10° and a speed of 13 m/min for 5 min, followed by increments of 4 m/min every 2 min until 17 min was reached, and then increments of 4 m/min every 1min30s. The test was conducted until the rats could no longer keep up with the treadmill speed, despite 2 consecutive stimulations with compressed air sprays. The test was therefore stopped and the last fully sustained incremental speed was defined as the rat's MAS.

A MAS test was also performed at the end of the exercise protocols to validate the exercise program.

Treadmill running protocol

The 8-week exercise protocol consisted of different running modalities depending on the group involved. Continuous running consisted of running at a constant pace for 45 min per day at 70% MAS (Fig. 1 (A)), intermittent running consisted of 7 repetitions of 3-min blocks at 50% MAS, followed by 2 min at 100% MAS and 1 min of passive rest (Fig. 1(B)), and combined exercise was built on an alternation of continuous and intermittent running over the week (Fig. 1C).

Average intensity training.

It is considered that rats are walking at a speed under 10 m/min (Bourrin et al. 1994) , are running at light speed until 15 m/min (Patch et Brooks 1980), at moderate speed until 20 m/min (Chae et Kim 2009; Ip et al. 2009), and at intense speed above 20 m/min (Bourrin et al. 1994). Intensity scores were adapted in order to obtain three intensity levels (I#1, I#2 and I#3) corresponding to three coefficients (I#1: low speed ≤ 15 m/min, coefficient 1; I#2

moderate speed: 15 to 20 m/min, coefficient 2; I#3 intensive speed: >20 m/min, coefficient 3). Then, scores were calculated based on the following equation (Arnaud Boudenot et al. 2015; Mujika et al. 1995):

$$\text{Intensity score} = \frac{(\text{time spent at I\#1}) \times 1 + (\text{time spent at I\#2}) \times 2 + (\text{time spent at I\#3}) \times 3}{\text{total time}}$$

The intensity score (in arbitrary unit) was calculated each week for the three training groups. Then, the averaged intensity score was calculated as the mean value of each week's score. This score gives information about the difficulty of the exercise: the higher the score, the more difficult the exercise. The averaged intensity score was 1.64 ± 0.5 , for the interval exercise group, 1.71 ± 0.7 for the continuous exercise group and 1.68 ± 0.6 for the combined exercise group. No difference was observed between groups.

Blood collecting

During exsanguination, cardiac blood was collected on heparinized tubes, immediately cooled and centrifuged at 700 g at 4°C for 10 min and plasma stored frozen at -80°C until assays were performed.

Total alkaline phosphatase (ALP), a bone formation marker, was assayed using standard laboratory analyzer (Selectra xl, Elitech) and enzymatic kinetic kit (ALP (DEA) SL, Elitech), following the manufacturers' instructions. The limit of detection was equal to 6 U/L.

Cross linked N-Telopeptide of type I collagen (NTx), a bone resorption marker, and intact parathyroid hormone (PTH), a key regulator of the phospho-calcic metabolism, were measured using established rat enzyme linked immunosorbent assay kits (Antibodies-online GmbH, Aachen, Germany, and Immutopics, Inc., San Clemente, CA, USA, respectively) following the manufacturers' instructions. The intra-/inter-assay coefficients of variation and limit of detection for NTx were $< 12\%$ and 2.47 ng/mL respectively. The intra-/inter-assay coefficients of variation and limit of detection for PTH were $< 8.9\%$ and 3 pg/mL respectively.

Bones collecting and parameters

Femurs were retrieved and cleared of soft tissue then stored and fixed in 4% v/v paraformaldehyde at 4°C until further analysis.

Bone morphometric parameters

Immediately after sacrifice and removal of connective tissue, the length of the left femurs, the antero-posterior (AP) and the latero-medial (LM) diameters were measured using a digital caliper (resolution 0.1 mm: Werckmann®, Netherlands).

Bone analysis by in-vitro micro-Computed Tomography (μCT)

The left distal and proximal femur of each rat were imaged using a high-resolution in-vitro μCT (Skyscan 1172, Bruker, Belgium). For micro-architectural parameters and trabecular bone mineral density, the μCT images were acquired at the following setting: source voltage 80 kV, source current 100 μA , matrix 1000x668, exposure time 175 ms, rotation step 0.3-degree, frame

averaging 10, filter aluminum 0.5 mm thick, pixel size 9.81 μm . Images were reconstructed using NRecon software, 16 bits (v1.7.0.4, Skyscan). They were then segmented into binary images using simple global thresholding methods, because of the low noise and good resolution of the data sets.

Image analysis was performed using CTAn software (v1.6.1, Skyscan). For trabecular micro-architectural and BMD evaluation, trabecular volumes of interest (VOI) were determined by manually drawing a region of interest (ROI) following the endocortical boundaries every 20 images on a subset of 200 images located immediately distal to the ventral border of the femoral head for the proximal part of the femur, and immediately proximal to the distal growth plate for the distal part of the femur. The software extrapolated the ROI between drawn ones. Trabecular BMD (Tb.BMD, gHA/cm^3) was computed from the VOI using hydroxyapatite phantoms for calibration (Bouxsein et al. 2010). The trabecular bone variables retrieved also included trabecular bone volume fraction Tb.BV/TV (bone volume/tissue volume, %), trabecular number Tb.N (1/mm), trabecular spacing Tb.Sp (mm) and trabecular thickness Tb.Th (mm) (Bouxsein et al. 2010).

Bone histology and immunohistochemistry analysis

After chemical fixation, the diaphysis of the right femurs and the whole left femurs were decalcified with EDTA 177 g/L, pH 7.0–7.3 (Osteosoft, Merck KGaA, Darmstadt, Germany) during 4 weeks, then embedded in paraffin, and cut to a thickness of 4–5 μm with a microtome (cross sections for the right femur diaphysis, and longitudinal sections for the left femurs). Before staining, all tissue sections were deparaffinized in xylene and rehydrated in a series of graded alcohols (100, 90, 80, 70, and 40%), with a final rinse in water.

Bone histomorphometrical analysis

Right femur cross-sections were stained with Harris hematoxylin (10047107, VWR Chemicals) and eosin (117081, VWR Chemicals), to measure the cortical bone thickness.

The diaphyseal cortical thickness was evaluated by histomorphometric analysis. Images were obtained using a digital microscope (Keyence, VHX-2000) at 10x magnification. Using the dedicated Keyence toolbox, the cortical thickness was measured at 8 different locations on 3 consecutive sections in distal diaphysis. The results obtained were averaged to obtain a mean cortical thickness per rat, and a mean cortical thickness for the cranial, caudal, lateral and medial regions.

Bone histochemical analysis (TRAP)

Longitudinal sections from the left femurs were used for histochemical labelling of the Tartrate-Resistant Acid Phosphatase activity (TRAP labelling).

TRAP labelling is a histochemical method that produces staining using reaction of Naphthol AS-TR substrate (Filgueira 2004). The enzymatic detection of acid phosphatases was performed with a defined concentration of naphthol substrate (naphthol AS-TR) (16mg in 1mL of dimethylformamide) and of tartaric acid (100mg of tartaric acid per 100mL of prepared solution). After coating all samples with our solutions, they were incubated in a humidity chamber at 37°C for one hour. The counter-staining was performed with hematoxylin for one minute. The TRAP labelling, when positive, gave a red fading, indicating osteoclastic bone resorption.

Images were obtained using a digital microscope (Keyence, VHX-2000) at 200x magnification. Six images, i.e. 3 images from both proximal and distal epiphyses (each set of 3 being on a transversal line without overlap), were obtained for each of two longitudinal sections per rat. Using the image processing software “Image J” (National Institute of Health, 1987), and through color convolution, contrast adjustment, threshold and noise suppression tools, the TRAP labelling was quantified for all samples. We set as a quantification parameter “the area of osteoclastic bone resorption”, testifying to osteoclastic bone resorption activity. This parameter is calculated as the percentage of resorption area (red discoloration area) related to the total area of the images, which was the same in the different experimental groups.

Bone immunohistochemistry analysis

Longitudinal sections of the left femur were used to evaluate the osteocyte apoptosis, using immunostaining for cleaved caspase-3. Cleaved caspase-3 is known to be an effector caspase in the cell death process, and therefore an important marker of the initiation of cell apoptosis (Nicholson et al. 1995). Samples were incubated overnight at 4°C with an anti-cleaved caspase-3 antibody (cleaved caspase-3 (Asp175) (5A1E) Rabbit mAb #9664, Ozyme, 1/400 dilution), (Fan et Bergmann 2010). Samples were then incubated for 30 min in a humid chamber at room temperature with the secondary antibodies (SignalStain Boost IHC Detection Reagent, HRP, Rabbit, Ozyme, diluted).

The revelation was performed with DAB (SignalStain DAB Substrate Kit #8059, Ozyme, diluted) incubation for 10 min (for adequate staining intensity). The chromogen DAB reacts chemically with the HRP enzyme coupled with cleaved caspase-3 secondary antibody, therefore inducing a brown fading. Finally, the counter-staining was performed with hematoxylin for one minute. Images were obtained using a digital microscope (Keyence, VHX-2000) at 500x magnification. A total of 12 images was obtained from each of two longitudinal sections per rat, as follows: 3 images from each of the proximal and the distal epiphysis (each set of 3 images being on a transversal line without overlap), and 3 images for each of the lateral and medial aspects of the diaphysis (each set of 3 images being on a longitudinal line without overlap). Using Image J (National Institute of Health, 1987), the numbers of full (living osteocyte, counterstained with hematoxylin), apoptotic and empty lacunae were determined in an area of 400 x 400 μm^2 located in the center of each image. The number of each type of lacunae counted was reported on the counting area for each image. Then the values were summed for all 12 images and was added together for the 2 sections for a single rat. Three parameters were retrieved from the images: the numbers of apoptotic lacunae, the osteocyte occupancy rate (“full lacunae/total lacunae” x 100), and the apoptosis rate (“apoptotic lacunae/total lacunae” x 100).

Statistics

Statistical analyses were performed using GraphPad Prism 6 software. Descriptive data were presented as mean \pm standard deviation (SD) for the micro-architectural parameters, and as mean \pm standard error of the mean (SEM) for the histological parameters. A “Shapiro-Wilk” test was used to assess the normality of the data distribution.

Since MAS data (along with weight, length, and BMI) were assessed longitudinally, a repeated measures approach (group by time) was used.

For the other parameter, since the data were not normally distributed, non-parametric tests were used to compare groups: the Kruskal-Wallis test was used to compare the four groups and a U-Mann-Whitney test was then performed, when applicable, to compare groups 2 by 2. The level of significance was set at $p < 0.05$.

Results:

Body and femurs parameters

Results are summarized in Table 1. The weight, the length and the BMI were similar between groups at the end of the protocol. The lengths of the femur were similar between the different experimental groups.

The weight gains were not statistically different between groups at the end of the protocol (T8).

Maximal aerobic speed

Maximal aerobic speeds (MAS) did not statistically differ between groups at the beginning of the study. Compared to respective results at T0, an increase in the MAS at T8 was observed in each exercise group, whereas the MAS in the sedentary group remained similar (Table 1), validating the exercise program.

Femoral trabecular micro-architecture parameters

Results are summarized in Table 2. In the combined exercise group, the trabecular thickness in the proximal femur was higher compared to respective results in the other two exercise groups and SED group. In the distal femur, however, the trabecular thickness of COME was higher compared to respective results in the IE group only.

In contrast, the Tb.BMD in the proximal femur was higher in IE compared to the respective results of all other experimental groups (SED, CE and COME) ($p < 0.05$). In addition, a lower value in this parameter was also recorded in CE compared to COME and SED.

Bone histomorphometrical parameters

Femoral cortical bone thickness

The mean cortical bone thickness did not significantly differ between the groups. Looking at the various anatomical regions, the cortical thickness only differed in the medial region, where it was lower ($p < 0.05$) in the IE groups than the respective results in the CE and SED groups (Fig 2).

Osteocyte lacunar density

The osteocyte occupancy rate statistically differed neither in the whole femur nor in the different anatomical regions (proximal epiphysis, diaphysis, and distal epiphysis) between the groups (Table 3). A representative image of the immunohistochemical staining is given in the Fig.3 (A).

Bone histochemical and immunohistochemical results

Osteocyte apoptosis rate

The total numbers of apoptotic lacunae assessed by cleaved caspase-3 immunostaining were not significantly different between the groups (Fig. 3 (B)). However, a higher number of apoptotic osteocytes was observed in the diaphysis of CE rats compared to the respective results of SED rats (+150 %, $p = 0.008$) and COME

(+114%, $P < 0.05$) (Fig. 3 (C)). The osteocyte apoptotic rate for the whole femur did not statistically differ between the groups. A higher rate of apoptosis, however, was observed in the diaphysis of CE rats, compared to respective results from both COME and SED rats ($p = 0.005$ and $p = 0.03$ respectively).

Osteoclastic bone resorption (TRAP labelling)

The representative images of the TRAP labelling are given in Fig. 4 (A). The overall surface of osteoclastic bone resorption and the surface of osteoclastic bone resorption in both the proximal and distal epiphyses, measured following TRAP labelling, were not significantly different between the groups (Fig. 4 (B)).

Systemic bone turnover markers

At the end of the protocol, the total plasmatic concentrations ALP, NTx and PTH were not statistically different between the experimental groups.

Discussion:

To our knowledge, the present study is the first to compare the effects of three different running modalities (intermittent running, moderate continuous running, and combination of both) on various bone quality parameters.

The results of this study showed that combined exercise increased the trabecular thickness, only in the proximal femur, while intermittent exercise improved trabecular BMD. In contrast, in the distal femur, intermittent running activity decreased the trabecular thickness. The other bone parameters (cortical thickness, osteocyte density and viability, osteoclastic resorption activity) did not differ between groups in the whole femur. Some significant differences were, however, observed between groups, according to the anatomical regions of the femur. Because there was no difference in the average intensity (work load) between the three exercise groups, results obtained from the 3 exercise groups are directly comparable. The results from the studies on the effect of running exercise on bone are very heterogeneous and not comparable because the protocols carried out are not identical in terms of overall intensity, duration and frequency (Portier, Benaitreau, et Pallu 2020; Arnaud Boudenot et al. 2015; Joo et al. 2003; Yao et al. 2004). Continuous running is well known in the literature to improve trabecular bone structure and microarchitecture in rodents. For instance, this type of training induces adaptations in the tibial trabecular bone after 3 weeks of exercise in young male rats (Bourrin et al. 1994). Compared to sedentariness, it also increases the tibial trabecular volume and number by 25% and 21%, respectively (Yao et al. 2004). These increases are associated with thicker trabeculae and reduced trabecular spacing (Yao et al. 2004). In the femur, 10 weeks of continuous endurance exercise improves bone strength and positively alters trabecular bone microarchitecture (Joo et al. 2003). More specifically, it increases the trabecular thickness, trabecular numbers and trabecular bone volume in the distal metaphysis of the femur (Joo et al. 2003). Similarly, continuous training for 12 weeks increases the trabecular bone volume in the tibia in rats (Iwamoto et al. 1999).

Taken together, these data show that the length of running protocols is a key factor affecting the adaptive responses in the bones. In the context of bone healing, a recent study in rats showed that 8 weeks of preventive continuous running improves BV/TV in a healing tibial defect as early as 2 weeks following a non-critical

bone defect surgery, whereas it improves BV/TV in the healing femur defect only after 4 weeks (Bourzac et al. 2020). Another key factor is the anatomical site within a bone, as reported by Iwamoto et al., 1999, who showed that continuous running exercise improves the trabecular bone parameters in the distal tibia to a greater extent than in the proximal tibia (Iwamoto et al. 1999). The aforementioned results suggest that the adaptation of trabecular bone to continuous moderate treadmill exercise is site-specific. This highlights that the adaptation of bone tissue to mechanical stresses is highly variable depending on the anatomical location. This is likely due to variations in the distribution of mechanical stresses according to the site. This 'variation in the distribution and the intensity of mechanical stresses according to the anatomical site' could explain our result concerning the Tb.BMD (Georges et al. 2022), which was also different between the groups, in the proximal but not in the distal femur.

The Tb.BMD was the highest in the intermittent running group, whereas the lowest value was recorded in the continuous running group. Data in the literature show that compared to sedentariness, intermittent running consistently improves femoral (Nakajima et al. 2001; Chen, Aoki, et Fukui 2004) and tibial (Boudenot et al. 2014) BMD. In addition, in the study by Joo et al., 2003, the increase in BMD was recorded in the proximal and distal femoral metaphysis but not in the epiphyses (Joo et al. 2003).

In a previous study comparing intermittent and continuous running protocols by Boudenot et al. the intermittent running protocol improved BMD of the whole body and femur in rats by more than 20% compared to moderate continuous running even with a 30% shorter training period (Boudenot et al. 2015). This could be explained by the important and adequate stimulation by intermittent running as shear stress of cell communication via interstitial bone fluid flow (Bonewald et Johnson 2008). Indeed, bone cell communication is mediated by molecules that are released in greater quantities with fluid flow (Bonewald et Johnson 2008). These molecules are known to promote bone formation and bone remodeling and are modified by exercise. For example, compared to sedentary rats, a decreased level of sclerostin (secreted element inhibiting bone formation) is recorded in rats subjected to cycles of intermittent running exercise and free-fall exercise (Boudenot et al. 2021).

Our results showed no effect of different exercise protocols on other femoral micro-architectural parameters (Tb.N and Tb.Sp). Previous studies in the literature report no effect of exercise on femoral bone microarchitecture (Hamann et al. 2012), or even a deleterious effect of continuous running in the tibia (Bourrin et al. 1995).

Considering cortical bone parameters, our results showed no significant differences in whole cortical bone thickness between groups. Although not statistically significant, continuous and combined running decreased, however, the osteocyte occupancy rate, both in the whole femur and at each of the proximal and distal epiphyses, and diaphysis, compared to respective results in the intermittent and sedentary groups. Continuous running has usually no effect or even a negative effect on cortical bone in short duration protocols (Maurel et al. 2013; Gao et al. 2017). In contrast, extending this protocol over longer periods of time (10-12 weeks) allow for cell responses that translate into higher numbers of osteocytes (Vicente et al. 2013; Li et al. 1991). This reinforces the hypothesis that important adaptive responses are conditioned by more enduring and longer running protocols. As for intermittent

running, recent studies have demonstrated its effect in increasing femoral cortical thickness (Scott et al. 2021) and osteocyte occupancy rate by more than 60% compared to sedentariness (Boudenot et al. 2014). To date, only one study compared intermittent and continuous running, showing a 50% higher osteocyte occupancy rate in the tibia in the intermittent running group compared to respective results in the continuous running group (George et al. 2022). This is in contrast with the study herein and may be explained by the differences in intensity and distribution of stresses between the femur and tibia due to the relative orientation of these bones when the limb contacts the ground, and resulting in an attenuation of the mechanical stimulus in the femur compared to the tibia (Bourzac et al. 2020). This might suggest that, to study the effects of running in rat models and to better understand site and load-specific stresses, analysis of the tibia would be more relevant than that of the femur.

Moreover, the difference in the effect of these two running protocols (intermittent and continuous running) could be explained by the difference in the mechanical loads applied during each protocol. The continuous load applied to the osteocyte can be considered highly stressful and exhausting, thus causing an imbalance in the remodeling process. However, the qualitative notion of rest inserted during intermittent and combined running seems to be an indicator and a criterion of physical practice performance. The benefit of this resting time can be summed up in its necessity to regain sufficient sensitivity to mechanical strains in order to benefit as much as possible from the exercise performed (Umemura et al. 2002). Indeed, it has been shown in the literature that after a few minutes of exercise, bone sensitivity to stress decreases. Therefore, passive recovery phases (resting time) in intermittent running are desirable to restore the osteocyte's receptivity and adaptation to the induced constraints (Umemura et al. 1997). This may therefore also explain the propensity of moderate continuous and combined exercises to have more apoptotic osteocyte lacunae compared to sedentariness and intermittent exercise.

No differences were observed in the whole femur or the proximal and distal femur for osteoclastic activity assessed by TRAP labelling between the groups. Although not statistically significant, greater osteoclastic activity was recorded in the combined exercise group for the whole femur and the proximal femur.. Previous studies have reported osteogenic and positive effects of continuous moderate (Bourrin et al. 1995; Iijima et al. 2015) and intermittent (George et al. 2022) running exercise on osteoclastic cells in the tibia. These positive effects on the osteoclastic lineage have been previously reflected by a decrease in the number of these cells (Bourrin et al. 1995; Iijima et al. 2015), or by a decrease in the osteoclastic resorption surface (George et al. 2022). A significant increase in osteoclastic resorption activity has been observed in the continuous running group compared to respective results in the intermittent running group by George et al., 2022 (George et al. 2022). This suggests again a variability in mechanical constraints applied to the bone between these two protocols, and consequently, a variability in bone adaptive responses according to the running protocol.

The total plasmatic concentrations of systemic bone turnover markers (ALP, NTx and PTH) were not statistically different between the experimental groups in our study. The change in

levels of these markers in response to exercise in rodent is not always provided in the literature (Bourzac et al. 2020).

To our knowledge, unfortunately there are no studies in the literature about the effect of a combined exercise on cortical parameters in order to discuss the results obtained with regard to this type of exercise in the best possible way. Such a training program was chosen and defined in the present study in order to imitate the reality of sports practice in athletes and patients.

The moderate results showed by our study obtained regarding this training method (absence or few effects of this modality on bone), could be explained by the increased mechanical stress for the animals while the alternation between the two different protocols. Indeed, the difficulty of the rats in this group to perform this combined running protocol prevented the practice of the exercise in a regular way. As a consequence, a dispersion of the results within the group was observed. A prolonged rest period should have been implemented to avoid putative mechanical overload by the combined running protocol. To expect greater adaptive responses with respect to all exercise modalities, it probably makes sense to accumulate these stresses over much longer durations to show changes in BMD and microarchitectural changes. The COME protocol could be considered as challenging, because it is closer to the reality of athlete training and/or programming in rehabilitation centers. In fact, the protocols used in the literature (only intermittent running or only continuous running) do not correspond to the reality of human practice.

Study limitation

In this study, body composition was not assessed, which could be a limitation in interpreting the results. It has been shown that exercise can vary body composition, i.e. increase in lean mass and reduction in fat mass and also have an effect on bone tissue (Iwamoto 1999, Maurel, 2011, Boudenot 2015).

Conclusion:

This study confirms that the femur is not the most relevant bone for studying running in rats particularly for short-duration protocols. This could be due firstly to the distance between the site and the point of impact, and secondly to the position of the femur during impact (horizontal position) (Bourzac et al. 2020). Therefore, we believe that the adaptive responses at the tibial level will be more important than at the femoral level.

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Author contributions:

The authors would like R.W., C.B., S.P., C.J., and H.P. contributed to the design of the study. R.W., C.B., P.G., S.A. and H.E. contributed to the acquisition, collection, and assembly of data. R.W., C.B., P.G., S.A., H.E., S.P., C.J., and H.P. contributed to reagents/ materials/analysis tools. R.W., C.B., S.P., C.J., and H.P. wrote the main manuscript text. All authors contributed to revising the manuscript and approved the final version to be submitted.

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The authors declare no competing financial interests.

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Corresponding author:

Dr Hugues Portier

Laboratory of Bioengineering and Biomechanics for Bone and Articulations; UMR 7052, CNRS, Paris Diderot University, Sorbonne Paris-Cité, 10 avenue de Verdun 75010 Paris, France

E-mail: hugues.portier@univ-orleans.fr

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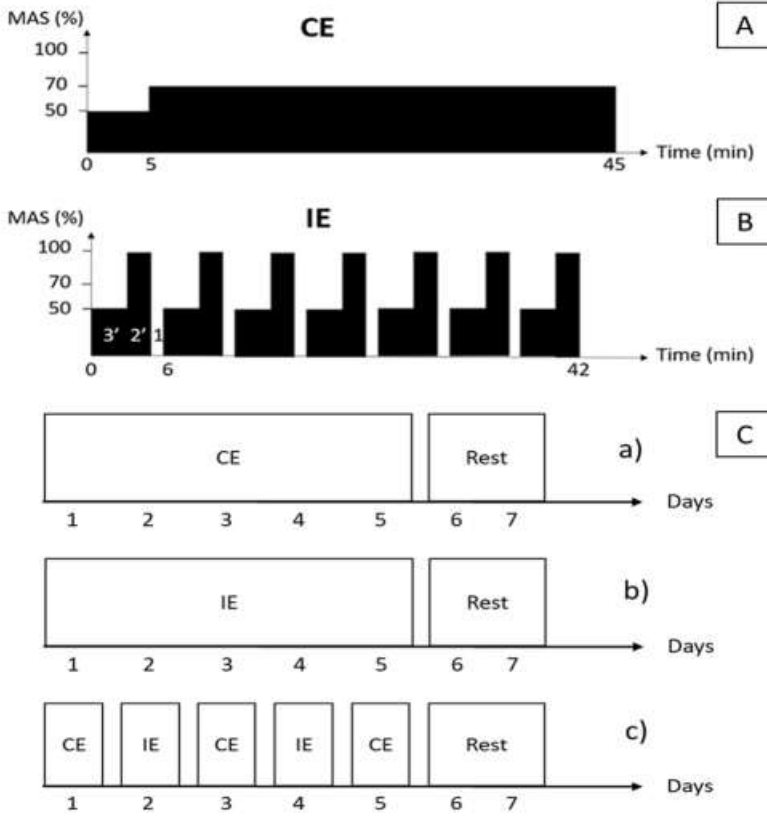


Figure 1: Running exercise protocols. A) Continuous exercise (CE) and B) Intermittent exercise (IE) modalities. C) Running protocols for the different groups. a) Protocol for CE group, b) protocol for IE group and c) protocol for combined exercise group (COME) consisting of alternation of running modalities (IE and CE) during the week.

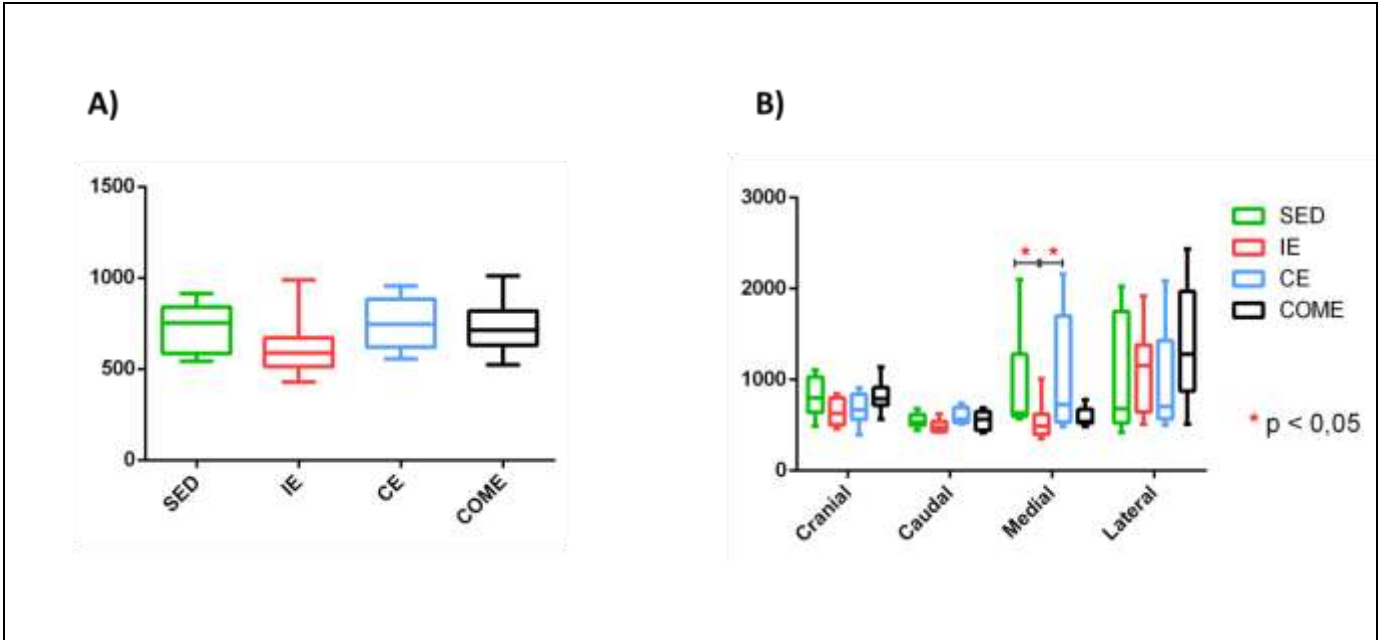


Figure 2: Cortical bone thickness in the femoral diaphysis from sedentary (SED) and exercise groups (IE: intermittent exercise; CE: continuous exercise; COME: combined exercise). A) Mean cortical thickness for the whole diaphysis, and B) Mean cortical thickness per anatomical region, measured on transversal sections (n=10 for SED; n= 10 for IE, n=10 for CE and n=9 for COME). Data are presented as means \pm SEM.

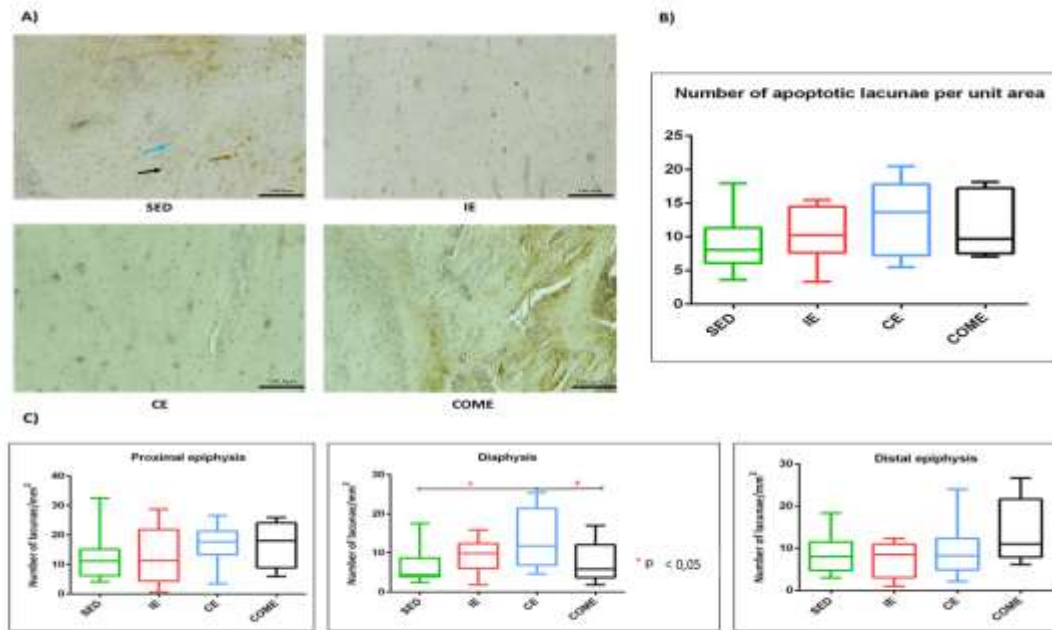


Figure 3: Osteocyte lacunae apoptosis in the femur (longitudinal sections) from sedentary (SED) rats and rats subjected to intermittent running (IE), continuous running (CE) and combined running (COME). A) Representative picture fields for the quantification of apoptotic lacunae for the different groups by cleaved caspase-3 immunohistochemical staining. Black arrows indicate apoptotic lacunae; blue arrows indicate full lacunae containing an osteocyte. B) Relative result for the total number of apoptotic lacunae in the different groups. C) Relative result for the number of apoptotic lacunae in the different anatomical locations of the femurs (proximal epiphysis, diaphysis and distal epiphysis); n=10 for SED, n= 10 for IE, n=10 for CE and n=8 for COME. Scale bar = 100µm. Data are presented as means ± SEM.

The osteocyte apoptotic rate for the whole femur did not statistically differ between the groups. A higher rate of apoptosis, however, was observed in the diaphysis of CE rats, compared to respective results from both COME and SED rats ($p=0.005$ and $p=0.03$ respectively)

A)

a) TRAP labelling in proximal epiphysis of femurs



b) TRAP labelling in distal epiphysis of femurs



B)

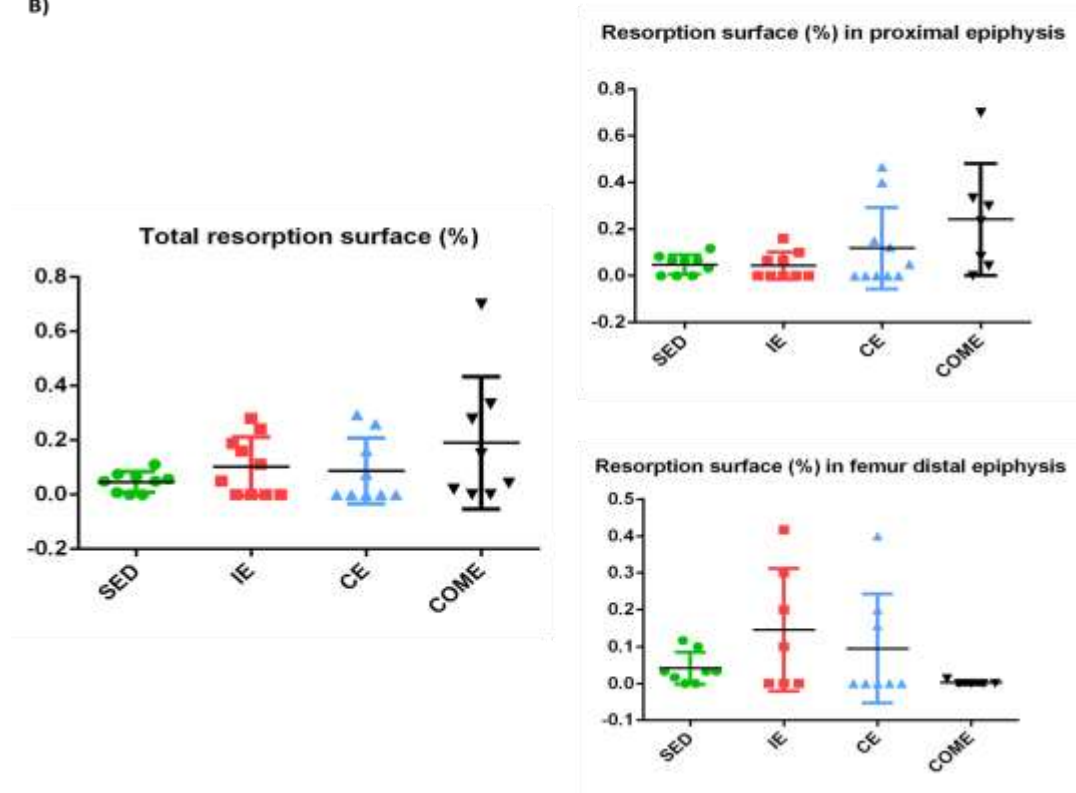


Figure 4: Osteoclastic bone resorption surface in the femur (longitudinal sections) from sedentary (SED) and rats subjected to intermittent running exercise (IE), continuous running exercise (CE) and combined running exercise (COME). A) Representative picture fields for the quantification of TRAP labelling in the proximal and distal epiphyses of the femurs in the different groups. Black arrows indicate osteoclastic bone resorption surface. B) Relative results of resorption surface (%) in whole femurs (total resorption surface) and in the proximal and distal epiphyses; n=10 for SED, n= 10 for IE, n=10 for CE and n=8 for COME. Scale bar = 100µm. Data are presented as means ± SEM.

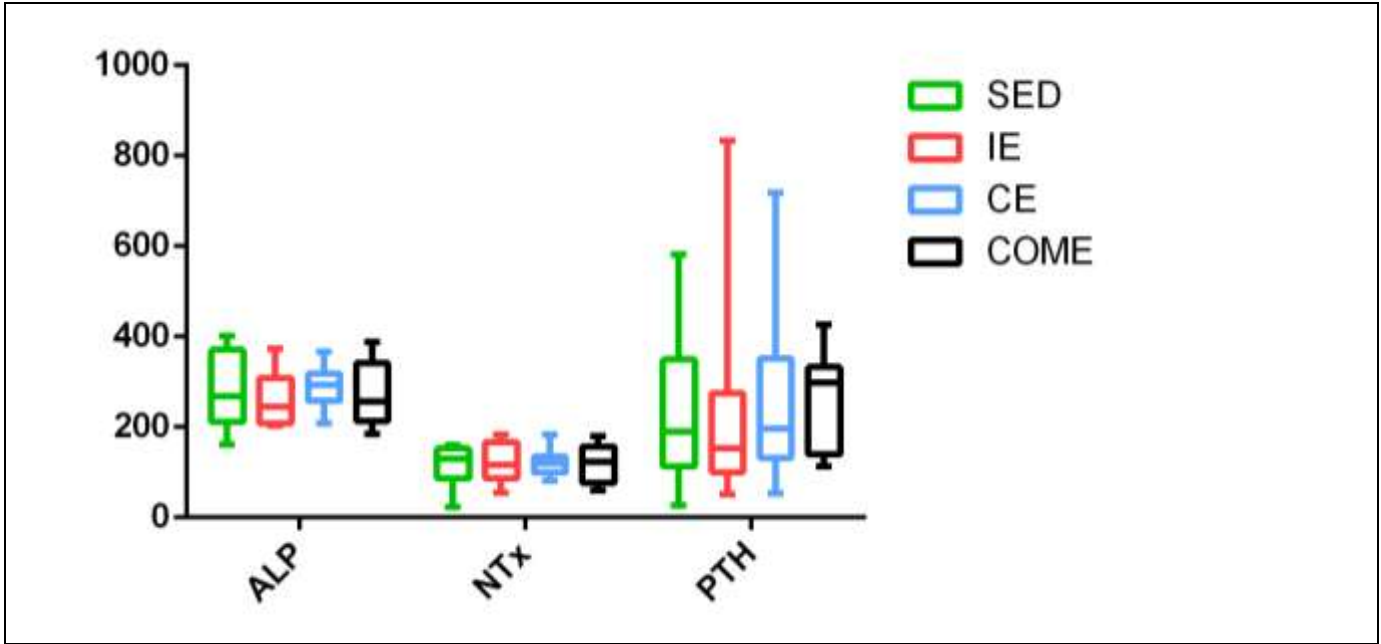


Figure 5: Relative results of bone turnover markers ALP, NTx and PTH for sedentary (SED) and rats subjected to intermittent running exercise (IE), continuous running exercise (CE) and combined running exercise (COME). n=10 for SED, n= 10 for IE, n=10 for CE and n=8 for COME. Data are presented as means \pm SEM.

Parameters	Time	SED	IE	CE	COME
Weight (g)	T0	199 ± 8	197 ± 7	192 ± 6* [‡]	201 ± 9
	T8	492 ± 36	475 ± 31	486 ± 33	491 ± 16
Rat Length (cm)	T0	19.8 ± 0.5	19.8 ± 0.4	19.7 ± 0.4	19.6 ± 0.5
	T8	26.7 ± 1.1	26.4 ± 0.6	26.4 ± 0.6	26.5 ± 0.7
Rat BMI (g/cm ²)	T0	0.51 ± 0.02	0.5 ± 0.02 [‡]	0.49 ± 0.01 [‡]	0.53 ± 0.03
	T8	0.69 ± 0.01	0.68 ± 0.02	0.69 ± 0.03	0.70 ± 0.04
MAS (m/min)	T0	28 ± 9	32 ± 5	33 ± 6	31 ± 8
	T8	23 ± 6	34.7 ± 11*	33 ± 8*	37 ± 3*
Femurs parameters	Time	SED	IE	CE	COME
Length (cm)	T8	37.9 ± 1.3	38.6 ± 0.9	38.2 ± 1.4	37.7 ± 1.3
AP diameter	T8	3.2 ± 0.7	3.2 ± 0.6	3.6 ± 0.5	3.6 ± 0.9
LM diameter	T8	3.9 ± 0.6	3.9 ± 0.6	4 ± 0.9	4.2 ± 1

Data are presented as mean ± SD
BMI body mass index, *MAS* maximal aerobic speed, *AP* anteroposterior, *LM* lateromedial
n = 10 for SED, *n* = 10 for IE, *n* = 10 for CE and *n* = 9 for COME.
T0: beginning of the study; T8: end of the running protocol
**p* < 0.05 vs SED
[‡]*p* < 0.05 vs COME

Table 1: Body and femurs parameters at T0 (beginning of the study), and T8 (end of the running protocol); MAS at T0 and T8 in SE and exercised groups (IE, CE, COME).

Parameters	SED	IE	CE	COME
Proximal femur				
BV/TV (%)	43.57 ± 5.99	42.05 ± 4.02	43.12 ± 3.43	46.63 ± 3.88
Tb.N (1/mm)	3.72 ± 0.40	3.63 ± 0.49	3.72 ± 0.36	3.41 ± 0.33
Tb.Th (mm)	0.11 ± 0.01 ^Ω	0.11 ± 0.007 [¶]	0.11 ± 0.008 [¶]	0.13 ± 0.006*
Tb.Sp (mm)	0.06 ± 0.007	0.07 ± 0.004	0.06 ± 0.008	0.07 ± 0.007
Tb.BMD (gHA/cm ³)	0.60 ± 0.12	0.68 ± 0.04* ^{§,¶}	0.52 ± 0.05* [¶]	0.59 ± 0.06 [#]
Distal femur				
BV/TV (%)	39.42 ± 6.8	35.93 ± 5.10	39.88 ± 6.97	42.83 ± 8.08
Tb.N (1/mm)	4.11 ± 0.54	4.04 ± 0.56	4.27 ± 0.63	4.36 ± 0.55
Tb.Th (mm)	0.09 ± 0.006	0.08 ± 0.006 [¶]	0.09 ± 0.004	0.09 ± 0.007
Tb.Sp (mm)	0.09 ± 0.01	0.11 ± 0.01	0.08 ± 0.01	0.08 ± 0.01
Tb.BMD (gHA/cm ³)	0.40 ± 0.06	0.40 ± 0.07	0.41 ± 0.08	0.46 ± 0.12

Data are presented as mean ± SD

BV/TV bone volume fraction, *Tb.N* trabecular number, *Tb.Th* trabecular thickness, *Tb.Sp* trabecular spacing, *Tb.BMD* trabecular bone mineral density

n = 10 for SED, *n* = 10 for IE, *n* = 10 for CE and *n* = 9 for COME

**p* < 0.05 vs SED

§*p* < 0.05 vs CE

¶*p* < 0.05 vs COME

Ω*p* < 0.05 vs COME

#*p* < 0.05 vs CE

Table 2: Femoral micro-architectural and trabecular bone mineral density parameters in SED and the different exercise groups (IE, CE, COME) at proximal and distal parts.

Osteocyte occupancy rate	SED	IE	CE	COME
Whole femur				
Total	87.63 ± 3.5	84.27 ± 3.4	81.41 ± 6.8	82.69 ± 7.9
Femur locations				
Proximal epiphysis	83.65 ± 5.8	81.82 ± 7.4	78.32 ± 4.4	77.64 ± 10
Diaphysis	90.9 ± 4.57	86.08 ± 4.1	81.57 ± 8.5	86 ± 10.7
Distal epiphysis	85.01 ± 8.9	84.82 ± 6.3	82.75 ± 10.9	81.58 ± 9.29

Data are presented as mean ± SD

$n = 10$ for sedentary group (SED); $n = 10$ for intermittent exercise group (IE); $n = 10$ for continuous exercise group (CE) and $n = 8$ for combined exercise group (COME)

Table 3: The osteocyte occupancy rate in the whole femur and in the different anatomical locations of the femur in SED and the different exercise groups (IE, CE, COME).