Marrow aspiration in aged mice: intramedullary osteogenesis, reduced mechano-adaptation, increased marrow fat

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Marrow aspiration in aged mice: intramedullary osteogenesis, reduced mechano-adaptation, increased marrow fat

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Abstract

With age, the number of adipocytes and osteoclasts increases, the number of osteoblasts decreases, and mechano-adaptation is impaired. Using marrow aspiration, which has a known osteogenic effect in young mice, we sought to recruit osteoblast progenitors to mediate the mechano-adaptive response to *in vivo* tibial loading. First, we assessed bone formation and marrow adiposity in the tibiae of old mice (>20 months) sacrificed 1, 2, and 4 weeks after unilateral marrow aspiration. Then, we examined the effects of marrow aspiration on mechano-adaptation in aged mice using tibial loading. Two weeks after aspiration, aspirated tibiae had more bone than contralateral tibiae due to the formation of bone in the medullary canal. Two weeks and four weeks after marrow aspiration, the volume of marrow adipose tissue was higher in the aspirated tibiae, compared to contralateral tibiae. Histomorphometry indicated that aspiration increased non-periosteal (endosteal, intracortical, intramedullary) bone formation, compared to the contralateral tibia. Mice with marrow aspiration had reduced periosteal bone formation in the contralateral tibia, compared to mice that had loading alone. Loading-induced periosteal bone formation was higher in mice that had loading alone, compared to mice that had aspiration + loading, indicating that aspiration further reduced the mechano-adaptive response. These data demonstrate that, in old mice, bone forms in the medullary canal following aspiration. Adiposity is increased following marrow aspiration, and periosteal mechano-adaptation is reduced.

20 **Keywords**

21 Marrow aspiration, BMAT, tibial loading, bone adaptation, histomorphometry

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23 **Running head:** “Marrow aspiration in aged mice”

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1. Introduction

Bone is a dynamic tissue that adapts to external loads. Reduced loads promote bone resorption by osteoclasts, moderate loads maintain bone mass, and high loads promote bone formation by osteoblasts. *In vivo* uniaxial tibial loading is a common model used to study mechano-adaptation in mice. While mice are under anesthesia, the tibia is fixed in loading cups at the ankle and knee and subjected to cyclic compressive loads. This loading is in near-physiological direction and promotes bone formation in the cortical and trabecular compartments, which can be evaluated with dynamic histomorphometry and quantitative micro-CT imaging [1,2]. Increasing cycle repetitions and load magnitude further augments bone formation. Bone's adaptive response to *in vivo* tibial loading model is reduced in aged mice, compared to young and mature mice. In aged bone, higher strain magnitudes are required to promote a mechano-adaptive response [3–5]. Among many other changes in bone biology with aging, the shift in cell populations is suspected to impair the mechano-adaptive response. With age, the number and activity of osteoclasts is increased [6–8]. Concurrently, progenitor cells tend to differentiate into adipocytes rather than cells of the osteoblastic lineage [9–11], and the number and activity of osteoblasts is decreased [12–15].

The present work aims to prime old bone to mediate a stronger mechano-adaptive response, by utilizing the cells recruited in response to bone marrow aspiration. In young mice, marrow ablated by aspiration, reaming, or flushing, is regenerated within weeks (4 weeks in 6-8 week-old mice [16]). The transient formation and resorption of intramembranous bone in the medullary canal, which takes place after marrow ablation, has been thoroughly characterized (in rats [17], in mice [18]). Briefly, the response to marrow injury/aspiration in the medullary canal is the following: clot formation, capillary invasion, appearance of mesenchymal stem cells and then differentiation to osteoblasts, formation of intramembranous bone, reappearance of hematopoietic tissue, and finally bone resorption by osteoclasts. Systemic bone formation (not restricted to the ablated bone) has been observed (in rats [19]). This model

is widely used in young (and rarely in mature) rodents because it is highly reproducible, and because it gives the opportunity to study how different factors are involved bone formation and resorption in vivo, within a short time period.

Few studies have looked at the effects of marrow aspiration in skeletally mature rodents (rats [20–22], mice [23]). In 24-month-old rats, the number of osteoblasts and the volume of mineralized bone was lower compared to 6-month-old rats [20]. To our knowledge, no study has examined the potential osteogenic effects of marrow aspiration and the subsequent effects on mechano-adaptation in old osteoporotic mice (over 20 months of age).

The first objective of this study is to characterize the response of old murine bone to marrow aspiration, by quantifying the amount of bone and marrow adipose tissue following marrow ablation. We hypothesize that even in aged animals, bone forms in the medullary canal in response to marrow aspiration. The second objective of this study is to examine the effects of marrow aspiration on bone mechano-adaptation, with the rationale that cells recruited following aspiration can mediate the mechano-adaptive response. We hypothesize that marrow aspiration augments the mechano-adaptive response of aged bone.

2. Materials and methods

2.1 Animals

Female C57BL/6J retired breeders were obtained from Jackson Laboratory at 6-9 months of age, and aged until 20-23 months old. Mice were housed in groups of five (individually ventilated cages, sterilized corn cob bedding with sterilized nestlets and Enviro-Dri), with a 12-hour light/dark cycle, and fed maintenance mouse diet (Iso-Pro RMH 3000 and filtered water). All animal procedures received approval from Northeastern University's Institutional Animal Care and Use Committee (IACUC, project 18-0105R). All live animal experiments were performed in the animal facility at Northeastern University. Over the course of the experiment, animal welfare was assessed daily (external appearance, abnormal activity, answer to external stimulation, weight, body condition score).

Mice were randomly allocated to 6 different groups (Figure 1B and Figure 2B): (1) marrow aspiration, sacrifice after 1 week (n=5); (2) marrow aspiration, sacrifice after 2 weeks (n=7); (3) marrow aspiration, sacrifice after 4 weeks (n=7); (4) marrow aspiration, sacrifice after 6 weeks (n=7) ; (5) loading for 2 weeks (n=7); (6) marrow aspiration, followed after 4 weeks by 2 weeks of tibial loading (n=8) (see weights in supplementary table S1). Sample sizes were based on a previous study from our group using tibial loading in old mice, which indicated that 5 mice per group were needed to obtain statistical significance with histomorphometric assessment [24], and groups with a longer experimental time were assigned additional mice in case of animals dying due to old age. Groups 1-3 examined the osteogenic and adipogenic response to marrow aspiration, and groups 4-6 examined the effects of marrow aspiration on mechano-adaptation. Experiments in the 6 groups were run in parallel to minimize age difference at sacrifice, and samples were assessed at the same time.

2.2 Tibial marrow aspiration

On day 0, at 20-23 months of age, mice were anesthetized with 5% isoflurane, the advantages of inhalation anesthesia being that anesthesia depth is easy to steer, metabolism is limited, and recovery is fast. Mice were kept on a warm pad during surgery, and later during recovery. The right hindlimb

underwent marrow aspiration (Figure 1A) [18]. A small incision was performed on the skin that covers the knee (about 2 mm in length). The 25g needle of a saline-filled syringe was inserted into the tibial cavity, under the patella, parallel to the longitudinal axis of the tibia. The motion of the saline exiting the needle locally disrupted the marrow. The plunger was moved back to create negative pressure, to aspirate bone marrow (reddish coloration in the syringe). This operation was repeated with clean saline-filled syringes that had a smaller diameter (28.5g) to minimize damage to the needle track. Saline was gently pushed into the tibial cavity and aspirated, until the liquid aspirated came out clear (using overall 2 mL of saline). After aspiration, the skin of the knee was closed with Monocryl sutures and Vetbond. The analgesic drug Meloxicam (NSAID, 5 mg/kg/day, recommended dosage in mice) was administered subcutaneously once a day until day 3. This procedure did not achieve total aspiration of the marrow, in particular the distal end of the tibia was not reached by the needle because of the tibial curvature, but it achieved a reproducible injury to the proximal end of the marrow.

2.3 *In vivo* tibial loading

Groups 4-5 and 5-6 were subjected to *in vivo* tibial loading, 3 times a week for 2 weeks using an Instron 5942, starting 4 weeks after marrow aspiration for group 5-6. Mice were anesthetized with 3% isoflurane. The right ankle and knee were fixed in custom-made loading cups and held with a preload of 1.5N, and loading was applied vertically through the knee (Figure 2A). The right tibia was subjected to 100 compressive cycles, with a magnitude of 9 N, a rise and unloading rate of 20-30 N/s, and a 1 s rest period inserted between each cycle. This loading protocol induced small but significant bone formation in aged mice in a previous study (20-22 months, [24]).

2.4 Fluorochrome labeling for dynamic histomorphometry

Mice in groups 4-6 were injected with a dose of 20 mg/kg calcein (Sigma C0875) intraperitoneally on two consecutive days, 2 weeks before sacrifice, and with a dose of 40 mg/kg demeclocycline (Sigma D6140) and 10 mg/kg calcein on two consecutive days before sacrifice (see * on Figure 2B), in order to quantify

the amount of bone formed over the last two weeks of the experiment (standard protocol at Harvard School of Dental Medicine, e.g. used in [24]).

2.5 Sacrifice and tibiae fixation

Mice were sacrificed by CO₂ inhalation and cervical dislocation (see weights at sacrifice in supplementary table S1). Tibiae in groups 1, 2, and 3, were dissected from the surrounding muscles, and fixed in 10% neutral buffered formalin. In groups 4, 5, and 6, surrounding muscles were kept to preserve periosteal surfaces for histomorphometry, and fixed in 70% ethanol.

2.6 Calcified scans

Calcified samples were imaged in a desktop micro-CT scanner (Scanco μ CT40, 10 μ m isotropic voxel size, 70 kVp, 114 μ A, 8 W, 200 ms). The portion of tibia located between the tibia-fibula junctions was analyzed, as the most distal part was not reached by marrow aspiration. Tibia-fibula junctions were defined as the locations where the tibia and the fibula split on micro-CT images (approximately 10% and 60% of total tibial length). Bone cross-sectional area along that portion was obtained using the BoneJ plugin in ImageJ [25] (threshold = 4,500).

In each pair of tibiae, intramedullary bone was segmented out from cortical bone using Mimics (see Figure 1C and Figure 2C). Intramedullary bone was defined as bone present inside the medullary canal, which did not have the typical structure of the very few trabeculae present in old mice (Figure 3).

2.7 Osmium staining and imaging

After scanning the calcified bones, tibiae in groups 1, 2, and 3, were decalcified for 21 days in a 4% EDTA solution, and were then incubated for 4 days in a 1% osmium tetroxide solution as described in [26]. Osmium tetroxide fixes into lipid and is radio-opaque, therefore allowing the visualization of adipocytes after micro-CT imaging. Following osmium staining, decalcified bones were imaged in a desktop micro-CT (Scanco μ CT40, 10 μ m isotropic voxel size, 55 kVp, 145 μ A, 8 W, 300 ms). Osmium also stains remaining bits of soft tissue, so adipose volume (stained by osmium) was segmented in Mimics to ensure no stain outside the marrow cavity would be included (see Figure 1C, threshold =

20,928), and quantified with BoneJ. The volume between tibia-fibula junctions was analyzed, as the distal part is always filled with constitutive adipose tissue in mice (as opposed to “regulated” adipose tissue in the proximal end [27]). 2 animals were excluded because muscle dissection was incomplete in one of their tibiae, resulting in improper decalcification and Osmium staining. Therefore, 4/5 animals in group 1, 6/7 animals in group 2, and 7/7 animals in group 3 were included.

2.8 Histomorphometry

Tibiae and surrounding muscles in groups 4-6 were embedded in MMA for histomorphometric analysis. Sections were cut at 30% (see Figure 2C) of the distance between tibia-fibula junctions, approximately in the middle of the portion affected by surgical marrow aspiration. The sections were cut with a 4- μ m thickness for optimal osteoblast and osteoclast visualization. In each tibia, one slide was left unstained to measure dynamic parameters with fluorochrome labeling. A second slide was stained with tartrate-resistant acid phosphatase (TRAP) to count osteoclasts, and a third slide was stained with 2% toluidine blue to measure osteoid and osteoblasts (please read Supplementary Methods for more information on staining protocol).

Sections were analyzed with OsteoMeasure software (OsteoMetrics, Atlanta, GA, USA). Overall, mineral apposition rate (MAR), mineralizing surface (MS), bone formation rate (BFR), number of osteoblasts and osteoclasts (N.Ob and N.Oc), as well as osteoid width and surface (O.Wi and OS) were measured. MAR was calculated as $MAR = Ir.L.Wi / t$, where Ir.L.Wi is the interlabel width, t is the time interval between the first and second sets of fluorochrome injections. MS was calculated as $sLS/2 + dLS$, where sLS is the length of single label marking, and dLS is the length of double label marking. BFR was calculated as $MAR \times BS$. Parameters were analyzed as periosteal or non-periosteal, which comprised endosteal, trabecular, intramedullary, and intracortical labels or cells. Non-periosteal parameters were grouped together, because the endosteal perimeter was not clearly defined in many animals (see Supplementary Figure S1). Therefore, conventional classification would be too dependent on personal interpretation and location of the section. What appears as an intracortical cavity may be interpreted as trabecular bone a

few sections away. Histomorphometric results are usually reported normalized to the bone surface BS, which may or may not be labeled. In this study, histomorphometric parameters were not normalized to bone surface (MS was reported, instead of MS/BS, BFR instead of BFR/BS, N.Ob instead of N.Ob/BS, and N.Oc instead of N.Oc/BS), because normalizing would not capture the absolute and variable formation of bone inside the medullary canal. Many bones with high bone formation activity also have increased bone surface compared to bones with lower amounts of activity. Normalizing by bone surface does not capture the increase in overall amount of bone formation activity.

2.9 Statistical analysis

Micro-CT data: For total bone sectional area, we compared, within each group, bone area (continuous data) along the tibia-fibula junctions (normalized field) in the right versus left tibia. To allow multiple comparisons of these data along the field, we used one-dimensional statistical parametric mapping (Spm1d [28]). This method uses random field theory to perform statistical tests on normalized sets of 1D measurements that are continuous in space or in time, and corrects for the multiple comparison problem [29]. We used two-tailed two-sample t-tests in Spm1d, to test the null hypothesis that mean bone area in the left and right tibiae are equal. We reported the location of the clusters where the null-hypothesis was rejected, as well as the associated p-value. We also compared, within each group, intramedullary bone volume (groups 1-6) and adipose tissue volume (groups 1-3) in the right versus left tibiae. The difference (right - left) was not always normally distributed according to Kolmogorov-Smirnov tests, therefore non-parametric Wilcoxon sign-rank tests were used, with the null hypothesis that left and right distributions were the same within each group.

Histomorphometry: Within a group, we compared histomorphometric parameters in the right versus left tibiae using Wilcoxon signed-rank tests, with the hypothesis that distributions were equal. This determined the effect of aspiration/loading relative to the contralateral leg. Then, because aspiration promoted some systemic changes in bone formation, we compared histomorphometric parameters across groups. We used Kruskal-Wallis tests to compare left tibiae, and then right tibiae, across the 3 groups,

196 with the null hypothesis that distributions are equal across groups. When Kruskal-Wallis tests indicated
197 that distributions were not equal across groups, we performed post-hoc analysis to determine which group
198 was different from the others. Post-hoc analysis was performed with Wilcoxon rank-sum tests, using the
199 Bonferroni correction for multiple comparisons, with the null hypothesis that distributions are equal.

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3. Results

3.1 Effects of marrow aspiration on bone and marrow adipose tissue

2 weeks after aspiration, bone cross-sectional area was higher in the right aspirated tibiae around 12-13% of the normalized location on tibia, compared to the left contralateral tibiae (Figure 4, group 2, $p < 0.01$). This increase in bone amount was in part caused by formation of bone in the medullary canal: after 2 weeks, the volume of intramedullary bone was significantly higher in the right than in the left tibiae (Figure 5, $p < 0.05$). 1, 4, and 6 weeks after aspiration, bone cross-sectional area was not different in the left and right tibiae (Figure 4, groups 1, 3, and 4). 3 mice out of 22 that had aspiration presented elevated levels of intramedullary bone in the contralateral tibiae as well (Figure 5, more than 0.25 mm^3 , which is more than ten times the mean intramedullary bone volume in the left tibia of mice that did not have marrow aspiration, groups 3, 4, and 6). Supplementary Figure S2 shows examples of intramedullary bone distribution after marrow aspiration, in the left and right tibiae.

2 weeks and 4 weeks after marrow aspiration, the amount of adipose tissue increased in the right tibiae, compared to left (Figure 6, groups 2 and 3, $p < 0.05$, 140% increase in the right compared to left tibia at 2 weeks, 39% increase at 4 weeks). Supplementary Figure S3 shows examples of marrow adipose tissue distribution after marrow aspiration, in the left and right tibiae.

3.2 Combined effects of aspiration and loading

Loading the right tibiae increased bone cross-sectional area at 47%-49% between tibia-fibula junctions, compared to the left contralateral tibiae (Figure 4, group 5). This corresponds to the region where strains are highest and where loading-induced formation is usually observed (about 37% of the total tibial length, [30–32]). Right tibiae that had aspiration + loading had higher bone cross-sectional area at 1% between the tibia-fibula junctions, compared to left tibiae (Figure 4, group 6). This increase was located at the very proximal end, where a lot of intramedullary bone was present 6 weeks after marrow aspiration.

Intramedullary bone volume was no different between the left and right tibiae within groups 4, 5, and 6 (Figure 5).

3.2 Histomorphometry

3.2.1 Effects of aspiration and/or loading in the right tibia, compared to left (contralateral)

Aspiration alone increased non-periosteal bone formation in the right tibia, compared to the left (Figure 7). Non-periosteal mineral apposition rate and bone formation rate were higher in the right tibia, compared to the left (Table 1A, group 4). No difference was observed in cell numbers (osteoblasts and osteoclasts) or in osteoid parameters. Supplementary figure S1 shows sections of a mouse with very high amounts of bone formation in the medullary canal and high intracortical remodeling, and illustrates why it is difficult in this experiment to discriminate non-periosteal parameters (endosteal / intracortical / trabecular / intramedullary). This mouse in particular had elevated amounts of intramedullary bone (navy blue six-pointed star on figure 5, group 4).

Loading alone overall increased bone formation in the right tibia, compared to the left (Figure 8).

Periosteal mineral apposition rate, bone formation rate, and osteoid surface, as well as non-periosteal bone formation rate and osteoid surface, were higher in the right tibia compared to the left (Tables 1A and 2A, group 5). No difference was observed in cell numbers (osteoblasts and osteoclasts).

When aspiration and loading were combined, the response was a mix of what was seen in aspiration and in loading, as both periosteal and non-periosteal bone formation were increased in the right tibia, compared to the left (Figure 9). Periosteal mineralizing surface, mineral apposition rate, bone formation rate, osteoid width, osteoid surface, as well as non-periosteal mineral apposition rate were higher in the right tibia, compared to the left (Tables 1A and 2A, group 6). No difference was observed in cell numbers (osteoblasts and osteoclasts).

3.2.2 Mice that had marrow aspiration in the right tibia had lower periosteal formation in the left tibia

We compared left (contralateral) tibiae across groups. Mice that had marrow aspiration (groups 4 and 6) had lower periosteal mineralizing surface, mineral apposition rate, and bone formation rate in the left tibia, compared to mice that had loading alone (group 5) (Table 1B).

3.2.3 Following marrow aspiration, the periosteal response to loading was decreased

We compared all right tibiae (aspiration and/or loading) across groups. Mice that had loading (groups 5 and 6) had higher periosteal and non-periosteal bone formation in the right tibia, compared to mice that had aspiration alone (group 4). Similarly, mice that had loading had less periosteal osteoclasts, compared to mice that had aspiration alone. For instance, periosteal and non-periosteal mineral apposition rate in the right tibia were higher in groups 4-5 and 6, compared to group 4 (Table 1B). Loading-induced periosteal bone formation was even higher in mice that had loading alone (group 5), compared to mice that had marrow aspiration + loading (group 6). For instance, periosteal mineralizing surface and bone formation rate in the right tibia were higher in group 5, compared to group 6 (Table 1B).

4. Discussion

To our knowledge, this is the first study to examine marrow aspiration in old mice (20-23 months of age). These data indicate bone formation in the medullary canal 2 weeks following aspiration, indicating aged bone has the cellular machinery to make bone. Interestingly, the increase in bone formation was accompanied by an increase in marrow adiposity. While the increase in adipocytes following aspiration has been reported based on histological sections in young mice [33] and in 1-month old immuno-compromised rats (but no increase was observed in 3- and 10-month-old animals) [21], no study has examined the effects of aspiration on marrow adipose tissue in old rodents. In particular, no study has quantified the total volume of marrow adipose tissue following aspiration in both tibiae. Given the variability in volume and distribution of regulated marrow adipose tissue, results from histological sections might depend greatly on the location of the section, while 3D osmium imaging captures marrow

adipose tissue in its totality. Using osmium staining, and despite high variability in marrow distribution and low sample size, the present study found that marrow adipose tissue increased following aspiration. Cell fate is altered with age, and common progenitors tend to differentiate into adipocytes rather than osteoblasts [9–11]. The increase in marrow adiposity following marrow aspiration might be due to the recruitment of stem cells, part of which differentiated into adipocytes. Future studies could examine senescence markers to provide valuable information on bone environment after marrow aspiration.

Interestingly, marrow aspiration reduced periosteal bone formation in the contralateral tibiae of mice that had aspiration, compared to contralateral tibiae of mice that did not have aspiration. This decrease in periosteal formation was captured during the last 2 weeks of the experiment, starting 4 weeks after aspiration, and suggests that bone anabolism is reduced in a systemic manner. Further studies are needed to investigate this mechanism. Measuring serum bone turnover markers for instance would provide insight on the systemic response in this study.

Tibiae that had aspiration + loading had lower periosteal formation compared to tibiae that had loading alone. The same load magnitude (9 N) was applied to both groups, because the transient formation of bone in the medullary canal close to the neutral axis in bending (induced by tibial loading) likely did not alter much the mechanical cues experienced in the cortex, especially close to the periosteal surface.

Additionally, even mice that did not present intramedullary bone following aspiration had lower levels of periosteal bone formation, compared to mice that did not have aspiration. Marrow aspiration might have reduced baseline periosteal formation in the aspirated tibiae (like it did in the contralateral tibiae), and the mechano-adaptive response to loading was further reduced. Moreover, initiating loading sessions earlier after marrow aspiration might yield a different outcome, for instance 2 weeks after aspiration, when the aspirated tibia still had more bone than the contralateral.

Interestingly, the decrease in periosteal mechano-adaptation coincided with the increase in bone marrow adiposity following marrow aspiration. High marrow adiposity has been linked with skeletal fragility [34–36], and bone marrow adipose tissue can modulate osteoblast and osteoclast differentiation [37–43].

Communication across the cortex between the endosteal and periosteal surfaces might mediate the decrease in periosteal mechano-adaptation, but further studies would be required to elucidate this mechanism.

Histomorphometric sections showed varying levels of intracortical remodeling in all groups (with or without aspiration). Intracortical remodeling in old mice has been evidenced recently [44]. The amount of intracortical remodeling was however variable across animals and even within the same animal, and we found no correlation to periosteal bone formation (analysis presented in Supplementary material).

The lack of a group of baseline controls (age-matched, non-loaded, non-aspirated) is a limitation in this study. However, the mechano-adaptive response to *in vivo* tibial loading for cortical and trabecular bone is confined to the loaded leg [45], and most studies employing tibial loading use the contralateral leg as internal control [1–5]. Therefore, contralateral limbs in group 4-5 (no loading, not affected by marrow aspiration) provide baseline information for this experiment. We decided to reduce our sample size (supporting the 3Rs in animal research) by using the contralateral leg as a control rather than having a separate control group as it provides the same information. This study can help to inform sample size in future studies.

Marrow aspiration in the proximal site of the tibia might have been incomplete in these mice, and the osteogenic response observed could have been a response to marrow injury by the needle and turbulent motion of saline, rather than the actual removal of marrow by aspiration. Marrow aspiration is always accompanied by injury, and we do not know if injury alone would produce the same results. In young mice (15 weeks of age, BMP2 BAC transgenic males), reaming of the tibial marrow with needles was sufficient to induce an osteogenic response (increased BMP2 expression and CD73 positive cells within 2 weeks following injury) [46]. However, whether it is caused by injury or aspiration and regeneration, the procedure results in an osteogenic response along with increased marrow adiposity.

5. Conclusion

In old mice, there was transiently more bone formed in the medullary canal of the aspirated tibiae compared to contralateral tibiae, while marrow adiposity was increased. After marrow aspiration, the periosteal mechano-adaptive response to tibial loading was decreased as evidenced by dynamic histomorphometry. In the contralateral tibiae, periosteal bone formation was reduced. These intriguing results indicate that old bone is capable of forming transiently in the medullary canal in response to marrow aspiration. However, periosteal formation and mechano-adaptation in response to loading are reduced. This might have implications for bone health in elderly patients with marrow injury.

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479 **Figures**

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Figure 2: Experimental groups 4-6. (A) In vivo axial loading of the tibia. (B) Mice in group 4 were sacrificed 6 weeks after marrow aspiration. Mice in group 4-5 had 2 weeks of tibial loading, and mice in group 5-6 had marrow aspiration followed after 4 weeks by two weeks of loading, and mice in group 6 were sacrificed 6 weeks after marrow aspiration. Aspiration and/or loading was performed on right tibiae and left tibiae were kept intact. * indicate injection of fluorochromes (C) Bone area was analyzed along the tibia, between tibia-fibula junctions. Total volume of intramedullary bone between tibia-fibula junctions was measured. Sections were taken at 30% of the normalized length between tibia-fibula junctions, and were analyzed with static and dynamic histomorphometry.

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Figure 5: Amount of intramedullary bone in all groups, in the left (L, contralateral controls) and right (R, aspiration and/or loading) tibiae. Horizontal bars represent the mean of each distribution. Each mouse is represented with a different marker type and color. Intramedullary bone volume within each group was tested with a Wilcoxon signed-rank tests, with the null hypothesis that distributions are equal. *p<0.05
All animals are included.

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Figure 8: Histomorphometric sections of a mouse that had loading on the right tibia (group 5). Loading increased endosteal and periosteal mineralization. This figure is a mosaic of smaller fields of view arranged together to show a global image of bone activity. Measurements in OsteoMeasure were made consecutively in each field of view (450 μm x 650 μm).

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Figure 9: Histomorphometric sections of a mouse that had marrow aspiration and loading on the right tibia (group 6). Both tibiae present bone formation in the medullary canal. The contralateral tibia shows important intracortical remodeling. Some periosteal formation was observed in the loaded tibia. This figure is a mosaic of smaller fields of view arranged together to show a global image of bone activity. Measurements in OsteoMeasure were made consecutively in each field of view (450 μm x 650 μm).

510 **Supplementary Figures**

Supplementary figure S1: Histomorphometric sections of a mouse that had marrow aspiration in the right tibia (group 4). In this mouse, the aspirated tibia presented impressive bone formation in the medullary canal, as well as intracortical remodeling. No periosteal formation was observed on either tibia. This figure is a mosaic of smaller fields of view arranged together to show a global image of bone activity. Measurements in OsteoMeasure were made consecutively in each field of view (450 μm x 650 μm).

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523 Tables

DYNAMIC HISTOMORPHOMETRY												
MS (mm)			Non-periosteal					Periosteal				
Wilcoxon A signed- rank tests	(4) aspiration (5) loading (6) aspi+loading	left		right		p-value	left		right		p-value	
		mean ± std	median	mean ± std	median		mean ± std	median	mean ± std	median		
		2.77 ± 0.83	2.56	4.23 ± 3.11	3.41		0.36 ± 0.22	0.45	0.70 ± 1.18	0.19		
		2.98 ± 0.41	2.90	3.81 ± 1.44	3.14		2.02 ± 1.07	1.88	3.23 ± 0.39	3.20		
Kruskal- B Wallis tests		3.21 ± 1.89	2.90	3.30 ± 0.89	2.98	0.844	0.43 ± 0.54	0.22	2.08 ± 1.30	1.94	0.016	
		p-value	post-hoc p-values	p-value	post-hoc p-values	p-value	post-hoc p-values	p-value	post-hoc p-values			
		0.662		0.867		0.005	4 v 5: 0.004 4 v 6: 0.536 5 v 6: 0.004	0.009	4 v 5: 0.007 4 v 6: 0.072 5 v 6: 0.040			

MAR (µm/day)			Non-periosteal					Periosteal				
Wilcoxon A signed- rank tests	(4) aspiration (5) loading (6) aspi+loading	left		right		p-value	left		right		p-value	
		mean ± std	median	mean ± std	median		mean ± std	median	mean ± std	median		
		0.79 ± 0.13	0.82	0.89 ± 0.13	0.89		0.031	0.14 ± 0.20	0.00	0.26 ± 0.33		0.26
		1.08 ± 0.45	1.04	1.65 ± 1.17	1.47		0.078	0.54 ± 0.23	0.55	1.62 ± 1.58		1.08
Kruskal- B Wallis tests		0.85 ± 0.17	0.91	1.28 ± 0.39	1.15	0.008	0.10 ± 0.16	0.00	0.69 ± 0.58	0.50	0.016	
		p-value	post-hoc p-values	p-value	post-hoc p-values	p-value	post-hoc p-values	p-value	post-hoc p-values			
		0.209		0.023	4 v 5: 0.026 4 v 6: 0.014 5 v 6: 0.536	0.004	4 v 5: 0.006 4 v 6: 0.736 5 v 6: 0.002	0.005	4 v 5: 0.002 4 v 6: 0.042 5 v 6: 0.054			

BFR (mm.µm/day)			Non-periosteal					Periosteal				
Wilcoxon A signed- rank tests	(4) aspiration (5) loading (6) aspi+loading	left		right		p-value	left		right		p-value	
		mean ± std	median	mean ± std	median		mean ± std	median	mean ± std	median		
		2.22 ± 0.87	2.03	3.66 ± 2.55	2.73		0.016	0.07 ± 0.09	0.00	0.48 ± 1.12		0.05
		3.22 ± 1.31	2.97	5.38 ± 2.14	4.64		0.016	1.22 ± 0.85	0.89	5.54 ± 6.02		3.42
Kruskal- B Wallis tests		2.74 ± 1.75	2.18	4.25 ± 1.75	3.86	0.109	0.10 ± 0.25	0.00	1.88 ± 2.46	1.05	0.016	
		p-value	post-hoc p-values	p-value	post-hoc p-values	p-value	post-hoc p-values	p-value	post-hoc p-values			
		0.375		0.070		0.002	4 v 5: 0.004 4 v 6: 0.736 5 v 6: 0.001	0.005	4 v 5: 0.004 4 v 6: 0.057 5 v 6: 0.021			

Table 1 – Dynamic histomorphometry. MS = mineralizing surface, MAR = mineral apposition rate, BFR = bone formation rate.

(A) Wilcoxon signed-rank tests, comparing right to left tibiae within each group. Marrow aspiration alone (group 4) promoted non-periosteal bone formation in the right tibia, compared to the left. Loading alone (group 5) promoted periosteal and non-periosteal bone formation in the right tibia, compared to the left. When marrow aspiration and loading were combined (group 6), bone formation was increased in the periosteal and non-periosteal compartments in the right tibia, compared to the left.

(B) p-values for Kruskal-Wallis (KW) tests, comparing values across groups, with the null hypothesis that distributions area equal. If significance was found with the Kruskal-Wallis test (p<0.05), post-hoc analysis was run with multiple comparisons (group 4 vs 5, group 4 vs 6, and group 5 vs 6), using Wilcoxon rank-sum tests and the Bonferroni correction, with the null hypothesis that distributions are equal.

In mice that had marrow aspiration (groups 4 and 6), periosteal bone formation was lower in the contralateral (left) tibia, compared to mice that had loading alone (group 5). Mice that had loading (groups 5 and 6) had higher periosteal and non-periosteal bone formation in the right tibia, compared to mice that had aspiration alone (group

4). Loading alone (group 5) promoted more periosteal bone formation in the right tibia, compared to mice that had aspiration + loading (group 6).

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STATIC HISTOMORPHOMETRY

O.Wi (μm)				Non-periosteal					Periosteal				
A	Wilcoxon signed-rank tests	(4) aspiration (5) loading (6) aspi+loading	left		right		p-value	left		right		p-value	
			mean ± std	median	mean ± std	median		mean ± std	median	mean ± std	median		
			2.90 ± 0.95	2.54	2.58 ± 0.54	2.70		2.51 ± 0.56	2.57	2.81 ± 1.05	3.08		
			2.99 ± 0.99	2.76	2.56 ± 0.56	2.82		2.43 ± 0.48	2.41	2.19 ± 0.55	2.01		
			2.80 ± 0.67	2.47	2.66 ± 0.63	2.59	0.844	4.85 ± 4.10	3.27	2.14 ± 0.32	2.10	0.008	
B	Kruskal-Wallis tests		p-value	post-hoc p-values	p-value	post-hoc p-values		p-value	post-hoc p-values	p-value	post-hoc p-values		
			0.919		0.979			0.066		0.099			

OS (mm)		Non-periosteal						Periosteal					
A	Wilcoxon signed-rank tests	(4) aspiration (5) loading (6) aspi+loading	left		right		p-value	left		right		p-value	
			mean ± std	median	mean ± std	median		mean ± std	median	mean ± std	median		
			1.15 ± 0.75	1.42	1.15 ± 0.59	1.00		0.19 ± 0.09	0.00	0.50 ± 0.58	0.37		
			0.40 ± 0.27	0.37	0.98 ± 0.45	1.07		0.17 ± 0.14	0.55	0.93 ± 0.55	0.89		
			1.41 ± 1.11	1.07	0.98 ± 0.53	0.98	0.461	0.19 ± 0.10	0.00	0.83 ± 0.48	0.80	0.008	
B	Kruskal-Wallis tests		p-value	post-hoc p-values	p-value	post-hoc p-values		p-value	post-hoc p-values	p-value	post-hoc p-values		
			0.060		0.943			0.710		0.155			

N.Ob (count)			Non-periosteal					Periosteal				
A	Wilcoxon signed-rank tests	(4) aspiration (5) loading (6) aspi+loading	left		right		p-value	left		right		p-value
			mean ± std	median	mean ± std	median		mean ± std	median	mean ± std	median	
			44.1 ± 33.6	48.0	44.4 ± 24.3	33.0		4.1 ± 4.1	3.0	7.3 ± 9.7	4.0	
			24.1 ± 16.6	25.0	49.0 ± 31.7	54.0		9.4 ± 9.4	8.0	23.4 ± 17.1	19.0	
			53.6 ± 44.0	37.5	32.4 ± 18.4	29.5	0.250	4.5 ± 4.5	2.5	8.1 ± 7.8	5.5	0.297
B	Kruskal-Wallis tests		p-value	post-hoc p-values	p-value	post-hoc p-values		p-value	post-hoc p-values	p-value	post-hoc p-values	
			0.275		0.431			0.391		0.109		

N.Oc (count)		Non-periosteal					Periosteal				
Wilcoxon A signed-rank tests	(4) aspiration (5) loading (6) aspi+loading	left		right		p-value	left		right		p-value
		mean ± std	median	mean ± std	median		mean ± std	median	mean ± std	median	
		24.1 ± 10.6	23.0	37.3 ± 19.5	32.0		2.4 ± 2.3	1.0	2.4 ± 1.7	2.0	
		16.3 ± 4.2	16.0	23.9 ± 17.2	17.0		0.4 ± 1.1	0.0	0.1 ± 0.4	0.0	
		27.8 ± 12.6	30.0	18.3 ± 14.0	12.0	0.156	1.8 ± 2.5	1.0	1.3 ± 1.4	1.0	1.000
Kruskal-Wallis tests B		p-value	post-hoc p-values	p-value	post-hoc p-values		p-value	post-hoc p-values	p-value	post-hoc p-values	
		0.045	4 v 5: 0.165	0.073		0.056		0.021	4 v 5: 0.005	4 v 6: 0.144 5 v 6: 0.103	
			4 v 6: 0.955								
			5 v 6: 0.189								

Table 2 – Static histomorphometry. O.Wi = osteoid width, OS = osteoid surface, N.Ob = number of osteoblasts, N.Oc = number of osteoclasts.

(A) Wilcoxon signed-rank tests, comparing right to left tibiae within each group. Loading alone (group 5) increased periosteal and non-periosteal osteoid surface in the right tibia, compared to the left. Aspiration + loading (group 6) increased periosteal osteoid surface and width in the right tibia, compared to the left.

(B) p-values for Kruskal-Wallis (KW) tests, comparing values across groups, with the null hypothesis that distributions area equal. If significance was found with the Kruskal-Wallis test ($p < 0.05$), post-hoc analysis was run with multiple comparisons (group 4 vs 5, group 4 vs 6, and group 5 vs 6), using Wilcoxon rank-sum tests and the Bonferroni correction, with the null hypothesis that distributions are equal. Mice that had aspiration alone (group 4) had more periosteal osteoclasts in the right tibia, compared to mice that had loading alone (group 5).

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Supplementary Table

experimental group		initial weight (g)			final weight (g)		
1	1 week post-aspi	33.2	±	2.5	31.6	±	2.4
2	2 weeks post-aspi	30.8	±	6.5	31.1	±	6.2
3	4 weeks post-aspi	32.0	±	3.3	30.4	±	2.3
4	6 weeks post-aspi	30.3	±	4.5	30.6	±	2.6
5	loading	30.2	±	3.2	30.8	±	2.9
6	aspi + loading	32.8	±	3.4	32.3	±	3.0

Supplementary table S1: Initial and final weights in all groups.

Supplementary methods: section preparation for histomorphometry

We did not deplasticize slides for dynamic histomorphometry. Instead, slides were dipped three times in toluene (3 seconds for each dip) and mounted with toluene-based mounting media (Thermo Scientific, 4112). For static histomorphometry, sections were deplasticized with two successive baths in acetone (10 minutes for each bath, second acetone bath must be fresh), followed by a bath in 70% ethanol for 3 minutes and a bath in distilled water for 3 minutes.

Toluidine blue staining: Sections were stained with toluidine blue to identify osteoblasts and osteoid (Skingle and Compston, 2011). Toluidine blue buffer was prepared with 1.58 g citric acid (Sigma, 251275, MW 192.12), 0.75 g sodium phosphate dibasic (Fisher, 3828-01, FW 141096), 1000 ml distilled water. The buffer can be used for a month. A 2% toluidine blue staining solution was prepared with 2 g toluidine blue (Sigma, 198161-25g) and 100 ml of buffer. Solution was stirred for several hours, and filtered through Whatman filter paper. pH was adjusted to 3.7 before each use (staining solution was changed every 6 months). Sections were immersed in staining solution for 3 minutes, rinsed in buffer for 3-5 seconds, air dried, quickly dipped in butanol, and supplied with a coverslip using toluene-based mounting media (Thermo Scientific, 4112).

TRAP staining: Following deplasticizing, sections were incubated in 0.2 M acetate buffer for one hour at 37 °C. Close to the end of incubation time, TRAP solution was prepared in acetate buffer in the following order. (1) 1000 ml of 0.2 M acetate buffer with 16.4mg sodium acetate (Sigma, S7545, FW 82.03) and 11.6 mg sodium tartrate dibasic dehydrate (Sigma, 228729, FW 230.1), stirred until dissolved and adjusted pH to 5.0 with acetic acid. (2) TRAP staining solution with 12.5 ml of 0.2 M acetate buffer, 6.25 mg of Naphthol AS-MX phosphate (Sigma, N4875), and 13.75 mg Fast Red TR salt (Sigma, F6760). When section incubation was over, slides were removed from 0.2 M acetate buffer. Sections were incubated in TRAP staining solution in humidity dish, with TRAP solution dropped on the top of tissue sections. Sections were incubated for ~2 hours at 37°C, monitoring after the first half hour, until osteoclasts were bright red. The reaction was stopped before staining of the background occurred. Slides

were rinsed in distilled water. Sections were counterstained for 15 seconds in toluidine blue solution, then washed by dipping several times in toluidine blue buffer and distilled water. Sections were air-dried and mounted with Histomount (HS-103, National Diagnostics) diluted 3:1 with Histo-Clear (HS-200, National Diagnostics).

Reference: Skingle, Linda, and Juliet Compston. "Bone histomorphometry." Vitamin D. Academic Press, 2011. 845-859.

Supplementary analysis of intracortical remodeling:

We analyzed the amount of intracortical remodeling using a semi-quantitative score (because it is difficult to discriminate intramedullary / endosteal / intracortical parameters). We divided each section into 4 quarters (posterior cortex, lateral cortex, anterior-medial cortex, and posterior-medial cortex) (as described in [32]). For each cortex quarter, we attributed a grade: 0 for no intracortical remodeling, 0.5 for some intracortical remodeling (less than 10% of the surface), 1 for important remodeling. We then added up this score on all 4 quarters to reach a grade from 0 to 4 (with increments of 0.5). We tested the distribution with Kolmogorov-Smirnov tests and found that distributions were normal. We then compared left and right tibiae within a group using paired t-tests, with the null hypothesis that left and right tibiae had equal means. All p-values were higher than 0.05. We concluded that, within a group, there was no difference in intracortical remodeling between left and right tibia. Even some of the unloaded tibiae of mice without aspiration presented intracortical remodeling.

	left		right		paired t-test
	mean	± std	mean	± std	p-value
(4) 6 weeks aspiration	0.71	± 0.59	1.29	± 1.16	0.356
(5) loading	0.36	± 0.35	1.14	± 1.12	0.130
(6) aspiration + loading	1.19	± 1.09	0.75	± 0.35	0.247
p-value for one-way ANOVA	0.170		0.571		

Intracortical remodeling scores in the left and right tibiae. Paired t-tests were used to test the null hypothesis that means are equal in the left and right tibiae within each group. One-way ANOVA tests were also used to test the null hypothesis that means are equal across groups within left tibiae, and within right tibiae.

We also looked for correlations between the intracortical remodeling score and the periosteal bone formation in the left contralateral tibiae, with the idea that perhaps the more intracortical remodeling, the smaller the periosteal formation. We used linear regression between the intracortical remodeling score and the periosteal mineralizing surface, and found no correlation ($p > 0.05$).

	Ps.MS		Ps.MAR		Ps.BFR	
	R ²	p-value	R ²	p-value	R ²	p-value
(4) 6 weeks aspiration	0.052	0.622	0.144	0.402	0.176	0.348
(5) loading	0.001	0.952	0.185	0.335	0.018	0.776
(6) aspiration + loading	0.014	0.780	0.000	0.974	0.002	0.912
Linear regression between intracortical remodeling scores and periosteal dynamic histomorphometry parameters (periosteal mineralizing surface, mineral apposition rate, bone formation rate).						

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The ARRIVE Guidelines Checklist

Animal Research: Reporting In Vivo Experiments

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	ITEM	RECOMMENDATION	Section/ Paragraph
Title	1	Provide as accurate and concise a description of the content of the article as possible.	Yes, title
Abstract	2	Provide an accurate summary of the background, research objectives, including details of the species or strain of animal used, key methods, principal findings and conclusions of the study.	Yes, abstract
INTRODUCTION			
Background	3	a. Include sufficient scientific background (including relevant references to previous work) to understand the motivation and context for the study, and explain the experimental approach and rationale. b. Explain how and why the animal species and model being used can address the scientific objectives and, where appropriate, the study's relevance to human biology.	Yes, throughout intro
Objectives	4	Clearly describe the primary and any secondary objectives of the study, or specific hypotheses being tested.	Yes, last paragraph of intro
METHODS			
Ethical statement	5	Indicate the nature of the ethical review permissions, relevant licences (e.g. Animal [Scientific Procedures] Act 1986), and national or institutional guidelines for the care and use of animals, that cover the research.	Yes, paragraph 2.1
Study design	6	For each experiment, give brief details of the study design including: a. The number of experimental and control groups. b. Any steps taken to minimise the effects of subjective bias when allocating animals to treatment (e.g. randomisation procedure) and when assessing results (e.g. if done, describe who was blinded and when). c. The experimental unit (e.g. a single animal, group or cage of animals). A time-line diagram or flow chart can be useful to illustrate how complex study designs were carried out.	Yes, paragraph 2.1 and time-line diagrams in figures 1 and 2
Experimental procedures	7	For each experiment and each experimental group, including controls, provide precise details of all procedures carried out. For example: a. How (e.g. drug formulation and dose, site and route of administration, anaesthesia and analgesia used [including monitoring], surgical procedure, method of euthanasia). Provide details of any specialist equipment used, including supplier(s). b. When (e.g. time of day). c. Where (e.g. home cage, laboratory, water maze). d. Why (e.g. rationale for choice of specific anaesthetic, route of administration, drug dose used).	Yes, paragraphs 2.2-2.5
Experimental animals	8	a. Provide details of the animals used, including species, strain, sex, developmental stage (e.g. mean or median age plus age range) and weight (e.g. mean or median weight plus weight range). b. Provide further relevant information such as the source of animals, international strain nomenclature, genetic modification status (e.g. knock-out or transgenic), genotype, health/immune status, drug or test naïve, previous procedures, etc.	Yes, paragraph 2.1 and supplementary table 2.1

The ARRIVE guidelines. Originally published in *PLoS Biology*, June 2010¹

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Housing and husbandry	9	Provide details of: a. Housing (type of facility e.g. specific pathogen free [SPF]; type of cage or housing; bedding material; number of cage companions; tank shape and material etc. for fish). b. Husbandry conditions (e.g. breeding programme, light/dark cycle, temperature, quality of water etc for fish, type of food, access to food and water, environmental enrichment). c. Welfare-related assessments and interventions that were carried out prior to, during, or after the experiment.	Yes, paragraph 2.1
Sample size	10	a. Specify the total number of animals used in each experiment, and the number of animals in each experimental group. b. Explain how the number of animals was arrived at. Provide details of any sample size calculation used. c. Indicate the number of independent replications of each experiment, if relevant.	Yes, paragraph 2.1
Allocating animals to experimental groups	11	a. Give full details of how animals were allocated to experimental groups, including randomisation or matching if done. b. Describe the order in which the animals in the different experimental groups were treated and assessed.	Yes, paragraph 2.1
Experimental outcomes	12	Clearly define the primary and secondary experimental outcomes assessed (e.g. cell death, molecular markers, behavioural changes).	Yes, paragraphs 2.6-2.8
Statistical methods	13	a. Provide details of the statistical methods used for each analysis. b. Specify the unit of analysis for each dataset (e.g. single animal, group of animals, single neuron). c. Describe any methods used to assess whether the data met the assumptions of the statistical approach.	Yes, paragraph 2.9 (statistical analysis)
RESULTS			
Baseline data	14	For each experimental group, report relevant characteristics and health status of animals (e.g. weight, microbiological status, and drug or test naïve) prior to treatment or testing. (This information can often be tabulated).	Yes, supplementary table S1
Numbers analysed	15	a. Report the number of animals in each group included in each analysis. Report absolute numbers (e.g. 10/20, not 50% ²). b. If any animals or data were not included in the analysis, explain why.	Yes, see legend of figures 4-6
Outcomes and estimation	16	Report the results for each analysis carried out, with a measure of precision (e.g. standard error or confidence interval).	Yes, see tables 1 and 2
Adverse events	17	a. Give details of all important adverse events in each experimental group. b. Describe any modifications to the experimental protocols made to reduce adverse events.	No adverse events
DISCUSSION			
Interpretation/scientific implications	18	a. Interpret the results, taking into account the study objectives and hypotheses, current theory and other relevant studies in the literature. b. Comment on the study limitations including any potential sources of bias, any limitations of the animal model, and the imprecision associated with the results ² . c. Describe any implications of your experimental methods or findings for the replacement, refinement or reduction (the 3Rs) of the use of animals in research.	Yes, in discussion
Generalisability/translation	19	Comment on whether, and how, the findings of this study are likely to translate to other species or systems, including any relevance to human biology.	Yes, in conclusion
Funding	20	List all funding sources (including grant number) and the role of the funder(s) in the study.	Yes, included

References:

1. Kilkenny C, Browne WJ, Cuthill IC, Emerson M, Altman DG (2010) Improving Bioscience Research Reporting: The ARRIVE Guidelines for Reporting Animal Research. *PLoS Biol* 8(6): e1000412. doi:10.1371/journal.pbio.1000412
2. Schulz KF, Altman DG, Moher D, the CONSORT Group (2010) CONSORT 2010 Statement: updated guidelines for reporting parallel group randomised trials. *BMJ* 340:c332.

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