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Cite this article: Müller R *et al.* 2019 Analysis of microscopic bone properties in an osteoporotic sheep model: a combined biomechanics, FE and ToF-SIMS study. *J. R. Soc. Interface* **16**: 20180793.
<http://dx.doi.org/10.1098/rsif.2018.0793>

Received: 24 October 2018

Accepted: 14 January 2019

Subject Category:

Life Sciences—Physics interface

Subject Areas:

biomechanics, biophysics, computational biology

Keywords:

sheep model, osteoporosis, biomechanics, μ -CT, ToF-SIMS, finite-element analysis

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Electronic supplementary material is available online at <http://dx.doi.org/10.6084/m9.figshare.c.4373525>.

Analysis of microscopic bone properties in an osteoporotic sheep model: a combined biomechanics, FE and ToF-SIMS study

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The present study deals with the characterization of bone quality in a sheep model of postmenopausal osteoporosis. Sheep were sham operated ($n = 7$), ovariectomized ($n = 6$), ovariectomized and treated with deficient diet ($n = 8$) or ovariectomized, treated with deficient diet and glucocorticoid injections ($n = 7$). The focus of the study is on the microscopic properties at tissue level. Microscopic mechanical properties of osteoporotic bone were evaluated by a combination of biomechanical testing and mathematical modelling. Sample stiffness and strength were determined by compression tests and finite-element analysis of stress states was conducted. From this, an averaged microscopic Young's modulus at tissue level was determined. Trabecular structure as well as mineral and collagen distribution in samples of sheep vertebrae were analysed by micro-computed tomography and time-of-flight secondary ion mass spectrometry. In the osteoporotic sheep model, a disturbed fibril structure in the triple treated group was observed, but bone loss only occurred in form of reduced trabecular number and thickness and cortical decline, while quality of the residual bone was preserved. The preserved bone tissue properties in the osteoporotic sheep model allowed for an estimation of bone strength which behaves similar to the human case.

1. Introduction

Osteoporosis is a widespread disease characterized by loss of bone mass and a reduction in trabecular number and thickness that leads to a significantly increased fracture risk [1]. For an optimized bone defect treatment with next-generation implant materials, a detailed knowledge of the mechanical and physiological properties of the osteoporotic bone and its composition is essential.

Several *in vivo* and *ex vivo* techniques are applied to describe bone quality from the macroscopic to the microscopic scale [2]. Throughout the manuscript, a length scale pertaining to bone samples or whole bones is referred to as *macroscopic*, while a length scale below the size of a trabecula is referred to as *microscopic*. Thus, macroscopic properties and measurements on trabecular bone are dependent on both the effects of trabecular structure (e.g. relative bone volume) and

tissue properties. By contrast, microscopic properties and measurements are dependent on tissue properties only. In order to be consistent, in the following the apparent bone strength is denoted as macroscopic bone strength.

On the macroscopic scale, the diagnosis of osteoporosis is mainly made by dual-energy X-ray absorptiometry (DEXA) measurements [3]. The obtained bone mineral density (BMD) and the *T*- and *Z*-score values derived from BMD are the prevailing parameters in clinical routine [4]. DEXA measurements do not reveal any insights into the trabecular structure or the microstructure of bone. Quantitative computed tomography (QCT) offers three-dimensional information at the macroscopic level and the local volumetric BMD, but at the expense of higher X-ray dose. The continuous change of the mechanical bone properties under osteoporotic conditions on the macroscopic scale is mostly measured by bending and compression tests [1].

Micro-computed tomography (μ -CT) with a resolution of 10 μ m or better provides insights into the microscopic three-dimensional bone structure [2,5]. To assess bone tissue quality on the microscopic scale, time-of-flight secondary ion mass spectrometry (ToF-SIMS) is a suitable technique [6,7]. Owing to the high spatial resolution of up to 100 nm and the simultaneous detection of calcium and collagen distributions [8], ToF-SIMS complements the μ -CT measurements. In a comprehensive study on rodents with induced osteoporosis, ToF-SIMS enabled the determination of the osteoporotic stage in combination with a semi-quantitative evaluation of the mineral content. Within this study, pathological changes and significant reduction in the mineral content under osteoporotic conditions and a strong heterogeneity of mineralization were observed [9].

As bone remodelling is activated by mechanical load, the elastic properties also have to be considered [10,11]. The microscopic mechanical properties are accessible by a number of direct measurement methods like bending and tensile tests [12,13] or nano-indentation [14–16]. In addition, ultrasonic methods are used [13,17]. The indirect estimation of an averaged microscopic Young's modulus by a combination of compression tests and finite-element analysis (FEA) was demonstrated by Ladd *et al.* [18] for human bone. Using a similar procedure, recently, remarkable osteoporosis-induced decay of the microscopic Young's modulus was found in an osteoporotic rat model [19].

Knowledge of mechanical and chemical bone properties is important in human patients as well as for animal models resembling human osteoporosis. Basic research on osteoporosis is usually done on rodent models, but large animal models are needed to reach experimental and mechanical conditions close to the human situation [20]. Besides undemanding keeping and handling [21], sheep turned out to be the most applicable animals as the bone forming units, the harversian system and remodelling process patterns are similar to those in humans [22]. It is the non-rodent large animal model recommended by the Food and Drug Administration (FDA) to study postmenopausal osteoporosis [23]. Furthermore, sheep models have been established to study osteoporosis and osteoporotic fractures after induction via ovariectomy combined with calcium and vitamin D2/3 deficient diet as well as steroid administration [24–26]. The sheep model described in this study was recently used to assess the role of osteocytes in bone remodelling in loaded and unloaded bone regions [27]. While

osteoporosis-induced changes of macroscopic properties in the sheep model are well known [24,25,28], the development of microscopic bone properties during osteoporosis is scarcely documented.

Therefore, the aim of the present study is to evaluate the evolution of bone properties during osteoporosis in a sheep model at the microscopic tissue level rather than the macroscopic scale. Herein, we present a combination of biomechanical testing, μ -CT and FEA, to evaluate the elastic properties of trabecular bone tissue in a sheep model of postmenopausal osteoporosis. In addition, for the first time, ToF-SIMS is applied to gain spatially resolved information about the inorganic and organic composition of the sheep bone.

As guidance, table 1 provides descriptions of terms and variables used throughout the following sections.

2. Material and methods

2.1. Osteoporotic sheep model

The study was conducted in strict accordance with the European Union legislation for the protection of animals used for scientific purposes and approved by the district's Animal Ethics Committee 'government presidium of Darmstadt, Germany; permit no. Gen. Nr. F31/36'.

2.2. Experimental design

Twenty-eight sheep were divided into four groups with a mean age of 5.5 years: (i) non-operated sham group (control, $n = 7$), (ii) bilaterally ovariectomized group (O, $n = 6$), (iii) bilaterally ovariectomized and treated with special diet deficient of calcium and vitamin D group (OD, $n = 8$), and (iv) triple treatment group, which received glucocorticoid treatment (ODS, $n = 7$) in addition to the treatment received in OD. A detailed description of the treatment can be found elsewhere [27]. After eight months, animals were euthanized and lumbar vertebral samples were explanted. A detailed description of the experimental design can be found in the electronic supplementary material.

2.3. Dual-energy X-ray absorptiometry measurements

An *in vivo* DEXA scan of the lumbar vertebrae in the anterior–posterior direction was obtained directly prior to euthanasia using the scanner and software from Lunar Prodigy version 13.40; GE Healthcare, Darmstadt, Germany. Data and further details were published earlier in [32] and can be found in the electronic supplementary material.

2.4. Micro-computed tomography protocol

Cylindrical samples from the centre of the vertebra body (L1, length $h = 10$ mm, diameter $d = 8$ mm) were stored in phosphate buffer and imaged using the μ -CT system SkyScan 1173 (Bruker Micro-CT, Kontich, Belgium). Post-processing was done following the guidelines for assessment of bone microstructure [5]. Scanning and reconstruction parameters can be found in the electronic supplementary material.

Treatment-induced changes in bone morphology were quantified by relative bone volume, trabecular separation, trabecular thickness, trabecular number, surface to volume ratio, and structure model index (cf. table 1). Visualization of μ -CT data was done using Paraview software [33]. After μ -CT imaging, samples were stored at -20°C .

2.5. Compression tests

Subsequent to μ -CT imaging, uniaxial compression tests were conducted on the samples. They were defrosted for 1 h and

Table 1. Definition and description of terms and variables used in the article.

term, variable	description	abbreviation	unit
stiffness constant	measure of the rigidity of a <i>body</i> . $k = dF/ds$ (Hooke's Law, force F , deformation s) [29]	k	$N\ m^{-1}$
Young's modulus	measure of the ability of an isotropic <i>material</i> to withstand changes in length under lengthwise tension or compression [29]	E	Pa
Poisson's ratio	ratio between transversal and axial strain [29]	ν	dimensionless
ultimate stress	stress at which a material or structure breaks down	σ_{ultim}	Pa
structure length	geometrical coupling between averaged Young's modulus E and stiffness k . $S = k/E$ [19]	S	m
Pistoia criterion	fracture is assumed to occur if 2% of the bone tissue is strained beyond a critical limit of 7000 microstrain [30]		
relative bone volume	ratio between bone tissue volume and total volume of the region of interest [5]	BV/TV	%
bone surface to bone volume ratio	ratio of bone tissue surface to its volume [5]	BS/BV	$1\ mm^{-1}$
trabecular thickness	mean thickness of trabeculae [5]	Tb.Th	mm
trabecular separation	mean distance between trabeculae [5]	Tb.Sp	mm
trabecular number	average number of trabeculae per unit length [5]	Tb.N	$1\ mm^{-1}$
structure model index	an indicator of the structure of trabeculae; SMI will be 0 for parallel plates and 3 for cylindrical rods [5]	SMI	dimensionless
Pearson correlation coefficient	measure for linear correlation between two variables [31]	R	dimensionless

then processed in wet state at 25°C. Samples were placed between the plate and the punch of the testing machine and compressed in longitudinal direction until failure (materials testing machine Z10, Zwick, Ulm, Germany).

A typical force–displacement curve obtained from the compression test is shown in figure 1 (red). The curves for compression of trabecular bone samples can be divided into three parts: in the first nonlinear part (I), loose tissue is compressed and remaining non-alignment between sample surface and compression stamps is eliminated. Then a linear part (II) with mainly elastic deformation follows. In the last part (III), non-linear behaviour is observed, where irreversible destruction of tissue starts. In the linear part, the bone sample exploits linear elastic behaviour. The slope $k = dF/ds$ remains approximately constant and represents the stiffness.

The macroscopic ultimate strength of the sample is approximated by

$$\sigma_{ultim} = \frac{4F_{max}}{\pi d^2}. \quad (2.1)$$

The macroscopic Young's modulus of the cylindrical sample with height h and diameter d is

$$E_{macro} = k \frac{4h}{\pi d^2}. \quad (2.2)$$

For more details and limits of this test procedure, see the electronic supplementary material.

2.6. Time-of-flight secondary ion mass spectrometry

In ToF-SIMS, the sample surface is bombarded with a Bi_3^+ -primary ion beam, leading to the emission of secondary ions which are analysed by a ToF analyser. By scanning the primary ion beam over the sample surface, the distribution of the chemical compounds is obtained. Measurements were performed with a TOF-SIMS 5–100 machine (ION-TOF Company, Germany) equipped with a 25 kV Bi-cluster ion gun for surface analysis. Measurements were recorded with spectrometry mode, which

implies high mass resolution (full width half maximum of about 8000 at $m/z = 29.003$) and a lateral resolution of about 10 μm . For data evaluation, the Surface Lab 6.5 software of ION-TOF Company was used. For a more detailed description of the method, measurement parameters and details of the semi-quantitative evaluation, see the electronic supplementary material.

2.7. Finite-element analysis

The effective microscopic Young's modulus of trabecular vertebrae tissue was determined by *virtual compression tests* on the cylindrical samples. The actual compression experiment was reconstructed *in silico* by linear elastic FEA based on the μ -CT of the bone sample (figure 2). By comparing the computational stiffness k with the experimental stiffness, an effective microscopic Young's modulus of the bone tissue can be extracted. The FEA was done with parallel in-house software [19,34,35] using the library PETSc [36].

Assuming linear elastic behaviour and fixed Poisson's ratio, the sample stiffness k is determined by bone morphology and the microscopic Young's modulus. These quantities can be combined by defining the structure length S as the sample stiffness normalized by the microscopic Young's modulus E_{mic}

$$S = \frac{k}{E_{mic}}. \quad (2.3)$$

S is a measure for the influence of the bone structure on the stiffness k . It can be obtained from equation (2.3) by calculating the sample stiffness by FEA while setting the microscopic Young's modulus E_{mic} to 1. For more details of the numerical procedure and a discussion on the basic elasticity model of bone tissue, see the electronic supplementary material.

2.8. Predictors for bone strength

Bone strength is defined as the bone's resistance to mechanical load and is closely related to fracture risk. The maximum tolerated force in a compression test of a cylindrical bone sample

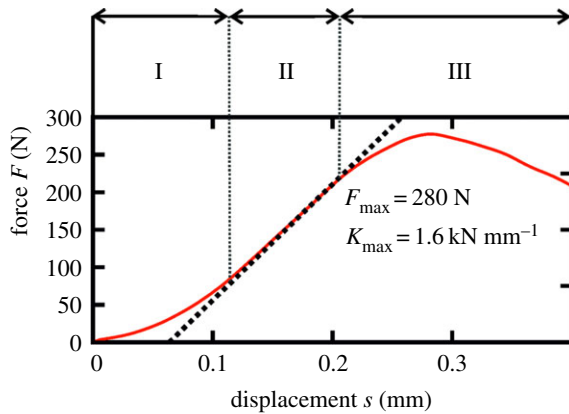


Figure 1. Force–displacement curve (red) for a standard compression test of sheep bone samples (animal ODS2 from the ODS treatment group). Black dotted line represents the highest slope of the force–displacement curve, and is used as a measure for bone stiffness.

with radius r (cf. figure 1) normalized by its base area πr^2 can be considered as a measure for macroscopic bone strength. As the base area of the samples is approximately constant, throughout the paper, we use the term maximum tolerated force synonymous to macroscopic bone strength.

Indicators for the bone strength, measured by the maximal compressive force, were tested in the sheep model. Measured bone sample stiffness, BMD obtained by *in vivo* DEXA, the FEA-based Pistoia criterion [30] and the FEA-based structure length were compared with respect to their predictive power for bone strength.

The clinical gold standard for estimating bone quality is DEXA, which yields the BMD (unit g/cm^2). FEA-based approaches using μ -CTs have been established for human bone [30,37]. Compared to DEXA, μ -CT measurements require more effort and cause more radiation exposure, but bone strength prediction is more accurate [37]. In the presented work, two different FEA-based indicators were tested: the Pistoia criterion [30] and the structure length S (see the last paragraph). For quantification, the Pearson correlation coefficient R between each indicator and the measured maximum force for the samples was calculated. For more details, see the electronic supplementary material.

2.9. Statistics

Statistical evaluation was done by software R [38], with the Shapiro–Wilk normality test, Levene’s test for homogeneity of variance, analysis of variance and multiple pairwise comparisons using the t -test. The significance codes are: *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$.

3. Results

3.1. Imaging and morphometry of trabecular structure

From the μ -CT datasets, representative stacks of $n = 50$ horizontal cross-sections were cut out and visualized using a volume rendering technique (cf. figure 3). The images indicate nearly no bone loss in the O and OD groups (cf. figure 3*b,c*) and a pronounced loss of bone material in the ODS group, with most of the struts still existing (cf. figure 3*d*). For quantitative comparison of the different treatment groups, morphometric indices were evaluated for the complete volume of interest, consisting of voxels of isotropic side length (cf. figure 4). The mean values and standard deviation are listed in table 2.

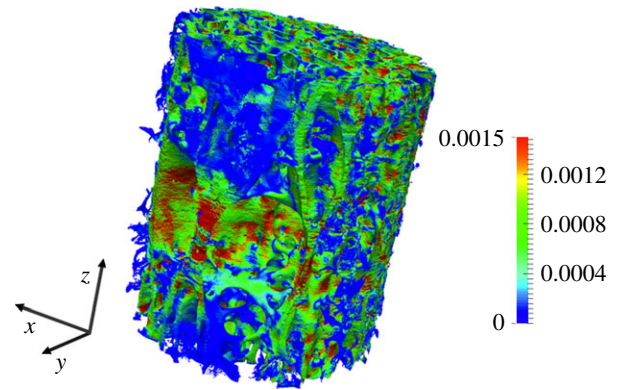


Figure 2. Deformation field calculated with FEA of sample from animal K1 (control group). Encoded in colour is the volumetric strain (magnitude of trace of strain tensor). In the middle of the image, the entrance for the blood vessel vena ventralis is visible. Owing to artificial loading (force applied on a cylindrical cut out of the vertebra), mechanical deformations in the surrounding of vena ventralis are quite inhomogeneous (sample height 10 mm, colour bar saturated). (Online version in colour.)

Relative bone volume (BV/TV) shows a mild, but statistically significant bone loss both in the O and OD groups (cf. figure 4*a*). In the ODS group, BV/TV drops down to 50% of the control group value (cf. figure 4*a*). Consequently, the surface to volume ratio of mineralized bone (BS/BV, cf. figure 4*b*) shows the inverse behaviour with an increase of 108% in the ODS group. Trabecular thickness (Tb.Th) exhibits a characteristic similar to bone volume with a decrease in trabecular thickness by 50% in the ODS group (cf. figure 4*c*).

Contrary to the loss of bone tissue in the ODS group, the overall network topology is preserved in all treatment groups (cf. figure 3). This is quantified by the absence of drastic changes in trabecular separation (Tb.Sp), trabecular number (Tb.N) and structure model index (SMI). In Tb.Sp, only a small increase is observed in the O and OD groups (cf. figure 4*d*). Besides a small decrease for the O group, Tb.N also shows no statistically significant change (cf. figure 4*e*). SMI shows a tendency to higher values in the treated animals, which is statistically significant only in the case of the O group (cf. figure 4*f*).

In summary, pronounced bone loss in the ODS group indicates an osteoporosis-like bone status, while changes in the trabecular topology are mild.

3.2. Compression tests

Compression tests confirm the results of bone imaging and bone morphometry. The mean of the maximum force until structural failure in the control (O, OD, ODS) group was determined as $F = 1.1$ (0.84, 0.99, 0.36) kN (cf. table 3 and figure 5*a*). According to equation (2.1), the average macroscopic ultimate stress σ_{ultim} follows as $\sigma_{\text{ultim}} = 22$ (17, 20, 7.2) MPa. The mean stiffness of the bone samples k in the control (O, OD, ODS) group was determined as $k = 8.7$ (6.7, 8.0, 2.9) $\text{N } \mu\text{m}^{-1}$ (cf. table 3 and figure 5*b*). According to equation (2.2), the average macroscopic Young’s modulus E_{macro} follows as $E_{\text{macro}} = 1.7$ (1.3, 1.6, 0.58) GPa. Strength values as well as stiffness values in the O and OD groups do not differ significantly from those of the control group. For the triple treated ODS group, the mean stiffness value dropped by 66%, while the bone strength dropped by 69%.

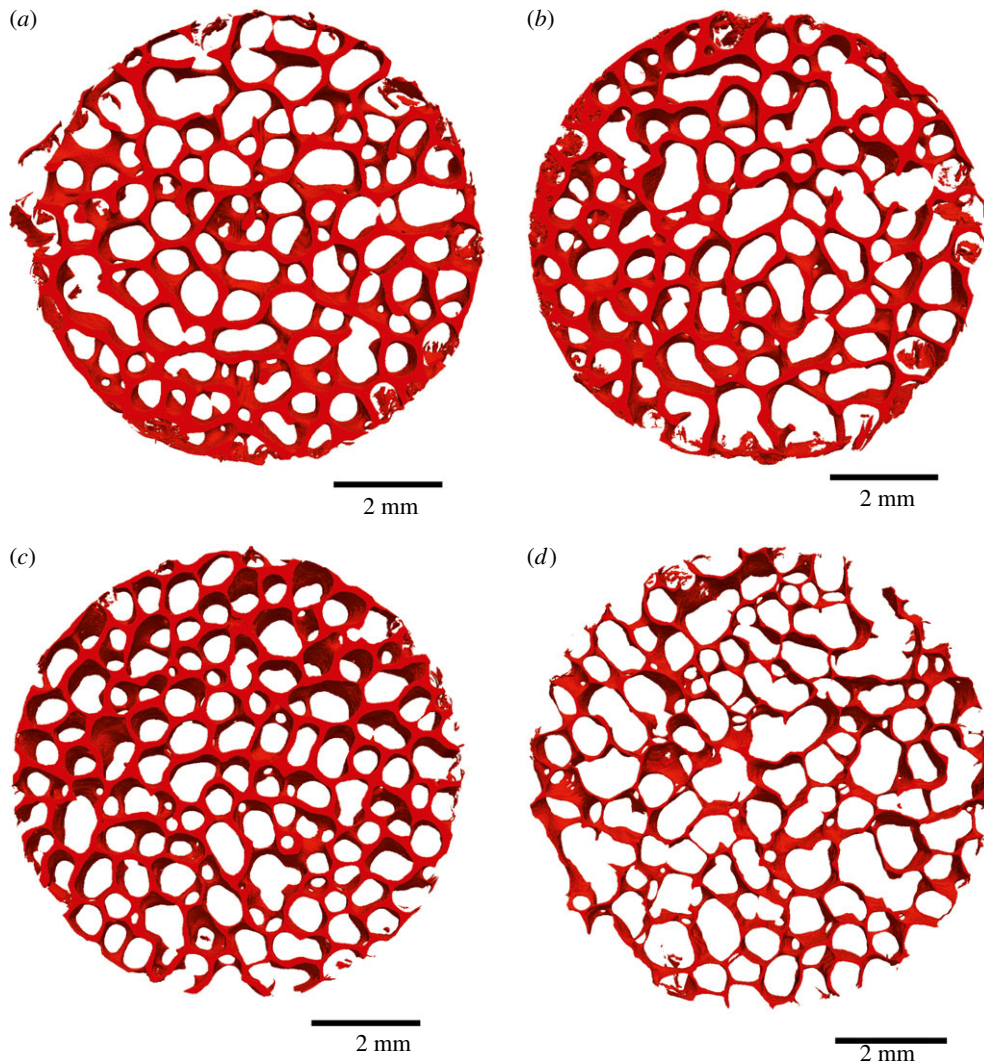


Figure 3. Typical cross-sections through cylinder samples from sheep vertebrae. Compared to the animal from the control group (a), only a small decrease in bone volume is visible in the O (b) and OD sheep (c), while a dramatic loss of bone is observed in the ODS animal (d). (Online version in colour.)

Interestingly, the scattering of stiffness and strength data in the ODS group is lower compared to the control group. This indicates a strong influence of the threefold treatment on bone metabolism, overriding the individual variability of the animals to a certain extent.

3.3. Finite-element analysis

The microscopic Young's modulus E_{micro} (cf. figure 6a and table 4) is one parameter to characterize the quality of bone tissue. No significant difference in E_{micro} among the four groups was found. The lowest value of all animals was even detected in the control group. Note the broad range of values in the control (ODS) group with almost a factor of 2.6 (2.3) between the strongest and the weakest animal of each group. Data scattering seems to be lowest in the O group.

The structure length S (cf. figure 6b and table 4) describes the hypothetical stiffness of the trabecular bone while setting Young's modulus to 1 and is an indicator for the preservation of the trabecular structure in the treated groups. Compared to the control group, slightly lower values of S can be seen in the O and OD groups. This indicates mild loss of bone material. In contrast with the O and OD groups, in the ODS group, the mean value of the structure length is decreased by 67%.

3.4. Time-of-flight secondary ion mass spectrometry

Figure 7 exemplarily shows ToF-SIMS mass images of entire sheep vertebrae, while figure 8 presents close-ups of the regions chosen for the compression tests. The collagen matrix is imaged by the $\text{C}_4\text{H}_8\text{N}^+$ signal that derives from the amino acid proline, one of the main components of collagen type I [9]. Assessing the whole vertebrae as shown in figure 7, the collagen has a homogeneous distribution and the Ca^+ images reveal a homogeneous mineralization for both groups. However, trabecular number and thickness seem to be slightly reduced in the case of the treated animal.

The detailed image of the biopsy region (cf. figure 8) shows a deterioration of the trabecular network in the case of the ODS vertebra. While the collagen signal is depicted in blue, the calcium signal is given in green. The overlay of both shows a slight inhomogeneous mineralization. The heterogeneity is even more pronounced at the single trabecular level. Nevertheless, an ordered fibril superstructure can be assumed for the control group, with increased calcium content in the centre of the trabecula, while a more disordered fibril superstructure and mineralization can be found in the case of the ODS group. Integral evaluation of the local calcium and collagen content within the biopsy region revealed a slight, but not statistically relevant decrease for the ODS group (cf. figure 9 and table 5).

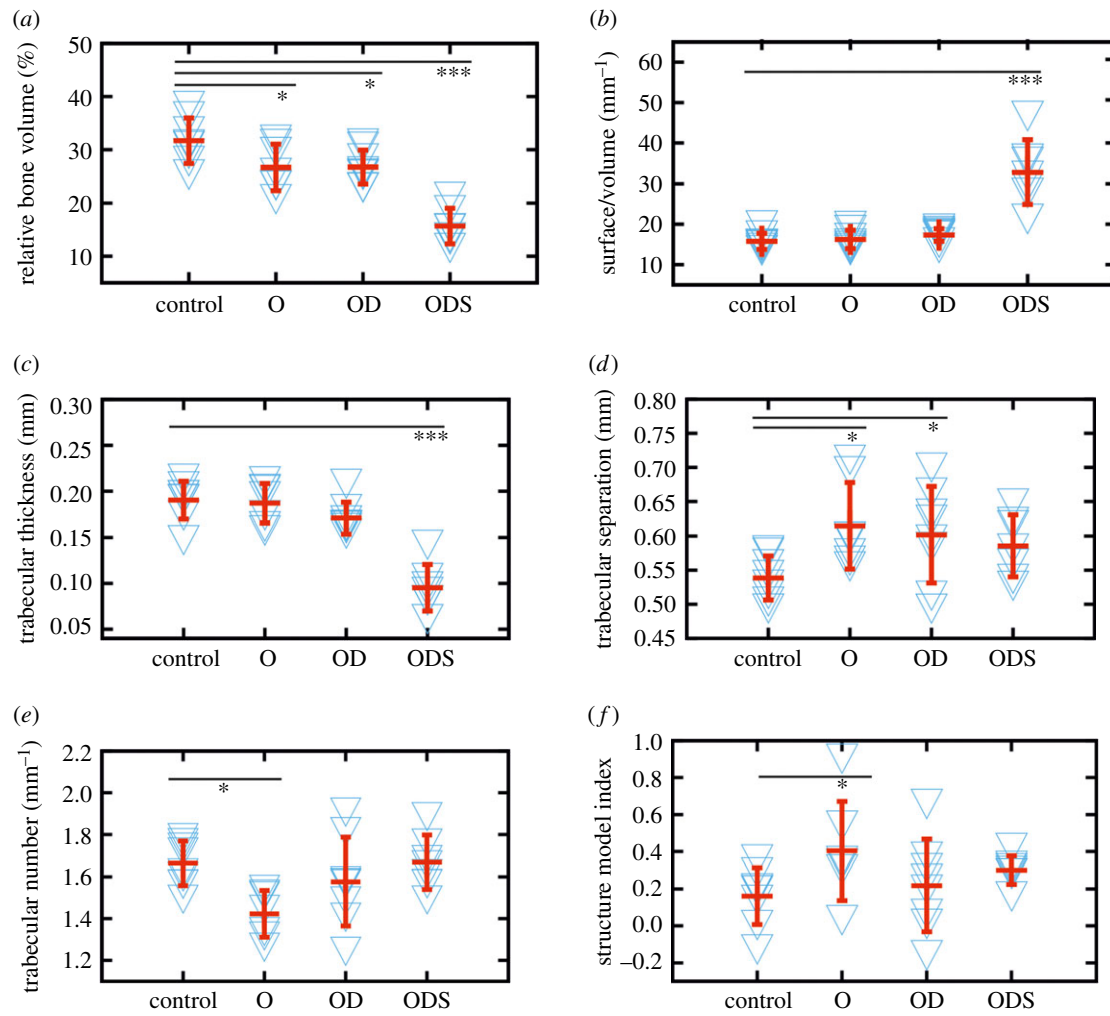


Figure 4. Morphological indices from sheep bone samples: (a) relative bone volume, (b) bone surface to bone volume ratio, (c) trabecular thickness, (d) trabecular separation, (e) trabecular number and (f) the structure model index. Data of single animals (blue triangles) as well as the mean values with standard deviations (red) are shown. Stars denote significance level. (Online version in colour.)

Table 2. Morphometric indices. Mean $\pm$ s.d.

group	BV/TV (%)	BS/BV (1 mm^{-1})	Tb.Th (mm)	Tb.Sp (mm)	Tr.N (1 mm^{-1})	SMI
control	31.7 ± 4.3	15.8 ± 2.0	0.190 ± 0.021	0.539 ± 0.032	1.66 ± 0.11	0.161 ± 0.154
O	26.7 ± 4.3	16.2 ± 2.3	0.187 ± 0.021	0.681 ± 0.063	1.42 ± 0.11	0.404 ± 0.267
OD	26.8 ± 3.1	17.3 ± 1.6	0.171 ± 0.017	0.602 ± 0.070	1.58 ± 0.21	0.218 ± 0.249
ODS	15.7 ± 3.4	32.8 ± 8.0	0.095 ± 0.025	0.585 ± 0.045	1.67 ± 0.13	0.300 ± 0.076

Table 3. Results of compression tests. Maximum force F and stiffness k . Mean $\pm$ s.d.

group	F (N)	k ($\text{N } \mu\text{m}^{-1}$)
control	1129 ± 268	8673 ± 2446
O	839 ± 225	6651 ± 1657
OD	991 ± 133	7958 ± 1743
ODS	355 ± 124	2930 ± 1324

3.5. Bone strength predictors

The Pearson correlation coefficients R between macroscopic bone strength and stiffness measurements, BMD measurements, Pistoia's criterion as well as structure length (the

latter two obtained by FEA of μ -CT data) were calculated. Experimentally determined values for both stiffness and strength span almost one order of magnitude. Nevertheless, there is a strong linear correlation ($R = 0.92$) between the measured sample stiffness and its strength over all groups (cf. figure 10a). By contrast, BMD shows only a moderate linear correlation to bone strength ($R = 0.65$, figure 10b). The Pearson correlation coefficient R between the maximum force predicted by the FEA-based Pistoia criterion and bone strength over all animal groups is $R = 0.87$ (cf. figure 10c). Nearly, the same correlation ($R = 0.86$) over all groups was found between structure length calculated by FEA and bone strength (cf. figure 10d). The overall correlation between bone volume BV/TV and bone strength was measured to $R = 0.83$ (cf. figure 10e).

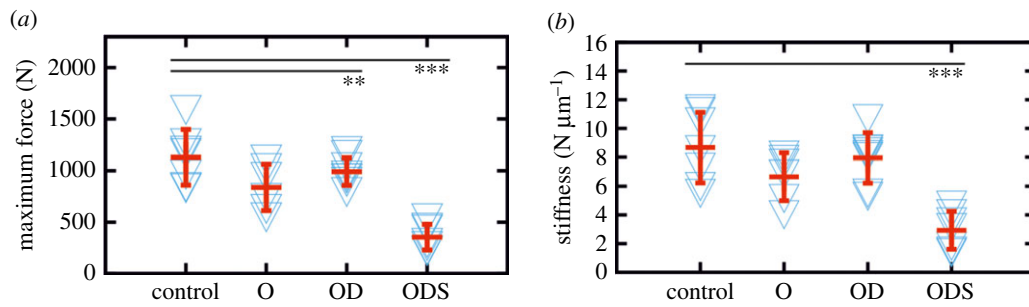


Figure 5. Macroscopic mechanical properties of bone samples obtained by compression tests. (a) Measured maximum force (strength) of sheep bone samples and (b) measured stiffness of sheep bone samples. Data of single animals (blue triangles) as well as the mean values with standard deviations (red) are shown. Stars denote significance level. (Online version in colour.)

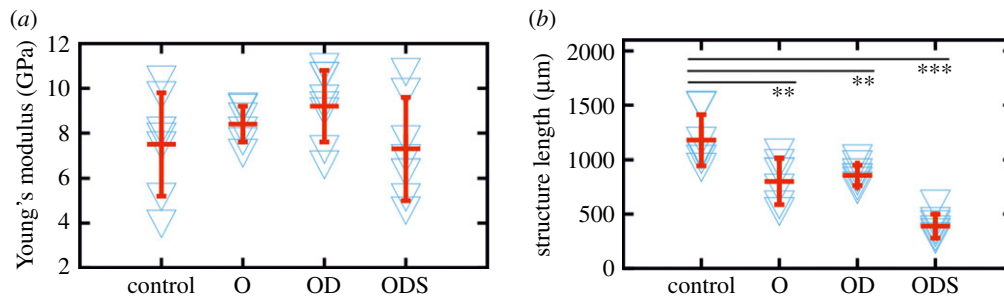


Figure 6. Microscopic mechanical properties of bone tissue obtained by FEA. (a) Microscopic Young's modulus and (b) structure length. Data of single animals (blue triangles) as well as the mean values with standard deviations (red) are shown. Stars denote significance level. (Online version in colour.)

Table 4. Results of FEA. Structure length S and microscopic Young's modulus E . Mean $\pm$ s.d.

group	S (μm)	E (GPa)
control	1179 ± 234	7.5 ± 2.3
O	801 ± 213	8.4 ± 0.8
OD	858 ± 94	9.2 ± 1.6
ODS	390 ± 110	7.3 ± 2.3

4. Discussion

A detailed analysis of the macroscopic and microscopic elastic properties of mineralized tissue is imperative for the adaption of implant materials for osteoporotic bone. Several groups have reported on successful induction of osteoporotic conditions in a sheep model. By ovariectomy combined with a diet and steroid medication, they observed a significant reduction in overall bone mineral content and mineral density within six months [27,28,39].

While other groups conducted biomechanical tests for assessing the macroscopic properties of bone only [27,28,39], the study presented herein used a combination of macroscopic and microscopic analyses to distinguish the pure bone loss caused by osteoporosis from tissue changes induced by the disease. Averaged microscopic elastic properties as well as detailed information obtained by ToF-SIMS on the calcium and collagen content and distribution were evaluated in a sheep model of postmenopausal osteoporosis.

4.1. Bone properties in the ODS treatment group

μ -CT imaging revealed a dense trabecular network in the samples of the control group as well as in the O and OD

groups. Correspondingly, the mean of relative bone volume (BV/TV), Tb.Sp as well as Tb.Th and BS/BV did not change significantly between the control and the O and OD groups. This is in accordance with previous reported observations on osteoporotic sheep [27,28,40]. Due to the different hormonal cycles of sheep and humans, oestrogen deficiency caused by ovariectomy has less influence on female sheep. Shown by the so-called 'rebound' effect, where the initial BMD is reached again after six months, ovariectomy alone is not decisive within eight months [41,42]. The additional treatment with hormones is known to be more effective [43]. Consequently, BV/TV as well as Tb.Th decreased by about 50%, while BS/BV increased by 108%. ToF-SIMS images in figure 8a,b also visualize an almost intact trabecular network topology, but with reduced trabecular thickness and some deterioration in the ODS sample. Overall mineral content showed only a slight, insignificant tendency towards lower values in the ODS group.

After eight months of steroid treatment, struts in the trabecular network show thinning (cf. figures 3d and 4c). However, Tb.Sp, Tb.N and SMI suggest an intact topology of the network. This indicates, that even after eight months of steroid treatment, the animals are still in an early phase of osteoporosis. Nevertheless, BMD is usually used as criterion for the diagnosis of osteoporosis in clinical routine, when a deviation of more than 2.5 from the standard value (Z-score) is observed. In the study of Khassawna *et al.* [27], a significant decrease in the BMD value compared to the age-matched controls of O as well as OD groups is obtained for the same animal experiment. This is not in contradiction to the presented changes in morphometric indices during treatment.

The influence of observed bone loss on mechanical stability of the vertebra was investigated by compression tests. The average macroscopic ultimate stress σ_{ultim} was estimated to $\sigma_{\text{ultim}} = 22$ (17, 20, 7.2) MPa for the control (O, OD, ODS)

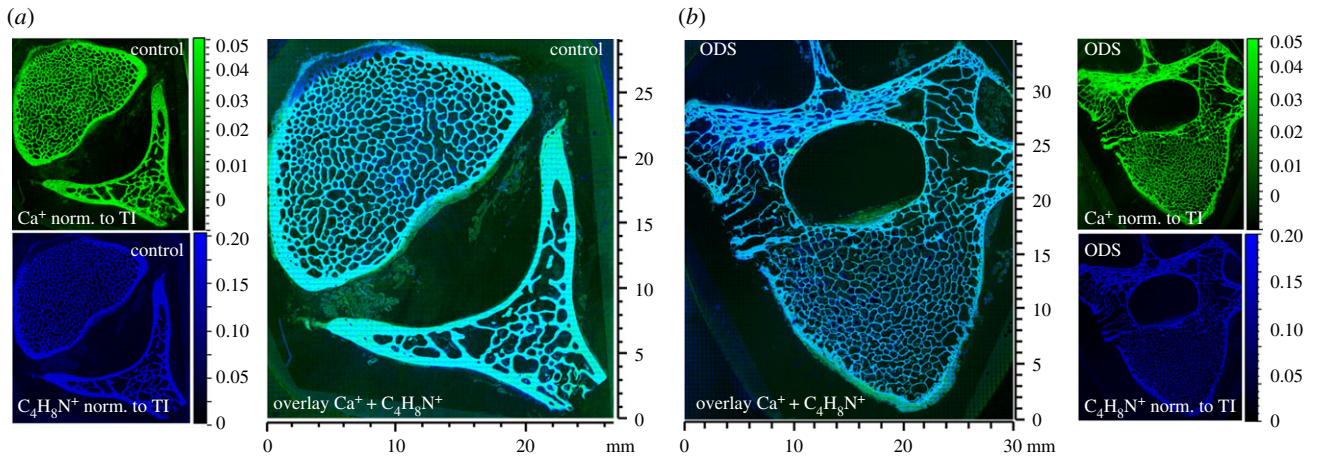


Figure 7. ToF-SIMS images showing the inorganic (Ca^+) as well as the organic compounds ($\text{C}_4\text{H}_8\text{N}^+$) of the bone. (a) Healthy sheep (K1, control group) and (b) treated sheep (ODS1, ODS group). (Online version in colour.)

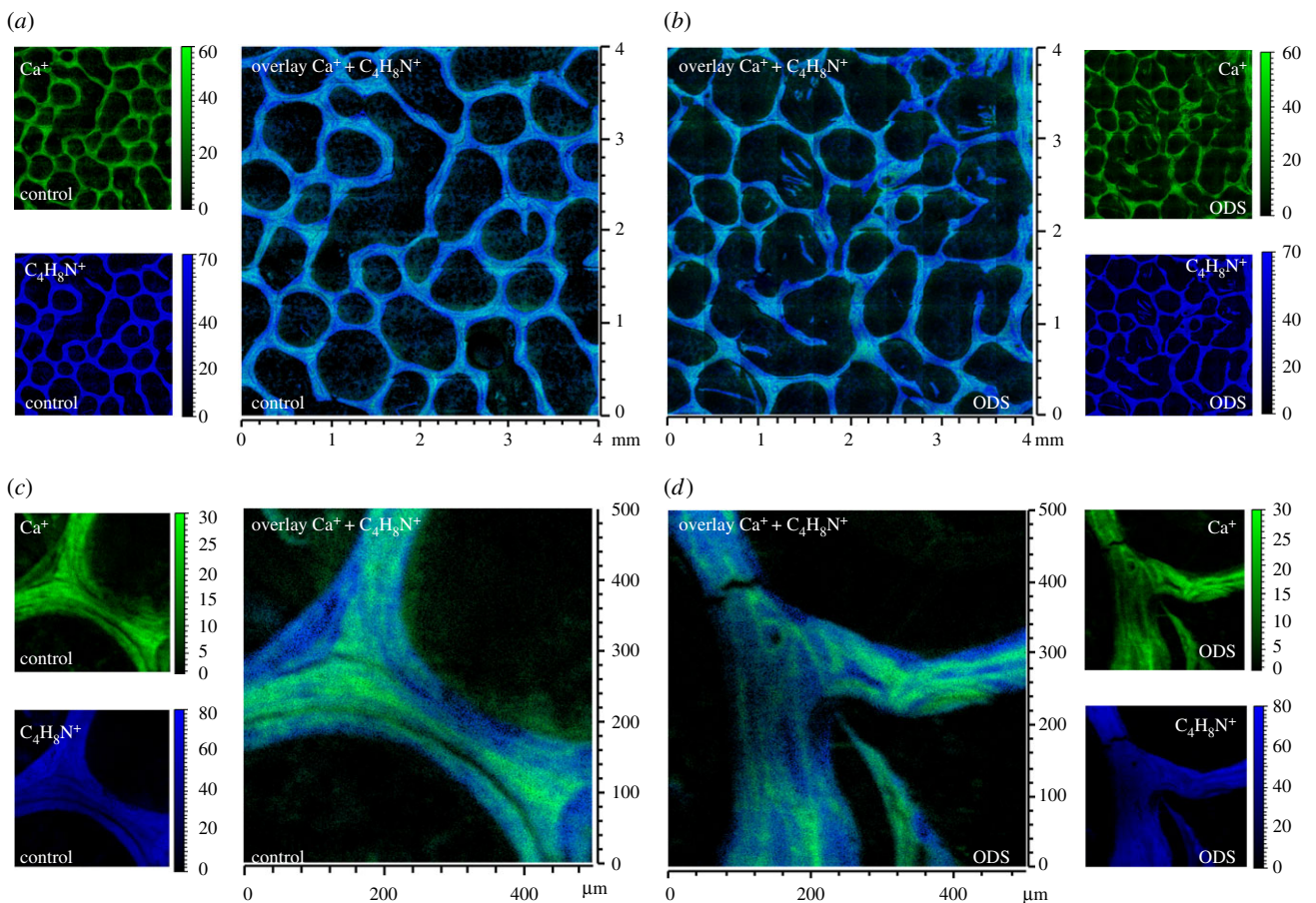


Figure 8. ToF-SIMS images showing inorganic as well as organic compounds of the bone in high magnification as overlay of Ca^+ in green and $\text{C}_4\text{H}_8\text{N}^+$ in blue. (a,c) Healthy sheep (K1, control group) and (b,d) treated sheep (ODS1, ODS group). (a,b) The biopsy of the vertebrae shown in figure 7. (c,d) The close-up views of single trabeculae.

group. For healthy animals, Mitton *et al.* [44] obtained a similar result of $\sigma_{\text{ultim}} = 23$ MPa. The average *macroscopic* Young's modulus E_{macro} was estimated as $E_{\text{macro}} = 1.7$ (1.3, 1.6, 0.58) GPa for the control (O, OD, ODS) group. This is in agreement with the values obtained for healthy animals by Mitton *et al.* [44] (1.3 GPa) and Schorlemmer *et al.* [40] (1.7 GPa), using similar compression tests on trabecular bone samples from healthy sheep vertebrae.

As it is expected for porous or cellular structures in general [45], stiffness and macroscopic strength data showed very similar behaviour. In the OD group, a small but

statistically significant decrease can be observed. Accordingly, the relative bone volume both in the OD and the O groups shows a small decay. In the ODS group, the mean of strength and stiffness strongly drop down by 69% respective 66%, while loss in bone volume is only 50%. This indicates non-trivial changes in the morphology of the trabecular structure. From μ -CT data, the structure length was computed by FEA. Similar to strength and stiffness, the structure length shows mild decay in the O and OD groups. For the ODS animals, a drop of the mean value of the structure length by 67% was found. This strong change matches the

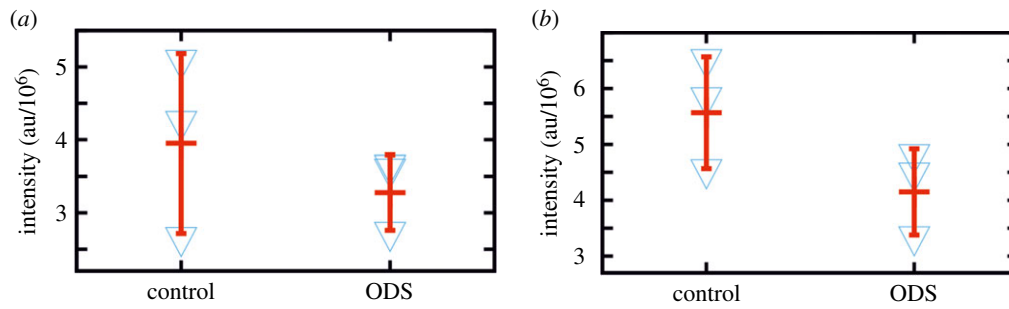


Figure 9. Semi-quantitative evaluation of (a) the integral calcium signal intensity and (b) the integral $C_4H_8N^+$ signal intensity representing the trabecular calcium and collagen content of the control and ODS group. The mean of data of single animals (blue triangles) as well as the mean values with standard deviations (red) after normalization to primary ion current are shown. Description of the semi-quantitative analysis can be found in the electronic supplementary material. (Online version in colour.)

Table 5. Results of semi-quantitative evaluation of calcium and collagen content given in arbitrary units. Mean $\pm$ s.d.

group	Ca^+ (au)	$C_4H_8N^+$ (au)
control	$(4.0 \pm 1.2) \cdot 10^6$	$(5.6 \pm 1.0) \cdot 10^6$
ODS	$(3.3 \pm 0.5) \cdot 10^6$	$(4.8 \pm 0.8) \cdot 10^6$

decay in stiffness and strength but not in pure bone volume and again emphasizes that the observed changes in bone structure are not solely due to bone volume reduction.

In summary, the results of μ -CT imaging and morphometry as well as the compression tests indicate an almost healthy bone status for the O and OD groups. By contrast, additional administration of steroids in the ODS group leads to significant changes towards an osteoporotic bone status in the reported parameters. The observed changes on the macroscopic scale correspond qualitatively to the observations in the osteoporotic rat model, with the only exception being the structure length [9,19,46,47].

4.2. Preservation of microscopic bone properties

The microscopic composition of the bone material from the control and ODS groups was analysed. ToF-SIMS mass images depict the organic matrix as well as the distribution of Ca to assess the mineralization state. While the trabecular network of the biopsy region gives the impression of a homogeneous mineralization in figure 8a,b, at the single trabecular level, the fibril structure is visible and the mineralization appears to be more heterogeneous in the case of the ODS group. The control group reveals increased calcium content in the centre of the trabecula and a less mineralized edge region, which might be the collagenous zone of new bone formation. A tightly controlled mineralization along the collagen fibrils can be seen in the overlay. By contrast, a more disturbed fibril superstructure and mineralization can be recognized for the ODS group. A disorder of collagen fibrils in osteoporotic bone has already been reported in former studies for ovariectomized rats and sheep or osteoporotic humans [28,48–51]. Nevertheless, semi-quantitative evaluation of the integral calcium and collagen content of the sheep trabeculae showed only a non-significant difference between the healthy-control and the osteoporotic-ODS groups. This might reveal an early stage of osteoporosis which is in accordance with studies of Brennan *et al.* [49,52]

who stated an increased bone turn over after 12 months and a clear osteoporotic bone status only after 31 months, however, only related to ovariectomy and diet-induced changes. As our study includes steroid medication, it is reliable to assume an early stage of osteoporosis after eight months as reported elsewhere [28,53,54], but with minor changes in the microstructure. FEA-based determination of the microscopic Young's modulus could confirm these findings. Despite the observed dramatic drop of strength and stiffness in the ODS group, no significant change in the microscopic Young's modulus was found among all groups.

In contrast with the sheep model, the osteoporotic rat model presented by Govindarajan *et al.* [46] showed a significant inhomogeneous calcium distribution at the trabecular level: large parts of the bone structure were basically composed of collagen [9]. Correspondingly, FEA revealed a strong decay in the microscopic Young's modulus by a factor of three [19]. In addition to the macroscopic bone loss, also the remaining material showed severe decay in the treated rats, making it rather a model for osteomalacia than osteoporosis.

In summary, in the sheep model, apart from the disturbed fibril structure, no significant changes in the microscopic properties of the bone material were detected in the ODS group. The disordering of the fibrils has no significant influence on the calcium and collagen content, and no influence on the microscopic Young's modulus was observed. According to equation (2.3), this means, changes in stiffness and strength are predominantly caused by changes in the trabecular morphology, quantified by the structure length. From the observed changes in macroscopic stiffness and strength, it becomes clear that these changes in morphology cannot be exclusively be explained by reduced bone volume BV/TV or reduced trabecular thickness. Homminga *et al.* [55] found only negligible changes in the mean microscopic elastic properties of bone tissue from human patients suffering from typical osteoporotic fractures. Changes in macroscopic mechanical bone properties could be attributed dominantly to structural changes.

4.3. Bone strength estimation in the control and treatment groups

Different predictors for bone strength were tested. The stiffness–strength correlation is very strong ($R = 0.92$, cf. figure 10a), but of extremely limited practical usability. The clinical gold standard DEXA (BMD) is easily applicable to

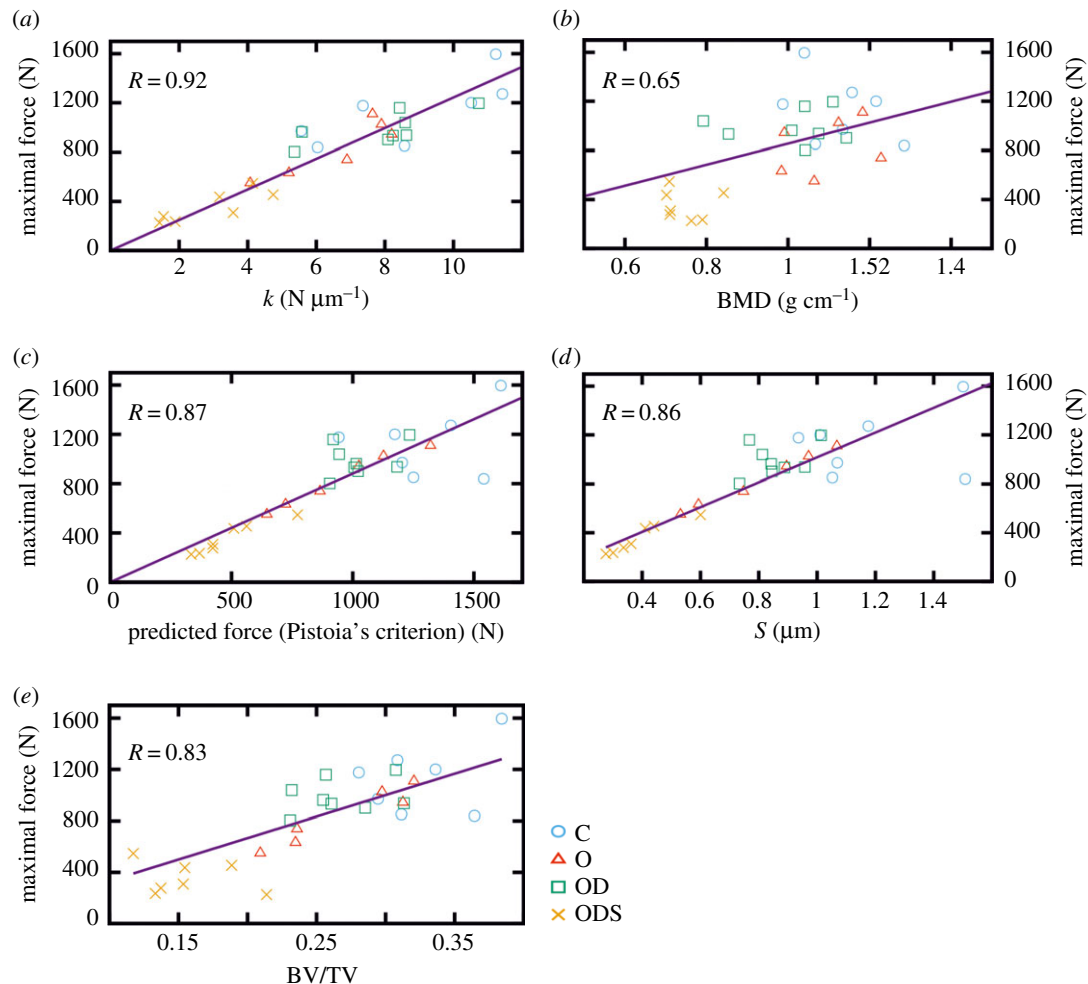


Figure 10. Correlations between bone strength and different predictors for bone strength. (a) Sample stiffness, (b) BMD, (c) Pistoia criterion, (d) structure length, and (e) bone volume over tissue volume. Symbols mark individual animals; lines show the linear fits between predictor and maximum force obtained in compression tests of the bone samples. (Online version in colour.)

living animals, but BMD shows only a moderate correlation with strength ($R = 0.65$, cf. figure 10*b*). BMD is mainly influenced by the density of bone mineral at the region of interest and therefore closely related to the bone volume BV/TV, respectively, the Tb.Th obtained from μ -CT. Interestingly, the correlation between bone volume BV/TV and macroscopic bone strength is much higher ($R = 0.83$, cf. figure 10*e*). One of the reasons is, probably, that the BMD measurement procedure integrates over several lumbar vertebrae, whereas the samples are all explanted from vertebra body L1. Further, DEXA measurements cannot distinguish between cortical and trabecular bone. The anterior–posterior projection might emphasize this effect, as the radiation has to pass the posterior part of the vertebra, including arch and process. Our findings are in accordance with literature data for long bones [56], which are also composed of trabecular and cortical bone, but in a different ratio than vertebrae.

The two predictors based on the combination of μ -CT and FEA showed a correlation to the macroscopic bone strength almost as strong as the stiffness–strength relation ($R = 0.87$ for the Pistoia criterion, $R = 0.86$ for the structure length S). FEA-based predictions of human bone strength with comparable precision can be found in the literature [30]. As FEA models are based on the knowledge of the elastic bone properties, similar correlations do not exist in the case of the rat model with osteoporosis-induced strong changes in bone tissue properties [9].

The correlation between bone volume BV/TV and macroscopic bone strength is almost as high as between the FEA-based predictors and strength ($R = 0.83$, cf. figure 10*e*). This is especially interesting as the overall structure of the samples is not homogeneous due to the large cavity caused by the vena ventralis (cf. figure 2). However, the most interesting ODS group shows a much higher deviation from the linear relation between BV/TV and strength opposed to the relation between S and strength (cf. figure 10*e,d*). This again indicates that loss in strength indeed is due to loss in BV/TV, but in addition influenced by changes in the trabecular architecture.

4.4. Summary

Microscopic properties of bone samples from an osteoporotic sheep model were tested by compression tests, μ -CT imaging, FEA and ToF-SIMS. Bone loss and changes in bone morphology parameters revealed an osteoporotic bone status in the ODS treatment group. At the microscopic level, a disturbed fibril superstructure and a more heterogeneous mineralization in the ODS group were observed. Unlike a previously studied rat model, the microscopic Young's modulus as well the mineral content in the remaining bone tissue did not change in the osteoporotic sheep.

The preserved microscopic Young's modulus enabled the prediction of macroscopic bone strength in the sheep model

for treated and untreated animals based on FEA of μ -CT data without the necessity for determining elastic properties of treated animals. As in human bone, this prediction turned out to be much more precise than the estimation based on *in vivo* DEXA measurements. These microscopic findings show a certain similarity of osteoporotic sheep to osteoporotic human bone and makes our sheep model suitable to mimic human osteoporotic properties [28].

Ethics. The study was conducted in strict accordance with the European Union legislation for the protection of animals used for scientific purposes and approved by the district's Animal Ethics Committee 'government presidium of Darmstadt, Germany; permit no. Gen. Nr. F31/36'.

Data accessibility. Original data obtained and used in the study (except micro-computed tomography data files) are included in the electronic supplementary material.

Authors' contributions. R.M., A.H. and A.D. designed the overall study and wrote the manuscript. C.H. and T.E.K. designed and conducted

the animal sheep study. R.M., H.J.W. and A.I. performed and evaluated the compression experiments. M.K. and A.C.L. conducted μ -CT imaging and morphometry. A.H., M.R. and J.J. performed the ToF-SIMS measurements and did the data evaluation. R.M. conducted FEA. A.V. and J.H. supported design and verification of FEA. All authors discussed and edited the manuscript.

Competing interests. We have no competing interests.

Funding. R.M., A.H. and T.E.K. are funded by the German Research Foundation (DFG). A.D. and A.V. are funded by the Free State of Saxony. M.R., J.J., M.K., A.C.L. and C.H. are funded by the State of Hesse. H.J.W. and A.I. are funded by the State of Baden-Württemberg.

Acknowledgements. This study is part of the project M5, M8, T1 and Z3 within the collaborative research centre SFB/TRR 79 'Materials for tissue regeneration within systemically altered bone', funded by the German Research Foundation (DFG). We gratefully acknowledge the financial support within this project. We thank Dr N. Steiner, University of Giessen, for her help with the statistical analysis and G. Martels for technical assistance and Daniel Bürgener for help in preparation of bone samples. We thank ZIH, TU-Dresden, for providing computational resources.

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