

1 Original article  
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3 **Fatigue-caused damage in trabecular bone from clinical,**  
4 **morphological and mechanical perspectives**  
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## Abstract

1  
2 Bone quantity and quality are considered the main predictors of bone mechanical properties (*i.e.*,  
3 strength and fracture resistance). These factors deal with the morphology and chemical  
4 composition of bone and can be assessed by non-invasive techniques such as dual-energy x-ray  
5 absorptiometry (DXA), providing the bone mineral density (BMD) and the trabecular bone score  
6 (TBS). These parameters, and in particular BMD, are currently used as clinical predictors of  
7 fracture risk but do not provide information regarding the fatigue life. Bone is continuously  
8 subjected to fatigue loading and fatigue-induced damage can be crucial in fragility fractures. To  
9 probe the effect of fatigue-induced damage on bone microarchitecture and elucidate the effect of  
10 such damage on the bone clinical parameters, we combined fatigue testing on *ex-vivo* porcine  
11 trabecular bone samples with DXA measurements and  $\mu$ CT imaging. In addition, we performed  
12 interrupted cyclic tests at different load levels and measured fatigue-induced damage  
13 accumulation in the form of stiffness degradation. We also highlighted the change of clinical and  
14 microstructural parameters during the accumulation of fatigue-induced damage in interrupted  
15 fatigue tests. Our results suggest that the parameters obtained from the current non-invasive  
16 diagnostic protocols (*i.e.*  $\mu$ CT and DXA) are not able to assess the amount of fatigue-induced  
17 damage. This can be due to the fact that such techniques provide global parameters, whereas  
18 fatigue-induced damage is a local phenomenon, closely connected to the microarchitecture.

19

20 **Keywords:** Fatigue loading, Bone damage, Trabecular bone score, Bone mineral density, DXA,  
21  $\mu$ CT-imaging.

## 1 1. INTRODUCTION

2 Bone provides support to human and animal bodies, also protecting organs and enabling  
3 mobility. It is well known that aging and diseases or traumatic events [1-3] or static and fatigue  
4 loadings [4] significantly affect the fracture resistance of bone. Yet, it is not completely  
5 understood whether bones have more tendency to fracture due to fatigue or static loadings [5].  
6 The first clear evidence of fatigue-induced fractures emerged about one century ago [1-3] when  
7 fractures occurred in athletes [6] or military recruits [7] exposed to periods of long and hard  
8 training. Currently, another type of fatigue-induced fracture is becoming more common owing to  
9 an ever-aging population. In healthy bones, accumulation of microdamage under cyclic loading  
10 is a slow process, which also stimulates the remodeling, promoting repair [5]. Conversely, in  
11 elder or osteoporotic bones (*i.e.*, a bone disease with significant bone loss) the faster growth of  
12 fatigue microdamage or the slower repair causes failures [8]. Accumulation of microcracks and  
13 diffuse damage, which are the consequences of both single static overloading events [9-17] and  
14 repetitive physiological loadings [2, 18-22] can lead to the degradation of mechanical properties  
15 [11, 23-26]. Understanding the different kinds of damage and their effect on bone strength and  
16 life is complex, yet, the knowledge of the fatigue behavior of bones has become a paramount  
17 concern owing to the population's longer lifespan.

18 Fatigue-induced damage can impair bone strength and play a crucial role in fragility fractures.  
19 *In-vivo* fatigue (destructive) testing are precluded and *ex-vivo* experiments are generally used to  
20 evaluate fatigue-induced damage for bones. Previous studies have addressed this topic  
21 considering the whole vertebra [27] or focusing on the cortical bone [22, 28-30]. Few studies  
22 [31, 32] focused on the fatigue behavior of the cancellous tissue showing that the fatigue curve

1 (S-N curve) of trabecular bone tissue is not dependent on the site and the species [31], and that  
2 the fatigue strength is correlated with age and BMD (bone mineral density) [32].  
3 Today, the most widely adopted clinical parameters for the assessment and prediction of bone  
4 fracture risk are BMD and TBS (Trabecular Bone Score [33-35]) measured via the dual-energy  
5 x-ray absorptiometry (DXA) and bone quantity and quality, respectively. Although BMD and  
6 TBS are currently used as predictors of bone fragility [36-38], there is no clear evidence of  
7 significant correlation between these parameters and the fatigue life. Also, it is not clear how  
8 these parameters are affected by cyclic loading and fatigue-induced damage. A low BMD is  
9 generally considered a predictor of bone fragility. Yet, both younger and more senior individuals  
10 with the same level of BMD have shown different fracture risks [39, 40] and over half of all non-  
11 vertebral fractures observed in people above 55 occurred to those with a normal BMD [41].  
12 Thus, bone quality, which can be influenced by the amount of microdamage and its accumulation  
13 with aging [19], can also play a crucial role in the occurrence of bone fatigue fractures [42, 43].  
14 In this study, to demonstrate the effect of fatigue-induced damage on both the 3D-bone  
15 microarchitecture and the bone clinical parameters, we combined fatigue testing under uniaxial  
16 compressive loading on *ex-vivo* porcine trabecular bone samples with DXA measurements and  
17  $\mu$ CT imaging. Additionally, we performed interrupted cyclic loading at different load levels, to  
18 highlight the life-trend of the microstructural and clinical parameters. We hypothesized that  
19 fatigue-induced continuum damage in the form of stiffness degradation is significantly correlated  
20 to the microarchitectural morphology of trabecular bone. Moreover, based on Wöhler analysis,  
21 we developed two fatigue models accounting for the following parameters: *i*) specimen-specific  
22 effective area, measured via  $\mu$ CT, *ii*) specimen-specific initial elastic modulus, and *iii*) BMD.

1 The choice of testing porcine vertebrae was motivated by the fact that the morphology is similar  
2 to the human one [44] and spines are amongst the skeletal sites, which are more commonly  
3 affected by fractures.

## 4 **2. MATERIALS AND METHODS**

5 We focused on trabecular tissue taken from porcine vertebrae of similar age and weight and  
6 unknown sex. The experimental procedures followed in this study are listed below and described  
7 in different subsections:

- 8 1) Sample preparations from lumbar vertebrae taken from porcine lumbar spines;
- 9 2) Analysis of undamaged samples via
  - 10 a) DXA scanning;
  - 11 b)  $\mu$ CT imaging;
- 12 3) Mechanical testing on bone samples
  - 13 a) Preliminary quasi-static compression tests, to obtain an initial indication of the  
14 appropriate force amplitudes and to define the stop criterion in the fatigue tests;
  - 15 b) High-cycle fatigue tests;
  - 16 c) Interrupted cyclic tests followed by DXA scanning and  $\mu$ CT imaging;
- 17 4) Statistical analysis;

18 Thirty specimens were tested in this study and divided into three groups, *i.e.* three specimens for  
19 the quasi-static compression tests, twenty-one specimens for fatigue tests and six specimens for  
20 the interrupted cyclic tests. One specimen, subjected to interrupted cyclic loading (force level  
21 360 N), was removed from the data analysis due to catastrophic damage during the test.

22

### 23 **2.1 Sample preparation**

1 Six porcine lumbar spines with six lumbar vertebrae (L1 to L6) were collected from a local  
2 butcher. The spines belonged to 12-18-month-old animals, of unknown sex, and about 120 kg  
3 weight. The spines were stored at  $-18^{\circ}\text{C}$  until sample preparation and experimental tests. Each  
4 vertebra was separated from the spine by handsaw cutting at the inter-vertebral discs. Using a  
5 manual hand drill, a cylindrical specimen (diameter,  $D$ , 16 mm and length 35 mm) was cored out  
6 of each vertebra, along the vertebrae axis. The bone sample was then transferred to a lathing  
7 machine to reduce the diameter,  $D$ , to 14 mm. The length of the specimen,  $L_0$ , was reduced to 22  
8 mm with a circular saw (Hitech Europe, rpm =  $1000\text{ min}^{-1}$ ). During the preparation steps, the  
9 specimens were kept hydrated by adding the saline solution. Thirty cylindrical specimens were  
10 prepared for mechanical testing. To eliminate the boundary effects and improve the force  
11 transmission, the specimens were glued into custom-made aluminum end caps using Loctite  
12 496<sup>®</sup>. Before the adhesive bonding was applied, the end caps were cleaned for about two minutes  
13 in an ultrasonic bath, then rinsed with acetone. The internal surfaces of the end caps were  
14 roughened using sandpaper (grit size #320) to increase the bonding strength between the internal  
15 surface of the end caps and the bone tissue. Both ends of the bone specimens were defatted with  
16 the acetone before glueing. After glueing the bone specimen ends into the end caps, the samples  
17 were kept at ambient temperature for 24 hours to ensure a complete curing of the glue. Finally,  
18 the specimens were frozen again at  $-18^{\circ}\text{C}$  until the next steps. The end caps, having an internal  
19 diameter of 14 mm and an external diameter of 20 mm, covered 3 mm of the cylindrical-shape  
20 specimens. The effective length between the gripping holders was  $L_{eff} \approx 16\text{ mm}$ .

21

## 22 **2.2 Analysis of undamaged samples**

### 23 **2.2.1 DXA scanning**

1 DXA scanning was performed on the trabecular bone specimens at the Bone Metabolic Unit of  
2 Nuclear Medicine of the Fondazione IRCCS Ca' Granda-Ospedale Maggiore Policlinico, using a  
3 Hologic Discovery A system (Hologic Inc, Marlborough, Massachusetts, USA, software version:  
4 13.3.0.1.3). BMD was measured through the APEX software installed on the same machine.  
5 TBS was automatically calculated using the software provided by the Medimaps Group  
6 (Wilmington, US) and on the same machine. The scans were performed using the posterior-  
7 anterior lumbar spine option with a pixel size of  $0.5 \times 0.5 \text{ mm}^2$ . Four trabecular bone specimens  
8 were placed in the scanning machine in each batch, and the total scanning time was about two  
9 minutes. The samples were kept frozen ( $-18^\circ\text{C}$ ) before and after scanning, to prevent any  
10 deterioration of the bone microstructure. In a post-processing analysis, BMD and TBS were  
11 calculated by the manual selection of the region of interest of the bone scans. This setup resulted  
12 in the exclusion of the aluminum stand from the bone scans. The segmentation method in DXA  
13 scanning being a manual procedure, we performed the measurement three times to evaluate the  
14 segmentation effect on our results. From each set of scans, BMD and TBS were obtained for the  
15 bone specimens. DXA scanning was performed on all the samples before and after the  
16 mechanical fatigue tests, and at the beginning of each interrupted fatigue step.

17

### 18 **2.2.2 $\mu\text{CT}$ imaging**

19  $\mu\text{CT}$  imaging of the bone specimens was performed on trabecular bone specimens using an X-  
20 ray Metrology CT system (X25, North Star Imaging Inc., Buckinghamshire, UK) with the spatial  
21 resolution of  $25.6 \text{ }\mu\text{m}$ . The scanning parameters were fixed at 60 kV and  $150 \text{ }\mu\text{A}$ . Three  
22 specimens, submerged in the saline solution, were placed simultaneously in the scanning  
23 machine and each scan took 110 minutes. Image reconstruction was performed with the x-view

1 CT software. Post-processing of the images was carried out using ImageJ [45] and BoneJ plugin  
2 [46]. A Gaussian blur filter (standard deviation of the Gaussian distribution 1.5) was used to  
3 remove the noise from the images. Afterward, the scans were converted to gray-level 8-bit  
4 images. The gray-level images were segmented by the Otsu local thresholding method [47],  
5 resulting in binary images with the voxel value of 1, for bone, and 0, for the empty spaces.  
6 Conventional morphological parameters were calculated for each specimen for a cylindrical  
7 region of interest (ROI). These parameters included: bone density ( $BV/TV = \frac{\text{bone volume}}{\text{total volume}}$ ); bone  
8 porosity, ( $\rho_p = 1 - \frac{BV}{TV}$ ); trabecular thickness ( $Tb.Th$ ) and trabecular spacing ( $Tb.Sp$ ),  
9 calculated based on the conventional definition of the greatest sphere that fits within the structure  
10 [48]; bone surface ( $BS$ ), defined as the inner surface of the bone material and calculated by the  
11 summation of the triangulated mesh area obtained from the isosurface creation (with a  
12 resampling equal to 1) [49]; trabecular ellipsoid factor ( $Tb.EF$ ), which locally distinguishes  
13 between rod- or plate-like trabeculae [50]; connectivity density ( $Conn. D.$ ), which determines the  
14 number of 3D-connected trabeculae; the degree of anisotropy ( $DA$ ), which defines the main  
15 microstructural orientation of bone [51, 52]

16

## 17 **2.3 Mechanical testing**

### 18 **2.3.1 Quasi-static compression tests**

19 Three specimens were tested under monotonic compression loading (MTS machine Alliance,  
20 RF/150 with a load cell of 150 kN, class 1 ISO 7500-1) to obtain the yield stresses and strains  
21 based on the 0.2%-strain criterion. In the light of these results, a displacement stop criterion,  
22 equal to 2 mm, was set to end the fatigue tests. The corresponding yield forces were calculated



1 from the measured yield stresses. A typical force-displacement curve for the monotonic  
2 compression tests is shown in Figure S1 of the supplementary document.

3

### 4 **2.3.2 Fatigue tests**

5 High-cycle fatigue (HCF) testing was performed under force control using an MTS 810 servo-  
6 hydraulic testing system equipped with an MTS 661.20F-03 load cell (maximum load capacity  
7 100 kN), with an accuracy of 20 N. The fatigue testing time being demanding, we created a  
8 proper testing environment: during loading, specimens were submerged in saline solution (NaCl  
9 0.9%) using a custom-designed aquarium. The gripping set-up was endowed with a pressure disc  
10 and pressure stamp. The pressure disc had a polished and greased spherical surface fitted to the  
11 pressure stamp. Three circumferential pre-tension springs were used to pull the pressure disc into  
12 the stamp. This allowed the pressure disc to be moved before the test and to be adaptively  
13 aligned to the specimens during mechanical testing. It also resulted in smooth force transmission  
14 and reduced peak stress, due to partial contact between the end cap and the pressure disc  
15 surfaces.

16 After performing the preliminary tests, we set a load frequency of 10 Hz, which is higher than  
17 the frequency of normal walking (*i.e.* 2 Hz [53, 54]). Indeed, in our preliminary tests, we  
18 observed a significant increase in the temperature of the saline solution when the test lasted  
19 longer than 15 hours. The increase in temperature could go beyond 37 °C, which is higher than  
20 the body temperature, for the samples tested at the frequency of 2 Hz. Therefore, we chose 10 Hz  
21 load frequency to avoid the significant change of temperature during fatigue loading. The  
22 tolerated change in the temperature was 2K for these fatigue tests.

1 Specimens were randomly chosen for being tested in the fatigue loading, and various force  
2 amplitudes were considered for such tests. Four levels of force amplitude,  $F_a$ , (*i.e.*, 360, 450, 540  
3 and 720 N) were chosen. These values were selected to be below the yield forces determined in  
4 monotonic compression tests. Compressive cyclic loading was determined by the force  
5 amplitude,  $F_a = \frac{F_{max} - F_{min}}{2}$ , the mean force,  $F_m = \frac{F_{max} + F_{min}}{2}$ , and the force ratio,  $R = \frac{F_{min}}{F_{max}} =$   
6 0.1, where  $F_{max}$ , and  $F_{min}$  were the maximum and minimum force, respectively.

7 From  $\mu$ CT imaging, the nominal area,  $A_{n,i}$ , calculated by using the diameter at  $i^{\text{th}}$  stack of the  
8 image, and relative bone density,  $BV/TV_i$  at the same  $i^{\text{th}}$  stack were calculated. Thereafter, for  
9 each stack of the image the effective area was calculated as  $A_{eff,i} = A_{n,i} \times BV/TV_i$ . That led to  
10 the definition of the nominal stress,  $\sigma_n = \frac{F}{A_n}$ , and the effective stress,  $\sigma_{eff} = \frac{F}{A_{eff}}$ , where  
11  $A_n = \min \{A_{n,i}\}$  and  $A_{eff} = \min \{A_{eff,i}\}$ . The nominal and the effective stress amplitudes were  
12 calculated as  $\sigma_{a,n} = \frac{F_{max} - F_{min}}{2A_n}$  and  $\sigma_{a,eff} = \frac{F_{max} - F_{min}}{2A_{eff}}$ , respectively.

13 The longitudinal strain was defined as  $\varepsilon = \frac{\delta}{L_{eff}}$ , where  $\delta$  is the measured displacement from the  
14 actuator linear variable differential transformer (LVDT). We performed a preliminary study  
15 where we removed the saline solution tank and attached an extensometer (MTS 632.26F-23) to  
16 the specimen. In this test, the bone sample had a total length of 25 mm, whereas the  
17 extensometer on the specimen was installed over a length of 8 mm. The comparison between the  
18 axial strain, obtained from the direct measurement of the extensometer, and the axial strain,  
19 obtained from LVDT, showed a 3.5% difference. It was not possible to install an extensometer  
20 due to space constraints in the experimental set-up. During the fatigue loading, the displacement  
21 amplitude varied from 0.05 to 0.10 mm, on the edge of the resolution of the LVDT (0.01 mm).

1 The elastic modulus was calculated as follows,  $E_i = \frac{\sigma_{max} - \sigma_{min}}{\varepsilon_{max} - \varepsilon_{min}} = \frac{F_{max} - F_{min}}{\delta_{max} - \delta_{min}} \times \frac{L_{eff}}{\bar{A}_{eff}} = \frac{K_i L_{eff}}{\bar{A}_{eff}}$ ,  
2 where  $K_i$  is the stiffness at the  $i^{\text{th}}$  hysteresis loop, measured from the slope of the force-  
3 displacement curve, between the maximum and minimum force, and the corresponding  
4 displacement at that hysteresis loop, and  $\bar{A}_{eff} = \text{mean}\{A_{eff}\}$ . The initial elastic modulus,  $E_0$ ,  
5 was calculated with the average effective bone area,  $\bar{A}_{eff}$ , since the total material response for  
6 the overall displacement of the sample was measured. The accumulation of damage at the  $i^{\text{th}}$   
7 hysteresis cycle,  $D_i$ , was calculated as  $D_i = 1 - \frac{E_i}{E_0}$ .  
8 Fatigue tests were stopped either when the maximum displacement reached 2 mm or when the  
9 cycles exceeded  $N > 8 \times 10^5$ . Force, displacement, and time were recorded from the machine  
10 with a sampling rate of 10 measurements per second. During testing, the temperature of the  
11 saline solution was kept approximately constant (34-36 °C). Finally, 21 specimens were tested  
12 under fully compressive fatigue loading.

### 13 2.3.3 Interrupted fatigue tests

14 In the interrupted fatigue testing, we stopped each test twice before reaching the life cycle end-  
15 condition. Three levels of force amplitude, *i.e.*, 360, 450, and 540 N (equivalent to nominal stress  
16 amplitude of  $\sigma_n = 2.37, 2.96, \text{ and } 3.56$  MPa, respectively) were selected. We adopted a cycle-  
17 based stop criterion depending on the chosen force amplitude:  $N_{stop} = 1 \times 10^3$ , for  $F = 360$  N;  
18  $N_{stop} = 3.6 \times 10^3$ , for  $F = 450$  N;  $N_{stop} = 2.4 \times 10^5$ , for  $F = 540$  N. At each stopping point,  
19 clinical and morphological parameters were measured via DXA and  $\mu$ CT, respectively, following  
20 the protocols described in Section 2.2.1 and Section 2.2.2. Six specimens were tested for the  
21 interrupted cyclic loading, *i.e.*, two specimens per each force level. The total number of life

1 cycles was then added to the data of fatigue life testing. Therefore, the total number of the  
2 specimens considered for the fatigue curve is 27.

3

#### 4 **2.4 Statistical analysis**

5 A balanced Latin square approach was used for the design of mechanical fatigue testing.  
6 Statistical analysis was carried out in MATLAB<sup>®</sup> (R2015a) and SAS 9.2, and a  $p$ -value  $< 0.05$   
7 was assumed as the significant level.

8

### 9 **3. RESULTS AND DISCUSSION**

#### 10 **3.1 Fatigue life**

11 Descriptive statistical data for morphological and clinical parameters for the pooled data are  
12 given in Table 1. Figure 1a shows the S-N curve of the trabecular bone samples, considering the  
13 nominal stress amplitude,  $\sigma_{a,n}$ , and the number of loading cycles to failure,  $N_f$ . The pooled data  
14 are given in Table 2. It is evident that the obtained data are scattered and do not have any specific  
15 trend across the loading cycles. To obtain a suitable description of the fatigue behavior of  
16 trabecular bone, we defined an effective area,  $A_{eff}$ , which takes into account the porosity of the  
17 bone tissue and, consequently, allowed us to evaluate an effective stress,  $\sigma_{a,eff}$ . We normalized  
18 the effective stress amplitude to the initial elastic stiffness of each specimen. The resulting  
19 normalized stress *vs.* life ( $\frac{\sigma_{a,eff}}{E_0} - N_f$ ) trend is depicted in Figure 1b, together with the literature  
20 trends [21, 31]. The comparison in Figure 1b shows similar fatigue life behavior for human,  
21 bovine, and porcine trabecular bone, which follow the relationship  $\frac{\sigma_{a,eff}}{E_0} = aN_f^b$  with  $a = -0.01$   
22 and  $b = -0.1$  ( $R^2 = 83.34\%$ ,  $p < 0.001$ ) (Figure 1b). The porcine results are, however, closer  
23 to the bovine ones.

1 We also calculated the stress amplitudes based on the mean effective area,  $A_{\text{eff,mean}}$ , and the  
2 nominal area,  $A_{\text{nominal}}$ , of the trabecular specimens (Figure S2 of the supplementary document).  
3 We found a higher correlation between the normalized stress amplitudes and the fatigue life of  
4 the trabecular bone when considering the minimum effective area (Figure 1b and Figure S2 of  
5 the supplementary document).  
6 Several authors [21, 27, 30, 32, 55, 56] have shown similar evidence of fatigue life trend for  
7 trabecular bone under compressive loading. The proposed models are based on Coffin-Manson  
8 equations, for low-cycle fatigue, and Wöhler or Basquin equations, for high-cycle fatigue. In  
9 some cases, the authors considered the F-N trend [27, 32] and normalized the force with respect  
10 to the ultimate tensile strength [27] or with respect to different correction factors (*i.e.*, sample  
11 area, sample strength estimation based on age and BMD, and applied load) [32]. In other cases,  
12 the authors considered the S-N curve and normalized the nominal stress by the pre-fatigue elastic  
13 modulus [31]. Instead, we proposed a model based on the effective stress,  $\sigma_{\text{eff}}$ , which showed a  
14 significant correlation with the fatigue life cycles. Similar normalizations were performed with  
15 respect to BMD (Figure 1c) and TBS (Figure 1d), where the effective stresses showed a  
16 significant correlation with the fatigue life cycles. The linear regression showed a significant  
17 correlation between BMD and the life cycles (Figure 2a) and no correlation between TBS and  
18 the life cycles was observed (Figure 2b).

### 19 **3.2 Failure modes and failure region**

20 We observed three principal types of fracture: *i*) a diagonal fracture, generally occurring in the  
21 top region of the sample and being 45°-oriented (Figure 3a), *ii*) an orthogonal fracture to the  
22 longitudinal direction, generally occurring in the mid-region of the sample (Figure 3b), and *iii*) a  
23 splitting fracture, generally occurring as a lateral separation of part of the trabecular bone

1 sample, causing a buckling-like failure (Figure 3c). These failure modes are in agreement with  
2 previous findings in literature [57]. In particular, the transverse failure represents a brittle-like  
3 failure and is generally associated with trabeculae orthogonally oriented to the loading direction.  
4 A diagonal failure can be considered as a ductile-like failure. Buckling-like failure, instead, is  
5 common to oblique trabeculae and may cause a longitudinal splitting.

6 We calculated the BV/TV trend along with the specimen height,  $L_{eff}$ , and showed its changes  
7 during the interrupted fatigue tests (Figure 4). We noticed that the location of the catastrophic  
8 failure corresponds to the zone with the lowest BV/TV value (Figure 4a). This finding also  
9 shows agreement with a previous literature study [58]. After the final rupture, the BV/TV  
10 increased suddenly in the region where the failure occurred, while its value remained almost  
11 constant in other parts of the specimen (Figure 4b). This can be explained as trabecular bone can  
12 recover large amounts of deformation after an overload [59-61].

### 13 **3.3 Variation of the clinical, morphological, and mechanical parameters**

14 The local increase in BV/TV, caused by failure (*i.e.*, the local collapse of the struts), also  
15 increased the mean value of BV/TV for each specimen. This finding is in line with the  
16 experimental results of our previous study where different morphometric parameters of pre- and  
17 post- quasi-static damage were measured in porcine trabecular bone [62]. This trend is more  
18 obvious for all the specimens subjected to the interrupted fatigue testing (Figure 5a): failure  
19 always corresponds to an increase in BV/TV. In particular, we measured an increase (about 20%)  
20 in the BV/TV between the failed samples and the undamaged ones. A 20% decrease in *Conn. D*  
21 was observed after a catastrophic failure (Figure 5b), which confirms the local breakage of the  
22 trabeculae and the damage accumulation under cyclic loading. The drop in *Conn. D* with  
23 increasing life cycles and damage was almost consistent for all the specimens. As expected, we

1 did not observe any change in BMD (Figure 5c) as we prevented any chemical deterioration  
2 during fatigue loading by keeping the specimen in wet conditions. TBS seemed decreasing  
3 (Figure 5d), which is consistent with the *Conn. D.* trend. Both the TBS and the *Conn. D.* can be  
4 considered morphometric parameters, which provide information on bone microarchitecture.  
5 Indeed, according to previous studies, there is a correlation between TBS and *Conn. D.*, where  
6 high values of both indicate the presence of a better skeletal tissue, whereas low values are a  
7 symptom of a weaker skeletal texture or a degraded microarchitecture [63, 64]. However, being  
8 TBS obtained from BMD, it is limited by its two-dimensional nature and cannot capture the  
9 three-dimensional microarchitecture. Besides, TBS is affected by the size and position of the  
10 samples [65, 66]. These limitations could explain the non-clear trend observed for the TBS with  
11 respect to the *Conn. D.*

12 The stiffness degradation was measured during the interrupted fatigue loading, providing a  
13 mechanical damage parameter,  $D$  (Table 2). Our results showed the bone samples reached about  
14 50% damage at the end of the interrupted tests (Figure 5e). Different models have been used, in  
15 the literature, for the prediction of the accumulated damage *vs.* life cycles, such as those  
16 proposed by Chaboche [67], Griffin et al. [68] and Pattin et al. [22]. These models showed that  
17 the (rate of) damage accumulation in bones could also be influenced by the level of applied  
18 cyclic stresses [69, 70] and other geometrical features of bone [71, 70]. From our experimental  
19 results, we found out that the level of applied cyclic stress could affect the rate of damage as  
20 fatigue cycles progressed. We calculated the parameters of the damage model using a similar  
21 procedure to the one proposed by Griffin et al. [68] for the prediction of damage in the interstitial  
22 bone of cortical tissue (Equation 1):

$$23 \quad D = k_2 \sigma_a^q [1 - \exp(-k_1 (N/N_f))] \quad (1)$$

1 Where,  $k_2 = 0.62, k_1 = 0.36, q = 0.36$  are coefficients of the model obtained from the non-  
2 linear least square fit on the interrupted fatigue tests (Figure 5e).

3 In this study, we measured an average BMD and TBS for each specimen and did not include the  
4 local variation of these measurements. This is due to the nature of the current densitometry  
5 technique that provides us average values. These results could be useful for life prediction if the  
6 microstructures of bone were uniformly distributed. The heterogeneity of BMD and its effect on  
7 the fatigue life of vertebra have been shown in a previous study [72], suggesting that a local  
8 BMD and a local minimum of BV/TV [58] could provide a better representation of the fatigue  
9 life estimation of trabecular bone.

10 The testing protocol used in this study was similar to those proposed in the literature [21, 2, 20,  
11 32, 31, 73] for the mechanical fatigue testing of trabecular bones. Therefore, during the fatigue  
12 testing, the trabecular specimens were kept in the normal saline solution. We did not  
13 quantitatively measure whether keeping the bone specimens in such solution during the fatigue  
14 testing could chemically degrade bones or influence their mechanical properties. Recent study  
15 has shown that storing bones for less than 3 days in the saline solution does not significantly  
16 affect the mechanical properties of bone [74]. From our results we also did not observe a  
17 significant change in BMD before and after testing showing no significant change in the  
18 chemical composition of bones.

19 Although it has been shown that microcracks are significantly correlated to the fatigue life of  
20 cancellous bone [2], we did not quantify the accumulated microdamage under fatigue loading  
21 owing to the intrinsic limitations of our instruments. Indeed, the micro damage cannot be  
22 captured by the clinical measurements as the microcrack average length is less than 100  $\mu\text{m}$  [75-  
23 77], which is below the resolution of DXA images. The fatigue-induced microdamage generally



1 accumulates in the older interstitial part of the cancellous bone and form longer microcracks  
2 [20]. Our results evidenced that other diagnostic methods, such as microindentation [78, 79] and  
3 high resolution quantitative computed tomography (HRQCT), are required for the invasive  
4 detection of microdamage in bone [80].

5

#### 6 **4. CONCLUSION**

7 This study aimed at investigating the effect of fatigue-induced damage on bone microarchitecture  
8 and the characteristic bone clinical parameters, by combining fatigue testing on *ex-vivo* porcine  
9 trabecular bone samples, DXA measurements, and  $\mu$ CT imaging.

- 10 • The predictive model for the description of the fatigue life of trabecular bone was  
11 obtained considering the specimen-specific effective area—measured by  $\mu$ CT—and the  
12 BMD showed a good comparison with recent literature results.
- 13 • The  $\mu$ CT imaging showed that the sub-regions with minimum BV/TV values are better  
14 predictors of the location of mechanical failure in trabecular bone than averaged-  
15 specimen BV/TV values, confirming that failure is a local phenomenon.
- 16 • Interrupted cyclic loading coupled with  $\mu$ CT showed that damage accumulation occurs  
17 locally, causing a sudden increase in the local and global BV/TV and a drop in *Conn. D*  
18 with increasing life. The local variations of bone volume fraction and bone  
19 microarchitecture, calculated from  $\mu$ CT images, suggest a need for the introduction of  
20 local sensitive parameters for BMD and TBS rather than an average value.

21 The outcome of this study suggests that the current invasive and non-invasive diagnosis  
22 protocols, *i.e.*,  $\mu$ CT and DXA, respectively, are not able to quantify the fatigue-induced damage.

23 Fatigue-induced damage is a local phenomenon, and for its characterization new local

1 parameters, able to detect punctual variations of bone mass and microarchitecture, need to be  
2 defined and validated.

3

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7 for helping in specimen cutting.

8

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9

1 **Figure captions**

2 **Figure 1.** a) Semi-log plot of four force (nominal stress) amplitudes applied for the prediction of  
3 the fatigue life. b) Fatigue life curve for the normalized stress amplitude with respect to the  
4 initial elastic modulus and its comparison with fatigue life of bovine trabecular [21] and human  
5 vertebrae [31]. The plot is shown in log-log and  $a = -0.01$ ,  $b = -0.1$  are the regression  
6 coefficients for  $\frac{\sigma_{a,eff}}{E_0} = aN^b$  (solid line). Three samples tested at the nominal stress amplitude  
7  $\sigma_{a,n} = 2.37$  MPa did not fail after 1 million cycles (runouts). The ratio of stress amplitude to the  
8 BMD (c) and TBS (d) exhibit a significant correlation with the fatigue life of the porcine  
9 trabecular bone.

10

11 **Figure 2.** Clinical parameters BMD (a) and TBS (b) and the fatigue life cycles shown in the log-  
12 log form. A significant linear correlation was found between BMD and fatigue life cycle while  
13 no significant correlation was found for TBS vs. fatigue life cycle.

14

15 **Figure 3.** Three different failure modes were observed in the mechanical fatigue loading where  
16 either oblique or straight macroscopic cracks propagated in the trabecular bones. The position of  
17 the fracture generally occurred at the top (a), center (b), or the lateral part (c) of the specimens.

18

19 **Figure 4.** a) The distribution of the BV/TV along with the effective length of the specimen,  $l_{eff}$   
20 before testing (left) and fractured specimen under interrupted fatigue tests. The fracture starts at  
21 the zones with the lowest BV/TV. b) BV/TV trend through the effective length between the  
22 grips,  $l_{eff}$ , after different interrupted cyclic loading, the local BV/TV increased after a  
23 catastrophic failure in the weakest regions. BV/TV as calculated as an average of every eight



1 stacks of images from  $\mu$ CT images. The interrupted-1, 2, 3 and 4 refer to the number of cycles at  
2 which the fatigue tests were stopped.

3

4 **Figure 5.** Change in a) BV/TV, b) connectivity c) BMD, d) TBS and e) accumulation of damage  
5 in the interrupted mechanical fatigue tests for five different samples under various force  
6 amplitudes and life fraction,  $\frac{N}{N_f}$ . The grey lines for (a-d) show the changes in the corresponding  
7 parameter in one specimen and are plotted to guide the eye. To eliminate the effect of load  
8 levels, the parameters in (a-d) are normalized to the effective stresses. The fitting line in (e) is  
9 based on Equation (1).

10

11

1 **Table captions**

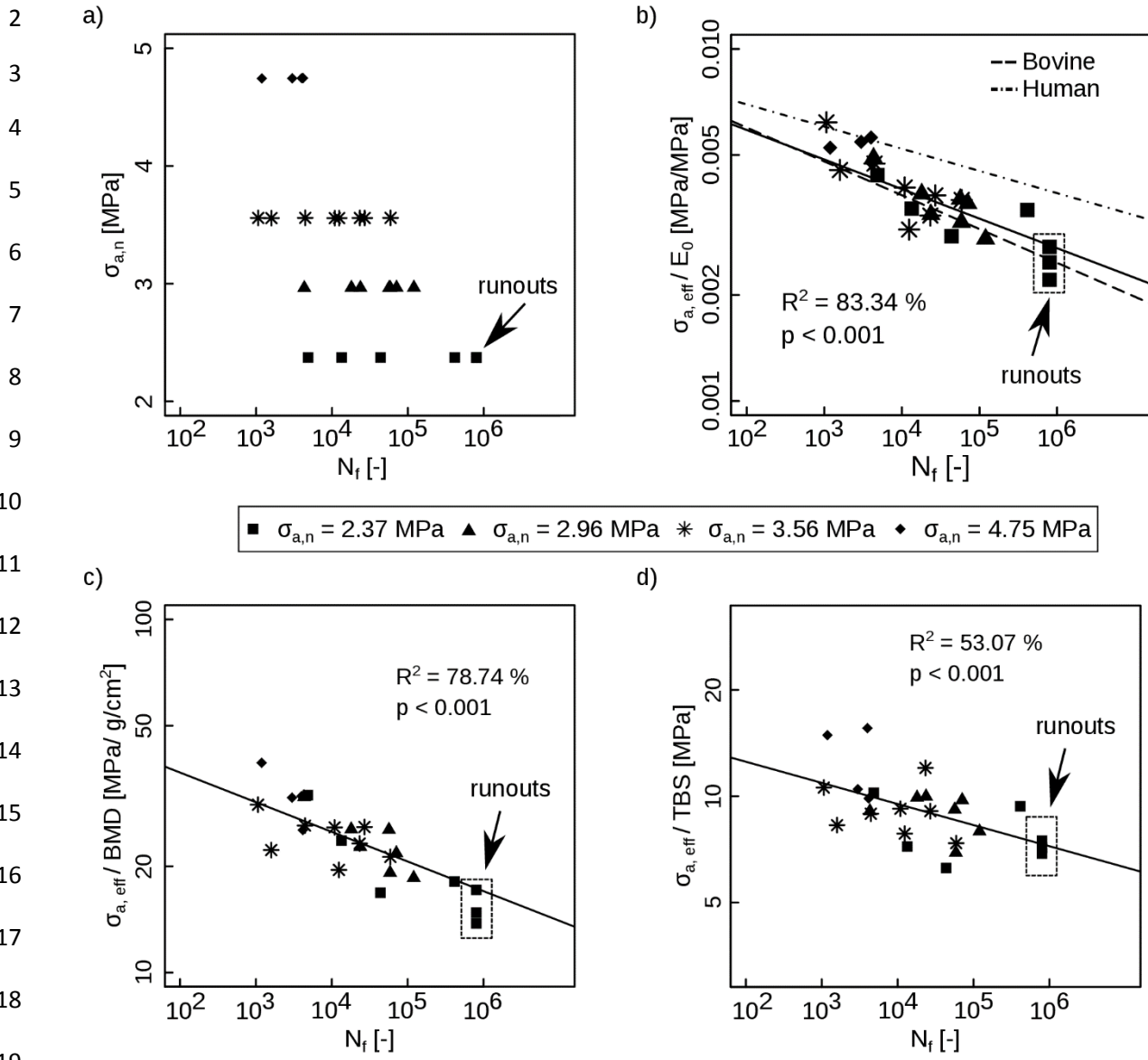
2 **Table 1:** The mean  $\pm$  standard deviation for the clinical and morphological parameters of pooled  
3 data.

4 **Table 2:** The mean, standard deviation (SD), minimum and maximum for the initial elastic  
5 stiffness,  $E_0$ , stress amplitude,  $\sigma_A$ , fatigue life,  $N_f$ , and accumulated damage,  $D_f$ .

6

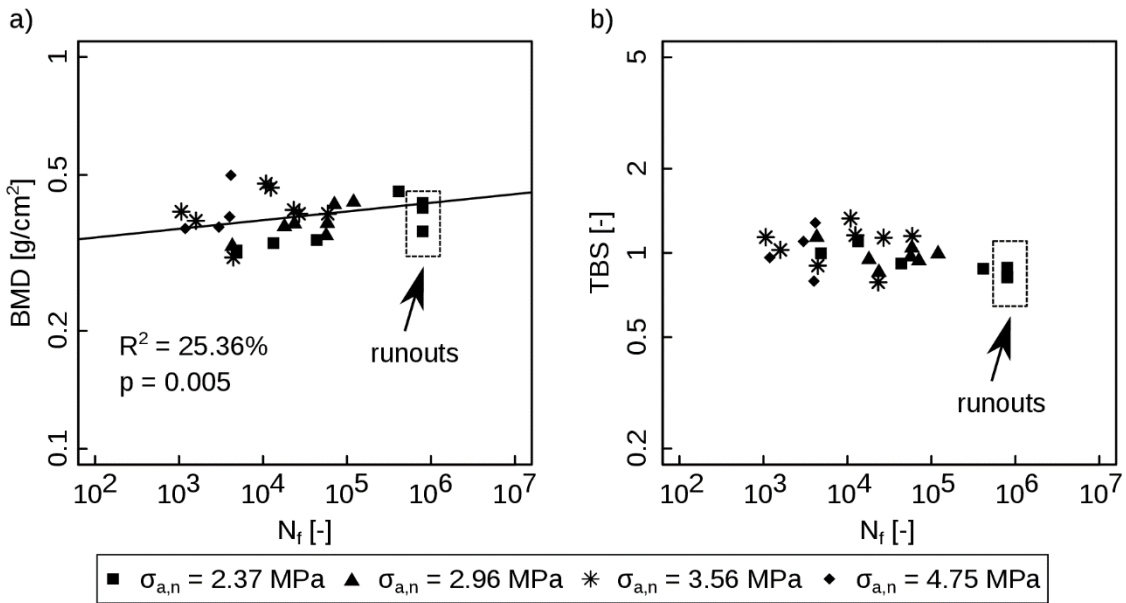
7

1 **Figure 1**



1 **Figure 2**

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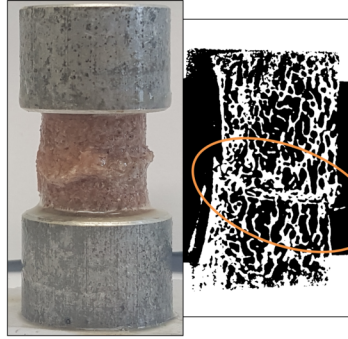
1 **Figure 3**

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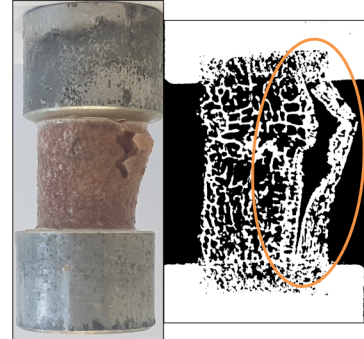
a) Diagonal fracture



b) Orthogonal fracture



c) Splitting fracture



1 **Figure 4**

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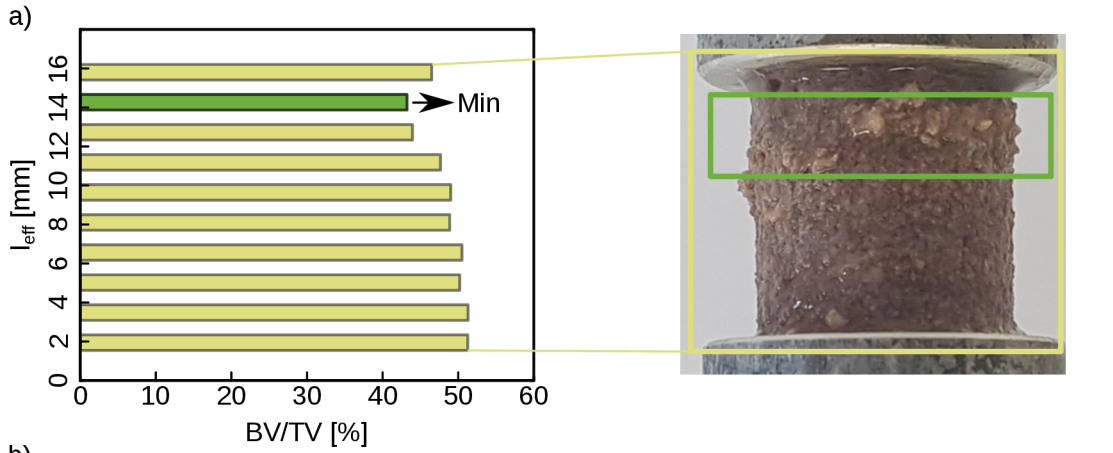
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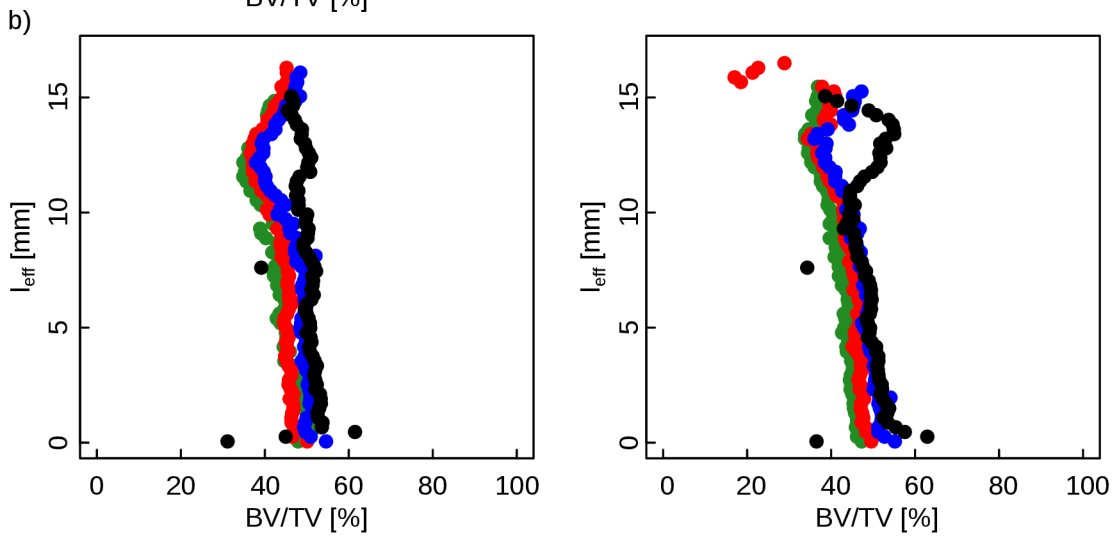
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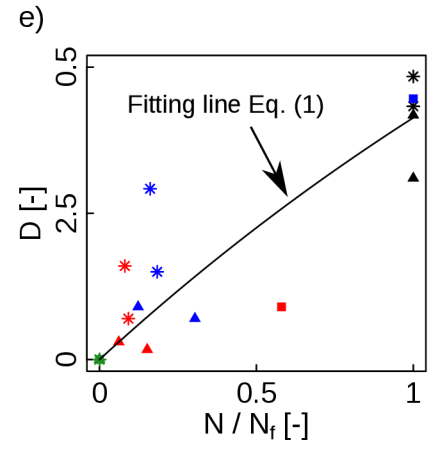
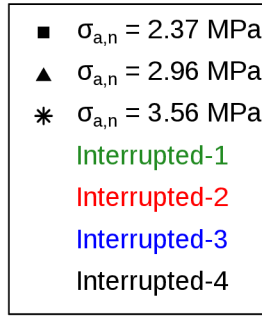
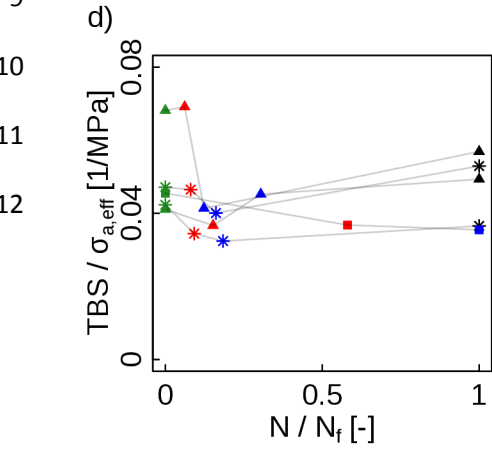
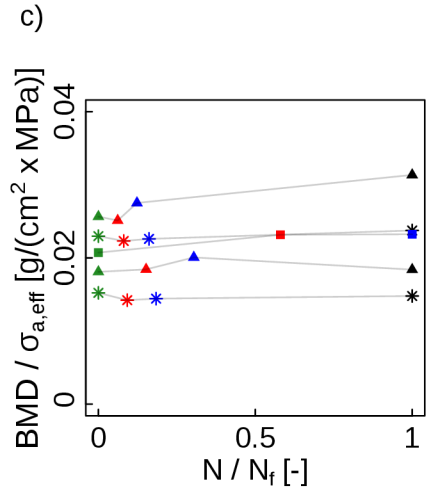
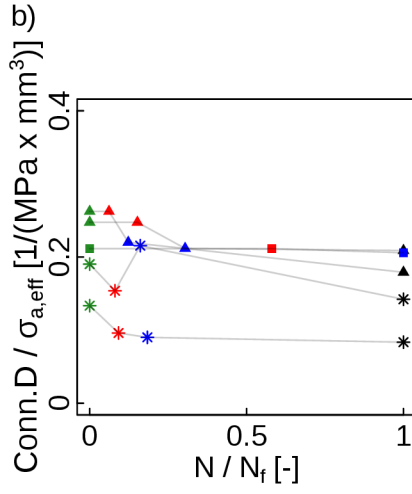
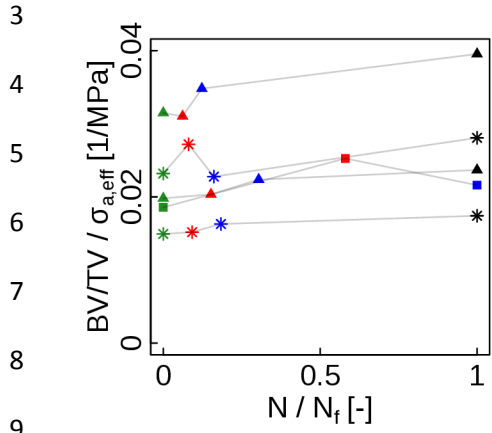


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1 **Figure 5**

2 a)



1 **Table 1**

Morphological parameters	
<i>BV/TV</i> [%]	43.60 ± 3.66
<i>BS/TV</i> [1/mm]	4.10 ± 0.36
<i>BS/BV</i> [1/mm]	9.44 ± 0.91
<i>Tb.Th</i> [mm]	0.27 ± 0.03
<i>Tb.EF</i>	-0.03 ± 0.03
<i>Conn.D</i> [1/mm <sup>3</sup> ]	2.78 ± 1.02
<i>DA</i> [-]	0.52 ± 0.09
Clinical parameters	
BMD [g/cm <sup>2</sup> ]	0.38 ± 0.05
TBS	1.03 ± 0.14

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1 **Table 2**

	Mean	SD	Minimum	Maximum
$E_0$ [MPa]	2360	340	1700	3030
$\sigma_a$ [MPa]	10	3.1	5.7	17.7
$N_f$	$1.4 \times 10^5$	-	1	$8.0 \times 10^5$
$D_f$	0.39	0.07	0.24	0.50

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