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

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Abstract: 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 fibril- and mineral-modulus and fibrillar-reorientation 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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13 October, 2019

To the Editorial Board of *Bone*

Manuscript submission: "*Reduction of fibrillar strain-rate sensitivity in steroid-induced osteoporosis linked to changes in mineralized fibrillar nanostructure*"-Revision

Dear Editor,

I am submitting, as corresponding author, our revised manuscript entitled "*Reduction of fibrillar strain-rate sensitivity in steroid-induced osteoporosis linked to changes in mineralized fibrillar nanostructure*" for your consideration for publication in *Bone*.

Our Reviewer Response is in the attached file, with Reviewer and Editor comments in red, our response in blue font, and changed text in blue highlight. In the revised manuscript, changed text is in blue highlight. This paper is submitted for the special issue honouring Prof. John Currey.

Sincerely yours, on behalf of all the authors,

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Editor comment 1:

Highlights consist of a short collection of bullet points that convey the core findings of the article and should be submitted in a separate file in the online submission system. Please use 'Highlights' in the file name and include 3 to 5 bullet points (maximum 85 characters, including spaces, per bullet point). See the following website for more information

Highlights – key findings

- Mineralized fibrils in healthy mouse bone show strain-rate dependent stiffening.
- The fibrillar stiffening is absent in steroid-induced osteoporosis.
- Modelling suggests altered collagen-mineral interactions may explain this change.
- Changed fibrillar stiffening may be relevant to altered mechanics in osteoporosis.

Author response to Reviewer comments:

We thank both Reviewers for their positive comments. e.g.

Reviewer #1: "*…This paper provides interesting information about the dynamic response of bone with and without GIOP at the nano-scale. I find no fault in the execution of the experiments which appear to have been thoughtfully planned and executed. …".*

Reviewer #2: *"…This is an interesting article and will make a significant contribution in this special issue of BONE dedicated to J Currey's inspiring life time of work on bone.…."*

as well as their insightful comments. Below we list the comments in red and our response in blue. New text additions to the MS are indicated in blue highlight both in the revised MS and in the current document.

Reviewers' comments:

Reviewer #1 (R1):

This paper provides interesting information about the dynamic response of bone with and without GIOP at the nano-scale. I find no fault in the execution of the experiments which appear to have been thoughtfully planned and executed. However, I have a few questions about analysis and interpretation:

R1-1: In the uCT data you clearly see that the endosteal region of the GIOP bone is significantly

more porous than the WT. Could you report the porosity of the bone? This would be useful along with the mean mineral concentration.

AR1-1: Yes, we added the porosity of bone in the revised paper. The porosity was calculated as (area of pores / bone cross section area) * 100%, as analyzed from backscattered electron (BSE) imaging of the cross section of femoral mid-diaphysis of wild-type and GIOP bone. The measurements were done following our previous study (X. Li et al Acta Biomater, 2018; e.g. Supplementary Figure S1 in this reference). We obtain 2D porosity p coefficient of 1.68 (± 0.26)% and 29.57 (± 1.74) % for wild-type and GIOP bone, respectively.

Changes made in text:

We have added a new subsection 2.6 "*Calculation of microscale porosity and stress*" to the *Materials and Methods*, where both the porosity and stress-calculation are discussed. We have also added text

"**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]).

R1-2: How did you calculate tissue level stress? Did you use calipers to determine the cross-section? Or did you do uCT? If so, what was your thresholding? With the increased porosity of the GIOP samples I wonder how accurate your area is when doing measurements and if that might affect your effective modulus values?

AR1-2: The tissue level stress 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 (L. Xi *et al*, Acta Biomat, 2018). SEM image was taken on the fracture surface while the fractured sample was mounted vertically, and the area of the fracture surface A_0 (i.e. the boundary of the whole tissue-cross section, including the pores) was measured from the SEM image using ImageJ. The corrected effective cross-sectional area is $A_0 (1 - p^{3/2})$.

Changes made in text:

In the new sub-section 2.6 referred to above, we have added the text on stress calculation, highlighted below.

"**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 [15].

R1-3: Does figure 5 have significance bars? Could you add them? Is there a reason these plots look so different from the ones in other figures?

AR1-3: Yes, there are significance bars for the experimental results of the effective fibril modulus, the effective mineral modulus and the fibrillar reorientation rate between WT and GIOP samples tested at different strain-rates in Figure 5, and we add that in the revised figure.

Changes made in text: As above.

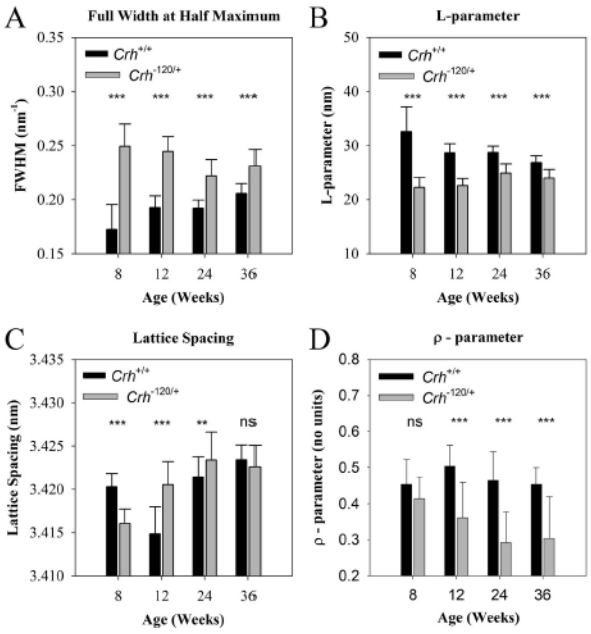
R1-4: In your model, do you ever try varying the collagen and mineral moduli? Both the collagen and the mineral have been shown to change significantly with disease due to substitution and cross-linking. Could GIOP be changing the mechanics of the basic components thus affecting the results?

AR1-4: This is an interesting point. Yes, we did try varying the collagen moduli in our first version of the model (a "biphasic" model, not presented in the paper). In this "biphasic" model, the strain-rate variation was taken to arise solely from the change in mechanics of the collagen phase (a strain-rate stiffening effect), analogous to the strain-rate stiffening of the extrafibrillar matrix (in the current paper).

We found that the biphasic model was not able to capture the change in "effective mineral moduli" at all; indeed, the predicted E_m was nearly constant, in contrast to the experimentally observed increase (Fig 5c). We therefore progressed to the newer version of the model with extrafibrillar matrix stiffening, presented in the paper, which shows better agreement with the three experimental parameters: E_f, E_m, and reorientation.

Our prior work (X. Li et al, Acta Biomater. (2018)) shows that there are small (but significant) differences in mineral crystallographic parameters as well, specifically (002) lattice spacing, FWHM of (002) mineral WAXD peak and L-parameter (from inverse of FWHM). Figure 6 from the paper is

shown below, where Fig. 6A-C shows that the mineral platelet in GIOP bone is slightly shorter that the WT-bone (in length, along the c-axis) and the lattice spacing slightly higher.



However, it is not clear to the current authors how and to what extent these changes in crystallographic structure affect the mineral elastic moduli. As the Reviewer is aware, even in healthy bone maturation the degree of crystallinity, carbonate substitution and other factors alter the local chemical structure of mineral, but the authors do not know of a good model to relate this to mechanical property changes. Perhaps, future *ab initio* molecular dynamics simulations of the change in mineral crystallite structure, linked to simulated mechanical testing at these small scales, could shed light on this question.

Changes made in text:
We have added the following text in the Discussion section:

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.

Reviewer #2:

This is an interesting article and will make a significant contribution in this special issue of BONE dedicated to J Currey's inspiring life time of work on bone.

The group used the latest gadgets and most up to date testing techniques to get to the very inner structure and deformation of bone at the micro-nanoscale, and argued that on the basis of understanding what is happening at this level one can then grapple with processes acting in the magnification levels above (meso- and macro-structure).

I accept their propositions with a caveat, they are as true as certain assumptions are fulfilled:

R2-1: data produced here is as valid and useful, for human GIOP effects, as far as this mouse model emulates human conditions, if the model does not, so do the results. I SUGGEST the authors make this clear in Abstract, Introduction and Discussion to the reader explicitly stating this very basic underlying assumption.

AR2-1: We agree with the Reviewer that a basic assumption of our work is that the mouse model of endogenous glucocorticoid production (Cushing's syndrome) is a relevant model for human GIOP where exogenous administration of glucocorticoids occurs.

Changes made in text:

Abstract: "… in a murine model of Cushing's syndrome, used to understand steroid-induced osteoporosis…."

Introduction: "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 … "

Discussion: paragraph 2, end: "…lowered mechanical competence. 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."

R2-2: mouse femurs are extremely slender and thin, as the figure sections themselves show about 200um thin. As such they don't support or allow the very basic model for bone remodelling we know, the best described one and fully prescribed the BMU driven osteonal remodelling. This illustrates the limitations of this model as a valid model for an equivalent human condition. As before I expect the authors to make this clear for the reader, in the 3 main sections in Abstract, Introduction and Discussion.

AR2-2: Please see changes made in response to **R2-1**, where we have included text to this effect.

R2-3: the authors point out that to achieve the SAXS/WAXS they required synchrotron radiation which provides brilliance and high flux. My question is, does this not then also burn (or cook) the collagen during the high strain rate experiments where in order to achieve the data collection the

demand is for the highest acquisition rate and most brilliant illumination? Is there evidence that the bone has not been damaged during these tests?

AR2-3: This appears to be a slight misunderstanding. We did not use "*the most brilliant illumination*" for the high strain-rate tests. We used the **same** X-ray energy level and exposure time per pattern for all WAXD/SAXD patterns collected from samples tested at three different strain rates. In the automated experimental protocol, the beam was blocked (shutter closed) between successive WAXD/SAXD patterns, so the total exposure time for each sample is proportional to the number of patterns per tensile test, which is of the same order of magnitude across strain-rates (accounting for some inter-sample variation). It is, of course, true that the fast strain-rate measurement gets over much quicker, but that is accounted for by varying the "waiting time" from larger to smaller values on increasing the strain-rate.

We added Figure S5 as an example, where it can be seen that the number of patterns per sample is of the same order of magnitude across strain rates. For the examples shown, the sample tested at strain rate of 0.02 s^{-1} actually had *less* exposure time (or number of WAXD/SAXD patterns) than samples tested at strain rate of 0.0004 s^{-1} and 0.01 s^{-1}. Figure S5 is reproduced below.

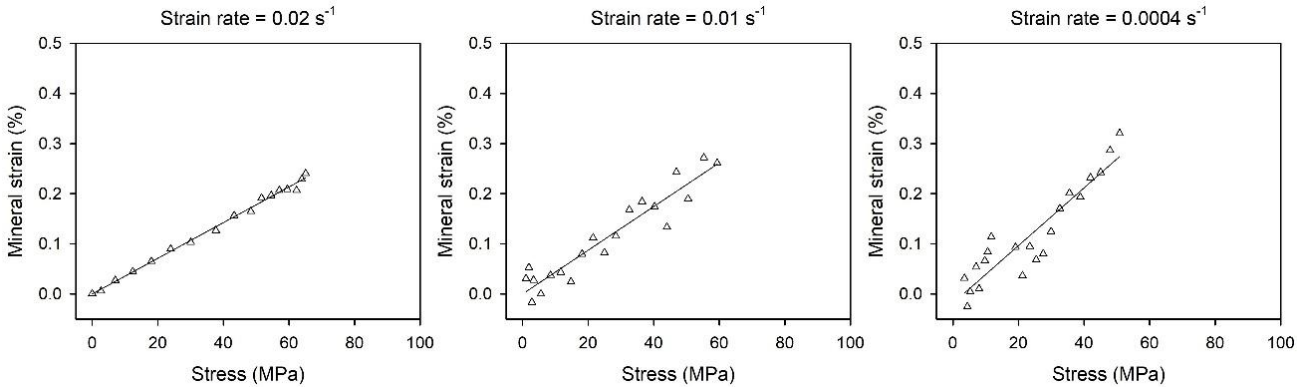


Figure S5: *Typical mineral strain versus stress curves for GIOP samples tested at three different strain rates.*

We have also added the following text in the Discussion:

"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 0.1s per point, the total X-ray dose is proportional to the number of SAXS patterns per tensile test. **Figure 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]."

R2-4: relevance of strain rates: the authors would be advised to specify that only 3 strain rates have been used in this work. Simply rephrasing sentences where their current expression alludes to a wider spread of strain rates, and using instead expressions which make it clear that 3 rates were indeed used, 2 which were near physiological (0.1s, 0.2 s are not high not even intermediate strains rates, simply physiological) and slow (3 orders of magnitude less).

AR2-5: As per the Reviewer's comment, we have rephrased sentences where it may be misinterpreted as a wider spread of strain-rates to be specific to "3" or "three" strain rates. We already had a sentence putting these strain-rates in context in the original MS (section 2.3, para 2).

R2-5: The crux of the results is in Figs 4 & 5. In lines 518-528 the authors describe how wild type bone shows a stiffening with loading rate and re-orientation but also lower maximal strain at $0.1s^{-1}$. Is the behaviour (on the whole) not counter-intuitive when most of us instinctively associate impact and disease with brittle and weak behaviour?

AR2-5: A very interesting observation, thank you. Please note that

i) We considered only data in the elastic regime (before any drops or nonlinearities in the stress/strain curve), so the "lowered maximal strain" is slightly misnamed – it should be lowered maximal strain (in elastic regime).

ii) Further, the lowered strain is at the fibrillar level, and does not account for any interfibrillar, interlamellar or larger-scale strains. Total strain at the tissue/organ level will be a complex sum of these quantities.

iii) Lastly, the maximal (macroscopic) stress is lower for GIOP bone compared to WT, as expected.

When i)-iii) are considered, it can be seen that the expected "weak" (lower strength) behavior in GIOP is still there, while the lower maximal fibril strain in WT- does not exclude that the maximal strain at macroscopic failure (sum of components as in ii)) will still be lower in GIOP than WT (possibly due to tissue-level defects and pores).

Changes made in text:

We have added the following text immediately after the referred text (lines 518-528).

"While the lower maximal fibril strain in WT relative to GIOP sounds counterintuitive 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 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)."

R2-6: lines 529-540: the authors make a sincere effort to alert us on the fact that the averaged behaviour does not allow specific effects to be analysed below or above the scale of fibril level magnification. That is all good, but they do not - later on in the ms - clarify how they are proposing to handle the problem (preferably) experimentally. Modelling is not a good recipe, a model is a tool which as good as its connection to a profound reality. If the connection breaks the model fails. I suspect the only unequivocal answer can be derived from an experimental solution to resolve this fibril scale problem, this will then be followed by an improved version of the model.

AR2-6: This is an important point. There needs to be an experimental way to (a) resolve spatial variations in fibrillar structure across the tissue (*above* the individual fibril-scale) and (b) identify the sub-fibrillar level variations (*below* the individual fibril-scale).

For (a), the authors believe newer technological advances in SAXS imaging, for example the 6D SAXS tensor tomography approach [Liebi et al, Nature 2015] can provide full-field maps of the fibrillar structure. Our understanding is that the current bottlenecks in these techniques are in speed of data processing and in potential radiation damage due to excessive exposure in scans + rotations. Advances in these and related-areas may enable spatially-resolved fibrillar-deformation. However dynamic and very rapid deformation studies will be still challenging.

For (b), with current techniques it would be suitable to model the covariation of angular intensity of the WAXD diffraction signal (from mineral) with the SAXS signal, which can provide some information on molecular-scale deformation of the mineral in relation to the fibrils. We are working on this. Another route would be to use contrast-variation neutron scattering to identify the molecular-level diffraction from collagen (we are not experts in this area, and size of beam relative to the sample may be an issue) and link to the fibril SAXS pattern. This would potentially give information on intrafibrillar rearrangement and dynamics. Also, the 6D and tensor tomography methods in a) can also be applied here.

Changes made in text:

At the end of the Discussion paragraph, starting "Fibrillar reorientation, as well, …"

"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 analyzed 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."

R2-7: .. line 539: as noted by the authors), so it is not likely that this second effect is playing a major role. "it is not likely" is this not an important assumption which needs some justification?

AR2-7: Yes, this was too terse in the original text. We overlooked to add additional sentences here which would have provided clearer justification.

In brief, the effective tilt angle (Figure 2 in Orgel et al (2006)) of the tropocollagen molecules are (estimate from the figure) about 4° (noting the factor of 5 compression in the c-axis direction specified by the authors). This value is much smaller (Figure 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.

Changes made in Text:

We have added the above text as a replacement for the existing single sentence (Discussion paragraph 4).

"... However, we note that the numerical value of the tilt inside microfibrils is small (~4° in Figures 2-3 in [70]) (noting the factor of 5 compression in the c-axis direction specified by the authors). This value is much smaller (Figure 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. …"

TYPOS:

80_ … properties of bone was pioneered by John Currey

85- … mechanical performance under three different loading speeds.

92- … scale[12, 13] and further aggregate into trabecular and cortical

112- … Glucocorticoids suppress bone formation through inducing osteoblast and osteocyte apoptosis and the inhibition of proliferation …

115- … osteoblastogenesis, but toward adipogenesis….

119- … Glucocorticoids directly affect osteoclasts resulting in decreased osteoclast apoptosis and increased osteoclast formation of a prolonged life span, which explains the observed enhanced and prolonged bone resorption [27]….

122-… Although excess of glucocorticoids leads to an increased osteoclast number, osteoclast function may be affected too, with impaired spreading and resorption of mineralized matrix. The osteoblast signals could also be impaired due to the abnormal osteoclast function [28].

136-… to controls [31].

145-… In this study, we examine the deformation of the mineralized fibrils in the bone matrix of a GIOP mouse model at 3 different strain rates …

156-… fixed age point (24 weeks) and at 3 strain rates to quantify the alterations

Changes made in Text:

Thank you for identifying these typos. As above, all typos were corrected in the revised paper.

Highlights – key findings

- Mineralized fibrils in healthy mouse bone show strain-rate dependent stiffening.
- The fibrillar stiffening is absent in steroid-induced osteoporosis.
- Modelling suggests altered collagen-mineral interactions may explain this change.
- Changed fibrillar stiffening may be relevant to altered mechanics in osteoporosis.

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

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Significance Statement

Biomechanically bone undergoes different loading-rates, from low (standing) to high (rapid loading during fracture), and its mechanical response varies with strain-rate. However, the role of the mineralized fibrillar matrix in contributing to the change in mechanical response is incompletely understood. In particular, the changes in bone matrix strain-rate sensitivity during metabolic bone disorders like osteoporosis are little studied. Here, we use rapid synchrotron X-ray imaging during variable strain-rate tests on cortical bone from a murine model of steroid-induced osteoporosis, to study the matrix-level response. We find that while control-samples showed an increase in effective fibrillar and mineral modulus with strain-rate, this effect is completely suppressed in osteoporotic bone. We model this effect by considering the matrix as a two-level fibrillar/lamellar composite, and find that the changes may be explained by an altered interaction between the collagen and mineral at the nanoscale. 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.

Abstract

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 fibril- and mineral-modulus and fibrillar-reorientation 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.

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 fibrils, carbonated apatite, noncollageneous proteins and water [10, 11], which are assembled into fibre-arrays at the micron-scale[12, 13] and further aggregate into trabecular and cortical bone types to form the organ bone [5]. Metabolic bone diseases may affect not only the macro- and microscale structure of bone, but also change the bone matrix-level quality [2], via altered cellular modelling and remodelling cycles. Alterations in matrix quality, such as collagen-cross linking [8, 9, 14] mineral-platelet structural changes [15] and the expression of noncollageneous proteins like osteopontin [16], have been shown to lead to deterioration or alteration in macroscopic mechanical properties, but the details of the nanoscale mechanisms are not completely understood. Understanding the nanostructural response of bone matrix to time-dependent loading in bone-disease types like osteoporosis is therefore of importance both to basic medical science as well as, eventually, to clinical practice.

In this regard, glucocorticoid induced osteoporosis (GIOP) is a prototypical secondary osteoporosis where BMD is known to be insufficient to explain mechanical changes. As the most common form of secondary osteoporosis, GIOP affects 1-3% of the general population and results in severe morbidity, especially in post-menopausal women and older men [17, 18]. GIOP usually develops in patients receiving glucocorticoids for the treatment of a variety of diseases like inflammatory and autoimmune disorders, and these underlying diseases themselves can also have negative effects on bone metabolism which constitute a risk of osteoporosis [19]. Glucocorticoids treatment results in

altered bone remodelling, early and rapid bone loss and increased fracture risk, through direct effects on bone cells and indirect effects through alteration of the neuromuscular system and gonadal hormones [20]. As a crucial process in GIOP, reduced bone volume is caused by osteoclastic activity (bone resorption) that cannot be matched by osteoblastic activity (bone formation) [21, 22]. Glucocorticoids suppress bone formation through inducing osteoblast and osteocyte apoptosis and the inhibition of proliferation, differentiation, maturation and activity of osteoblasts [23]. In the presence of glucocorticoids, the osteoblast precursor cells (mesenchymal cells) in bone marrow are not differentiated or directed toward osteoblastogenesis, but toward adipogenesis (cells of the adipocytic lineage) [24]. Glucocorticoids inhibit the differentiation of osteoblasts by a mechanism of opposing *Wnt/β*-catenin pathway, and *Wnt* signalling plays a critical role in increasing bone mass through induction of differentiation of bone-forming cells (osteoblasts), inhibition of osteoblast and osteocyte apoptosis, and suppression of the development of bone-resorbing cells (osteoclasts) [25, 26]. Glucocorticoids directly affect osteoclasts resulting in decreased osteoclast apoptosis and increased osteoclast formation of a prolonged life span, which explains the observed enhanced and prolonged bone resorption [27]. The proliferation of osteoclasts is inhibited by glucocorticoids in a dose dependent manner. Although excess of glucocorticoids leads to an increased osteoclast number, osteoclast function may be affected too, with impaired spreading and resorption of mineralized matrix. The osteoblast signals could also be impaired due to the abnormal osteoclast function [28].

However, the way these biological changes in GIOP affect the nano- and microscale mechanics is incompletely understood, especially in the area of time-dependent loading. Previous studies have showed that glucocorticoid therapy affects not only the amount of bone (bone quantity) but also the micro-architecture and other material level properties (bone quality) [17, 29, 30]. Micro-CT studies of trabecular and cortical bone with glucocorticoids treatment showed reduced trabecular bone volume, trabecular connectivity, trabecular number and cortical thickness as compared to control group [31, 32]. Glucocorticoid-treated mice showed increased size of osteocyte lacunae and there are "halos" of hypomineralized bone surrounding the lacunae, with corresponding reduced (~40%) 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, 3M 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 tap 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 ε_f (from the SAXD frames) and mineral strain ε_m (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 (M112.1DG; Physic Instruments, UK) and an 111N 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 corresponding 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 P3-2M 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 x 1679 pixels and pixel size of 172 x 172 μm^2. 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.

Fibril strain*:** The meridional stagger (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) (**Figure 1G**); this corresponds to considering an integrated averaged of fibrillar deformation in all directions. The fibril strain (ε_f) 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**).

***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 (ε_m) 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 (**Figure 1E**), similar to prior work [14, 15, 38, 45].

The *Processing* perspective of the data analysis software package DAWN [48] (*www.dawnsci.org*) was used for SAXD and WAXD data reduction. The integrated SAXD and WAXD 1D intensity

profiles (**Figure 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 unstrained (zero-stress) value for D-period and D(002). The collagen fibril strains ε_f and mineral strains ε_m were calculated from the percentage changes of collagen D-period and the (002) lattice spacing, respectively, relative to the unstrained state. 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 σ versus fibril strain and mineral strain, respectively, from the elastic region of deformation (**Figure S3-4, 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 fibril and mineral elastic modulus (hence the use of the qualifier "effective").

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 $I(\chi; q_0)$ were calculated over the full azimuthal range (360°) from the first-order collagen reflection (at $q = q_0 = 6\pi/D$). 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 $\Delta q=0.015$ 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 **Figure 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 × 15 × 15 μm^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 at 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 (**Figure 2 C1**)- 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 (**Figure 2E-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 for $p>0.05$).

2.8 Modelling of fibrillar and lamellar mechanics

To understand the structural mechanisms underpinning trends in E_f, 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 numerical (finite element) simulations performed in *Abaqus 6.14* [54] 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**.

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, φ_{EM} is the volume fraction of the extrafibrillar matrix and k is a factor defined in Equation S6.*

Nomenclature of the modulus	Experimental	Analytical/Numerical
Effective fibril modulus	$\frac{\text{Applied tissue stress}}{\text{average fibril strain}}$ Calculated via linear fitting of experimental data shown in **Figure 5A.**	$\frac{\text{Applied laminate stress}}{\text{average strain of the sublamellae}}$ Computed via laminate theory.
Effective mineral modulus	$\frac{\text{Applied tissue stress}}{\text{mineral strain}}$ Calculated via linear fitting of experimental data shown in **Figure 5B.**	$\frac{\text{Applied tissue stress}}{(afs * \varphi_{EM}) + (afs * \frac{(1-\varphi_{EM})}{k})}$ Computed via laminate theory.
ΔFWHM/fibril strain	ΔFWHM: variation of the FWHM of Gaussian fitting 'I vs χ' curves (more details in **Supplementary Information**). Fibril strain: average fibril strain, averaged from the volume of bone (beam size * sample thickness) measured by x-ray.	ΔFWHM: variation of the FWHM of the lamellar angular distribution (Gaussian distribution). The fibrillar reorientation leading to this variation was computed via FE simulations. Fibril strain: average strain of the sub-lamellae (computed via laminate theory).

2.9 Model structure and parameters

2.9.1 Analytical relations

Nanoscale force-balance relations: Stresses and strains on the fibril, mineral platelet and extrafibrillar matrix were calculated by considering the fibril as a staggered array of mineral particles embedded with a collagen matrix (**Figure 3A-I**), which is in turn embedded in an extrafibrillar 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 extrafibrillar matrix, whose constitutive law was taken as the Ramberg-Osgood law $\varepsilon = \sigma/(c\dot{\varepsilon}^d)$ [58, 59]. Most parameters were obtained from referenced literature (**Table 2**), with the exception of the Young's modulus and volume fraction of the extrafibrillar matrix, and the *k*-factor, which are obtained from nonlinear fitting to the experimental data (**Figure S6**) 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 $\varphi_m = 0.40$ for GIOP and $\varphi_m = 0.45$ for WT.

Plywood structural parameters: The bone lamella was modelled as a set of differently oriented fibril layers, with angular orientations at 0°, ±5°, ±10°, ±15°, ±30°, ±45°, ±60°, ±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 (**Figure S2**), in a manner similar to our previous work [15]. Details are provided in **Supplementary Information**.

Matching to experimental data: Least-squares minimizations was carried out by simultaneously fitting the experimental E_f and E_m data to the model expressions (**Figure 5** and **Figure 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**.**

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 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. Details are provided in **Supplementary Information**.

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 (Eqns. S2 and S6 in Supplementary Information).*

Young's moduli	**GIOP bone (GPa)**	**Wild-type bone (GPa)**
E_c = Young's modulus of collagen	2.5 [36]	2.5 [36]
E_m = Young's modulus of hydroxyapatite (mineral content)	100 [36]	100 [36]
E_{EM} = Young's modulus of extrafibrillar matrix	*k* = 1.58: low s.r. 163.8, medium s.r. 160.8, high 160.1 *k* = 1.6: low s.r. 107.6, medium s.r. 105.7, high 105.3 *k* = 1.7: low s.r. 53.0, medium s.r. 52.3, high 52.1	Extrafibrillar matrix: low s.r. 3.5, medium s.r. 159.0, high 370.0
Poisson's ratios		
ν_c = Poisson's ratio of collagen	0.3 [61]	0.3 [61]
ν_m = Poisson's ratio of hydroxyapatite (mineral content)	0.28 [61]	0.28 [61]
ν_{EM} = Poisson's ratio of extrafibrillar matrix	0.3 [61]	0.3
Volume fractions		
φ_c = volume fraction of collagen	0.6	0.55
φ_m = volume fraction of hydroxyapatite (mineral content)	*k* = 1.58: 0.37; *k* = 1.6: 0.34; *k* = 1.7: 0.27	0.45 - φ_{EM} = 0.37
φ_{EM} = volume fraction of extrafibrillar matrix	*k* = 1.58: 0.03; *k* = 1.6: 0.06; *k* = 1.7: 0.13	0.08 (from fitting)

3 Experimental Results and Model Fitting

3.1 X-ray Microtomography

X-ray microtomography was performed to investigate 3D micromorphometry, 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. **Figure 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 **Figure 2A** and **C**, are selected from mid-shaft of mice femora as indicated by red dash lines in **Figure 2 B** and **D**. Clear qualitative differences can be observed in the cortical microstructure of GIOP mice as compare with 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.

Histograms of degree of mineralisation and the mean mineral concentration of middle shaft femoral bone from wild-type (N=5) and GIOP (N=5) mice were measured using X-ray microtomography. Representative distributions of mineral concentration were plotted for mid-shaft femora from wild-type and GIOP mice (**Figure 2E**). While not clearly visible at the lower-magnification whole-bone CT slices in **Figure 2A-D**, our prior work using backscattered electron microscopy on GIOP vs WT-femora (at similar age-points) showed that the mineralization of the endosteal region is clearly lower than the periosteal region in GIOP, while it is similar across regions in WT [33]. Since two distinct regions of interest: endosteal region surrounded by less mineralized halos, and periosteal region, were observed in GIOP mice (**Figure 2 C, D**)[33], they were used separately for quantitative X-ray microtomography analysis (**Figure 2** inset **C1**). The weighted average mineral concentrations (denoted as the degree of mineralisation) measured as hydroxyapatite g/cm^3 were calculated from the frequency distribution of mineral concentration and plotted for different groups (**Figure 2 E-F**). One-way ANOVA test indicated that the mean mineral concentration among three groups were significantly different. The weighted average mineral concentration in wild-type mice is significantly higher than that in GIOP periosteal ($p < 0.01$) and endosteal regions ($p < 0.001$), and it is also significantly higher ($p < 0.01$) in GIOP periosteal regions compared to GIOP endosteal regions

(**Figure 2F**). The broad distribution of mineral concentration with a fat tail toward low mineral concentration in GIOP endosteal region indicated a microscale heterogeneous mineralisation.

3.2 *In situ* tensile testing with synchrotron SAXD and WAXD

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

Effective fibril moduli: To compare the fibrillar-deformation in mice femur tested at different strain rates (**Figure 5B** and **Figure 4A, D**), data for samples at each strain rate were combined and plotted (tissue stress vs. nanoscale fibrillar strain) in the elastic deformation region (**Figure 4A, D**), and show differences in the slope (effective fibril modulus $E_f = d\sigma/d\varepsilon_f$). Average effective fibril moduli from each group of samples were plotted as a function of strain rate in **Figure 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 fibril 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 fibril modulus remains nearly constant in GIOP mice bone (blue bars). The effective fibril 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 fibril modulus between wild-type and GIOP mice was found at strain rate 0.0004 s^{-1} (**Figure 4** and **Table 3**). Note that for the data plotted in **Figure 5 B-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 **Figure 4** are regressions through the pooled data points (tissue stress vs fibril 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 fibril moduli in GIOP is lowest at the highest strain rate (0.02 %.s^{-1}; **Figure 5B**) while the slope of the regression line for GIOP-bone in the fibril moduli plot in **Figure 4D** is lowest for the intermediate strain rate 0.01 %.s^{-1}.

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 (**Figure 4B, E**). Here, the effective mineral modulus ($E_m = d\sigma/d\varepsilon_m$) in wild-type mice bone increased with strain rate and the increase was significant (p = 0.026) as seen in **Figure 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 (**Figure 4** and **Table 3**).

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 (**Figure 4C**). Averaged values of the rate of fibrillar reorientation were plotted as a function of strain rate in **Figure 5D**, and showed a significant (p = 0.018) reduction. In wild-type mice bone, the rate of fibrillar reorientation (-40.8 ± 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 0004 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} (**Figure 4** and **Table 3**).

Table 3: *Effective fibril moduli, effective mineral moduli and fibrillar reorientation in WT- and GIOP-bone; p-values report differences between WT- and GIOP- in each group.*

	Strain rate (s^{-1})	Wild-type	GIOP	P-value
Effective fibril moduli (GPa)	0.004	13.60 ± 3.00	14.46 ± 2.66	0.876
	0.01	37.90 ± 9.90	13.02 ± 4.28	< 0.001
	0.02	65.60 ± 11.40	11.50 ± 3.58	< 0.001
Effective mineral moduli (GPa)	0.004	44.20 ± 7.29	17.90 ± 5.30	0.032
	0.01	70.50 ± 16.70	20.77 ± 1.42	< 0.001
	0.02	97.49 ± 28.38	26.66 ± 10.50	< 0.001
Reorientation rate (degree / %)	0.004	40.75 ± 23.22	2.18 ± 9.65	< 0.001
	0.01	4.90 ± 3.91	1.76 ± 5.63	0.703
	0.02	5.50 ± 4.94	1.24 ± 4.02	0.606

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 (**Figure 5A**) to be calculated. **Figure 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 $\dot{\varepsilon} = 0.0004\ s^{-1}$ (low strain rate) to 370.0 GPa at $\dot{\varepsilon} = 0.02\ s^{-1}$ (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**).

Figure 5B shows a comparison between the experimental and numerically computed effective fibril 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 ($\dot{\varepsilon} = 0.02\ s^{-1}$). For the wild-type model a stiffening effect with an increasing strain rate – as seen in experiment – was also found at the mineral level (**Figure 5C**). The effective mineral modulus, E_m, is overestimated at high and medium strain rates and slightly underestimate at low strain rate.

For the GIOP bone, both the effective fibrillar and mineral moduli confirm the constant trend found experimentally (**Figure 5B-C**) and show agreement with experimental values (average experimental 13.6 GPa vs 13.9 GPa). Indeed, the average experimental value of the effective fibril 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.

Figure 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 (**Figure 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 $k = 1.58$.

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 (**Figure 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 counterintuitive 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 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 0.1s per point, the total X-ray dose is proportional to the number of SAXS patterns per tensile test. **Figure 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 (**Figure 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 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 **Figure 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 (**Figure 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 Figures 2-3 in [70]) (noting the factor of 5 compression in the c-axis direction specified by the authors). This value is much smaller (**Figure 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 **Figures 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 (**Figure 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 fibril- 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 (**Figure 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 extrafibrillar 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 noncollageneous 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 $I(\chi)$. 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 (**Figure 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 normal murine bone with increasing strain-rate, and have found both a) clear changes with strain-rate for normal bone and b) a near constant-response across strain-rates for osteoporotic bone. Modelling the bone matrix as arrays of mineralized fibrils with intervening matrix, our results suggest alterations in extrafibrillar matrix stiffness and mineral-collagen reinforcement factors may be the underlying factors. Our results provide insight into the time-dependent nature of fibrillar mechanics in both normal and osteoporotic bone, and may be relevant in understanding the structural origins (in terms of bone quality) of the lower mechanical competence in osteoporosis.

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Figures:

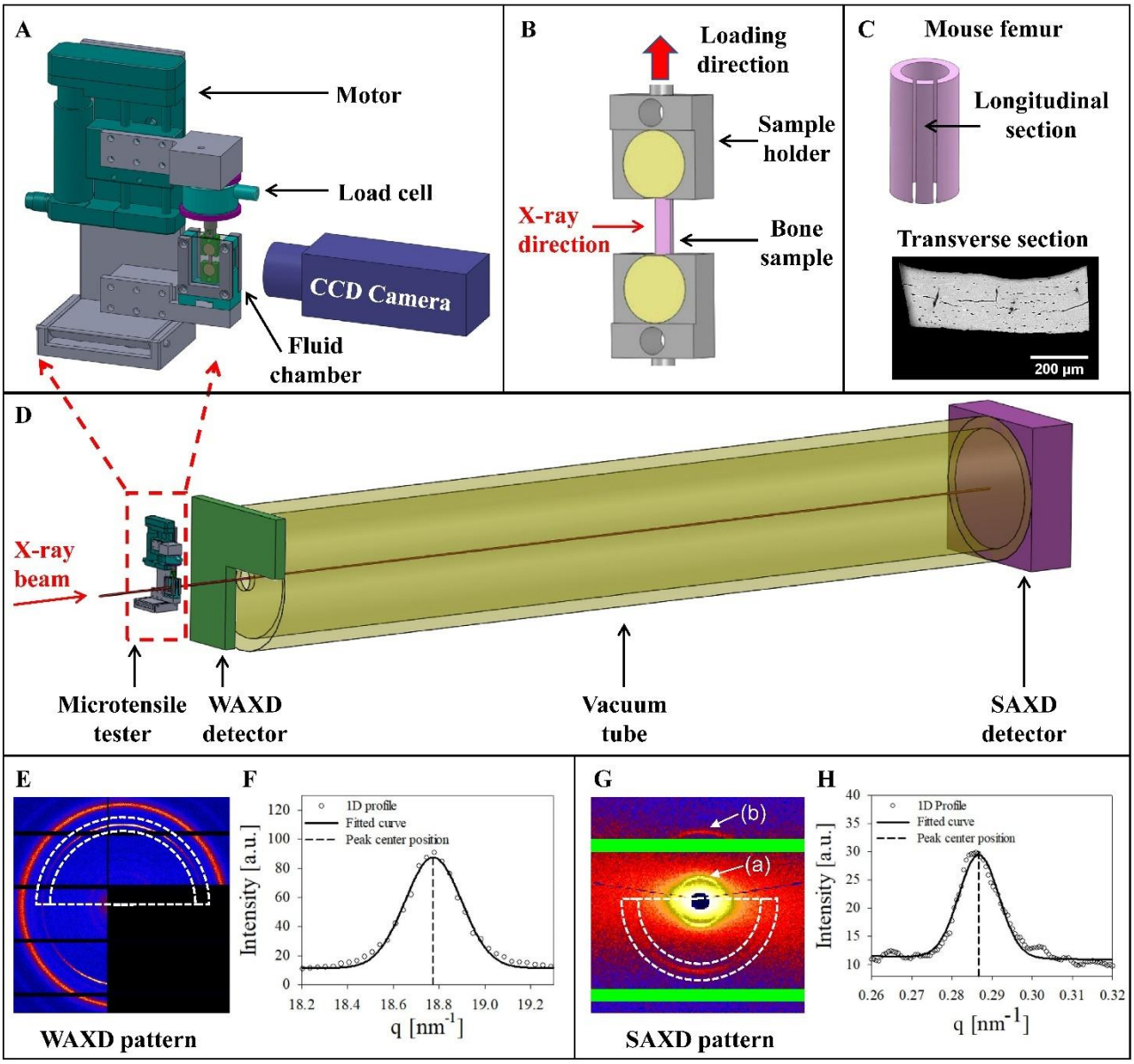

Figure 1: In situ nanomechanics with simultaneous synchrotron SAXD and WAXD. *(A) Customized tensile tester with bone sample mounted in a fluid chamber. (B) Magnified view of sample and tensile grips in fluid chamber, with tensile strain along the vertical direction. (C) Upper: Schematic of mouse femur, with tensile test specimen sectioned along the long axis of femur; lower: backscattered electron image of transverse section of specimen. (D) Experimental configuration: Tensile tester with specimen mounted along the X-ray beam path in transmission geometry; an L-shape WAXD detector, vacuum tube and SAXD detector were positioned along the X-ray beam path. (E) 2D WAXD pattern from bone apatite with predominant c-axis orientation vertical. Dotted lines denote the 180° region for azimuthal averaging of intensity around the (002) peak of apatite. (F) Azimuthally averaged radial intensity profile I(q) for the pattern in E. (G) 2D SAXD pattern from collagen fibrils in bone with predominant fibril orientation vertical. Dotted lines denote the 180° region over which the collagen reflection is averaged azimuthally; (a) the first-order and (b) the third-order collagen reflection. (H) Azimuthally averaged radial intensity profile I(q) for the pattern*

in G. For F and H, black solid line: peak fit with a Gaussian function plus a linear baseline; black dashed line: peak centre position.

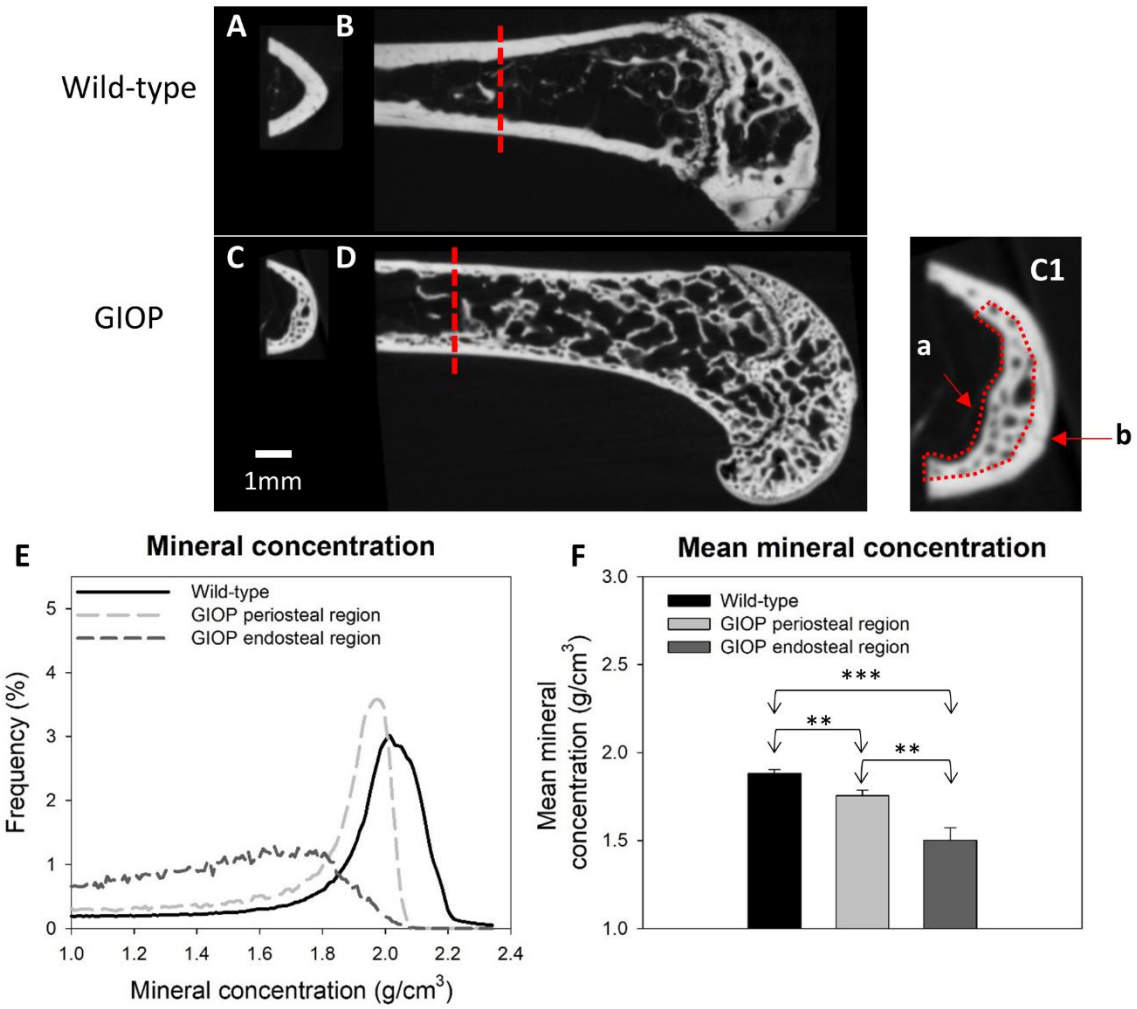

Figure 2: X-ray microtomography and degree of mineralisation. *Representative 2D slices from X-ray microtomography measurement for both transverse (A, C) and longitudinal (B, D) cross sections of femora from wild-type and GIOP mice. Red dash line indicated location where the 2D slice of transverse cross section was taken. C1: Inset on right shows an example 2D transverse slice, with (a) indicating the endosteal region and (b) the periosteal region. (E) Representative histograms of degree of mineralisation were plotted for wild-type (black), GIOP periosteal regions (light gray) and GIOP endosteal regions (dark gray). (F) Bar chart of the mean mineral concentration for wild-type cortex, GIOP periosteal regions and GIOP endosteal regions. Error bars shown are standard deviations. Statistical significances were denoted on the figures (*p < 0.05, **p < 0.01, ***p < 0.001, ns: not significant).*

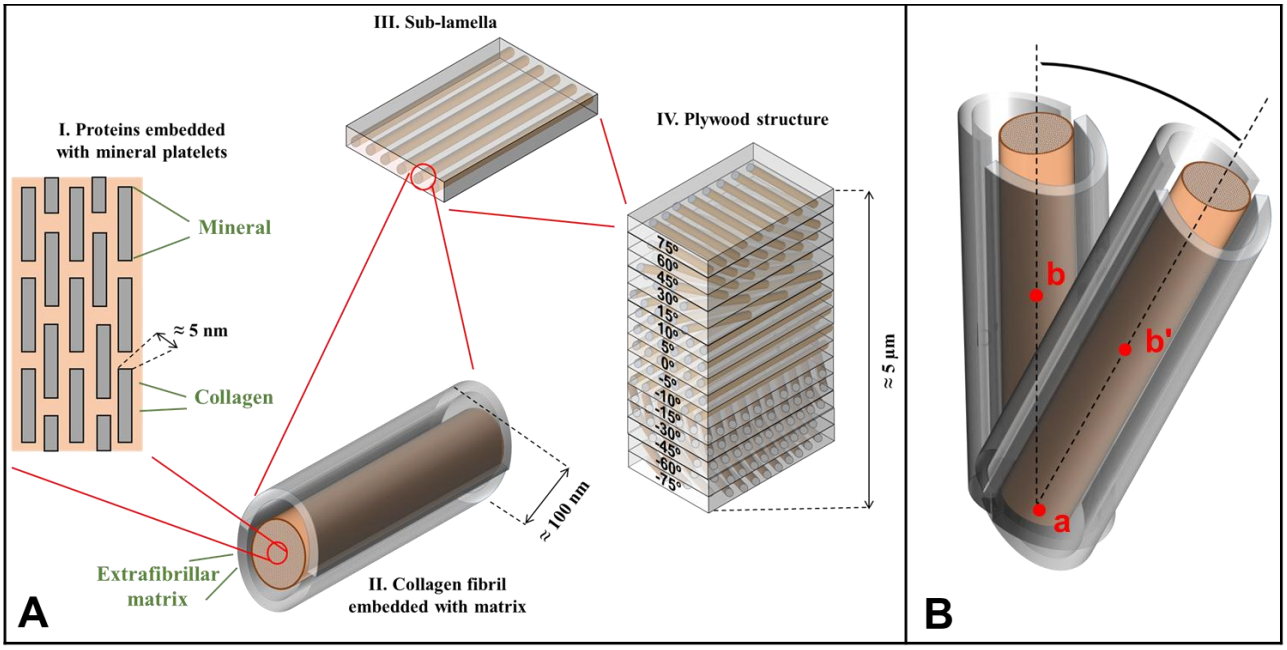

***Figure 3*: Schematic of the hierarchical structure of bone assumed for the modelling approach.** *A) I. At the lowest hierarchical scale, a staggered arrangement of hydroxyapatite mineral platelets and collagen [41] (left side of the figure) was considered. The material components are collagen, hydroxyapatite mineral and extrafibrillar matrix (which together form level II). A bunch of parallel collagen fibrils surrounded by an extrafibrillar 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 [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.*

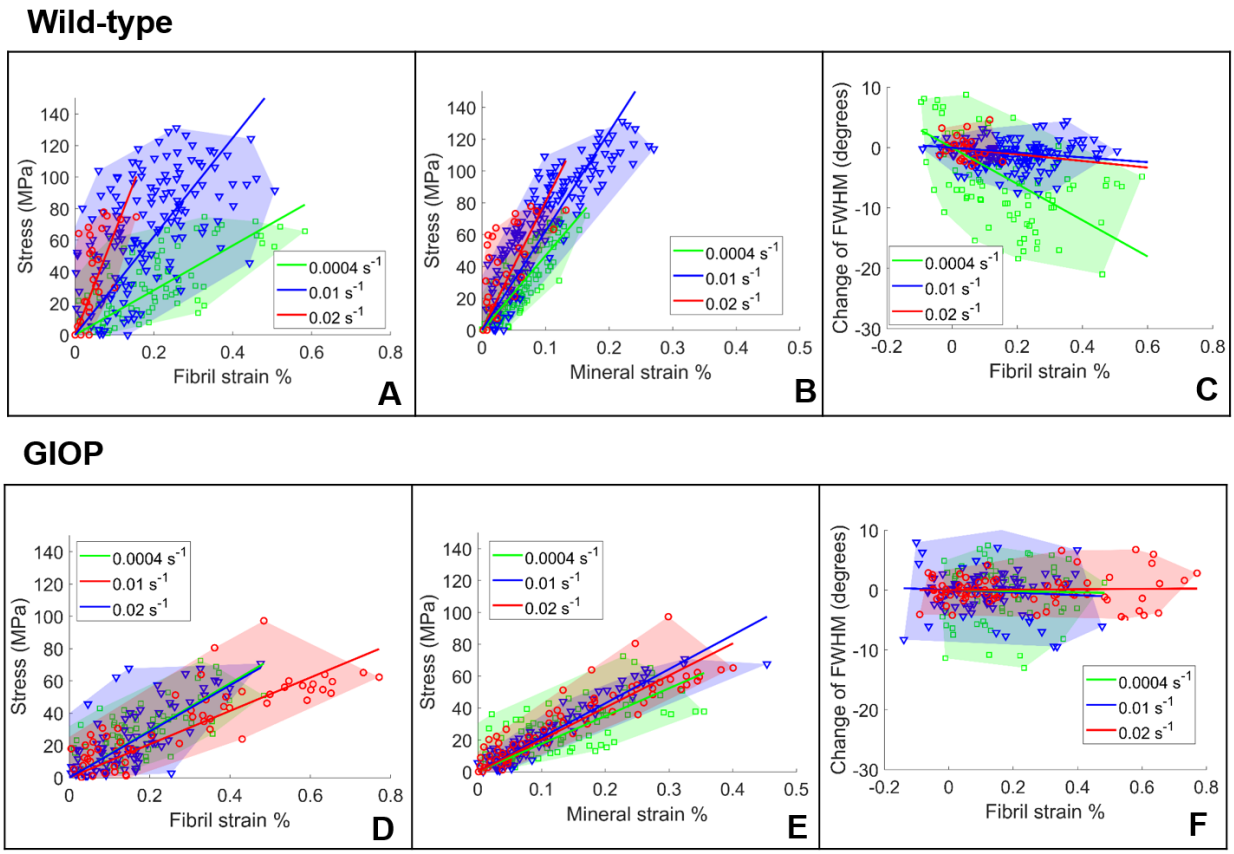

Figure 4: Fibril strain, mineral strain and change of FWHM from in situ synchrotron SAXD and WAXD: *Symbol code: Low strain rate (0.0004 s^{-1}, green squares), medium strain rate (0.01 s^{-1}, blue triangles) and high strain rate (0.02 s^{-1}, red circles). (A, D) Applied tissue stress vs average fibril strain. (B, E) Applied tissue stress vs average mineral strain. (C, F) 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.*

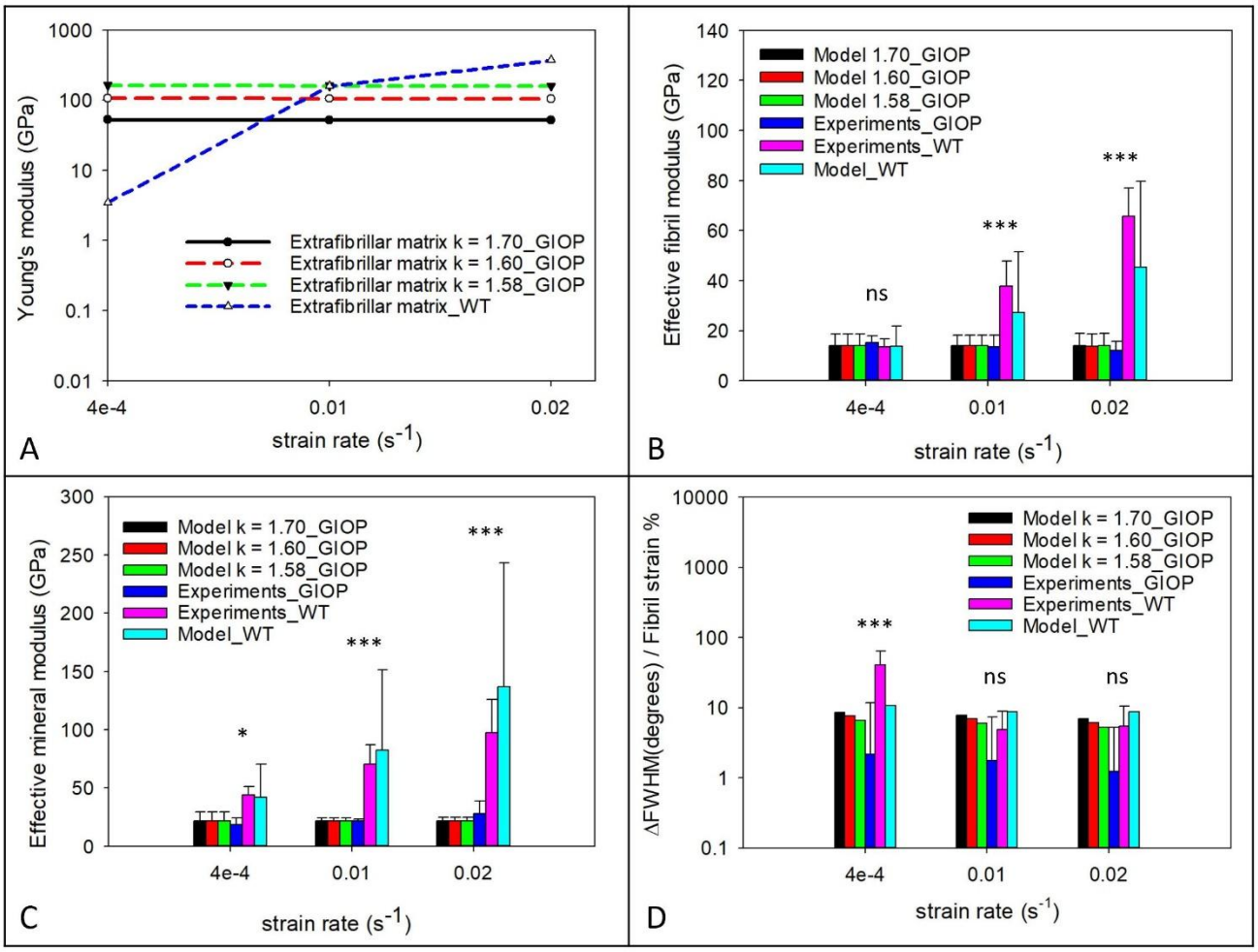

***Figure 5**: **Nanoscale structural parameters of bone mineral and fibrils from experiments and modelling**: (A) Young's modulus of collagen and extrafibrillar matrix at different strain rates from simulation results (in log scale). (B) Effective fibril modulus, (C) effective mineral modulus and (D) reorientation rate (in log scale) are plotted as a function of strain-rate. Error bars shown are standard deviations for experimental data while are 95% confidence interval from the fitting process. One-way ANOVA tests were performed to test for statistical differences in the experimental results of the effective fibril modulus, the effective mineral modulus and the fibrillar reorientation rate between samples tested at different strain-rates. Statistical significance is denoted (*p < 0.05, **p < 0.01, ***p < 0.001, ns: not significant).*

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Table(s)

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Table

	Strain rate (s^{-1})	Wild-type	GIOP	P-value
Effective fibril moduli (GPa)	0.004	13.60 ± 3.00	14.46 ± 2.66	0.876
	0.01	37.90 ± 9.90	13.02 ± 4.28	< 0.001
	0.02	65.60 ± 11.40	11.50 ± 3.58	< 0.001
Effective mineral moduli (GPa)	0.004	44.20 ± 7.29	17.90 ± 5.30	0.032
	0.01	70.50 ± 16.70	20.77 ± 1.42	< 0.001
	0.02	97.49 ± 28.38	26.66 ± 10.50	< 0.001
Reorientation rate (degree / %)	0.004	40.75 ± 23.22	2.18 ± 9.65	< 0.001
	0.01	4.90 ± 3.91	1.76 ± 5.63	0.703
	0.02	5.50 ± 4.94	1.24 ± 4.02	0.606

Figure 1

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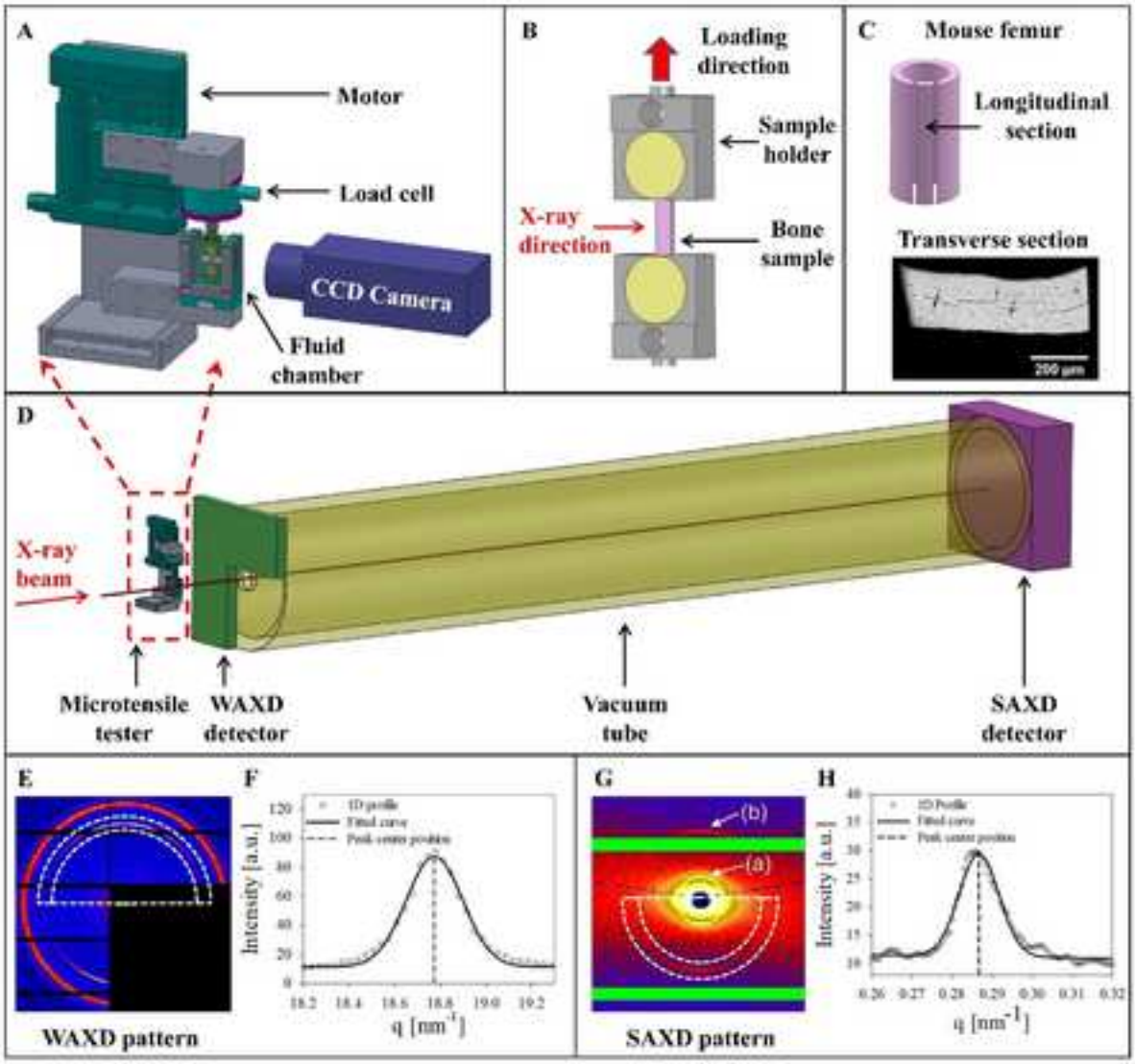

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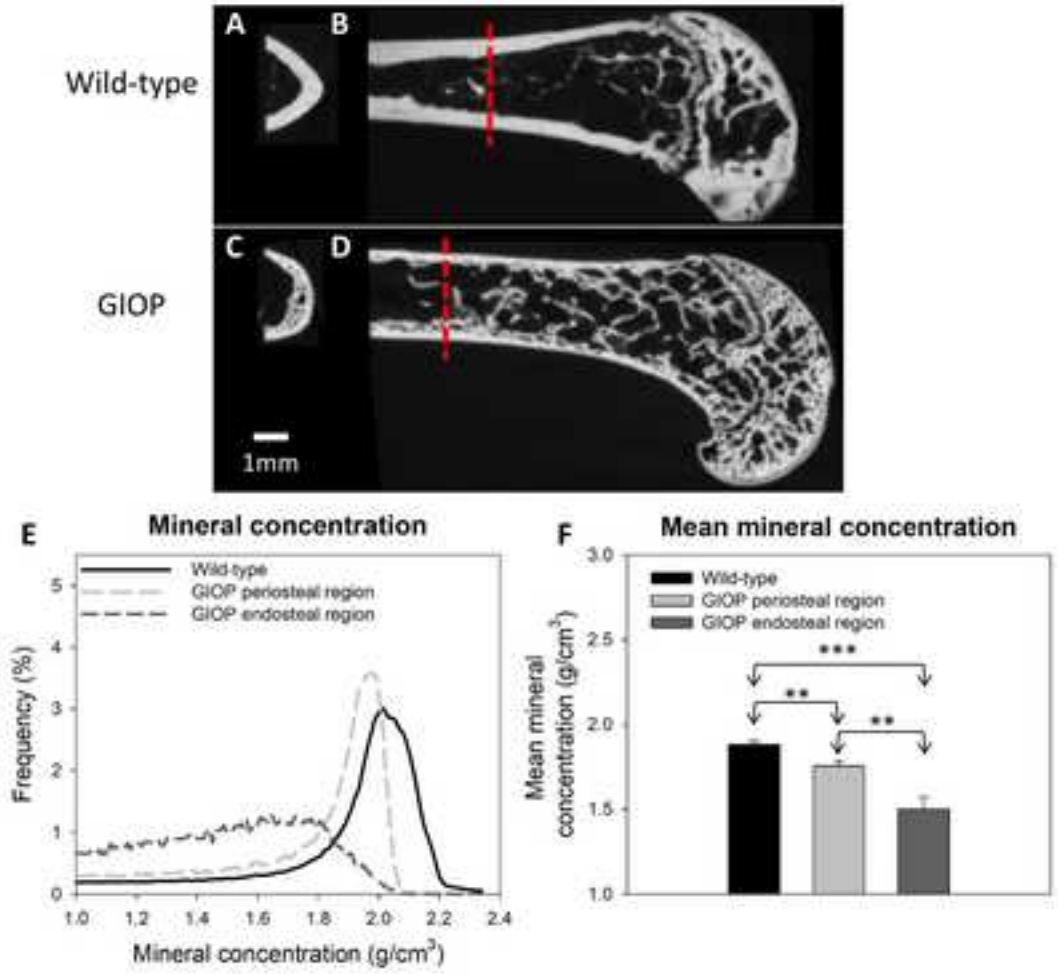

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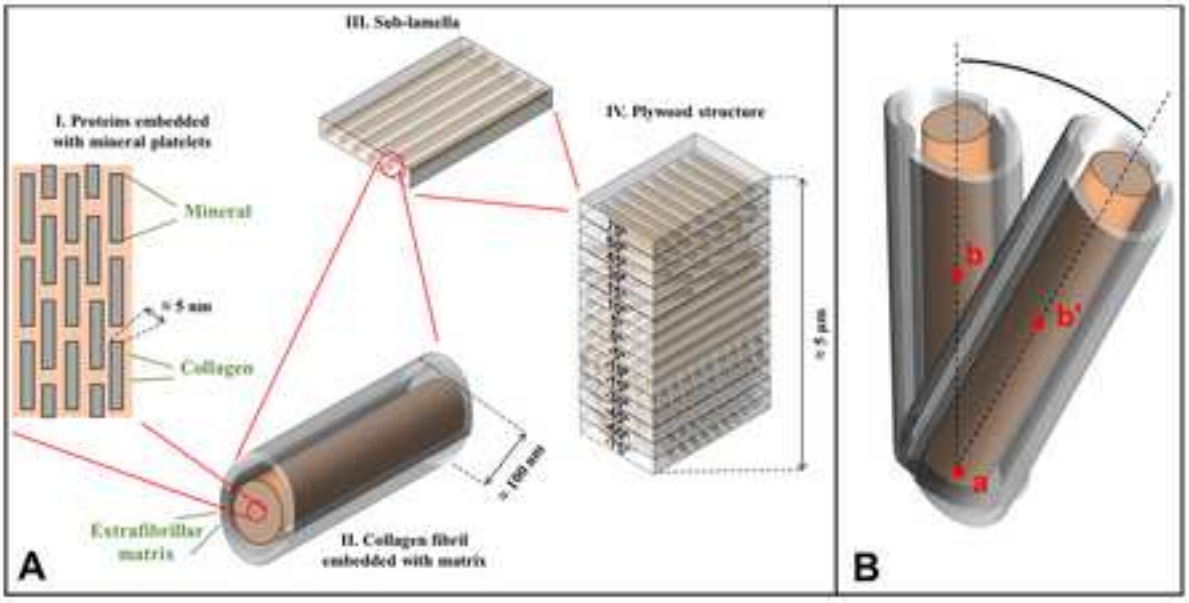

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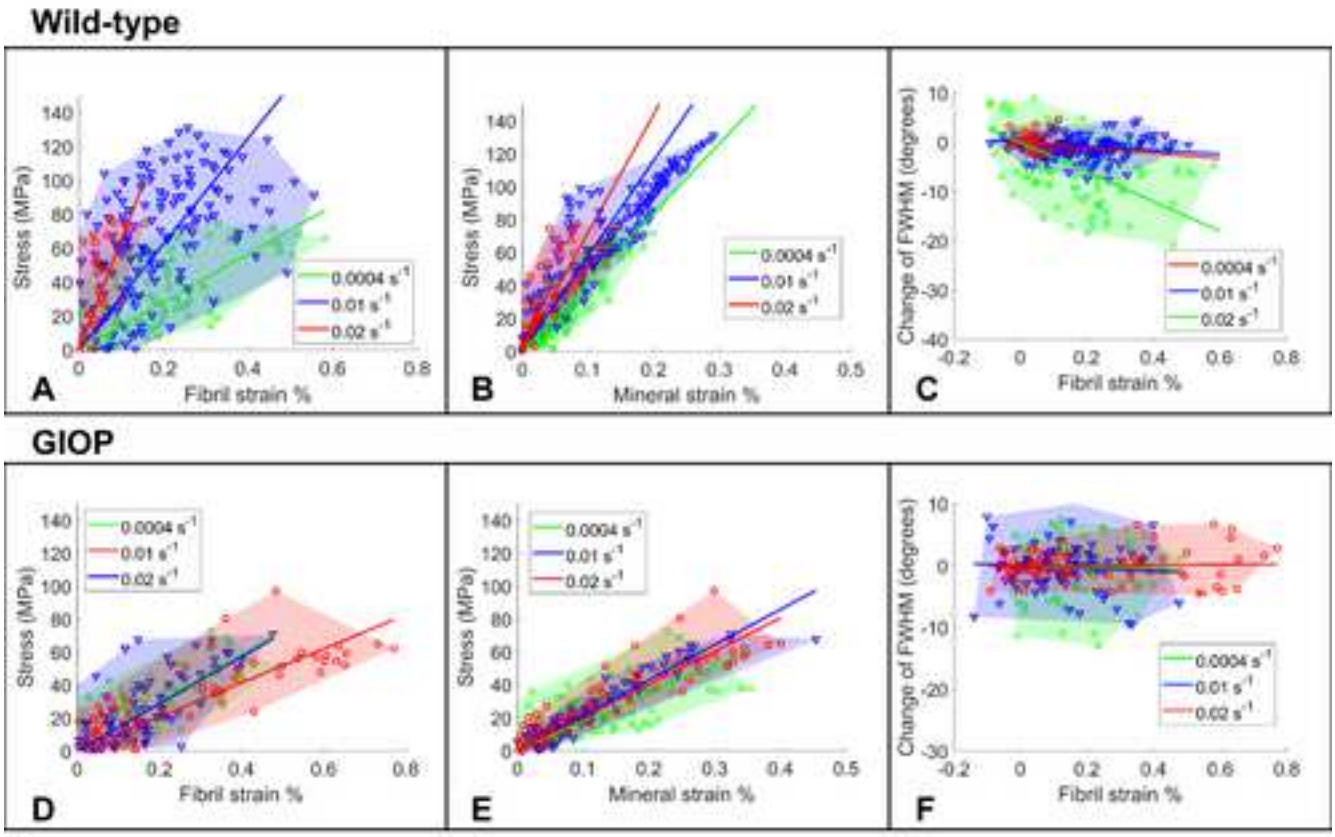

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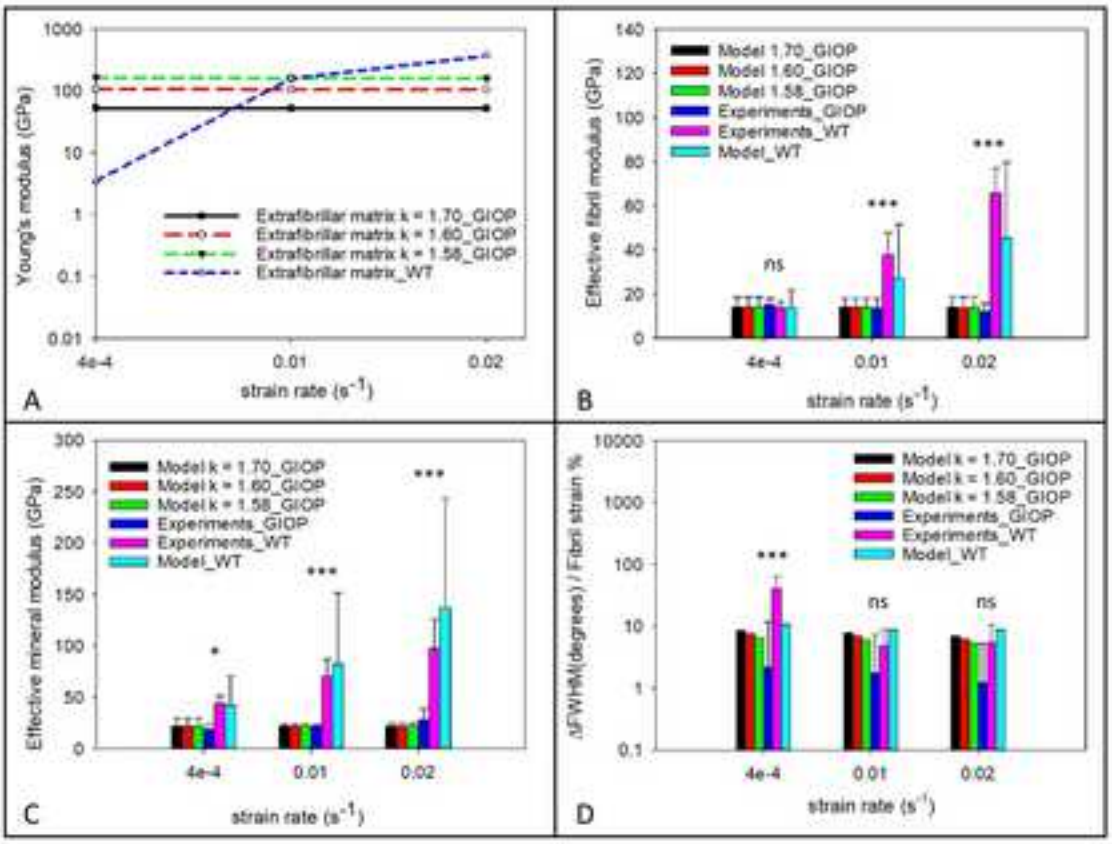

Supplementary Material

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