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Femoral stiffness and strength critically depend on loading angle: a parametric study in a mouse-inbred strain

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Abstract

Biomechanical tests of human femora have shown that small variations of the loading direction result in significant changes in measured bone mechanical properties. However, the heterogeneity in geometrical and bone tissue properties does not make human bones well suited to reproducibly assess the effects of loading direction on stiffness and strength. To precisely quantify the influence of loading direction on stiffness and strength of femora loaded at the femoral head, we tested femora from C57BL/6 inbred mice. We developed an image-based alignment protocol and investigated the loading direction influence on proximal femur stiffness and strength. An aluminum femoral phantom and C57BL/6 femora were tested under compression with different loading directions. Both tests, with the aluminum phantom and the murine bones, showed and quantified the linear dependence of stiffness on loading direction: a 5° change in loading direction resulted in almost 30% change in stiffness. Murine bone testing also revealed and quantified the variation in strength due to loading direction: 5° change in loading direction resulted in 8.5% change in strength. In conclusion, this study quantified, for the first time, the influence of misalignment on bone stiffness and strength for femoral head loading. We showed the extreme sensitivity of this site regarding loading direction.

Keywords: aluminum phantom; biomechanical testing; loading direction; mouse strain; proximal femur.

Introduction

Many elderly people suffer from osteoporosis, a skeletal disorder characterized by compromised bone strength leading to an increased risk of fracture. Osteoporosis primarily induces bone fragility resulting from decreased bone mass, altered microarchitecture and impaired bone quality [45]. Any bone can be affected, but of special concern are fractures of the hip, the spine and the wrist. Vertebral fractures have serious consequences, including loss of height, severe back pain and deformity. A hip fracture almost always requires hospitalization and major surgery. It can impair a person's ability to walk unassisted and may cause prolonged or permanent disability and even death [12, 13, 19]. Fractures of the femoral neck mainly occur in two situations. The first and most common one is under impact loading during a fall [22]. The second situation is under non-traumatic loading [4]. In both situations, fracture risk is much higher for people suffering from osteoporosis [13].

As the femoral neck is a relevant and sensitive site for studying the degree of osteopenia, many researchers have investigated this location [4, 13, 19, 22]. The gold standard to determine bone strength associated with susceptibility to fractures is *ex vivo* biomechanical testing [7, 8, 14–16, 40, 42]. It has been shown that, in addition to bone structural parameters, such as bone density, bone architecture and bone geometry, the loading configuration also plays a non-negligible role in determining bone competence. The direction of the externally applied load on the femur may be an important factor in the etiology of hip fractures. In an *ex vivo* study on human femora, Pinilla et al. [37] showed that a moderate variation in the fall-related loading angle significantly reduced the failure load of the proximal femur. They found that failure load decreased by 24% as the loading angle changed from 0° to 30°. Using *in vivo* quantitative computed tomography (QCT)-based modeling of human femora, similar findings [10] showed that the bending strength of the femoral neck varied significantly for different external loading directions.

Although tests on human bones are the most pertinent when studying human bone properties and diseases, such as osteoporosis, the heterogeneity of the populations does not make them well suited to precisely assess the effects of loading direction on bone strength experimentally [2, 11, 14, 30, 31, 37]. The results are affected by differences in geometry and material properties between the samples. Indeed, the mechanical and material properties variability in human samples is often high, as they originate from people having different sizes, different ages, different lifestyles and different genetic backgrounds [33, 35, 39]. This heterogeneity can be largely avoided using animal models, especially when using inbred strains [3, 6, 17, 23, 29, 34, 41]. Animal models complement and extend human studies by allowing close control of environmental factors, by expanding the characterization of phenotypes underlying bone strength and by facilitating breeding strategies to identify genetic linkage [44]. Rat and mouse inbred strains with relevant biological phenotypes related to disease models may provide important genetic clues that will improve the efficiency of identifying genes underlying bone strength. Raising inbred strains for mechanical studies allows having populations with exactly the same age, identical genotypes and constant environmental factors, including diet

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and daily activity. This results in very low variability in bone properties. Consequently, the difference in mechanical properties as measured in experimental testing can be assumed to mostly depend on variations in the boundary conditions. As an illustration of the concept of using inbred strains, Wergedal et al. quantified the variations for bone properties within several inbred strains, including the B6 strain, and showed that they were very small [47, 48]. Furthermore, Jamsa et al. and Mashiba et al. also performed similar femoral head loading tests on B6 and other inbred strains femora and their results also showed small variations in measured mechanical properties, as well as in bone geometry [23, 32].

Although engineering principles convey that loading direction will affect bone stiffness and will most likely affect bone strength, this has never been quantified. Therefore, the aim of this study was to quantify the influence of sample positioning on stiffness and strength in the proximal femur. Specifically, we investigated inbred female C57BL/6He mice. This inbred strain is commonly used in bone phenotype studies [1, 9, 20, 24, 25, 27, 28, 32, 38, 45].

Materials and methods

Overall description

Femoral head loading tests were performed to quantify the effect of loading direction on biomechanical parameters of the proximal femur. First, we tested an aluminum phantom of the murine femur, in which the femoral head was loaded under different directions. The aluminum phantom permitted an excellent control of the testing variability, because this test was not affected by variations in material properties or geometry. Second, we developed an alignment device as well as a new positioning protocol for murine femora. After developing this protocol, we performed identical tests, as with the aluminum phantom, to investigate the influence of loading directions on real proximal femur stiffness and strength.

Mechanical testing of the aluminum phantom

An aluminum phantom of a mouse femur was machined using laser technologies (Figure 1). The phantom geometry was an extrusion along the anteroposterior axis of a 2D shape (Figure 1, bottom). The phantom was embedded in polymethylmethacrylate (PMMA) and mounted on a ball-socket joint support, which was locked during loading. This support allowed compressive tests of the proximal femur loaded at the femoral head with different angles of inclination in frontal and sagittal planes (Figure 1). Before testing the aluminum sample, the compliance of the fixture was tested. The deformation of the fixture during testing was two orders of magnitude smaller than the phantom deformation and therefore negligible. The tests were performed on a materials testing machine (model 1456, Zwick GmbH and Co., Zwick GmbH, Ulm, Germany). We preloaded the sample with 1 N, the loading rate was 0.5 mm/s and the maximal load was limited to 25 N in order not to exceed the elastic limit of the phantom and to prevent permanent damage while

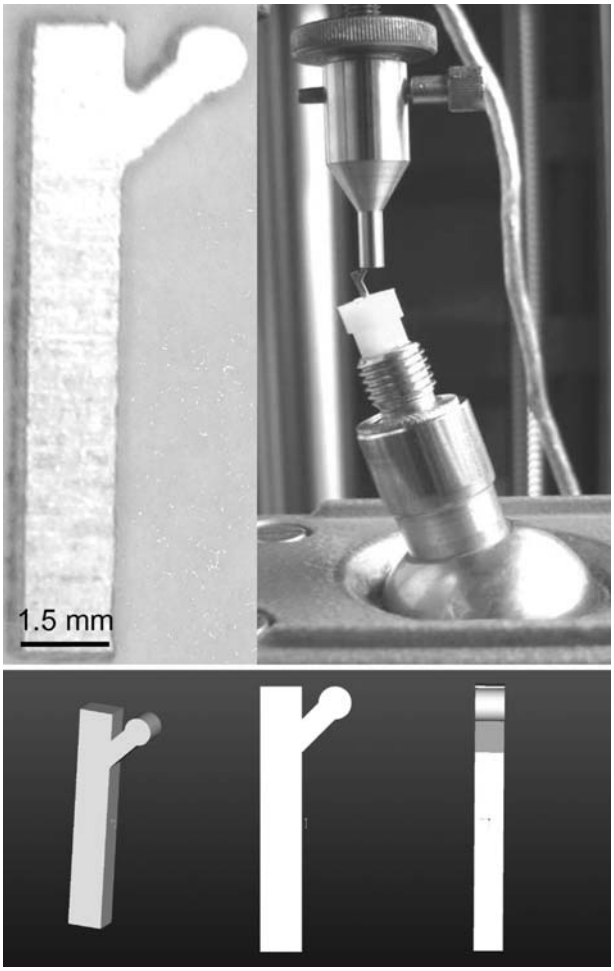

Figure 1 (Left) Aluminum phantom of a murine femur. (Right) Phantom embedded in PMMA and mounted on the ball-socket joint support. Here, the phantom is tested under a positive loading angle of 20°. The alignment in the second plane is neutral. (Bottom) 3D visualization of the phantom with two projections. The phantom geometry is an extrusion along the anteroposterior axis of a 2D shape.

repeating the loading test for each angular configuration. For each orientation of the phantom, tests were repeated three times. During the compressive loading of the aluminum phantoms, we measured force and displacement from which stiffness was computed. Due to the symmetry in the geometry of the aluminum phantom (Figure 1, bottom), along the anteroposterior direction in the sagittal plane, only inclination angles were tested from 0° to 45° with 5° increments in this direction. In the frontal plane, the inclination was varied from -40° to 40°, with 5° increments.

We also analytically computed the maximal bending moment along the midshaft of the phantom relatively to the loading angle. In the analytical model, the phantom was modeled as two beams, one for the midshaft and one for the neck.

Sample alignment

For sample alignment, we dissected left and right femora from 19 fresh frozen 16-week-old C57BL/6He (B6) female mice. Use of mice in this research project was reviewed and approved by the local Institutional Animal

Care and Use Committee (IACUC). Femora were cut above the condyles, resulting in a length of 11 ± 0.5 mm. Using a stereomicroscope, the femora were positioned in a custom-made Plexiglas alignment device (developed and manufactured in-house) (Figure 2). Online registration of the bones in the alignment device was made with a CCD camera (Canon, Tokyo, Japan) attached to the stereomicroscope and connected to a personal computer. Then, using a template that we applied directly on the computer screen, we checked and corrected the sample alignment until it matched the template.

We quantified bone alignment from the camera image on the computer screen. Quantitative assessment of bone alignment was performed in three measurements. First, we measured the angle α in the transverse plane of the femoral neck in the alignment device (Figure 3). The reference points to define this angle were the third trochanter and the center of the femoral head. Then, we determined the angles β and γ of the femoral shaft in the frontal and sagittal planes, respectively. To measure these two angles, we used as reference points the top of the main trochanter and the center of the shaft diameter at the cut position (Figure 3). Finally, the absolute angle δ between the vertical axis and the femoral shaft was trigonometrically computed from β and γ. After the bones were positioned, they were embedded with cyanoacrylate glue (Superglue, UHU Schweiz AG, Schönenwerd, Switzerland) into aluminum bone holders which could then be rigidly fixed in the testing device.

Biomechanical testing of murine proximal femur

For biomechanical testing, we dissected left and right femora from 29 fresh frozen 16-week-old B6 female mice. The animals were stored at -20°C and thawed at room temperature just before dissection of the femora. Left and right femora were meticulously prepared in exactly the same way as described in the "Sample alignment for mechanical testing" section. On the magnified images of the camera, we measured the femoral neck-

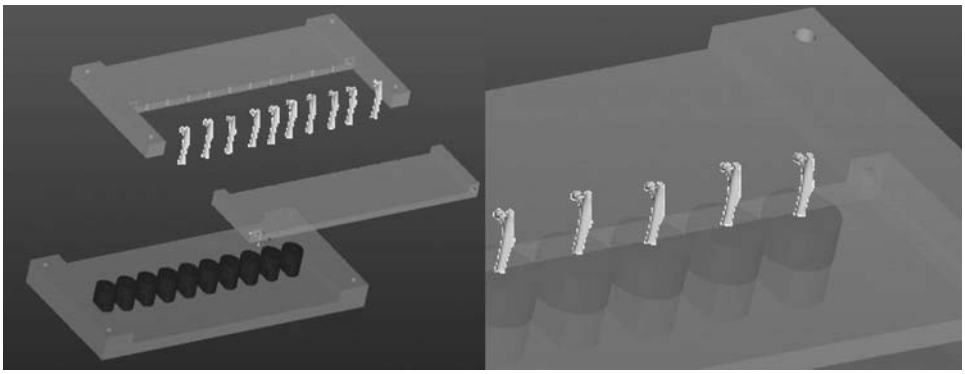

Figure 2 (Left) Exploded view of the alignment device with 10 murine femora ready to be aligned and 10 aluminum cylinders where the femora will be glued. (Right) Detail of the alignment device with aligned bones clamped between the two upper Plexiglas plates.

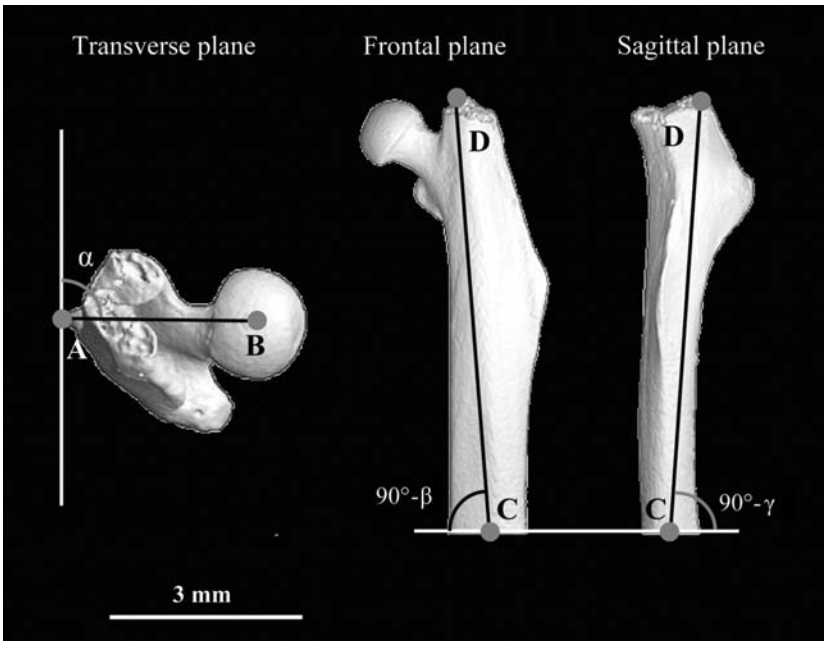

Figure 3 The angular alignment of each femur was measured in three planes.
(Left) Angular alignment α in the transverse plane. (Center) Angular alignment β in the frontal plane. (Right) Angular alignment γ in the sagittal plane. Anatomical landmarks were used as reference points to define femur orientation. A: Third trochanter; B: center of the femoral head sphere; C: center of the femoral shaft at the cut position; D: top of the trochanter.

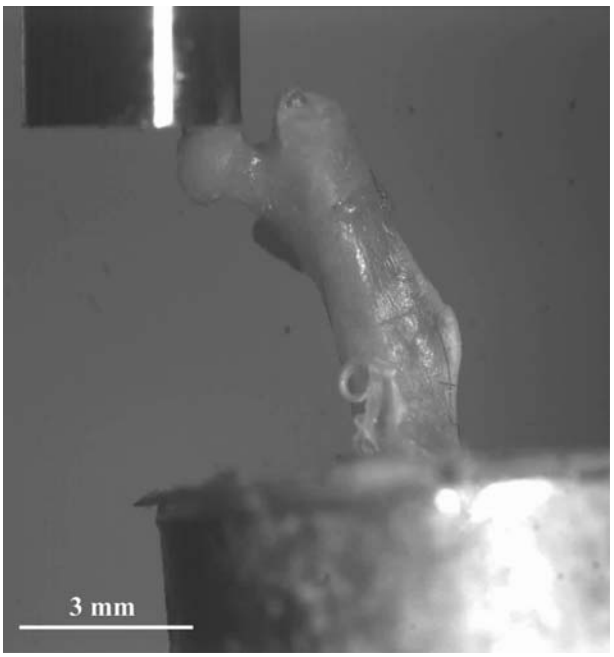

Figure 4 One B6 right femur positioned in the materials testing machine under a negative loading angle of -5° in the frontal plane.
The alignment in the second plane was neutral.

shaft angle (NSA angle) of the 58 B6 femora. The femora were then mounted on a ball-socket joint support, which allowed compressive tests of the proximal femur with different angles of inclination in the frontal plane (Figure 4). The ball-socket joint was locked during loading. Similarly as for aluminum phantom tests, the deformation of the fixture during testing was two orders of magnitude smaller than the bone deformation and therefore negligible. The 58 samples were divided into five groups. Each group was tested with a different inclination angle in the frontal plane ranging from -10° to 10°, with 5° incrementing. The samples were randomly distributed to each group. Originally, each group had similar numbers of left and right femora, but due to loss of samples during the experiments, the population in each group varied finally from 8 to 13 samples. Some samples were broken during the positioning preparation and others were excluded from the testing results due to poor embedding. The mechanical tests consisted of loading the femoral heads until fracture of the femoral neck occurred, using the same materials testing machine as for the aluminum phantom tests. The samples were pre-loaded with 1 N and load-displacement curves were recorded at a cross-head speed of 0.5 mm/s [43] until failure of the femoral neck. Stiffness was calculated as the slope of the linear part of the load-displacement curve. Strength was also measured as the maximum of the load-displacement curve.

Statistical analysis

In both aluminum phantom and murine bone testing, regression lines were computed to show the linear relationship between stiffness and angular position. In murine bone testing, such a regression line was also computed to illustrate the relationship between bone strength and angular position. The error in positioning alignment using the newly developed device was calculated with the root mean square (RMS) formula. Stiffness and strengths were compared between the five murine bone groups, with inclination angles ranging from -10° to 10°, by one-way analysis of variance (ANOVA) with a least significant difference (LSD) post-hoc analysis. All statistical analysis was performed with the statistical program SPSS 13.0 (The Apache Software Foundation, Chicago, USA).

Results

The compressive loading tests of the aluminum phantom showed an excellent reproducibility of the three measurements for each angle [coefficient of variation (CV) $=0.4\%$]. The relationship between stiffness and angular position in the sagittal plane was linear (Figure 5, $R^2=1$, $p<0.01$); 5° off-axis loading resulted in a stiffness loss of 8.5%. In the frontal plane, the stiffness had a maximum at 20° inclination (Figure 5). Around the neutral position of 0°, between -5° and 10°, the stiffness related linearly to inclination angle ($R^2=0.99$, $p<0.01$) and a change in inclination of 5° indicated a change in stiffness of 50%.

The Plexiglas alignment device was developed to simultaneously align 10 left or 10 right murine femora. Using this new device, the angle in the transverse plane was $\alpha=90°\pm3.2°$ (mean$\pm$SD) and the axial angle relatively to vertical was $\delta=89.6°\pm1.5°$ (mean$\pm$SD). The reproducibility errors (RMS) of α and δ were 3.2° and 1.5°, respectively.

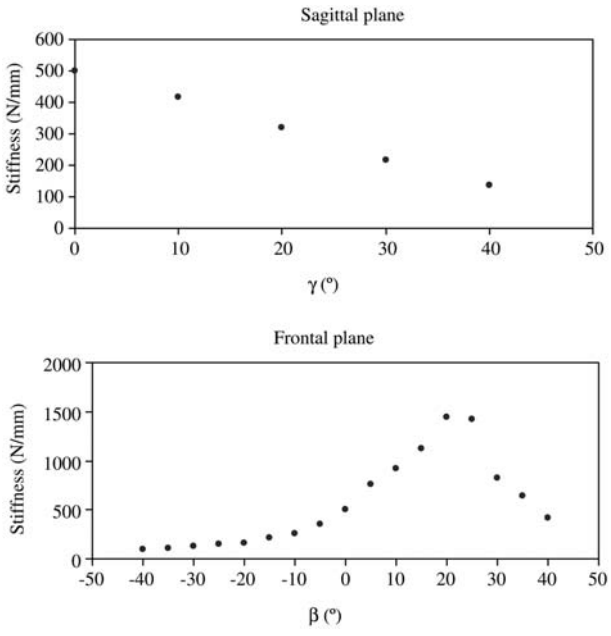

Figure 5 Stiffness of the aluminum phantom in the sagittal plane (top) and in the frontal plane (bottom).
The reproducibility of the measurements was so good that the error bars fall within the data point. (Top) The data were linearly distributed ($R^2=1.00$, $p<0.01$). Due to symmetry of the aluminum phantom, measurements were performed only for positive angles. (Bottom) The data were linearly distributed around the neutral position [-5°; 10°] ($R^2=0.99$, $p<0.01$). Stiffness was maximal at 20° inclination.

Measuring the NSA angle in 58 C57BL/6 femora, we found an average angle of 119.17°±1.96° and a CV of 1.64%.

The murine femora showed a significant influence of the inclination angle on the measured bone stiffness and strength in the frontal plane. Stiffness was significantly different between each group ($p<0.05$) (Table 1). The relationship between stiffness and angular position showed a strong linearity ($R^2=0.98$, $p<0.01$), just as for the aluminum phantom. Thus, the bones and the aluminum phantom demonstrated similar linear trends. The proximal femur stiffness rose linearly with the absolute femoral shaft angle δ (Figure 6). A 5° inclination in axial positioning around the neutral position of the B6 femora caused a 28.5% change in stiffness.

The maximal bending moment along the femoral shaft is illustrated in Figure 7.

Further, all the samples fractured at the femoral neck. Bone strength also showed a linear relationship with angular position ($R^2=0.86$, $p<0.01$). A 5° inclination in axial position around the neutral position of the B6 femora caused an 8.5% change in strength. So, strength was also affected by change in angular position, but less than stiffness. Further, strength was less variable than stiffness and ranged from 12.8 to 17.5 N, with a maximum CV of 10.5% at the neutral position 0° (Figure 6, Table 1).

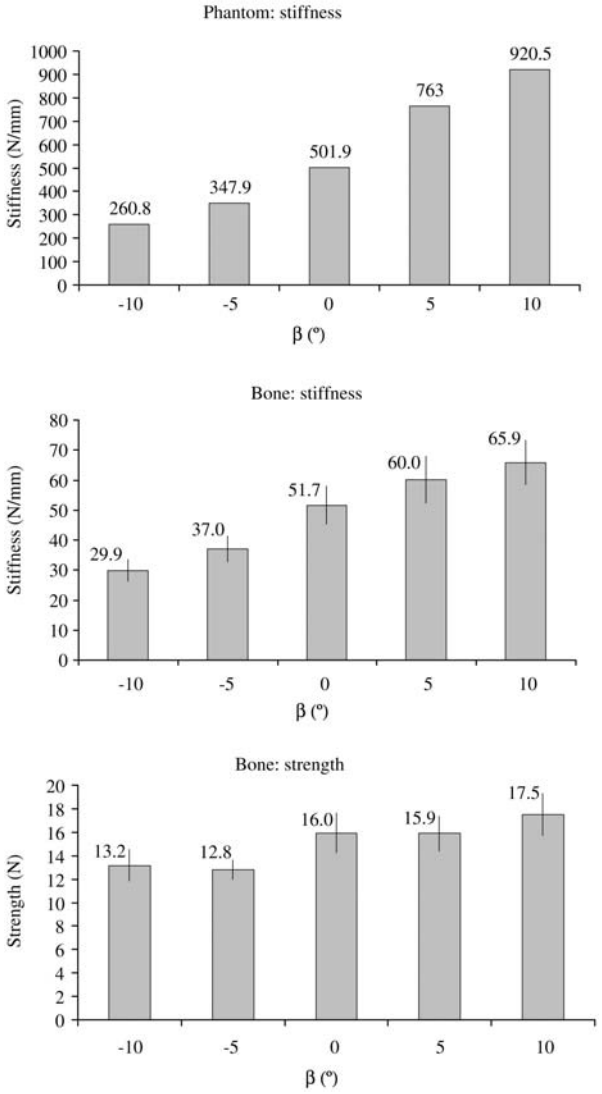

Figure 6 (Top) Stiffness of aluminum phantom plotted against the loading angle β in the frontal plane. The data are linearly distributed around the neutral position [-5°; 10°] ($R^2=0.99$, $p<0.01$). (Center) B6 proximal femur stiffness plotted for the five tested loading angles β in the frontal plane. The data were linearly distributed around the neutral position ($R^2=0.98$, $p<0.01$). (Bottom) B6 femoral neck strength plotted for the five tested loading angles β in the frontal plane. The data were linearly distributed around the neutral position ($R^2=0.86$, $p<0.01$).

Discussion

The aim of this study was to investigate the influence of sample alignment on experimentally determined bone stiffness and strength. We showed that accurate alignment is of utmost importance for accurate and precise determination of bone stiffness and strength; a 5° deviation from perfect vertical alignment led to a 28.5% deviation in estimated stiffness. The effects on strength were less dramatic, but with a change of 8.5% per 5° still remained considerable. To limit alignment errors, we developed and designed a new sample alignment protocol which was able to position murine femora with an absolute error (RMS) of only 1.5°.

For our parametric analyses, we used a mouse-inbred strain, which has the advantage that the individuals are genetically identical; hence, the femora are very similar.

As an example of the low variability in inbred strains, we measured the NSA angle. This measurement showed lower variety of geometrical properties in B6 inbred strains than in human populations (B6: 119.17°±1.96° and a CV of 1.64%, human: 127°±7° and a CV of 5.5% [46]). Therefore, NSA of B6 is lower than the general

Table 1 Femoral stiffness and strength of the proximal femur measured in five groups loaded under five different angles in the frontal plane.

Groups (loading directions)	-10°	-5°	0°	5°	10°
Stiffness					
Mean±SD (N/mm)	29.9±3.7	37±4.3	51.7±6.3	60±7.8	65.9±7.4
CV (%)	12.5	11.6	12.3	13	11.2
Strength					
Mean±SD (N/mm)	13.2±1.3	12.8±0.8	16±1.7	15.9±1.5	17.5±1.8
CV (%)	10.1	6.5	10.5	9.3	10.3
Population (n)	8	8	9	13	10

Stiffness and strength of the murine bones depended strongly on loading direction. All groups were significantly different for stiffness ($p<0.05$), as assessed by one-way ANOVA with LSD post-hoc analysis.

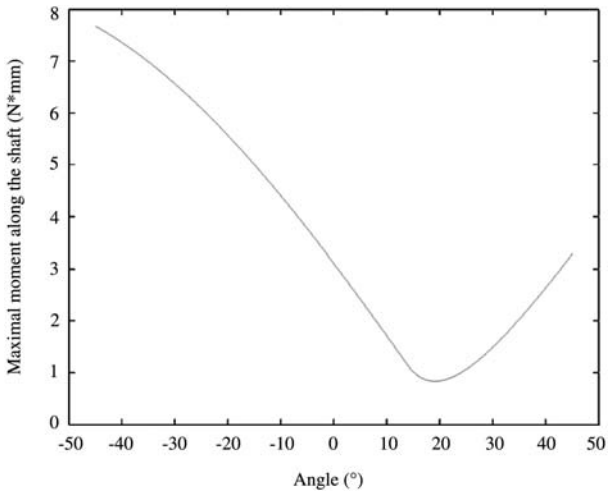

Figure 7 Maximal bending moment along the femoral shaft.

human population average; this is expected considering the differences in the human and mouse morphology and gait. Even more striking is the difference in the standard deviations. The variability is reduced almost four times compared to human populations. This further illustrates the lower variability in an inbred strain model than in human populations and the advantages that come with using this model.

But even femora from inbred mice show some biological variability. To exclude any variability, we also used an aluminum phantom of a mouse femur. Its material properties are well known and remain constant through a whole test series, as long as the deformations stay within the elastic domain. Therefore, the variations in the test results were only dependent on the changes in loading direction and were not disturbed by any other parameters.

The head of the aluminum phantom was loaded under different directions. The direction range was not chosen to address any physiological relevancy. Our testing setup allowed testing our samples in these extreme conditions. Testing between -20° and +20° would have been enough to observe the misalignment effects. Nevertheless, we presented the whole set of data for the sake of completeness. As expected, reproducibility of the phantom tests was close to perfection. The changes in stiffness due to variations of the loading direction were remarkably pronounced. For the same inclinations, the metallic phantom showed more marked variations in stiffness than the murine bones. Its custom-machined geometry as well as its different material properties made it stiffer than murine bones. Nevertheless, the phantom showed very similar behavior to different loading configuration as real bones. Furthermore, the perfect reproducibility of the tests on one unique sample made this model a very useful tool for our parametric research. Indeed, the changes in stiffness were directly related to changes in loading directions without any additional disturbance due to differences of geometry or material properties from one test to the other.

Stiffness was maximum when the phantoms were tested under 20° (Figure 5) The existence of a maximum coincides well with theories which maintain that femoral geometry and structures are optimized for very precise loading configurations [5, 21, 26, 49], and suggest that overall geometry of the femur is adapted to best resist physiological loading. At this maximum stiffness angle, the maximal bending moment along the femoral shaft had a minimum (Figure 7). This supports the hypothesis that anatomy and function of the hip mechanical system is optimized in a way that limits the highest bending stresses in the femoral shaft [5, 18, 36]. Moreover, Bergmann et al. [5] showed that the loading directions in the human femur for all daily activities varied in a very small range. In this study, we demonstrated a somewhat different view; the femur seems to have an optimized geometry for one specific loading direction. For this loading direction, the femur behaves stiffer and bending stresses in the whole bone are lower.

The angular error in the transverse plane, using the alignment device, is not so important for typical vertical compressive tests of the proximal femur, because, in this configuration, the femur is vertically oriented and the mechanical setup is axis-symmetric around the vertical axis. Of much more importance is the error (RMS) in the axial alignment angle δ of 1.5°. Taking the linear regression equation as calculated from stiffness against β (Figure 6), a 1.5° axial error resulting from the alignment procedure was then responsible for a 5% error in stiffness. This means that when testing proximal femur stiffness, variability of 5% is explained by the misalignment and the rest of the variability comes from actual differences in bone geometry and material properties. The use of our alignment device showed clear improvements in reproducibility as compared to other studies. The stiffness CV of the bone mechanical tests was between 11% and 13% for the different inclination groups (Table 1). These values mean that variability of 6–8% (11–13% minus 5%) comes from differences in bone geometry and material properties between the samples, as 5% are due to sample misalignment in the mechanical setup. This CV of 11–13% is much lower, hence, better than previously reported for this site in mice from the same inbred strain [43]. This previous study reported a CV of 27.5%, more than two times higher than in our study. Not only the stiffness, but also the strength had a smaller CV in our study (Table 1). For different loading angles, the CV of the strength was between 6.5% and 10.5%, which was better than the 13.5% in the aforementioned study [43]. These numbers demonstrate that a good alignment significantly lowers variability in stiffness and strength measurements.

We tested our bone samples from -10° to +10° of inclination in the frontal plane. Physiologically, it would make more sense to test from -5° to +15°. Nevertheless, this study did not aim to have any physiological meaning but strictly concentrated on possible misalignments during *in vitro* testing. Hence, we tried to show the influence of misalignment when testing the proximal femur under compression. We decided to test symmetrical misalignment, as during experiments, misalignment angles can randomly be either positive or negative. Our experimental tests revealed that small variations in alignment induced large changes in the measured stiffness. Around the neutral position of 0°, we measured a change in stiffness of

almost 30% for 5° axial inclination in the frontal plane (Figure 6). Small variations in alignment also provoked significant changes in bone strength. This narrow relationship between loading directions and stiffness or strength is particularly relevant, as femoral bone competence in inbred mice is a phenotype that is widely assessed. In such studies, large populations of mice are usually experimented within the same strain or between different strains. Comparing results between predefined groups and showing significant differences between treated or untreated animals, between different strains or between different models is often the main goal of these studies. Reducing variability in the results within each group makes the results more powerful, increases the significance of the study and therefore its impact. In the case of femoral bone testing, it is exactly what we achieved in reducing the variability caused by poor sample positioning.

Additionally, this study showed a predictable influence of the loading angle on sample stiffness. In the sagittal plane, the phantom showed a linear dependence between stiffness and loading angle (Figure 5). In the frontal plane, around the neutral position from -5° to 10°, stiffness also linearly correlated to inclination angle (Figure 5). Similar linear correlations were found for B6 proximal femur stiffness and loading angle around the neutral position (Figure 6). Strength also linearly correlated to inclination. Hence, we are now able to predict the influence of loading direction on femoral stiffness and strength. We showed this predictable influence for murine bone testing.

Because it is not possible to perform such a parametric study on human bones for population heterogeneity reasons, we proposed, in this study, to show and quantify the effects of misalignment, when testing the proximal femur, using a femur phantom and an animal model. These models permitted to drastically decrease the variations of most factors (morphometry and material properties) influencing the mechanical parameters so that the effect of misalignment could only be directly quantified. We do not claim that our results are directly relevant to human femoral testing. Nevertheless, our model demonstrated a linear relationship between misalignment and proximal femur stiffness and strength and indicated that such an influence should also be possible when testing other types of bone, e.g., human bone. Indeed, with the commonly accepted hypothesis that the murine model is an excellent model for bone testing and phenotyping [1, 9, 20, 24, 25, 27, 28, 38, 43, 45], such a predictable influence of loading inclination on bone stiffness is also likely to exist in the case of human femora.

Future studies, determining bone mechanical properties of healthy and osteoporotic human femora, should therefore include very accurate and reproducible sample preparation and alignment protocols, similar to the ones presented in this publication. In combination with results from Carpenter et al. or Pinilla et al. [10, 37], this study highlights the importance of loading direction in proximal femur mechanical testing. Changes in loading direction lead to drastic but predictable changes of bone structural capacity. Exact angles therefore need to be reported to be able to compare different studies.

In summary, the proximal femur is a very sensitive site regarding load direction in femoral head compressive tests. Small alignment errors generate significant changes in the femoral stiffness and strength. Furthermore, loading direction on the femoral head showed a very predictable influence on femoral stiffness. This influence plays a key role in femoral biomechanical testing, as different loading directions can potentially have higher effects on mechanical results than intrinsic differences in bone mechanical and material properties. This study was able to quantify these effects in a murine model and also highlighted the importance of accurate control of loading direction also in other bones, such as human femora.

Acknowledgements

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