Full length article

Reduction of fibrillar strain-rate sensitivity in steroid-induced osteoporosis linked to changes in mineralized fibrillar nanostructure


A R T I C L E I N F O

Keywords:
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Synchrotron X-ray nanomechanical imaging
Nanoscale deformation mechanisms
Multiscale Mechanical modelling

A B S T R A C T

As bone is used in a dynamic mechanical environment, understanding the structural origins of its time-dependent mechanical behaviour – and the alterations in metabolic bone disease – is of interest. However, at the scale of the mineralized fibrillar matrix (nanometre-level), the nature of the strain-rate dependent mechanics is incompletely understood. Here, we investigate the fibrillar- and mineral-deformation behaviour in a murine model of Cushing's syndrome, used to understand steroid induced osteoporosis, using synchrotron small- and wide-angle scattering/diffraction combined with in situ tensile testing at three strain rates ranging from \(10^{-4}\) to \(10^{-1}\) s\(^{-1}\).

We find that the effective fibrillar- and mineral-modulus and fibrillar-orientation distributions show no significant increase with strain-rate in osteoporotic bone, but increase significantly in normal (wild-type) bone. By applying a fibril-lamellar two-level structural model of bone matrix deformation to fit the results, we obtain indications that altered collagen-mineral interactions at the nanoscale – along with altered fibrillar orientation distributions – may be the underlying reason for this altered strain-rate sensitivity. Our results suggest that an altered strain-rate sensitivity of the bone matrix in osteoporosis may be one of the contributing factors to reduced mechanical competence in such metabolic bone disorders, and that increasing this sensitivity may improve biomechanical performance.

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1. Introduction

Determining the mechanically-critical structural and compositional alterations of bone matrix in metabolic bone disorders, such as osteoporosis or osteogenesis imperfecta, is essential to understand origins of the reduced mechanical competence exhibited in such disorders [1–3]. A systematic characterization of the mechanical properties of bone was pioneered by John Currey [4]. Among his many contributions to biomechanics, he found that stiffness, strength and toughness of bone depend on biological factors such as anatomical specialisation [5] and species [6], as well as on factors related to materials-composition and structure, such as mineral [7] and collagen content [8,9]. The research presented here was performed in the spirit of his systematic approach, but focusses not on quasi-static mechanical properties, but on changes of the mechanical performance under three different loading speeds. As bone is used under time-dependent loading in a dynamic mechanical environment, linking the viscoelastic and strain-rate dependent behaviour of bone matrix to such alterations is important. However, clinical measures assessing bone state (such as bone mineral density (BMD)) capture mainly changes in bone mass, and provide little information on alterations in quality of the bone matrix. The matrix of bone at the nanoscale is a composite of Type-I collagen (brillar matrix) and noncollagenous proteins and water [10,11], which are assembled into fibrils, carbonated apatite, noncollagenous proteins and water [10,11], which are assembled into fibrils, carbonated apatite, noncollagenous proteins and water [10,11], which are assembled into fibrils, and mineral [7], and correspond to changes in mineral to matrix ratio as measured by Raman microspectroscopy. A reduction in mineral concentration (by 45%) caused by glucocorticoids treatment is accompanied by reduced degree of bone mineralization, as compared to controls [31]. Our previous study on a mouse model of endogenous hypercorticosteronaemia (Cushing’s syndrome) shows a significant reduction (by 51%) of fibril modulus, larger fibril strain/tissue strain ratio and a disruption of intracortical architecture as compared with their wild-type littermates [33]. In relation to mechanics, bone fractures in healthy individuals usually happen with traumatic events at high strain rates, whereas in GIOP, bones are additionally involving fragility fractures with minimal trauma at relatively low strain rates [1,34,35]. Since the quasi-static fibrillar-level mechanics and structure are altered in GIOP-bone [15,33], it is therefore of interest to investigate, in this prototypical secondary osteoporosis, possible viscoelastic and strain-rate dependent effects in the mineralized fibrillar matrix.

In this study, we examine the deformation of the mineralized fibrils in the bone matrix of a GIOP mouse model at three different strain rates, using high-brilliance time-resolved synchrotron small-angle X-ray scattering (SAXS) and wide-angle X-ray diffraction (WAXD). These X-ray techniques provide information on the fibrillar- and mineral platelet-level strain in the bone matrix, induced by external mechanical loads. When combined with a high brilliance synchrotron source, SAXS/WAXD measurements can be carried out with time-resolution of the order of seconds [14,15,36–38], facilitating dynamic measurements. For the animal model of GIOP, we use a mouse model (Crh$^{-120/}$−) of endogenous hypercorticosteronaemia (Cushing’s syndrome), published as a model of endogenous GIOP [39]. Prior work has suggested that fracture risk in endogenous glucocorticoid production (Cushing’s syndrome) is similar to that in exogenous GIOP [40], although we acknowledge of the limitation of using mouse models to understand human GIOP, due to the absence of secondary osteonal remodelling. Our previous quasi-static (not time-dependent) SAXS/WAXD study, on the developmental changes in bone nanostructure in this model, provided evidence for increased fibrillar deformability, more random fibrillar orientation, and shorter/less stress-reinforcing mineral platelets in GIOP [15,33]. Here, we carry out tensile deformation on cortical GIOP mouse bone at a fixed age point (24 weeks) and at three strain rates to quantify the alterations in fibrillar mechanics in comparison to wild-type animals. Because SAXS/WAXD measurements are intrinsically volume-averaged measures of nanoscale deformation, the experimental data is combined with a multiscale model of the mechanics of the fibrils and fibril-arrays, developed from previous work [13,36,41], to help in the interpretation of the experimental results.
2. Materials and methods

2.1. Animals

Bone tissue from female GIOP mice (Crh−120/−) and wild-type (Crh+/+) littermates on a C57BL/6 genetic background (3rd generation) aged 24 weeks were used in this study. Mouse samples were stored at −20 °C before experiments. The mice were bred as part of a prior study [39], where all animal studies were carried out using guidelines issued by the UK Medical Research Council, in Responsibility in Use of Animals for Medical Research (July 1993) and Home Office Project License numbers 30/2433 and 30/2642.

2.2. Sample preparation for in situ tensile testing

Murine femora were dissected and longitudinally sectioned along the long axis using a water-irrigated low speed saw with a diamond-coated blade. The distal and proximal ends of anterior femora strips were embedded in dental ionomer (FiltekTM Supreme XT, 3 M ESPE, USA) such that samples could be mounted in the microtensile tester. The dental ionomer was exposed in UV light for 20 s, while the mid-diaphysis of femora bone was covered by lead tape during UV light exposure to prevent any UV-induced tissue alteration. The obtained femora strips for microtensile testing have typical gauge length, width and thickness of 5 mm, 1 mm and 0.2 mm, respectively. Samples were then wrapped in PBS-soaked tissue paper and stored at −20 °C before used for mechanical testing.

2.3. In situ micro tensile testing with simultaneous synchrotron SAXD/ WAXD measurements

Combining in situ tensile testing with real time synchrotron SAXD and WAXD, the load data (from load cell), fibril strain εf (from the SAXD frames) and mineral strain εm (from the WAXD frames) can be collected concurrently, as initially devised by Gupta et al. [37]. A customized microtensile tester was mounted in the path of synchrotron X-ray beam at beamline I22, Diamond Light Source (Harwell, UK), such that SAXD and WAXD frames were collected concurrently with mechanical loading of the sample. Samples were uniaxially loaded in tension using a customized microtensile tester equipped with a DC linear-encoder stage (MI12.1DG; Physic Instruments, UK) and an 111 N model SLC31/00025 tension/compression load cell (RDP Electronics Ltd, UK). A custom LabVIEW based software (LabVIEW 2013, National Instruments, UK) was used to control the microtensile tester and CCD camera. Samples were tested at room temperature and hydrated throughout each experiment in a fluid bath filled with physiological saline (PBS solution).

For the three different load rates used in the current study, the motor velocities were set to be 0.1, 0.05 and 0.002 mm/s, which correspond to motor strain rates of 0.02 s−1, 0.01 s−1 and 0.0004 s−1, respectively. Strain rates of 0.02 s−1 and 0.01 s−1 were used because they are in the range of physiological strain rates during walking and running, whereas a strain rate of 0.0004 s−1 representing the quasi-static loading was also examined as strain rates near this magnitude have been used in our previous studies [15,33,42,43]. The numbers of samples tested at strain rate of 0.02 s−1, 0.01 s−1 and 0.0004 s−1 were 4, 4 and 4, respectively, for wild-type mice; and 6, 5 and 4, respectively, for GIOP mice.

For the synchrotron SAXD and WAXD measurement, the X-ray wavelength λ was 0.8857 Å and beam cross section was ~240 × 80 μm at the sample. A Pilatus P3−2 M detector was used to collect the SAXD data, while a Pilatus P3-2M-DLS-L detector was used to collect the WAXD data; both detectors have a pixel resolution of 1475 × 1679 pixels and pixel size of 172 × 172 μm2. Note that in the concurrent SAXD/WAXD measurement protocol used, one quadrant (lower right) of the WAXD detector space is removed to allow for the remaining SAXD signal to transmit to the downstream SAXD detector; as a result, the WAXD pattern spans 3 out of 4 quadrants on the detector. The sample-to-detector distance was ~ 3727.0 mm for SAXD detector and ~ 175.3 mm for WAXD detector, as measured with Silver Behenate and Silicon standard, respectively. The X-ray exposure time was 0.1 s for both SAXD and WAXD patterns for samples measured at all strain rates. Due to the different durations of the mechanical tests at different strain-rates, the period between successive SAXD/WAXD acquisitions (with beam shutter closed) was controlled by the wait-time parameter (0.1 s: strain rate 0.01 s−1 and 0.02 s−1, and 3.4 s: strain rate of 0.0004 s−1). The beam shutter was closed between consecutive acquisitions of SAXD and WAXD patterns, to minimise the effect of X-ray irradiation on the mechanical properties of bone tissue [44].

2.4. SAXD and WAXD data analysis

Fibril strains and load-induced changes in fibrillar orientation distribution were measured from 2D SAXD patterns, and mineral strains were measured from 2D WAXD patterns.

2.4.1. Fibril strain

The meridional staggering (D-period) of collagen molecules inside the fibril leads to an axial diffraction pattern in the small-angle region of reciprocal space [45]. The third-order meridional collagen reflections were used to measure the D-period of collagen fibrils evaluating a radially-narrow semi-circular sector (180° angular width) (Fig. 1G); this corresponds to considering an integrated averaged of fibrillar deformation in all directions. The fibril strain (εf) was calculated from the percentage increases in D-period during tensile testing of samples [15,33,45,46]. SAXD patterns at different stress levels are shown in the Supplementary Information (Figure S1).

2.4.2. Mineral strain

For WAXD, the mineral particles consist of apatite (with a lattice structure of hexagonal closed-packed or hcp type) with the c-axis predominantly oriented along the fibril direction [47]. In a similar manner to the SAXD analysis, the mineral strain (εm) along the loading direction was measured from the percentage changes of lattice spacing, obtained from the (002) peak centre position of apatite averaged in a radially-narrow semi-circular (180° angular width) in the upper quadrant, in an analogous manner to SAXD (Fig. 1E), similar to prior work [14,15,38,45].

The Processing perspective of the data analysis software package DAWN [48] (www.dawnsac.org) was used for SAXD and WAXD data reduction. The integrated SAXD and WAXD 1D intensity profiles (Fig. 1F and H) were obtained from 2D SAXS/WAXD images as described above. Subsequently, the 1D profiles were fitted using a custom Python script. Both the 1D collagen SAXD data and the 1D mineral WAXD data were fitted to combinations of a Gaussian peak and a linear background term. To analyse the change of fibril and mineral strains during tensile loading, the obtained peak centre positions were used to calculate the D-period for the collagen fibrils and the (002) crystallographic lattice spacing for the mineral apatite. Linear regressions of D-period and D(002) were carried out versus macroscopic stress, and the intercept of each regression was taken as the unstressed (zero-stress) value for D-period and D(002). The collagen fibril strains εf and mineral strains εm were calculated from the percentage changes of collagen D-period and the (002) lattice spacing, respectively, relative to the unstressed sample. The effective fibril modulus (Ef = dσ/dεf) and effective mineral modulus (Em = dσ/dεm) were defined as the slope of tissue-level stress σ versus fibril strain and mineral strain, respectively, from the elastic region of deformation (Fig. S3 and 4, Supplementary Information), as described in prior work [15,33,45]. We note that the terminology (effective fibril modulus and mineral modulus) is used for consistency with prior work [15,33,49], and as will be discussed in the modelling section, these parameters are not equivalent to the actual...
2.4.3. Fibrillar orientation distribution

The changes in fibrillar orientation distribution with tensile load were analysed by observing the narrowing of the FWHM of the angular variation of SAXD intensity of the first-order collagen reflection, as described in our prior study on quasi-static deformation of glucocorticoid-induced osteoporotic bone [33]. Using the DAWN processing perspective, radially averaged azimuthal intensity profiles \( I(\chi) \) were calculated over the full azimuthal range (360°) from the first-order collagen reflection (at \( q = q_0 = \frac{6\pi}{D} \)). To subtract out the diffuse scattering background due to the mineral, similar azimuthal intensity profiles \( I_m(\chi; q_0-\Delta q) \) and \( I_m(\chi; q_0 + \Delta q) \) near the first-order collagen reflection, with \( \Delta q = 0.015 \text{ nm}^{-1} \) chosen to have \( q_0 \pm \Delta q \) outside of the first-order collagen peak, were calculated and averaged. The corrected azimuthal intensity profile \( I_c(\chi) \) was calculated as \( I_c(\chi) = I(\chi; q_0) - 0.5 \times [I_m(\chi; q_0-\Delta q) + I_m(\chi; q_0 + \Delta q)] \). The obtained \( I_c(\chi) \) was fitted with a pair of Gaussian peak functions separated by 180°. From the fit, the peak position indicates the predominant direction of fibril orientation, while the peak width (FWHM) is related to the extent of fibrillar alignment: larger FWHMs correspond to lower alignment (See Fig. S2 in the Supplementary Information). The rate of fibrillar reorientation was calculated from the slope of FWHM (degrees) versus fibril strain (%) curve for each sample [33], with units of degrees/%.  

2.5. X-ray microtomography

X-ray microtomography was used to study 3D micromorphometry and microscale mineralization distribution of bone tissue. Mice femora were longitudinally sectioned into two halves. Five samples from both wild-type and GIOP mice were used for X-ray microtomography measurements to obtain tomograms, which were used for quantitative analysis of microscale mineralization distribution in femoral mid-shaft from both wild-type and GIOP mice. Samples were mounted on the sample stage of a high-definition X-ray microtomography scanner.
(MuCat scanner) which equipped with an ultrafocus X-ray generator (Nikon Metrology (Leuven, Belgium)) and CCD camera (Spectral Instruments Inc (Tucson, Arizona, USA)) in a time-delay integration readout mode. An accelerating voltage of 40 kV was used to scan mice femora samples and a voxel size of $15 \times 15 \times 15 \, \mu m^3$ was obtained. The projection data were processed following a calibration procedure, in which the scanning data were corrected to an equivalence of 25 keV monochromatic X-ray source, and then a reconstruction procedure in which a cone-beam back-projection algorithm was used to generate 3D images (representing the absolute linear attenuation coefficient of 25 keV) of the scanned regions of samples. The 3D tomograms of samples were processed with an in-house software (Tomview, authored by GRD) to export a series of 8-bit grey level slices, multiplying the linear attenuation coefficient by a known constant to obtain an appropriate dynamic range. The histograms of grey levels for wild-type mice and two distinct regions of interest in GIOP mice - periosteal region and endosteal region (Fig. 2C1) - were generated from 2D slices using ImageJ software (ImageJ, NIH, USA). The histograms of grey levels for three data groups were converted into histograms of mineral concentration using published X-ray attenuation data [50], from which the average mineral concentrations (denoted as the degree of mineralisation) measured as hydroxyapatite (g/cm$^3$) were calculated and plotted for different bone regions (Fig. 2E and F). The mineral concentration is converted to mineral volume fraction as previously described [51,52]. For input of experimental mineral concentrations into the model (described below), the mineral concentration and volume fraction are taken as the average values across the cross-section of the tissue, similar to our prior work [15].

2.6. Calculation of microscale porosity and stress

The experimental stress data was calculated by the load values divided by the area of the fracture surface, and then corrected by the porosity of bone, following our previous study [15]. SEM image was taken on the fracture surface while the fractured sample was mounted vertically, and the area of the fracture surface was measured from SEM image using ImageJ (NIH, Bethesda, USA). The experimental stress data were post-multiplied by the coefficient $1/(1 - p^{3/2})$ to incorporate the effects – on the effective cross-sectional area – of a 3D isotropic distribution of internal porosity in bone [15]. In this case the 3D porosity is $p^{3/2}$, where $p$ is the 2D porosity coefficient ($p = 2D$ area of voids / 2D bone cross section area), as analysed from backscattered electron (BSE) imaging of the cross section of femoral mid-diaphysis of wild-type and GIOP bone, following our earlier work (Supplementary Information in [15]).

2.7. Statistical analysis

To test for statistical differences in bone mineralization and the nanoscale mechanical deformation behaviour between samples tested at three different strain-rates, one-way ANOVA tests with all pairwise multiple comparison procedures (Holm-Sidak method) were performed on the experimental measured results including the mean mineral concentration, the effective fibril modulus, the effective mineral modulus and the fibrillar reorientation rate. SigmaPlot (Systat Software Inc., USA) was used for the statistical analysis. The statistical significances were denoted on the figures (*: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, ns: not significant).

2.8. Modelling of fibrillar and lamellar mechanics

To understand the structural mechanisms underpinning trends in $E_t$, $E_m$ and fibrillar reorientation with strain-rate, we develop a two-level hierarchical model of the fibrils and fibril arrays, based on prior work, which is briefly summarized below (details in Supplementary Information). Analytical fitting (performed in Matlab [53]) and
Table 1 Description of the moduli introduced for the study of the bone mechanical properties at different length scales and of the fibrillar reorientation phenomenon. The term ‘effective’ indicates that the moduli result from the ratio of terms computed at different length scales. Specifically, they are calculated from the ratio of stresses applied at the macroscale and of strains computed at the microscale (effective fibril modulus) and at the nanoscale (effective mineral modulus). The equations used for the analytical calculation of these parameters are listed in Supplementary Information, Equations S1-S6. ‘afs’ is the average fibril strain, φEM is the volume fraction of the extracellular matrix and k is a factor defined in Equation S6.

<table>
<thead>
<tr>
<th>Nomenclature of the modulus</th>
<th>Experimental</th>
<th>Analytical/Numerical</th>
</tr>
</thead>
<tbody>
<tr>
<td>Effective fibril modulus</td>
<td>Applied tissue stress</td>
<td>Computed via laminate theory.</td>
</tr>
<tr>
<td></td>
<td>Calculated via linear fitting of experimental data shown in Fig. 5A.</td>
<td></td>
</tr>
<tr>
<td>Effective mineral modulus</td>
<td>Applied tissue stress</td>
<td>Computed via laminate theory.</td>
</tr>
<tr>
<td></td>
<td>Calculated via linear fitting of experimental data shown in Fig. 5B.</td>
<td></td>
</tr>
<tr>
<td>ΔFWHM/fibril strain</td>
<td>Applied tissue stress</td>
<td>Computed via laminate theory.</td>
</tr>
<tr>
<td></td>
<td>Calculated via linear fitting of experimental data shown in Fig. 5B.</td>
<td></td>
</tr>
<tr>
<td>Fibril strain: average strain of the sublamellae</td>
<td>Computed via laminate theory.</td>
<td></td>
</tr>
<tr>
<td>Fibril strain: average strain of the sublamellae</td>
<td>Computed via laminate theory.</td>
<td></td>
</tr>
</tbody>
</table>

Table 1 are used to fit the model to data. The experimental parameters are fitted to equivalent model parameters, summarized in the two columns of Table 1.

2.9. Model structure and parameters

2.9.1. Analytical relations

2.9.1.1. Nanoscale force-balance relations. Stresses and strains on the fibril, mineral platelet and extracellular matrix were calculated by considering the fibril as a staggered array of mineral particles embedded with a collagen matrix (Fig. 3A-4), which is in turn embedded in an extracellular matrix. The model follows earlier work on staggered model architecture of the mineralized fibrils in bone and related biomineralized tissues [11,36,41,55-57]. The mineral platelet aspect ratio was taken as 15 and 9.6 respectively for the wild-type and GIOP models, following our prior ultrastructural determination of mineral structure (L-parameter) using WAXD on GIOP- and WT-bone from the same cohort at a similar age-point [15]. A second parameter of note in the staggered model is the k-factor, which is inversely related to the stress transferred to the mineral via shear in the collagen matrix [11,36]. Mineral and collagen were taken as elastic, and the strain-rate sensitivity was incorporated into the material response of the extracellular matrix, whose constitutive law was taken as the Ramberg-Osgood law \( \varepsilon = \sigma/(E\varepsilon^*) \) [58,59]. Most parameters were obtained from referenced literature (Table 2), with the exception of the Young’s modulus and volume fraction of the extracellular matrix, and the k-factor, which are obtained from nonlinear fitting to the experimental data (Fig. 5) and will be reported in the Results. The tissue mineral volume fraction values were taken from the 24-week time-point values of volume fraction in GIOP- and WT-mice, in our recent work [15], with φEM = 0.40 for GIOP and φEM = 0.45 for WT.

2.9.1.2. Plywood structural parameters. The bone lamella was modelled as a set of differently oriented fibril layers, with angular orientations at \( 0°, \pm 5°, \pm 10°, \pm 15°, \pm 30°, \pm 45°, \pm 60°, \pm 75° \) and 90°. To determine the relative thicknesses of each layer, these were varied till the FWHM of the simulated fibril orientation distribution matched the experimental azimuthal intensity distribution of the meridional collagen SAXD peak (Fig. S2), in a manner similar to our previous work [15]. Details are provided in Supplementary Information.

2.9.1.3. Matching to experimental data. Least-squares minimizations was carried out by simultaneously fitting the experimental \( E_t \) and \( E_m \) data to the model expressions (Fig. 5 and Fig. S6 in Supplementary Information). Each fitted experimental point (at a given strain rate) was weighted by the inverse of its squared standard deviation [60]. The weighted fitting process was performed in MatLab with the function Nlinfit [53] (Table 1 and implementation in Supplementary Information). Table 2 describes the choice of the input parameters for the model.

Fig. 3. Schematic of the hierarchical structure of bone assumed for the modelling approach. A) I. At the lowest hierarchical scale, a staggered arrangement of hydroxyapatite mineral platelets and collagen [41] (left side of the figure) was considered. The material components are collagen, hydroxyapatite mineral and extracellular matrix (which together form level II). A bunch of parallel collagen fibrils surrounded by an extracellular matrix, forming a sublamella (III). A set of sub-lamellae, each with the longitudinal axis of fibrils pointing toward a specific direction, forms (IV) a plywood (or Bouligand [79]) system. For both modelling approaches the scheme in Ref. [13] with an angular distribution of sub-lamellae of the type: 0°, \(+/-5°, +/-10°, +/-15°, +/-30°, +/-45°, +/-60°, +/-75°\) (0° direction is along the applied loads). B) Schematic for reorientation in the model.
2.9.2. Finite element simulations of fibrillar and lamellar reorientation

To simulate the load-induced reorientation of fibrils toward the loading axis, an approximate method was used, based on finite element simulations. The reorientation of a fibril embedded in an extracellular matrix was determined (Fig. 3B), assuming isotropic material properties (Table S2), by applying a uniform traction of 10 MPa to the top edge of the fibril and calculating angular reorientation from the horizontal and longitudinal displacements. Details are provided in Supplementary Information.

3. Experimental results and model fitting

3.1. X-ray microtomography

X-ray microtomography was performed to investigate 3D micro-morphometry, microscale mineralization distribution and possible mineralization defects of femora from wild-type and GIOP mice. A series of 8-bit grey level slices were obtained from the 3D tomograms of samples. Fig. 2 showed representative 2D slices for both longitudinal and transverse cross sections of femora from wild-type and GIOP mice. The 2D slices of transverse cross sections of femora, as shown in Fig. 2A and C, are selected from mid-shaft of mice femora as indicated by red dash lines in Fig. 2B and D. Clear qualitative differences can be observed in the cortical microstructure of GIOP mice as compared to wild-type mice. Both of the transverse and longitudinal cross sections of femoral from GIOP mice showed a very large fraction of cavities with less mineralized bone tissue near the endosteal cortex, whereas no such cavities were found in the femoral mid-shaft of wild-type mice. The femoral cross section of GIOP mice showed a much thinner cortex compared to wild-type mice. This is in agreement with backscattered electron (BSE) imaging results of the cross section of mice femoral mid-diaphysis (as also carried out in [15]), which showed 2D porosity coefficients of 1.68 ± 0.26 % and 29.57 ± 1.74 % for wild-type and GIOP bone, respectively.

Table 2

Elastic material properties of the basic components and their volume fractions in the Wild and GIOP models at low, medium and high strain rate values. Red: values extrapolated from referenced literature; Blue (with light blue background): values obtained from the fitting process; Black with dark grey background: values that were assumed. The k-factor is linked to the reinforcement of the collagen fibrils by the mineral platelets (Eqs. S2 and S6 in Supplementary Information) (for interpretation of the references to colour in this table legend, the reader is referred to the web version of this article).

<table>
<thead>
<tr>
<th>Young’s moduli</th>
<th>GIOP bone (GPa)</th>
<th>Wild-type bone (GPa)</th>
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</thead>
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<tr>
<td>E&lt;sub&gt;c&lt;/sub&gt; = Young’s modulus of collagen</td>
<td>2.5 [36]</td>
<td>2.5 [36]</td>
</tr>
<tr>
<td>E&lt;sub&gt;m&lt;/sub&gt; = Young’s modulus of hydroxyapatite (mineral content)</td>
<td>100 [36]</td>
<td>100 [36]</td>
</tr>
<tr>
<td>E&lt;sub&gt;EM&lt;/sub&gt; = Young’s modulus of extracellular matrix</td>
<td>k = 1.58</td>
<td>k = 1.6</td>
</tr>
<tr>
<td>low s.r.</td>
<td>163.8</td>
<td>107.6</td>
</tr>
<tr>
<td>medium s.r.</td>
<td>160.8</td>
<td>105.7</td>
</tr>
<tr>
<td>high</td>
<td>160.1</td>
<td>105.3</td>
</tr>
<tr>
<td>Poisson’s ratios</td>
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<td></td>
</tr>
<tr>
<td>ν&lt;sub&gt;c&lt;/sub&gt; = Poisson’s ratio of collagen</td>
<td>0.3 [61]</td>
<td>0.3 [61]</td>
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<tr>
<td>ν&lt;sub&gt;m&lt;/sub&gt; = Poisson’s ratio of hydroxyapatite (mineral content)</td>
<td>0.28 [61]</td>
<td>0.28 [61]</td>
</tr>
<tr>
<td>ν&lt;sub&gt;EM&lt;/sub&gt; = Poisson’s ratio of extracellular matrix</td>
<td>0.3 [61]</td>
<td>0.3</td>
</tr>
<tr>
<td>Volume fractions</td>
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<td>φ&lt;sub&gt;c&lt;/sub&gt; = volume fraction of collagen</td>
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<tr>
<td>φ&lt;sub&gt;m&lt;/sub&gt; = volume fraction of hydroxyapatite (mineral content)</td>
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<td>0.34</td>
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<tr>
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<td>k = 1.6</td>
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<td></td>
<td>0.03</td>
<td>0.06</td>
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In situ tensile testing with synchrotron SAXD and WAXD

3.2.1. SAXD and WAXD patterns

Representative SAXD and WAXD patterns for femoral mid-shaft of wild-type mice aged 24 weeks are shown in Fig. 1E and G, and 1D intensity profiles of the third-order collagen reflection and (002) mineral reflections in mice femur mid-diaphysis are shown in Fig. 1F and H.

3.2.2. Effective fibrillar moduli

To compare the fibrillar-deformation in mice femur tested at different strain rates (Figs. 5B and 4 A, D), data for samples at each strain rate were combined and plotted (tissue stress vs. nanoscale fibrillar strain) in the elastic deformation region (Fig. 4A, D), and show differences in the slope (effective fibrillar modulus $E_f \approx \sigma_f/\varepsilon_f$). Average effective fibrillar moduli from each group of samples were plotted as a function of strain rate in Fig. 5B (pink bars). As strain rate increased from 0.0004 s$^{-1}$ to 0.02 s$^{-1}$, we observe a significant increase in the effective fibrillar modulus increased from 13.6 ± 3.0 S.D. GPa to 65.6 ± 11.4 S.D. GPa ($p < 0.001$) in wild-type mice bone.

In contrast, the effective fibrillar modulus remains nearly constant in GIOP mice bone (blue bars). The effective fibrillar modulus in wild-type mice are significantly ($p < 0.001$) higher compared to GIOP mice at strain rates 0.01 and 0.02 s$^{-1}$, no significant differences in the effective fibrillar modulus between wild-type and GIOP mice was found at strain rate 0.0004 s$^{-1}$ (Figs. 4 and Table 3). Note that for the data plotted in Fig. 5B–D, the parameters $E_f$, $E_m$ and rate of fibrillar reorientation are calculated per-sample and averaged within each strain-rate group, whilst the lines in Fig. 4 are regressions through the pooled data points (tissue stress vs fibrillar strain, mineral strain or reorientation) from all samples at that strain-rate. This difference accounts for slight differences in the slopes between the Figures: for example, the averaged fibrillar moduli in GIOP is lowest at the highest strain rate (0.02 %s$^{-1}$; Fig. 5B) while the slope of the regression line for GIOP-bone in the fibrillar moduli plot in Fig. 4D is lowest for the intermediate strain rate 0.01 %s$^{-1}$.

3.2.3. Effective mineral moduli

In a parallel manner, considering the mineral crystallite deformation, tissue stress versus mineral strain were grouped and plotted for three different strain rates (Fig. 4B, E). Here, the effective mineral modulus $(E_m = \sigma_m/\varepsilon_m)$ in wild-type mice bone increased with strain rate and the increase was significant ($p = 0.026$) as seen in Fig. 5C (dark blue bars). $E_m$ increased from 44.2 ± 7.3 S.D. GPa to 97.5 ± 28.3 S.D. GPa as strain rate increased from 0.0004 s$^{-1}$ to 0.02 s$^{-1}$ in wild-type mice bone. In contrast, $E_m$ remains nearly constant in GIOP mice bone (blue bars). The effective mineral modulus in WT mice were significantly higher compared to GIOP mice at all strain rates (Fig. 4 and Table 3).

3.2.4. Fibrillar reorientation

Considering the fibrillar orientation with respect to the direction of loading, the azimuthal intensity distributions of the first-order collagen reflection from mice femur were used to determine the degree of fibrillar orientation (FWHM) at unstrained state and the change of FWHM during tensile loading. Wild-type mice bone shows that 1) the FWHM consistently narrows with increasing strain, but 2) the percentage-change reduces dramatically as the strain rate increases (Fig. 4C). Averaged values of the rate of fibrillar reorientation were plotted as a function of strain rate in Fig. 5D, and showed a significant ($p = 0.018$) reduction. In wild-type mice bone, the rate of fibrillar reorientation (-0.08 ± 23.2 S.D. °.%-1) at low strain rate (0.0004 s$^{-1}$) is significantly higher as compared to strain rates of 0.01 s$^{-1}$ ($p = 0.034$) and 0.02 s$^{-1}$ ($p = 0.025$).

In contrast, for GIOP bone there are no significant differences in reorientation rate with strain rates. The reorientation rate in GIOP mice bone at strain rate 0.004 s$^{-1}$ is significantly lower than that in wild-type bone, whereas no significant differences in reorientation rate was found between wild-type and GIOP mice bone at strain rate 0.01 s$^{-1}$ and 0.02 s$^{-1}$ (Fig. 4 and Table 3).

3.3. Model fitting to experimental $E_f$, $E_m$ and reorientation

An initial fitting process for the two models allowed the Young’s moduli corresponding to the three analyzed strain rate values and the volume fraction of the extrafibrillar matrix (Fig. 5A) to be calculated. Fig. 5A shows the variation of the modulus of extrafibrillar matrix. In the wild-type case the extrafibrillar matrix stiffens by over a factor of 100 − from 3.5 GPa at $\varepsilon = 0.0004 \text{s}^{-1}$ (low strain rate) to 370.0 GPa at $\varepsilon = 0.02 \text{s}^{-1}$ (high strain rate). In the GIOP case, instead, depending on the imposed k-factor and on the strain rate, values of the extrafibrillar Young’s modulus can range between 52.1 and 163.8 GPa (Table 2).

Fig. 5B shows a comparison between the experimental and numerically computed effective fibrillar modulus $E_f$. For the wild model, the results show agreement within the experimental error bars, underestimation at medium and high strain rate values and overestimation at the low strain rate ($\varepsilon = 0.02 \text{s}^{-1}$). For the wild-type model a stiffening effect with an increasing strain rate – as seen in experiment – was also found at the mineral level (Fig. 5C). The effective mineral modulus, $E_m$, is overestimated at high and medium strain rates and slightly underestimated at low strain rate.

For the GIOP bone, both the effective fibrillar and mineral moduli confirm the constant trend found experimentally (Fig. 5B and C) and show agreement with experimental values (average experimental 13.6 GPa vs 13.9 GPa). Indeed, the average experimental value of the effective fibrillar modulus at the 3 strain rates is 13.6 GPa while the corresponding modelling value is 13.9 GPa. Corresponding values for the effective mineral modulus are respectively 22.8 GPa and 21.8 GPa.

Fig. 5D shows that for lamellar-level fibrillar reorientation – calculated via change of $\Delta$FWHM normalised by the fibril strain – the wild-type model reproduces the trend to reduced reorientation with increased stress. For the GIOP model a reduction of the k-factor (Equation S6) lead to a reduction of fibrillar reorientation (Fig. 5D). Our parametric analysis shows that the reorientation calculated via FE simulations matches the experimental reorientation (modelling values within the experimental error bars) for 3 strain rates assuming...
4. Discussion

Strain-rate dependent tensile tests were performed on small femoral samples of wild-type and steroid-induced osteoporotic (GIOP) mice. Our main findings can be summarized as follows:

- Under tensile testing with increasing strain rate, the fibrillar-level deformation of GIOP bone exhibits a contrasting behaviour to wild-type (WT; normal) murine bone – specifically, while WT-bone shows a significant increase in effective fibril- and mineral-moduli, this effect is absent in GIOP bone.

- On increasing strain-rate, WT-bone shows a significant reduction of extent of fibrillar reorientation toward the loading axis; in contrast, GIOP bone shows no change in reorientation with strain-rate.

- By comparing the volume-average SAXS- and WAXD-measures of fibril- and mineral-strain to the model predictions of a fibril/fibril-array model of bone matrix mechanics, the strain-rate dependent effects in WT-bone are explained via an increased extrafibrillar matrix stiffening.

- In contrast, for GIOP-bone, the experimental results can be matched to model predictions if the reinforcement between mineral- and collagen (via the k-factor; Table 2) at the nanoscale is taken higher for GIOP compared to WT, and no extrafibrillar matrix stiffening occurs in GIOP-bone.

The novelty of the current study is primarily in obtaining experimental data characterising how the strain-rate dependence of fibrillar deformation mechanics in osteoporotic bone differ from normal cortical bone, and as a secondary goal, to explore the underlying structural mechanism by fitting a multilevel model to the data. Prior work, by our group as well as others [14,15,33,42] have analysed alterations in fibrillar mechanics in metabolic bone disorders like rickets, GIOP, and ageing, but these have not studied strain-rate dependence in such pathological conditions. Because bone is used in a dynamic mechanical environment, understanding how the structural response of the bone matrix at the fibrillar level alters with increasing strain rate is of direct interest. From a materials-standpoint, for example, our observation that the fibril strain gradient (from $E_f$) is unchanged at different strain rates in GIOP-bone, but decreases in WT-bone (Fig. 4), provides insight into the altered biomechanical reinforcing efficiency of the collagen fibrils. Further, while the current work does not directly deal with fracture, prior work by other groups has shown that strain-rate influences work of fracture, with reduction of work of fracture and transition to unstable crack growth with increasing strain rate [62,63], as well as increase of elastic moduli and yield strength [64]. Indeed, if fibrils in osteoporotic GIOP bone show no change with increasing strain rate, while an effective “stiffening” is seen via the increased fibril modulus in normal (WT) bone, this may lead to a lower mechanical competence in GIOP at higher strain-rates compared to WT. When compared with the wild-type bone, the relationship between strain rate and increasing modulus breaks down for GIOP, indicating the mineral-collagen composite in GIOP failed to adequately stiffen with increasing strain rate, which is likely the cause of the lowered mechanical competence. While the lower maximal fibril strain in WT relative to GIOP sounds counter-intuitive when one associates disease with lowered strength and brittleness, we note that a) the total tissue strain is a complex sum of the fibril, interfibrillar, and interlamellar level strains and b) the maximal elastic stress level in GIOP is lower than WT. Therefore, the expected

$k = 1.58$. 

Fig. 4. Fibril strain, mineral strain and change of FWHM from in situ synchrotron SAXD and WAXD: Symbol code: Low strain rate (0.0004 s⁻¹, green squares), medium strain rate (0.01 s⁻¹, blue triangles) and high strain rate (0.02 s⁻¹, red circles). (A, D) Applied tissue stress vs average mineral strain. (B, E) Change of the FWHM of a Gaussian profile vs average fibril strain (see also text and Table 1 for parameter definitions). The symbols are experimental data points (pooled across samples for each strain rate) while the straight lines are linear regression lines for each group of data (regressions through pooled data points at a given strain-rate). The shadowed area in the six plots is a convex hull of the experimental data representing the region that numerical results are expected to intersect (for interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).
weak (lower strength) behavior in GIOP is present, whilst the lower maximal fibril strain in WT does not exclude that the maximal strain at macroscopic failure will still be lower in GIOP than WT (possibly due to tissue-level defects and pores). We note, however, an underlying assumption in our work is that the mouse model of endogenous glucocorticoid production (Cushing's syndrome) is a valid and relevant model for (exogenous) human GIOP [40]. As mouse models do not exhibit secondary remodelling, the bone structure at the tissue level will be different from human GIOP.

The strain-rate dependence of the mechanical properties of bone have been studied at the macroscopic level before [58,64–66], using phenomenological viscoelastic/viscoplastic models or relations such as the Ramberg-Osgood equation used earlier. The nature of the structural mechanisms in time-dependent mechanical loading is less studied. High strain-rate in situ SAXD measurements on human bone found a strain-rate induced stiffening of the fibril ductility associated with a loss in toughness in bone matrix [38], and compressive creep studies found the strain on both mineral and collagen phases in bone increase linearly with time, proposed as a load-shedding from collagen to mineral [67]. Stress-relaxation was observed to be more rapid in mineral than in collagen [68]. Molecular dynamics studies (e.g. [69]) have highlighted the role of rapidly breaking and reforming hydrogen bonds during deformation. Nevertheless, structural-mechanisms enabling viscoelasticity in the bone matrix are not clearly known, and the experimental data on the variation of the time-dependent behaviour in osteoporosis presented here may help toward that eventual goal. It is noted that the exposure of the samples to X-rays is consistent across three different strain-rates. By closing the shutter between acquisitions, and keeping acquisition time constant at 0.1 s per point, the total X-ray dose is proportional to the number of SAXS patterns per tensile test. Fig. S5 (Supplementary Information) shows that the number of patterns is of the same order of magnitude across strain-rates. Therefore, it is not likely that the high-strain rate tests are being exposed to much higher X-ray dosages compared to the low- and medium strain-rates, which would cause damage to the collagen matrix [44].

The experimental values for maximal fibril strain (Fig. 5A) at low strain rates (~0.4–0.6 %) are consistent with our prior quasi-static results on both murine [15,33,42] and bovine bone [37], and in the same range as those observed by others on human bone [14]. In WT-bone, the maximal fibril strain reduces consistently from ~0.6 % at the lowest

Table 3

<table>
<thead>
<tr>
<th>Strain rate (s⁻¹)</th>
<th>Wild-type</th>
<th>GIOP</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Effective fibril moduli (GPa)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.004</td>
<td>13.60 ± 3.00</td>
<td>14.46 ± 2.66</td>
<td>0.876</td>
</tr>
<tr>
<td>0.01</td>
<td>37.90 ± 9.90</td>
<td>13.02 ± 4.28</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>0.02</td>
<td>65.60 ± 11.40</td>
<td>11.50 ± 3.58</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Effective mineral moduli (GPa)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.004</td>
<td>44.20 ± 7.29</td>
<td>17.90 ± 5.30</td>
<td>0.032</td>
</tr>
<tr>
<td>0.01</td>
<td>70.50 ± 16.70</td>
<td>20.77 ± 1.42</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>0.02</td>
<td>97.49 ± 28.38</td>
<td>26.66 ± 10.50</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Reorientation rate (degree/%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.004</td>
<td>40.75 ± 23.22</td>
<td>2.18 ± 9.65</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>0.01</td>
<td>4.90 ± 3.91</td>
<td>1.76 ± 5.63</td>
<td>0.703</td>
</tr>
<tr>
<td>0.02</td>
<td>5.50 ± 4.94</td>
<td>1.24 ± 4.02</td>
<td>0.606</td>
</tr>
</tbody>
</table>
strain rate (0.0004 s$^{-1}$) to ∼0.1 % at the highest strain-rates (0.02 s$^{-1}$). However, a similar trend is not visible for GIOP; for intermediate strain rates (0.01 s$^{-1}$) in GIOP-osteoporotic bone – in Fig. 4D, maximum fibril strain can reach ∼0.6-0.8 % compared to the ∼0.4 % values for the lowest strain-rate, while for the highest strain rate the maximum fibril strain is again ∼0.4 %. Since maximum strains are linked to strength and failure of the entire bone, microstructural differences between GIOP- and wild-type bone (Fig. 2) may be relevant in explaining this behaviour, which is beyond the scope of the nano/microscale model presented and discussed below.

Fibrillar reorientation, as well, shows some notable differences between GIOP and WT. Here, it is important to note certain experimental limitations. As SAXD and WAXD provide volume averaged measures of fibrillar/mineral structure through the thickness of cortical bone specimens used in these tests, effects below and above the scale of the fibril cannot be excluded. Consequently, if the sample volume contained microscopically misaligned lamellae, these could undergo inter-lamellar reorientation, rather than the reorientation occurring at the fibril/interfibrillar matrix alone (this corresponds to phenomena above the scale of the fibril). Likewise, it is known that tropocollagen molecules inside microfibrils are arranged in a tilted geometry [70] and intrafibrillar rearrangement may also contribute, rather than fibrils rotating in a rigid-body manner. However, we note that the numerical value of the tilt inside microfibrils is small (∼4° in Figs. 2 and 3 in [70]) (noting the factor of 5 compression in the c-axis direction specified by the authors). This value is much smaller (Fig. 5D) compared to the ∼50° (FWHM change)/% strain reorientation seen for the lowest strain rate. Therefore, load-induced intrafibrillar rotation of the molecules, to remove the tilt, would be insufficient to explain the magnitude of the observed reduction in FWHM. To be able to overcome the averaging issue inherent in our experimental configuration, possible future routes may involve 6D SAXS tensor tomography [71], if challenges in data processing and potential radiation damage are overcome. Such methods can provide spatially-resolved 3D maps of the fibrillar nanostructure across the tissue, although time-resolved studies at the strain-rates proposed here (and above) will still be challenging. Subfibrillar-level deformation may be analysed by the covariation of changes in the angular intensities of the WAXD and SAXS patterns (which will provide information on how the mineral particles are reorienting relative to the fibrils), or possibly by contrast-variation neutron diffraction to resolve the changes in tropocollagen ordering.

While the empirical differences between the strain-rate dependencies in the GIOP- and WT-nanoscale parameters (E$_f$ and E$_m$) is clear from Figs. 4, 5, these numbers (averaged across scattering volume) by themselves do not provide a full structural explanation. From our earlier studies on GIOP-bone [15,33], the orientation distribution is wider in GIOP that WT. These facts imply that earlier simpler models, such as our prior work on antler [36], which modelled the uniaxial fibrils alone (oriented along the loading axis), are likely insufficient to explain the data. As a first step in this direction, we used a two-level multiscale model of bone nano- and microstructure to provide some insights into possible reasons for these changes. At the fibrillar level, the model is similar to prior staggered models of mineral-collagen interactions put forward [11,36,41,55-57,61,72], although the inclusion of the mechanics of the extrafibrillar matrix is an advance on our prior modelling [36]. At the fibril-array level (microscale), bone is known to have a lamellar structure although the precise details of the orientation (originally proposed as plywood or rotated plywood [13,73]) are still not fully clear, with recent revisions to the orientation scheme proposed [12] to incorporate a fraction (10 %) of disordered fibrils. The plywood scheme used in the original paper [13] is used here (also for consistency with prior modelling work [61]), but inclusion of more complex structures to model the experimental results is possible in the future. Further, the microstructure of rat and mice bone is different from human bone, which has extensive secondary remodelling and well developed secondary osteons, and these differences are not accounted for in the model. In addition, spatial variations in bone matrix parameters at larger length scales than the nano- and micro- (such as across cross-sections of cortical bone reported in rat bone [74]) are beyond the scope of the model, even though clear variations between endosteal and periosteal regions (Fig. 2) are visible. Parameter estimates from the model and their structural interpretation below need therefore to be considered as estimates rather than definitive values.

From optimizing the parameters for model predictions to agree with experimental values of effective fibrill- and mineral-moduli, it is observed that in normal WT cortical bone the stiffening of the extrafibrillar matrix with increasing strain-rate can lead to the increased fibril (and mineral) modulus seen experimentally (Fig. 5). Increased stress borne by the extrafibrillar matrix reduces the strain on the fibrils, which therefore increases the effective fibril modulus, which is a ratio of macroscopic stress to fibril strain. A similar process occurs for effective mineral moduli. The extrafibrillar space in bone contains extracellular mineral and non-collagenous proteins [75,76], and we can speculate that such a phase of mineral interlinked with protein may exhibit strain-stiffening behaviour with increasing strain-rate, being dominated by the moduli of the noncollagenous proteins (< 1 GPa) at low strain rates and by the modulus of the mineral at larger strain rates. However, we obtain unrealistically high values for the modulus of the extrafibrillar matrix (370 GPa) at the highest strain rate, well above the 100 – 110 GPa characteristic of hydroxyapatite mineral [36]. Possibly, these values arise from the extrafibrillar volume fraction or type of orientation distribution used here, and parametric-variation studies may be useful in future in this regard.

In contrast, the experimental data for the GIOP-bone can be fit to the model with essentially constant extrafibrillar matrix moduli (Table 2) but with a considerably lowered k-factor. The physical meaning of this difference compared to WT bone is not fully clear. The k-factor is inversely linked to the reinforcing efficiency of the mineral platelets inside the collagen fibril [11,36], and arises due to the load-transfer from the collagen matrix to the mineral platelet. Note that the effect of the more random fibril orientation in GIOP [15,33] has already been included via the wider FWHM from I(2θ). As the k-factor depends on the effectiveness with which loads are transferred to the mineral from the collagen, the differing k-factor in GIOP compared to WT suggests that possibly the orientation and/or interactions of intrafibrillar mineral with collagen may differ. However, this still does not explain why we do not obtain a similar strain-rate dependent stiffening as seen in WT-bone. We can speculate that these open questions are linked to limitations of our model. As the fibril orientation distribution is not precisely the multilayer lamellar structure described initially [13] but includes random fibril orientations [12], and the further differences in lamellar structure in GIOP have not yet been determined, it is likely that further alterations or refinements to the structural model will be needed, even though the experimental differences between GIOP- and WT-bone fibrillar strain-rate dependencies are not in question.

A limitation of the current work is that we did not report results of varying the collagen- and mineral-moduli in the model, both of which may change in disease due to substitution of ions and change in covalent crosslinking [14,77]. In this regard, we have observed (data not shown) that variation of collagen moduli cannot explain the increase in effective mineral moduli (Fig. 5C) with strain rate. Regarding the mineral phase, our previous study [15] showed that, compared to WT bone, the mineral platelet is slightly shorter (in length, along the c-axis) and the intra-platelet lattice spacing is slightly higher in GIOP bone, but the mechanical implications of these crystallographic changes is not clear to us at this point. Perhaps, future $ab$ $initio$ molecular dynamics simulations of the change in mineral crystallite structure [78], linked to simulated mechanical testing at these small scales, could shed light on this question.

In summary, we have analysed for the first time the fibrillar- and mineral-level strain changes in steroid-induced osteoporotic and


Supplementary Information:

Figure S1: Typical SAXD patterns of mice femur at different stress levels.

Figure S1: SAXD patterns of mice femoral mid-shaft at different stress levels $\sigma$. (A) $\sigma = 0$ MPa, (B) $\sigma = 36.8$ MPa, and (C) $\sigma = 74.5$ MPa. The first-order (a) and the third-order (b) collagen reflection were indicated in (C).

Figure S2: Azimuthal intensity profile of the first-order collagen peak of mice femoral mid-shaft at different stress levels.

Figure S2: The corrected azimuthal intensity profile (angle: azimuthal angle on the 2D SAXS detector plane, Figure 1E) of the first-order collagen peak from SAXD patterns of femoral mid-shaft at different stress levels.
Figure S3-4: The intercepts of linear regressions of D-period and D(002) versus macroscopic stress were taken as the unstrained (zero-stress) value for D-period and D(002), respectively. The effective fibril modulus ($E_f = d\sigma/d\varepsilon_f$) and effective mineral modulus ($E_m = d\sigma/d\varepsilon_m$) were defined as the slope of tissue-level stress $\sigma$ versus fibril strain and mineral strain, respectively, from the elastic region of deformation.

Figure S3: (A) D-spacing of collagen fibrils versus applied tissue stress; (B) applied tissue stress versus fibril strain. Black solid lines are linear regressions.

Figure S4: (A) (002) lattice spacing of apatite versus applied tissue stress; (B) applied tissue stress versus mineral strain. Black solid lines are linear regressions.
Figure S5: Typical mineral strain versus stress curves for GIOP samples tested at three different strain rates.

Figure S6 shows the simultaneous nonlinear fitting of the experimental effective fibrillar and mineral moduli that was performed in Matlab.

Figure S6: Results from the three-parameter fitting process for the wild-type bone (Section 2.8.1: Analytical Relations). The experimental data was fitted by the numerical model (solid curves). The dotted curves show the 95% confidence interval. The output of the fit was: $c = 3.4 \times 10^{13}$ GPa.s, $d = 1.19$ (no units) and $\varphi_{EM} = 0.08$. 
Fibrillar mechanics and structure-function relations:

Assuming each sub-lamella to be orthotropic, the longitudinal and transverse Young’s moduli and the shear modulus were determined by a combination of different variants of rules of mixtures. The longitudinal Young’s modulus ($E_1$) was taken as the axial fibril modulus. This fibril modulus is derived from a staggered arrangement of mineral platelets in a collagen matrix [1-3] and depends on intrafibrillar mineral volume fraction, mineral platelet aspect ratio, and Young’s moduli of collagen and mineral. Secondly, for the transverse modulus ($E_2$) and shear modulus ($G_{12}$) of the lamella, a Reuss (iso-stress) rule of mixtures was adopted [4, 5]. Finally, a Voigt (iso-strain) rule of mixtures was employed to calculate homogenized Poisson’s ratios ($\nu_{12}$).

Fibrils to mineral load transfer:

In order to estimate the longitudinal modulus of the fibrils a rule of mixture based on the Jager-Fratzl model was used [1] (Equation S2). Here, the difference with a standard Voigt rule is the presence of a $k$-factor at the denominator of the first term. This factor, reported in Equation S6, depends on the aspect ratio of mineral particles, on the Young’s modulus of both the mineral and the collagen content and on the mineral volume fraction. The $k$-factor corresponds to the ratio between the effective mineral and the fibrillar moduli providing an estimation of how much of the strain is transferred from the fibrils to the intrafibrillar mineral platelets. Furthermore, the Jager-Fratzl is based on the assumption that mineral platelets and fibrils are parallel to each other. While for the wild-type bone we found that the $k$-factor resulting from the model was very similar to the experimental ratio between effective mineral modulus and effective fibrillar modulus, for the GIOP bone the two values were quite different. Therefore, assuming that in the GIOP bone mineral particles are not fully parallel to the longitudinal axis of the fibril, we implemented a parametric study imposing three values of $k$ (1.7, 1.6, 1.58).

Analytical expressions for elastic moduli:

The main equations used for the calculation of the elastic moduli of a single sub-lamella are listed below. :

$$G_{m/c/EM} = \frac{E_{m/c}}{2(1 + \nu_{m/c})} \quad (S1)$$

$$E_1 = \frac{E_{m}\varphi_m}{k} + \varphi_c E_c + \varphi_{EM} c\dot{e}^d \quad (S2)$$

$$E_2 = \frac{E_{m}E_c c\dot{e}^d}{(\varphi_m E_c c\dot{e}^d) + (\varphi_c E_m c\dot{e}^d) + (\varphi_{EM} E_c E_m)} \quad (S3)$$
\[
G_{12} = \frac{G_m G_c G_{EM}}{(\varphi_m G_c G_{EM}) + (\varphi_c G_m G_{EM}) + (\varphi_{EM} G_m G_c)} \quad (S4)
\]

\[
\nu_{12} = \varphi_m \nu_m + \varphi_c \nu_c + \varphi_{EM} \nu_{EM} \quad (S5)
\]

\[
k = 1 + \left(\frac{4}{AR^2} \frac{1 - \varphi_m}{\varphi_m} \frac{E_m}{\gamma_c E_c}\right) \quad (S6)
\]

In the equations above, \(\varphi_m\) is the mineral volume fraction, \(E\) is the Young’s modulus, \(G\) is the shear modulus and \(\nu\) is the Poisson’s ratio. The subscript 1 indicates the longitudinal direction of sub-lamellae/fibrils while the subscript 2 the transverse direction. The subscripts \(m\), \(c\) and \(EM\) indicate respectively the mineral, collagen and extrafibrillar matrix contents. The aspect ratio of the mineral platelet is \(AR\) while \(\gamma_c\) is a constant coefficient equal to 0.4. The symbol ‘/’ indicates that Equation S1 can be used for the shear modulus of the mineral, collagen and extrafibrillar matrix contents (under the assumption of basic isotropic material).

The material and geometrical properties used as input of the FE simulations are listed in Table S2.

**Table S2: Geometrical and materials properties adopted for the FE simulations.**

<table>
<thead>
<tr>
<th>Wild-type bone</th>
<th>GIOP bone</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Young’s modulus of extrafibrillar matrix (GPa)</strong></td>
<td></td>
</tr>
<tr>
<td>low s.r.</td>
<td>3.5</td>
</tr>
<tr>
<td>medium s.r.</td>
<td>159.0</td>
</tr>
<tr>
<td>high</td>
<td>370.0</td>
</tr>
<tr>
<td>high</td>
<td>160.1</td>
</tr>
<tr>
<td><strong>Young’s modulus of fibril (GPa)</strong></td>
<td></td>
</tr>
<tr>
<td>((E_m \times \frac{\varphi_m}{1 - \varphi_{EM}}) + (E_c \times \frac{\varphi_c}{1 - \varphi_{EM}}) = (100 \times 0.402) + (2.5 \times 0.598) = 39.7 (\text{GPa}))</td>
<td>39.7</td>
</tr>
<tr>
<td><strong>Poisson’s ratio</strong></td>
<td>Extrafibrillar matrix: 0.3</td>
</tr>
<tr>
<td>Fibril: 0.3</td>
<td>Fibril: 0.3</td>
</tr>
<tr>
<td><strong>Surface area (per unit thickness)</strong></td>
<td>Fibril: 1 µm × 0.1 µm</td>
</tr>
<tr>
<td>Extrafibrillar matrix: 1.009 µm × 0.109 µm – 0.1 µm – 0.01 µm</td>
<td>Extrafibrillar matrix (µm):</td>
</tr>
<tr>
<td></td>
<td>k = 1.58</td>
</tr>
<tr>
<td></td>
<td>~ 0.003</td>
</tr>
</tbody>
</table>
Lamellar structure from angular SAXS intensity:

**Plywood structural parameters**: The experimental azimuthal intensity distribution of the meridional collagen SAXD peak (Figure S2) was used to determine the angular distribution of sub-lamellae in the model [6]. The thicknesses of differently oriented sub-lamellae (at 0°, ±5°, ±10°, ±15°, ±30°, ±45°, ±60°, ±75° and 90° [7]) were varied till the effective FWHM of the simulated lamella matched the experimental FWHM [6]. The average FWHM was found to be 72.3 ± 11.4° in wild bone, and 75.8 ± 5.5°. Therefore, the wild bone resulted in a plywood structure with 10% of sub-lamellae at 0°, 11% of sub-lamellae at 5°, 11% of sub-lamellae at 10°, 28% of sub-lamellae at 15°, 20% at ±30°, 12% at ±45°, 6% at ±60° and 2% at 75°. GIOP bone, instead, resulted in a plywood structure with 10% of sub-lamellae at 0°, 10% of sub-lamellae at 5°, 10% of sub-lamellae at 10°, 26% of sub-lamellae at 15°, 20% at ±30°, 12% at ±45°, 8% at ±60° and 4% at 75°.

**Finite-element simulation of load-induced reorientation.**

To simulate the load-induced reorientation of fibrils toward the loading axis, a method was used based on finite element simulations. The reorientation of a fibril embedded in an extrafibrillar matrix was determined (Figure 3B), assuming isotropic material properties (Table S2), by applying a uniform traction of 10 MPa to the top edge of the fibril and calculating angular reorientation from the horizontal and longitudinal displacements. The three Young’s moduli of extrafibrillar matrix were input into the simulations. The degrees of freedom of the bottom edge of the system were all constrained while at the top edge a surface traction load of 10 MPa was applied (Figure S7). The results do not depend on the specific load used, due to the linear elastic nature of the simulation, which implies the ratio of fibril reorientation to fibril strain is load-independent. The reorientation angle is arctangent \( \Delta Y/\Delta X \), where \( \Delta Y \) and \( \Delta X \) are, respectively, the relative horizontal and longitudinal displacements between the midpoint of the bottom edge (point ‘a’ in Figure 3B) and the mid-point of the mid-section (point ‘b’ in Figure 3B). The reorientation calculated from the displacements of the mid-point was taken as an approximation of the average reorientation of the entire system.

To compare the reorientation with the (experimentally observable) reduction in FWHM of the SAXD \( I(\chi) \) curves, the reorientation angles – over all sub-lamellae – calculated from FE simulations were input into Equation S7 to calculate the change of FWHM (\( \Delta FWHM \)):

\[
\Delta FWHM = \frac{\sum_{i=1}^{N} w_i (\alpha_i - \alpha_0) \delta \alpha_i}{FWHM_0}
\] (S7)

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In Equation S7, \( i \) denotes the index of the sublamella and \( N \) is the total number of sublamellae. \( \omega_i \) is the fraction of sub-lamellae initially at the angle \( \alpha_i \), \( \delta \alpha_i \) is the reorientation angle of each sub-lamella, \( FWHM_0 \) is the initial FWHM of the Gaussian distribution representing the angular lamellar distribution of bone and \( \alpha_0 \) is the center of this Gaussian distribution (0° in our convention). The number of finite elements included in the model was achieved after a convergence test (4767 CPS4R; 4-node bilinear, reduced integration with hourglass control elements).

**Figure S7**: The effect of different strain rate levels on the reorientation phenomenon of fibrils in bone. A) Example of FE model used to calculate the reorientation of the system ‘fibril + extrafibrillar matrix’ with its longitudinal axis oriented at 60° to the direction of the applied stress. The figure shows the deformation and reorientation of the system. Each represented colour is associated with a specific value of deformation of the finite elements. The magnified image shows the mechanism of deformation of the extrafibrillar matrix that accommodates the reorientation of the fibril. B) The viscoelastic extrafibrillar matrix is responsible for different levels of reorientation of fibrils in bone under different applied strain rate values. Indeed, our model assumes that the extrafibrillar matrix responds to an increasing applied strain rate with an increasing Young’s modulus (stiffening effect) accommodating higher (for low strain rates) or lower (for high strain rates) reorientation of the elastic fibrils.
References


