EFFECTS OF LONG-TERM ALENDRONATE TREATMENT ON BONE MINERALISATION, RESORPTION PARAMETERS AND BIOMECHANICS OF SINGLE HUMAN VERTEBRAL TRABECULAE

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Abstract

Due to their well-established fracture risk reduction, bisphosphonates are the most frequently used therapeutic agent to treat osteoporosis. Bisphosphonates reduce fracture risk by suppressing bone resorption, but the lower bone turnover could have a negative impact on bone quality at the tissue level. Here, we directly assess the structural and mechanical characteristics of cancellous bone from the lumbar vertebrae (L5) in non-treated osteoporotic controls ($n = 21$), mid-term alendronate-treated osteoporotic patients ($n = 6$), and long-term alendronate-treated osteoporotic patients ($n = 7$). The strength and toughness of single trabeculae were evaluated, while the structure was characterised through measurements of microdamage accumulation, mineralisation distribution, and histological indices. The alendronate-treated cases had a reduced eroded surface (ES/BS, $p < 0.001$) and a higher bone mineralisation in comparison to non-treated controls ($p = 0.037$), which is indicative of low turnover associated with treatment. However, the amount of microdamage and the mechanical properties were similar among the control and treatment groups. As the tissue mineral density (TMD) increased significantly with alendronate treatment compared to non-treated osteoporotic controls, the reduction in resorption cavities could counterbalance the higher TMD allowing the alendronate-treated bone to maintain its mechanical properties and resist microdamage accumulation. A multivariate analysis of the possible predictors supports the theory that multiple factors (*e.g.*, body mass index, TMD, and ES/BS) can impact the mechanical properties. Our results suggest that long-term alendronate treatment shows no adverse impact on mechanical cancellous bone characteristics.

Keywords: Osteoporosis, alendronate, histomorphometry, microcomputed-tomography, mineralisation, microdamage.

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Introduction

Vertebral fractures are the most common fragility fractures in patients with osteoporosis and potentially lead to adverse effects, such as acute or chronic back pain, disability, and impairment in quality of life (Oleksik *et al.*, 2000; Krause *et al.*, 2012b). Currently, bisphosphonates represent the most frequently used therapeutic agent to prevent osteoclast-induced bone loss, such as in post-menopausal osteoporosis. An average bisphosphonate treatment duration of 3 to 4 years is associated with a significant fracture risk reduction of up to 50 % for vertebral fractures and up to 55 % for non-vertebral fractures (Black *et al.*, 1996; Cummings *et al.*, 1998; Harris *et al.*, 1999); however, bisphosphonate treatment is often continued indefinitely. As the main effect of bisphosphonates is based on a continuous suppression of bone turnover, which could impair the structure and mechanical properties of the remaining bone tissue, there are reservations in the medical community regarding the use of bisphosphonates as a long-term therapy.

Currently, only a few clinical studies analyse the clinical consequences of long-term bisphosphonate therapy in humans (Bone *et al.*, 2004; Black *et al.*, 2006; Black *et al.*, 2012). Two studies have shown that continued bisphosphonate treatment over the average duration of 3-5 years lowers the risk of vertebral fractures and preserves the increased areal bone mineral density (aBMD) compared to patients who discontinue therapy after the average duration (Black *et al*., 2006; Black *et al*., 2012). Other than the benefits of a higher aBMD, factors relating to bone quality (*e.g.*, size, geometry, microarchitecture or microdamage accumulation) have not been completely evaluated although they could also explain the fracture risk reduction associated with bisphosphonate treatment (Ammann and Rizzoli, 2003). Some effects of long-term bisphosphonate therapy may be detrimental. For instance, an increase in the average degree of mineralisation due to suppression of bone turnover could lead to microdamage accumulation, resulting in reduced bone strength and toughness (Boivin *et al.*, 2000; Roschger *et al.*, 2001; Nyman *et al.*, 2004). An accumulation of microdamage due to the suppression of bone turnover is well established in animal studies (Mashiba *et al.*, 2000; Mashiba *et al.*, 2001; Allen *et al*., 2006), while data from human bone are inconsistent (Chapurlat *et al.*, 2007; Stepan *et al.*, 2007).

Nonetheless, even if microdamage increasingly occurs with long-term bisphosphonate therapy, the impact of

long-term bisphosphonate therapy on mechanical bone tissue characteristics and their clinical importance is largely unknown (Allen and Burr, 2011). In principle, bisphosphonate therapy may result in improved bone characteristics accompanied by changes in Young's modulus (Ding *et al.*, 2003; Day *et al.*, 2004) and reduced toughness (Komatsubara *et al.*, 2003; Allen and Burr, 2007; Wang *et al.*, 2008). While these principles have largely been established through animal models, studies on humans with severely suppressed bone turnover due to long-term bisphosphonate therapy have been more conflicting, indicating both improved (Tjhia *et al.*, 2011) and deteriorated (Bala *et al.*, 2011) mechanical properties in cortical and cancellous bone.

Indeed, inter-individual variations may explain the fracture risk reduction in some patients and the deterioration in mechanical properties of other patients. Specifically, patients with atypical femoral fractures (AFF) have been repeatedly documented after long-term bisphosphonate therapy (Vaishya *et al.*, 2013; Bottai *et al.*, 2014; Zanchetta *et al.*, 2014). A recent study using microindentation found deteriorated bone material properties in patients with both typical and atypical hip fractures in comparison to untreated controls. However, patients with long-term bisphosphonate therapy were not statistically different from untreated controls, which suggests that bisphosphonate treatment itself may not necessarily impair bone material properties (Guerri-Fernandez *et al.*, 2013).

To further investigate the complex effects of long-term bisphosphonate therapy, the aim of the present study was to directly assess the structural and mechanical characteristics of human cancellous bone. As bisphosphonates affect bone tissue properties on different structural levels, the effects of bisphosphonate treatment duration are investigated at the single trabeculae level by quantifying microdamage accumulation, bone turnover activity, bone tissue mineralisation and mechanical properties.

Materials and Methods

Materials

In this study, 35 patients were included from the *ex vivo "Biomechanically founded individualized osteoporosis assessment and treatment (BioAsset)"* study with the goal to elucidate the effects of bisphosphonate treatment on bone by combining imaging and biomechanical techniques (Glüer *et al.*, 2013; Krause *et al.*, 2014; Museyko *et al.*, 2014). Inclusion criteria were females with post-menopausal osteoporosis, which was diagnosed according to the World Health Organization definition, with a lumbar or femoral areal bone mineral density (aBMD) *T*-Score of -2.5 or less assessed by dual x-Ray absorptiometry (DXA). Exclusion criteria included bone cancer, immobility >1 year, renal transplantation or renal insufficiency III, strontium or fluoride therapy, specific anti-resorptive medication other than bisphosphonates and bisphosphonate therapy <1 year. Informed consent was obtained from the family members after comprehensive information on all related issues. The study was approved by the Ethics Committee of the Hamburg Chamber of Physicians (PV3486). Thirty-

five patients matching the inclusion criteria were included. One patient had to be excluded due to lumbar spinal fusion. Therefore, the lumbar spinal column was obtained from 34 patients (median age: 83 years [range: 65 to 90 years]) during autopsy at the Department of Forensic Medicine, University Medical Centre Hamburg-Eppendorf (Fig. 1). All patients were categorised according to bisphosphonate treatment duration prior to death as follows:
- **Control group** (*n* = 21), females with diagnosed osteoporosis but no documented history of bisphosphonate treatment
- **Mid-term bisphosphonate-treated group** (*n* = 6), females taking bisphosphonates for a duration of 1-5 years (mean 2.16 ± 0.99 years)
- **Long-term bisphosphonate-treated group** (*n* = 7), females taking bisphosphonates for a duration of 6 years.

The treatment for all patients on bisphosphonate therapy consisted of weekly alendronate.

Preparation of single trabeculae

To prepare single trabeculae for mechanical testing, the L5 vertebra was dissected along the sagittal direction in three sections (Fig. 1A). The trabeculae were singularised according to methods previously described in detail (Busse *et al.*, 2009). Briefly, the central 5 mm slice of L5 was soaked in hydrogen peroxide for 8 h and rinsed with demineralised water to remove soft tissue. According to guidelines established by previous studies (Busse *et al.*, 2009), trabeculae were removed from defined regions within the section. At least 20 single, rod-like trabeculae were harvested from each specimen using a headset magnifier (2.3, Carl Zeiss, Jena, Germany) and a mini scalpel (No. 11, Bahya, Tuttlingen, Germany). To ensure that only intact trabeculae were tested, the structural integrity was examined in a polarised light microscope.

Biomechanical testing

The strength of single trabeculae from the L5 vertebrae was assessed *via* three-point bending tests using a materials testing device with a 10 N force sensor (Z.2.5/TN1S, Zwick/Roell, Ulm, Germany). Prior to the testing procedure (Busse *et al.*, 2009), appropriate trabeculae were identified. Ideally, only trabeculae that are precisely cylindrically shaped should be tested. Therefore, in accordance with previous studies, trabeculae with a maximum ratio of 1:1.3 between minor and major cross-sectional axis were included (Busse *et al.*, 2009). Additionally, the tested trabeculae had a minimum thickness of 90 µm. All dimensions were determined with a stereomicroscope (SZH-ILLD, Olympus, Osaka, Japan). The specimens were dried to ensure that no adhesion phenomena occurred during the mounting and materials testing process. Each trabecula was placed on the custom-built three-point bending fixture. The fixture's bottom support consists of two pins with a diameter of 1.0 mm and a span of 600 µm. The top fixture consisted of a cross-head with a rounded-off tip of radius 0.1 mm that was positioned half way between the two pins on the lower fixture. The sample was placed on the bottom support's pins and load was applied to the sample *via* the cross-head at a speed of 2 mm/min. The
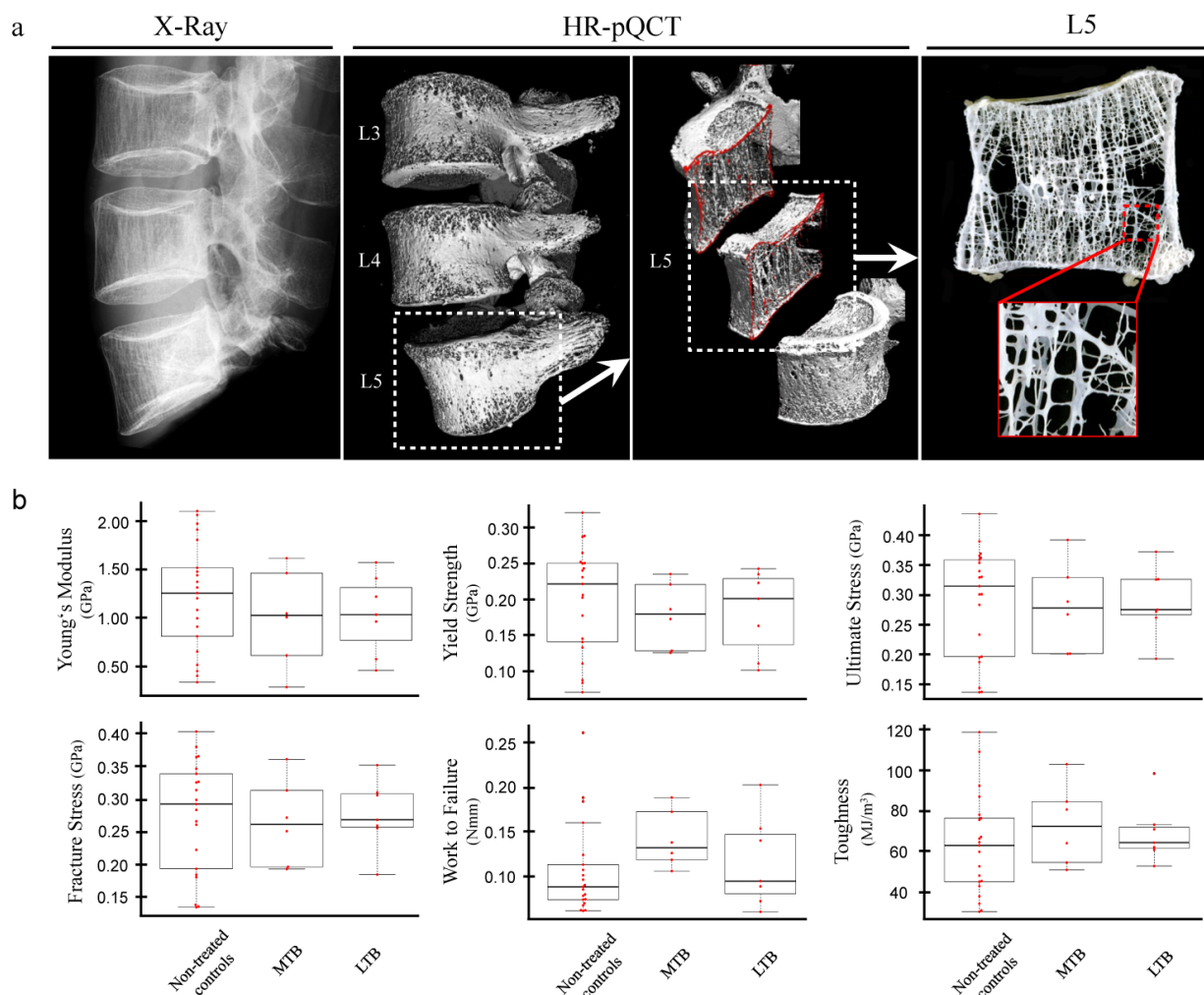
Fig. 1. (**A**) Procedure for preparation of the lumbar spine. X-ray of the lumbar spine L3-L5. HR-pQCT (high-resolution quantitative computer tomography, SCANCO Medical, Bruettisellen, Switzerland) of the lumbar spine L3-L5 with highlighted vertebra of interest (dotted line). Segmentation of L5 (HR-pQCT for illustration) with macerated L5 highlighting the trabecular network (3x magnification). (**B**) The mechanical properties measured during mechanical testing. Comparisons were performed by *t*-test and ANOVA or the non-parametric Mann-Whitney U test and Kruskal-Wallis test.

load and displacement data were collected during testing and the bending test ended after fracture of the trabecula. The ultimate load was taken as the highest load achieved during the test and the fracture load was taken as the final load at fracture. The load and displacement curve was normalised by the specimen geometry, such that the Young's modulus (GPa), yield strength (GPa), ultimate stress (GPa) and fracture stress (GPa) could be determined from the stress-strain curve. The work to failure (W, N mm) was measured from the area under the load-displacement curve. Then, the toughness was calculated according to Mashiba *et al*. (Mashiba *et al*., 2000) by normalising the work to failure (W, N mm):

$$\text{Toughness} = 0.75\, W\, b^2/(0.6\, I)$$

where b is the width of the trabecula. I is the cross-sectional moment of inertia with the assumption of a circular cross-section:

$$I = (\pi/4)\, r^4$$

where r is the radius of the trabecula.

Static 3D-histomorphometry and histological analysis

The second sagittal section of the L5 vertebrae was used for histological analysis. Prior to further preparation, the vertebral remnants were scanned in a micro-computed tomography (μCT) device (μCT40, SCANCO Medical AG, Bruettisellen, Switzerland) with an *in vitro* scanning protocol (55 kVp, 145 μA, 200 ms integration time) resulting in an isotropic 10 μm voxel size to assess the static 3D bone histomorphometry. The following histomorphometric parameters were measured according to American Society for Bone and Mineral Research (ASBMR) standards (Dempster *et al.*, 2013): the vertebral BV/TV (bone volume/tissue volume, %), Tb.Th (trabecular thickness, mm), Tb.N (trabecular number, /mm), Tb.Sp

(trabecular separation, mm) as well as SMI (structure model index, dimensionless). Additionally, the degree of mineralisation was determined with µCT measuring the bone tissue mineral density (TMD, mg hydroxyapatite [HA]/cm³) (Mulder *et al.*, 2004; Burghardt *et al.*, 2008). After completion of the 3D µCT measurements, the upper half of the specimens was subjected to an undecalcified infiltration process, plastic embedded, and cut into 5 µm sections. Then, the bone sections were stained using either the von-Kossa technique, toluidine blue or Goldner trichrome, as previously described (Breer *et al.*, 2012; Krause *et al.*, 2012a). The ratio of osteoid volume per bone volume (OV/BV, %), osteoid surface per bone surface (OS/BS, %) and eroded surface (ES/BS, %) were assessed using an automated computer-assisted image analysing system (OsteoMeasure, OsteoMetrics, Decatur, GA, USA).

Microdamage assessment

To evaluate microdamage, sections from the last third of the L5 vertebrae were prepared by bulk staining using calcein (Lee *et al.*, 2000; Aruwajoye *et al.*, 2013). Specimens were put in 0.9 % saline for 4 h after previous dehydration in alcohol (80 % ethanol). Subsequently, the specimens were soaked in calcein (0.3 g/100 mL saline) for 2 h under vacuum (20 mmHg), rinsed with reverse osmosis water for another 10 min and plastic embedded. Finally, central 5 µm sections were obtained. The microdamage assessment was performed in a region of interest (ROI) of at least 5 mm² using the OsteoMeasure system. Microdamage was defined according to the established criteria by Burr and Stafford (Burr and Stafford, 1990; Lee *et al.*, 2003), including intermediate size, sharp borders with increased fluorescence, deep staining after focus changing and linear shape. ROIs were scanned for the occurrence of microdamage at 100x magnification; when microdamage was detected, the number of cracks (Cr.N) and crack length (Cr.Le, µm) were measured at 200x magnification. In accordance with previous studies, crack density (Cr.Dn, #/mm²) and crack surface density (Cr.S.Dn, µm/mm², Cr.N*Cr.Le/BS) were determined (Aruwajoye *et al.*, 2013; Chapurlat *et al.*, 2007). Intra-observer reliability for the detection of microdamage was high (ICC: 0.996 [95 % confidence interval: 0.985, 0.999]) with a repeatability coefficient of 0.620.

Statistics

Statistical analysis was carried out using IBM® SPSS® Statistics 19 and the software package R (The R Foundation for Statistical Computing, Vienna, Austria). The distribution of categorical data is presented by absolute and relative frequencies. Descriptive statistics of continuous, normally and non-normally distributed data are given by mean ± standard deviation and median (range), respectively. Accordingly, comparisons of two or more groups were performed by *t*-test and analysis of variance (ANOVA) or the non-parametric Mann-Whitney U test and Kruskal-Wallis test. Spearman's correlation coefficient was used to assess the relationships among the TMD, bisphosphonate treatment duration and body mass index (BMI, kg/m²). In a univariate and multivariate analysis (ANCOVA) blind to treatment, possible predictors (age, body mass index, bisphosphonate exposition, TMD, osteoid parameters (OV/BV & OS/BS), Cr.Le, Cr.Dn and Cr.S.Dn) of the mechanical properties were identified using a stepwise forward variable selection based on Akaike's Information Criterion. All statistical tests were performed in an explorative manner at a 5 % significance level.

Results

Concerning baseline patient characteristics, parameters such as age and body mass index, which are known to independently influence mechanical properties of bone tissue, were not significantly different among the study groups (Table 1).

Histomorphometry

The histomorphometric analysis revealed that the overall bone volume (BV/TV) was marginally higher in controls (Table 1) due to the greater number of trabeculae (Tb.N; $p = 0.019$), which were slightly thinner (Tb.Th; Table 1) and significantly farther apart from each other (Tb.Sp; $p = 0.017$) in controls compared to the alendronate-treated groups. All three groups demonstrated an almost similar SMI with a tendency toward a more rod-like structure (mean: 1.92 ± 0.36). The amount of non-mineralised bone matrix (OV/BV, OS/BS) was marginally lower in patients with alendronate treatment compared to controls (OV/

Table 1. Patient characteristics and summary of histomorphometric parameters (mean ± standard deviation and median (range)). Comparisons were performed by *t*-test and ANOVA or the non-parametric Mann-Whitney U test and Kruskal-Wallis test.

Parameters	All	No treatment	Mid-term treatment	Long-term treatment	
	(*n* = 34)	(*n* = 21)	(*n* = 6)	(*n* = 7)	*p*-value
Age *(years)*	83 (65, 90)	84 (65, 90)	86 (75, 90)	82 (67, 86)	0.305
BMI *(kg/m²)*	21 (16, 34)	20 (16, 31)	22 (18, 28)	24 (17, 34)	0.439
BV/TV *(%)*	5.7 (3.0, 12.1)	6.3 (4.0, 12.1)	4.6 (3.3, 7.4)	4.7 (3.0, 7.7)	0.243
Tb.N *(/mm)*	0.91 ± 0.15	0.97 ± 0.15[a]	0.81 ± 0.09	0.82 ± 0.16	0.019
Tb.Th *(µm)*	117.3 ± 20.1	114.2 ± 20.5	124.1 ± 24.7	121.1 ± 14.7	0.500
Tb.Sp *(mm)*	1.10 ± 0.20	1.03 ± 0.17[a]	1.21 ± 0.13	1.23 ± 0.25	0.017
SMI	1.92 ± 0.36	1.84 ± 0.32	2.07 ± 0.33	2.06 ± 0.43	0.187

a: $p < 0.05$ for controls *vs.* long-term treatment.

Table 2. Univariate and multivariate analysis of mechanical properties. Univariate and multivariate analysis (ANCOVA) were performed blind to treatment.

Outcome	Predictor	univariate					multivariate				
		Intercept	Coefficient	SE	r	p	Intercept	Coefficient	SE	R²	p
Young's Modulus	Age	-0.152	0.016	0.013	0.217	0.218					
	BMI	**2.078**	**-0.042**	**0.018**	**-0.382**	**0.026**	2.062	-0.051	0.018	0.238	0.007
	TMD	**7.768**	**-0.007**	**5.326**	**-0.378**	**0.028**					
	ES/BS	1.312	-0.017	0.023	-0.131	0.459					
	Cr.Le	1.008	0.583	0.531	0.191	0.280		0.960	0.497		0.063
	Cr.Dn	1.078	0.015	0.042	0.063	0.723					
	Cr.S.Dn	1.067	0.066	0.072	0.159	0.369					
Yield Strength	Age	0.038	0.002	0.002	0.203	0.250	0.865	0.003	0.001	0.352	0.056
	BMI	0.250	-0.003	0.002	-0.184	0.297					
	TMD	**0.997**	**-0.001**	**0.0004**	**-0.360**	**0.036**		-0.001	0.0004		0.018
	ES/BS	0.220	-0.003	0.003	-0.165	0.351		-0.005	0.003		0.067
	Cr.Le	0.163	0.128	0.065	0.330	0.057		0.118	0.060		0.059
	Cr.Dn	0.184	0.002	0.005	0.065	0.716					
	Cr.S.Dn	0.175	0.015	0.009	0.291	0.095					
Ultimate Stress	Age	0.160	0.002	0.002	0.140	0.429	1.005	0.003	0.002	0.278	0.165
	BMI	0.297	-0.0004	0.003	-0.022	0.901					
	TMD	1.053	-0.001	0.0005	-0.282	0.107		-0.001	0.0005		0.059
	ES/BS	0.336	-0.005	0.004	-0.238	0.176		-0.007	0.003		0.043
	Cr.Le	0.259	0.130	0.080	0.275	0.115		0.123	0.077		0.120
	Cr.Dn	0.294	-0.001	0.006	-0.034	0.850					
	Cr.S.Dn	0.274	0.013	0.011	0.206	0.242					
Fracture Stress	Age	0.135	0.002	0.002	0.161	0.364	0.867	0.003	0.002	0.257	0,143
	BMI	0.268	0.0002	0.0028	0.015	0.932					
	TMD	0.934	-0.001	0.0005	-0.261	0.136		-0.001	0.0004		0.082
	ES/BS	0.315	-0.004	0.003	-0.219	0.213		-0.006	0.003		0.061
	Cr.Le	0.247	0.117	0.075	0.266	0.128		0.113	0.073		0.131
	Cr.Dn	0.284	-0.003	0.006	-0.075	0.673					
	Cr.S.Dn	0.261	0.011	0.010	0.183	0.301					
Work to Failure	Age	0.261	-0.002	0.001	-0.257	0.143					
	BMI	**-0.004**	**0.005**	**0.002**	**0.507**	**0.002**	-0.004	0.005	0.002	0.257	0.002
	TMD	-0.184	0.0003	0.0003	0.178	0.313					
	ES/BS	0.134	-0.002	0.002	-0.158	0.372					
	Cr.Le	0.114	0.004	0.052	0.013	0.941					
	Cr.Dn	0.135	-0.005	0.004	-0.214	0.225					
	Cr.S.Dn	0.120	-0.005	0.007	-0.116	0.512					
Toughness	Age	120.429	-0.666	0.537	-0.214	0.224					
	BMI	**26.856**	**1.773**	**0.767**	**0.378**	**0.027**	300.533	2.533	0.742	0.364	0.019
	TMD	119.690	-0.058	0.141	-0.072	0.686		-0.288	0.130		0.034
	ES/BS	83.365	-1.743	0.942	-0.311	0.074		-2.412	0.849		0.008
	Cr.Le	63.071	13.737	22.813	0.106	0.551					
	Cr.Dn	70.691	-1.054	1.764	-0.105	0.554					
	Cr.S.Dn	65.528	0.604	3.101	0.034	0.847					

Example of a regression formula and its interpretation emerging from multivariate regression analysis for Young's Modulus:

Young's Modulus = 2.062 - 0.051 x BMI + 0.960 x Cr.Le → With each unit of BMI the Young's Modulus decreases by 0.051 units. With each unit of Cr.Le the Young's Modulus increases by 0.960 units.

BV $p = 0.799$; OS/BS $p = 0.937$; Fig. 2). However, the resorption parameters (ES/BS) provided strong evidence of the alendronate treatment as resorption cavities were significantly lower in alendronate-treated patients (Fig. 2; $p < 0.001$). Also, the L5 vertebrae's degree of mineralisation (TMD) increased with alendronate therapy ($p = 0.037$) and body mass index (TMD, $r = 0.478$;

$p = 0.004$) (Fig. 3); however, the mineralisation parameters were not significantly different between the mid- and long-term alendronate-treated groups ($p = 0.668$).

Microdamage quantification
The crack density (*i.e.*, the amount of microdamage per mm bone area) was not different between the control and
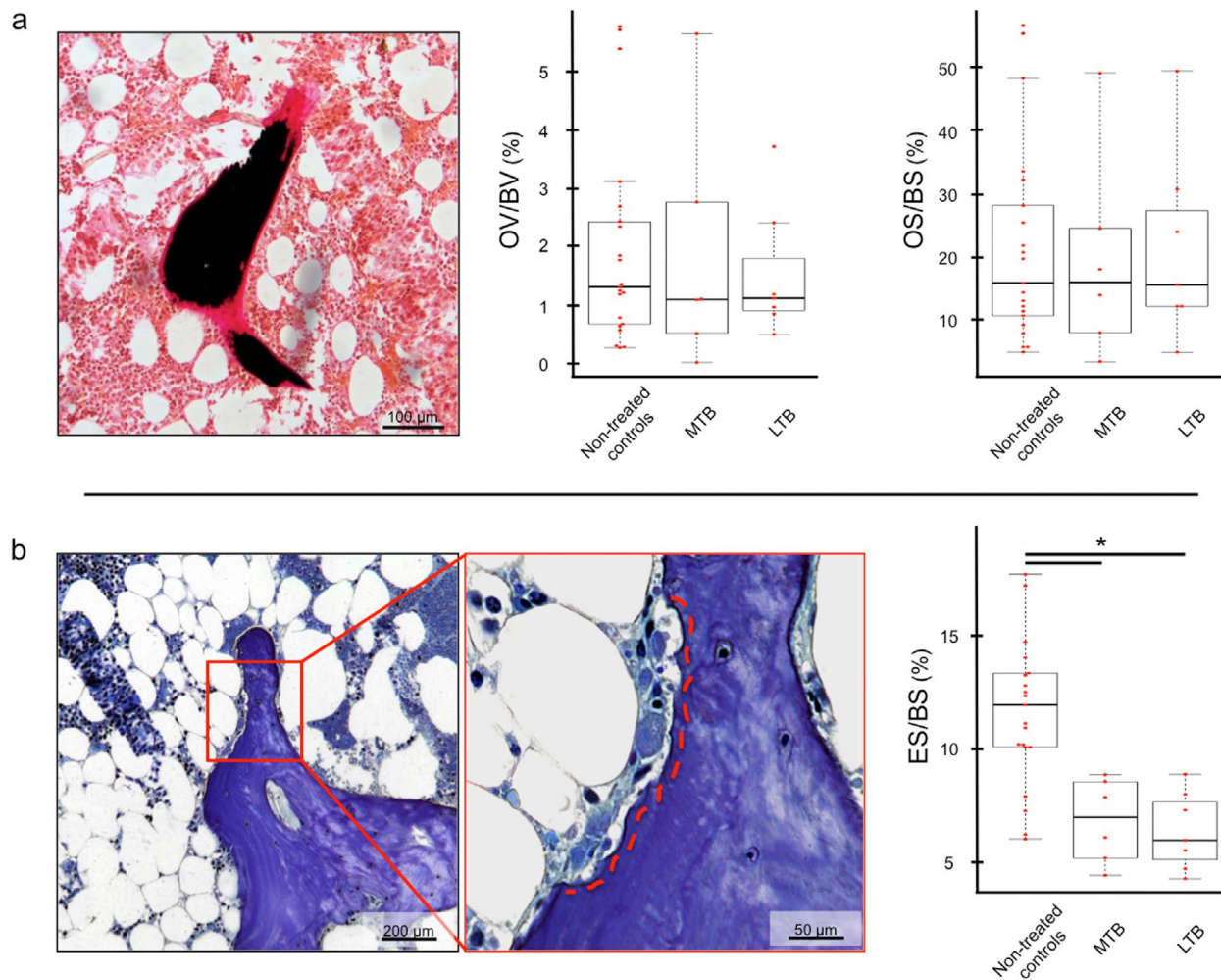
Fig. 2. (**A**, **B**) Histological analysis of undecalcified sections of L5. (**A**) Non-mineralised bone matrix (red) can be observed near the mineralised bone tissue (black, von Kossa staining). Osteoid volume (OV/BV) and osteoid surface (OS/BS) had a tendency to be lowest in long-term alendronate-treated patients. (**B**) Evidence of resorption activity in cancellous bone tissue (toluidine blue). Resorption cavities (dotted line) are considered stress raisers for single trabecular fractures. Eroded Surface (ES/BS) was lowest in alendronate-treated patients ($p < 0.001$). Comparisons were performed by *t*-test and ANOVA or the non-parametric Mann-Whitney U test and Kruskal-Wallis test.

alendronate-treated groups regardless of treatment duration ($p = 0.112$). The mean crack length had a tendency to be smallest in the long-term alendronate-treated group in comparison to the mid-term alendronate-treated group and controls ($p = 0.076$). Hence, crack surface density tended to be lower with long-term alendronate therapy in comparison with both the mid-term alendronate-treated group and controls ($p = 0.080$; Fig. 4).

Mechanical testing of single trabeculae
The Young's modulus, yield strength, ultimate stress, fracture stress, work to failure and toughness did not differ significantly among the three groups (Fig. 1).

Mechanical testing of single trabeculae – univariate analysis
In a univariate analysis blind to treatment group, potential influencing confounders such as age, eroded surface or microdamage parameters were not significantly associated with mechanical parameters (Table 2). TMD was the only univariate predictor of yield strength but demonstrated just a marginal negative association (coefficient -0.001,

$p = 0.036$). Also, Young's modulus decreased with increasing degree of mineralisation as assessed by μCT (TMD coefficient -0.007, $p = 0.028$). In addition, another independent predictor of Young's modulus was body mass index demonstrating a negative association (coefficient -0.042, $p = 0.026$). However, increasing body mass index resulted in a higher work to failure of the tested trabeculae, which was not associated with another influencing parameter (coefficient 0.005, $p = 0.002$). Also, a higher body mass index of osteoporotic patients was associated with a higher ability to absorb energy prior to material failure in single trabeculae (toughness: coefficient 1.773, $p = 0.027$). Interestingly, TMD showed no independent association with either work to failure or toughness. Ultimate stress and fracture stress showed no association with any potentially influencing confounder (Table 2).

Mechanical testing of single trabeculae – multivariate analysis
Bone and patient characteristics are interdependent and influence each other. Hence, mechanical properties might be influenced by a combination of bone and
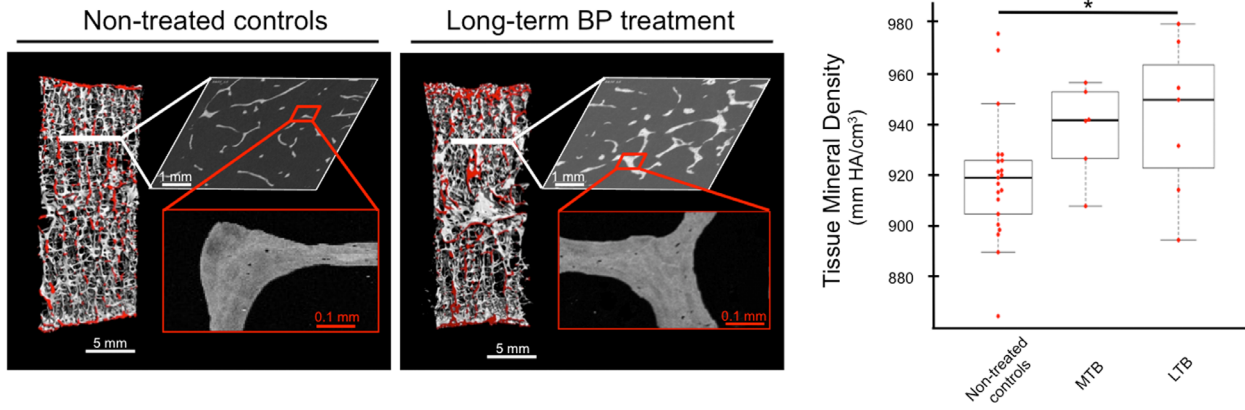
Fig. 3. Bone mineralisation assessment using µCT. Tissue mineral density of a non-treated osteoporotic control case demonstrating low mineralisation and of a long-term alendronate-treated case demonstrating higher mineralisation at higher magnification (scanning electron microscopy for demonstration). TMD was higher in both mid- and long-term alendronate-treated patients compared to non-treated osteoporotic controls ($p = 0.037$). Comparisons were performed by *t*-test and ANOVA.
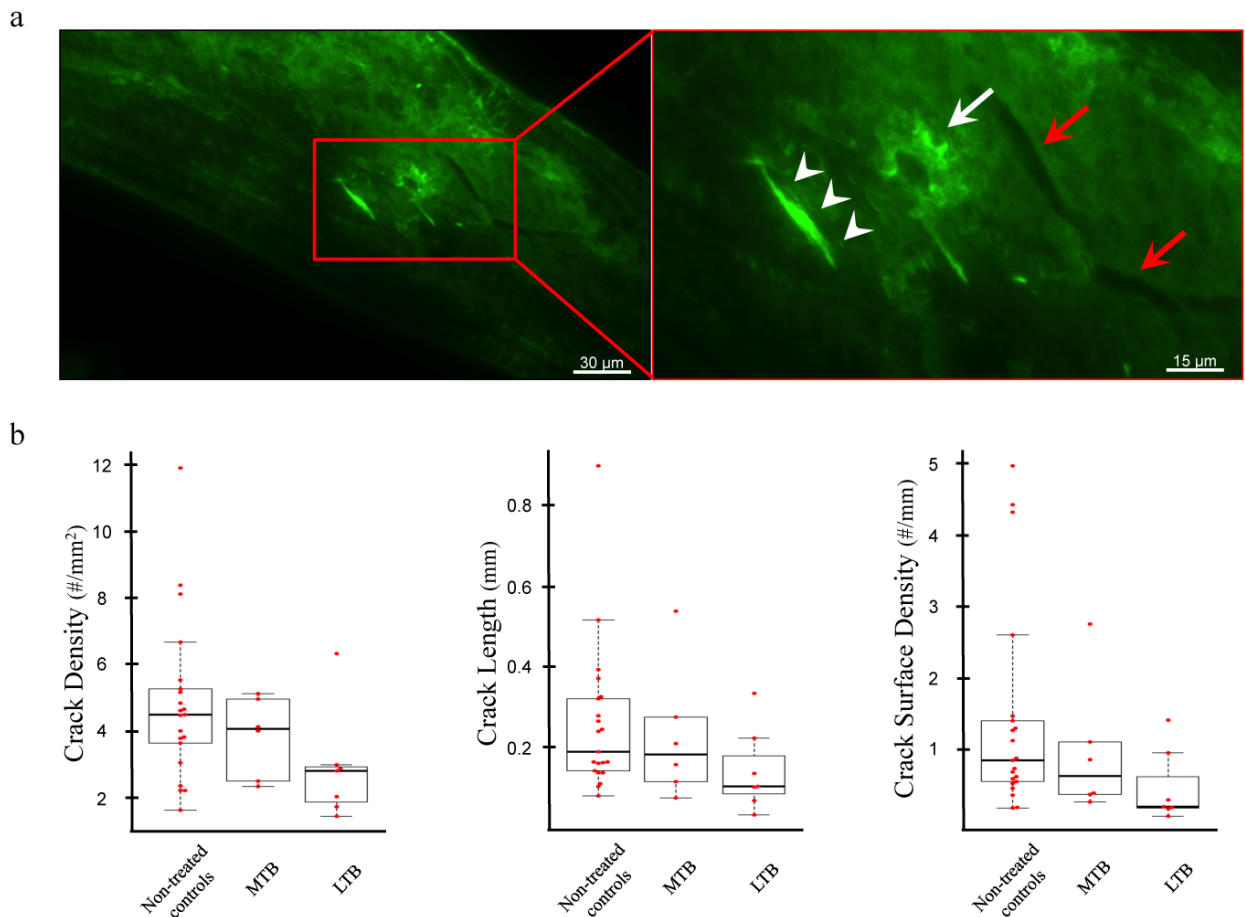

Fig. 4. (**A**, **B**) Microdamage assessment of cancellous bone. (**A**) Overview of single trabeculae with microdamage (calcein bulk stain). The white arrowheads indicate microdamage next to an osteocyte (white arrow) and the red arrows point to preparation artifacts. (**B**) Microdamage parameters have a tendency to be lower in patients with long-term alendronate treatment compared to non-treated osteoporotic controls and mid-term alendronate-treated patients but did not reach statistical significance. Comparisons were performed by *t*-test and ANOVA or the non-parametric Mann-Whitney U test and Kruskal-Wallis test.

patient predictors with an individually different impact. Thus, an additional multivariate analysis was performed including age, body mass index, TMD, eroded surface and microdamage indices. In a subsequent search for the best subset of influencing variables, the right-hand column of Table 2 shows the results of the multivariate analysis. Compared to the univariate analysis Young's modulus still decreased with increasing body mass index. However, considering all potentially influencing parameters tested this association was highest just in combination with increasing crack length (Cr.Le) resulting in a higher Young's modulus ($R^2 = 0.238$). Furthermore, yield strength, ultimate stress and fracture stress correlated with increasing age and greater crack length in combination with a lower degree of mineralisation and eroded surface. However, work to failure was only associated with body mass index, while toughness was associated with higher body mass index, higher TMD, but most importantly a lower eroded surface representing potential stress raiser in a single trabeculae (Table 2).

Discussion

Bisphosphonates act by suppressing bone turnover; hence, a major concern is that this mechanism of preserving bone quantity in disease such as osteoporosis negatively affects the tissue characteristics leading to an accumulation of microdamage, higher mineralisation profiles and in turn deteriorated mechanical properties. Suppression of bone turnover is of particular concern for long-term bisphosphonate-treatment plans due to the recent unexplained occurrence of atypical femoral fractures in some patients (Shane *et al.*, 2010; Donnelly *et al.*, 2012). Indeed, *in vivo* failure occurs through deformation and fracture of the entire vertebral body, and will depend on a multitude of factors including the bone quantity (*i.e.*, BV/TV) and bone quality (*e.g.*, architecture, composition, damage, mechanical properties) at multiple length-scales. In the present study, we analyse the quality of the bone tissue at the trabecular level in patients with long-term alendronate treatment, mid-term alendronate treatment and non-treated controls with osteoporosis to determine whether alendronate treatment affects microdamage accumulation, tissue mineralisation and mechanical properties at the single trabeculae level.

One of the main effects of alendronate treatment is attributed to the suppressed rate of remodelling. Here, alendronate-treated patients demonstrated a significantly elevated degree of mineralisation than non-treated controls in vertebral bone. However, the mineralisation profile was not significantly different between the long-term and mid-term alendronate treatments, which is in line with previous findings from other skeletal regions (Roschger *et al.*, 2010; Black *et al.*, 2012).

Along with a shift to higher mineralisation, microdamage has been observed to accumulate with bisphosphonate treatment in many pre-clinical studies (Mashiba *et al.*, 2000; Mashiba *et al.*, 2001; Allen *et al.*, 2006). Clearly, the accumulation of microdamage is a concern because bisphosphonates act by suppressing bone turnover, which would reduce the number of remodelling events. However, here, an accumulation of microdamage was not found in the alendronate-treated patients. Long-term alendronate-treated patients even showed a tendency towards a lower amount of microdamage compared to non-treated controls. A similar study on iliac crest biopsies also found no association between bisphosphonate treatment duration and microdamage accumulation in humans (Chapurlat *et al.*, 2007). Microdamage should accumulate in regions with higher mineralisation in comparison to the surrounding tissue (Wasserman *et al.*, 2005). Given that Roschger *et al.* (Roschger *et al.*, 2010) did not observe a higher incidence of abnormal highly mineralised bone areas after long-term bisphosphonate treatment in biopsies of human iliac crest bone, the lack of microdamage accumulation observed here in vertebral bodies is conceivable.

In accordance with the occurrence of microdamage, we were unable to find differences in the mechanical parameters of single trabeculae from alendronate-treated patients and non-treated osteoporotic controls. The mechanical properties of the single trabeculae here contrast with previous studies (computational and experimental in nature) using whole vertebral bodies that have found a higher vertebral compressive strength with bisphosphonate treatment (Mashiba *et al.*, 2001; Keaveny *et al.*, 2007). When testing whole vertebral bodies, the cortical thickness and trabecular architecture affect the mechanical properties. In addition to strength, toughness is another important material property in relation to bone quality (Launey and Ritchie, 2009) and defines the ability to absorb energy in the process before material failure. In cases where bone turnover is affected, such as osteoporosis and the treatment of osteoporosis, bone strength and toughness can become impaired (Busse *et al.*, 2009). Despite the potential of bisphosphonates to reduce the occurrence of vertebral and non-vertebral fractures, preclinical animal studies have suggested that bisphosphonate treatments are associated with a reduced toughness (Mashiba *et al.*, 2000; Mashiba *et al.*, 2001; Komatsubara *et al.*, 2003; Allen and Burr, 2007). However, in the present study, the mid-term and long-term alendronate treatments did not result in a reduced toughness of single lumbar trabeculae in comparison to untreated osteoporotic controls.

Therefore, even though the mid- and long-term alendronate treatments had a slightly higher mineralisation, there was no change in mechanical properties or microdamage accumulation in comparison to non-treated osteoporotic bone. While the larger-scale vertebral features (such as cortical shell, trabecular architecture) may contribute to fracture risk reduction, the data presented here demonstrate that histologically the alendronate-treated bone and osteoporotic controls are different through the higher mineralisation profile and a smaller eroded surface, which are both established effects of the suppression of turnover induced by alendronate (Balena *et al.*, 1993). Thus, we theorise that even though the alendronate-treated individuals have a higher TMD, which should decrease their toughness, the reduction in resorption cavities may counteract the TMD to maintain the bone toughness after alendronate treatment. Indeed, in past studies, resorption cavities were identified to predict fracture risk independent

of aBMD because they decrease the overall bone area and may be considered as stress concentrations, where fractures can initiate (Garnero, 2000; Hernandez, 2008; Slyfield *et al.*, 2012).

The univariate analysis between the mechanical properties and other possible predictors of fracture showed that the body mass index was associated with the mechanical properties. In clinical studies, the positive influence of the body mass index on fracture risk was primarily attributed to improved geometric strength and higher aBMD levels most probably due to adaptation to increased loading conditions (Beck *et al.*, 2001; Beck *et al.*, 2009). This was supported by our results as body mass index positively correlated with mineralisation data. However, the degree of mineralisation was not associated with toughness in the univariate analysis suggesting that body mass index has additional effects on bone material characteristics. As many of the bone characteristics are interrelated, the mechanical properties may be related to a joint contribution from multiple predictors. Thus, an additional multivariate analysis was performed. Here, toughness was influenced by a combination of TMD, body mass index and eroded surface. As discussed above, body mass index is commonly associated with improved geometric strength and higher aBMD levels (Beck *et al.*, 2001; Beck *et al.*, 2009) as well as TMD, in this study. Therefore, the multivariate analysis reflects the fact that combined changes in mineral density (through TMD or body mass index) and eroded surface influence the mechanical properties.

This study has some limitations. First, due to the retrospective study design, follow-up measurements were not possible, and there was no knowledge of the starting values for the parameters measured, which needs to be taken into account when looking at the restricted number of patients. However, the BioAsset study allowed a unique recruitment of patients with mid- and long-term alendronate treatments. The mechanical tests on single trabeculae from vertebral bodies also pose a few limitations. While testing material in its fresh state is optimal, special sample preparation techniques are required to singularise the trabeculae. In particular, the effect of hydrogen peroxide treatment to remove soft tissue is controversial; however, there is no strong evidence that the short hydrogen peroxide treatment affects the collagen or mineral properties due to the preservation of the collagenous matrix (osteoid) (Chng *et al.*, 2005; DePaula *et al.*, 2005; Joiner, 2007; Busse *et al.*, 2009; Li *et al.*, 2011). Second, specimens were also tested dry, which could influence the mechanical properties. Dry testing particularly influences the strength and toughness by reducing the plasticity generated in the sample through deformation of the organic components. However, as all groups were tested in a dry state, a comparison is valid. Third, the materials properties and geometry could change throughout the cross-section of the trabeculae, which would pose another limitation to precisely measuring mechanical properties, but this mechanical testing technique has been implemented numerous times to investigate this small but important feature within the cancellous bone structure (Carretta *et al.*, 2013; Ridha and Thurner, 2013). Fourth, an additional contribution to the trabecular mechanical

properties would be the inorganic component of bone. Indeed, increased levels of cross-linking within the organic component have been associated with fragility fractures (Saito *et al.*, 2006), as the cross-links presumably restrict deformation at small length-scales within bone (Zimmermann *et al.*, 2011). However, previous studies present in the literature do conflict over the effects of bisphosphonate treatment on the cross-linking profile within bone (Allen *et al.*, 2008; Boskey *et al.*, 2009; Gourion-Arsiquaud *et al.*, 2010; Donnelly *et al.*, 2012). Nonetheless, a drawback to the present study is that this level of the bone structure was not analysed.

In summary, here, we characterise the structure and mechanical properties of individual trabeculae from patients with long-term alendronate treatment, mid-term alendronate treatment and osteoporotic controls. From strength tests on single trabeculae, no differences were found between the alendronate-treated groups and the osteoporotic controls. The alendronate treatments did result in an elevated mineralisation profile in cancellous vertebral bone, which is a hallmark of bone turnover suppression due to treatment. However, the higher mineralisation profile's impact on the mechanical properties may be compensated by fewer resorption cavities (*i.e.*, more bone is present or potential stress raisers are eliminated) in the alendronate-treated bone. This counterbalance was supported by a multivariate analysis showing that multiple factors, such as body mass index, bone mineralisation and eroded surface, can together influence the trabeculae's mechanical properties.

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Discussion with Reviewers

S. Gamsjaeger: Can the results obtained in the trabecular bone of the spine transferred to other skeletal sites or is this a site-specific effect?

Authors: Previous studies have shown that the effect of bisphosphonate treatment is highly site specific (Smith *et al.*, 2003; Allen *et al*, 2010, additional references). In particular, the bisphosphonate-related reduction in bone resorption has been shown to be lower at peripheral sites than at the iliac crest or the vertebral body. In addition, the effects of BP treatment seem to depend also on the surface-to-volume ratio of the bone compartment (Hu *et al.*, 2002, additional reference) and its metabolic activity (Lin, 1996; Rodan, 1998; Rogers *et al.*, 2000, additional references). Hence, a transfer of the present results to other skeletal sites may not be appropriate.

S. Gamsjaeger: Does your study imply that the advantages of long-term bisphosphonate treatment in manifested osteoporosis, *e.g.*, preservation of increased bone mineral density, predominate possible effects on bone mechanical properties?

Authors: Here, we do not wish to make a claim that the advantages of long-term bisphosphonates outweigh possible impacts on the mechanical properties particularly because bisphosphonates have site-specific effects on the structural characteristics and mechanical properties of bone. However, specific to trabecular bone from the lumbar spine, our data showed that a preservation of increased bone mineral density in trabeculae does not result in an increased accumulation of microdamage. In addition, the reduced osteoclastic activity found in long-term bisphosphonate cases is associated with fewer resorptive lacunae, which could act as potential stress raisers for trabecular fractures. The lesser degree of resorptive lacunae seems to counterbalance potentially adverse effects on vertebral trabecular biomechanical properties.

A. Ignatius: The argument given for the use of peroxide is rather weak. Osteoid may remain intact, but the suspected effect of peroxide involves the dissolution of small immature crystallites. Please comment.

Authors: We agree that it is always a limitation when sample preparation techniques may endanger the physical integrity of the specimens. However, as we state in the limitations of the study paragraph, the effect of peroxide is minimal. In addition, prior to the experimental investigation, pilot tests were performed with quantitative backscattered electron imaging and did not indicate any significant change in the degree of mineralisation in bone samples before or after peroxide treatment, which is further supported by recent Raman spectroscopy analyses where peroxide treatments are common (Penel *et al.*, 2005; Petra *et al.*, 2005, additional references).

A. Ignatius: Are the μCT and qBEI results as expressed, influenced by a variable Ca/P ratio of the mineral crystallites? After all, bisphosphonates have been shown to directly affect the mineral properties through physical chemical mechanisms.

Authors: The µCT determines bone tissue mineralisation by measuring image intensity values (X-ray linear attenuation), which reflect compositional properties of the imaged field of view (FOV). These gray-scale attenuation values were calibrated against standard mineral density values using a phantom containing hydroxyapatite cylinders. Burghardt *et al.* have demonstrated high reproducibility of the assessment of bone tissue mineral density (Burghardt *et al.*, 2008, text reference). Given that non-destructive µCT is an established tool to measure the TMD, it is commonly used to monitor treatment effects in bone diseases (Nazarian *et al.*, 2008; Deuerling *et al.*, 2010; Shahnazari *et al.*, 2010; Wagner *et al.*, 2011, additional references).

H. Zahedmanesh: The number of patients reported for the control group is three-fold higher than the number of patients with bisphosphonate treatment. The authors need to justify that this does not compromise the main findings of the study.

Authors: The imbalance in group sizes does not affect the performed statistical analyses. From our point of view, the approach is in accordance with the statistical theory of the analyses used. The fact that the control group is bigger is even beneficial for the overall power of the statistical tests.

H. Zahedmanesh: Regarding Fig. 1a, the fracture toughness and work to fracture are lower in the long-term group compared to the mid-term group, although, not statistically significant. Albeit, given the limited number of patients, this visible trend does not corroborate the core result of the article if not compromise it.

Authors: We thank the reviewer for closely analysing the trends in the data, but we believe that these trends do not reflect a clear result. The work to failure and toughness values shown in Fig. 1b do not show any significant change between the compared groups.

H. Zahedmanesh: In the authors' opinion, do the trends identified based on micromechanical tests at the trabeculae level such as fracture toughness, predict similar trends at the tissue level (vertebrae)?

Authors: We believe that micromechanical tests on single trabeculae cannot reflect the mechanical properties of whole vertebral bodies. Apart from the inherent resistance of the bone tissue, an important aspect of trabecular bone's resistance to failure is the 3D network of trabeculae and the cortical shell. Thus, by using single trabeculae, we are using a rather idealised test specimen (small cylindrical) that is not influenced by any structural effects due to variations in orientation of trabeculae, trajectories, influence of the cortical shell, polyostotic heterogeneity in trabecular networks. Thus, by using somewhat idealised test specimen, we can determine directly whether the composition, microdamage distribution and/or structural changes to the trabeculae due to bisphosphonate treatment influence the mechanical properties of the bone tissue, while minimising confounding factors.

D. Ruffoni: Single trabeculae are neither homogenous in material properties nor uniform in cross section. Which is the impact of these "imperfections" on the material properties derived with three-point bending at the length-scale of single trabeculae? In addition, how large is the error on the measured elastic and failure properties? How can the authors be sure that the proposed method is accurate enough to detect possible difference in the mechanical competence of the trabeculae extracted from treated and non-treated bones?

Authors: It is correct that the inhomogeneous material properties and size of the sample could influence the mechanical property measurements. The data may have a greater statistical significance if the moment of inertia and the variation in the composition were accurately quantified prior to measurement. Unfortunately, accurate measurements of the composition and geometry would require techniques, such as µCT that would also damage the sample prior to mechanical testing. With these thoughts in mind, we acknowledge the limitations imposed by the technique in the limitations of the study paragraph. However, the testing of trabeculae in three-point bending is essential as they represent the smallest structural entity within the vertebral structure resisting load and this technique has been previously used in consideration of the limitations (Carretta *et al.*, 2013; Ridha and Turner, 2013, text references).

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