Micromechanical evaluation of cortical bone using *in situ* XCT indentation and digital volume correlation

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Abstract

The overall mechanical behaviour of cortical bone is strongly dependant on its microstructure. X-ray computed tomography (XCT) has been widely used to identify the microstructural morphology of cortical tissue (i.e. pore network, Haversian and Volkmann's canals). However, the connection between microstructure and mechanics of cortical bone during plastic deformation is unclear. Hence, the purpose of this study is to provide an in-depth evaluation of the interplay of plastic strain building up in relation to changes in the canal network for cortical bone tissue. *In situ* step-wise XCT indentation was used to introduce a localised load on the surface of the tissue and digital volume correlation (DVC) was employed to assess the three-dimensional (3D) full-field plastic strain distribution in proximity of the indent. It was observed that regions adjacent to the imprint were under tensile strain, whereas the volume underneath experienced compressive strain. Canal loss and disruption was detected in regions of higher compressive strains exceeding $-20000 \mu\epsilon$ and crack formation occurred in specimens where Haversian canals were running parallel to the indentation tip. The results of this study outline

the relationship between the micromechanical and structural behaviour of cortical bone during plastic deformation, providing information on cortical tissue fracture pathways.

Introduction

Cortical bone is a load-bearing hard tissue with hierarchical structure from nano- to macroscale (Jae-young et al., 1998; Tai et al., 2005), where the typical features of its structural organisation affect the overall mechanical behaviour and ability to resist fracture (Augat and Schorlemmer, 2006; Chapurlat, 2016). Assessing and preventing bone fracture has been one of the predominant aims in bone research (Hansen et al., 2008; Marshall et al., 2001; Raeisi Najafi et al., 2007; Unal et al., 2018; Zioupos et al., 2008); particularly, with regards to crack initiation and propagation to the point of failure. The natural ability of bone tissue to resist crack propagation and minimising microdamage is vital in sustaining load without fracturing and involves a variety of mechanisms at different structural levels (Hunt and Donnelly, 2016). Its mechanical behaviour in the macroscale is affected by the stiffness, toughness and strength in the mesoscale (millimetre-scale) (Grimal et al., 2011; Jae-young et al., 1998). For instance, investigation of the mesoscopic level has been useful in assessing stress and strain distribution in long bones with osteoporotic fractures (Rohrbach et al., 2015). Tissue porosity at the sub-micrometre scale in the cortical bone has been shown to be directly driving its mechanical behaviour (Donaldson et al., 2014) by largely affecting its ability to resist deformation (Hage and Hamade, 2017). Porosity in the cortical bone refers to the canal network (i.e. Haversian and Volkmann's canals), osteocyte lacunar and canalicular system (Cai et al., 2019; Cooper et al., 2016). In fact, differences in the cortical porosity have been associated with increased fracture risk (Bell et al., 1999). The microstructural properties influence fracture behaviour either by acting as constraints in the crack propagation or defects in stress concentration (Augat et al., 1998; Donaldson et al., 2014; Wolfram et al., 2016; Zioupos, 1998). This also depends on the orientation of the load to which cortical bone is subjected. Nalla et al. (2005) investigated strain-controlled mechanisms for the onset of

fracture in bone. It was observed that the impact of properties in the microstructure is more dominant in the transverse direction due to the fact that cement lines deflect the crack along the length.

Three-dimensional analysis (3D) of the microstructure of cortical bone has been successfully conducted with the use of either laboratory based or synchrotron radiation (SR) X-ray Computed Tomography (XCT) providing information on the porosity of the structure (Cooper et al., 2003). Cai et al. (2019) used SR-XCT to study the vascular pore and mineral content phenotypes in femoral cortical bone specimens. Moreover, micro-compression and SR-XCT have been combined to investigate the changes of canal network under loading in femoral cortical bones (Voide et al., 2009). Both studies concluded that the canal surfaces were acting as starting points for crack formation.

In the recent years, digital volume correlation (DVC) has been employed to compute the 3D full-field strain concentration in correspondence of bone microdamage using high-resolution XCT images (Gillard et al., 2014; Peña-Fernández et al., 2018b; Tozzi et al., 2016). DVC has also been used to assess the strain distribution around the intracortical canals in mice femoral bone under micro-compression during SR-XCT imaging (Christen et al., 2012). A cross-sectional notch was introduced in the diaphysis of the femur, similar to Voide et al. (2009), and the strain in two different regions of the bone containing microcracks was computed. In all specimens, cracks occurred at the canal surface and high shear and tensile strains were found in those areas.

Indentation has been extensively used to characterise bone mechanical properties at the micro- and nanoscale (Fan and Rho, 2003; Yin et al., 2012; Zhang et al., 2008). Plastic deformation due to indentation testing usually occurs when using pointed indentation tips (i.e. Vickers (Fischer-Cripps, 2006)) and is sustained by compressive and tensile strains in the whole volume, rather than just the area underneath the indenter (Fischer-Cripps, 1999). In that sense, *in situ* X-ray nanoCT indentation has been recently carried out on an elephant tusk to investigate the relation between plastic deformation and microstructure (Lu et al., 2019). Elephant dentin is an anisotropic material with hierarchical microstructure. At the mesoscale, it includes dentinal tubules that follow a wavy path.

The plastic deformation resulted in crack formation that was studied regarding the tubules location and orientation. DVC on the volume under the indentation surface showed the highest compressive strain with tubules at 45° angle and the lowest in those at 70°. However, as this could be somehow expected due to the relatively transverse isotropy of dentin (Seyedkavoosi and Sevostianov, 2019), plastic strain distribution and its effect on microdamage progression in more complex biological architectures (i.e. cortical bone) has yet to be established. In fact, to the best of the authors' knowledge, an investigation of crack propagation in cortical bone tissue due to plastic deformation via *in situ* XCT indentation and DVC has never been reported.

Therefore, the aims of this study were: to investigate the strain distribution in the cortical bone tissue due to plastic deformation accumulated during indentation; to correlate strain distribution and microdamage progression with microstructural changes of the canal and pore network under progressive load, both axially and transversely. A strong correlation of the strain distribution with microstructural phenotypes of crack formation and prevention was observed.

Methodology

Sample preparation

Bovine femoral bone harvested from a single animal between the age of 12 and 20 months was used in this study. The bone was cleaned and soft tissues removed using surgical tools. Cylindrical cortical bone specimens were extracted from the same region in the diaphysis of the femur ($D = 4 \text{ mm}$, $H = 6 \text{ mm}$), six in the transverse and six in the axial direction with respect to the long vertical axis of the bone. The specimens were cut using a diamond coring drill bit (Pacera, Meddings Machine tools, UK) under constant water irrigation and a 800-grade sandpaper was used to polish the top surface using distilled water as lubricant. All specimens were kept frozen at -20°C and thawed for approximately 2 h in saline solution at room temperature before testing. Brass endcaps aiming at stabilising the specimen during testing as well as reducing metal artifacts in XCT were then used to embed only the

distal and intracortical part for both axial and transverse specimens, respectively (Keaveny et al., 1999; Peña-Fernández et al., 2018b).

In situ Indentation and Imaging

A custom-made indentation set up was designed for the purpose of this experiment. The top plate of the XCT in situ loading stage (CT5000, Deben, UK) was adjusted to mount a Vickers' pyramidal diamond tip. A bottom cylindrical steel plate was designed to fit in the loading stage chamber with a recess in the middle to secure the sample with its endcap. Saline solution was used to keep the specimens hydrated throughout the duration of the experiment. The tip was fixed in the middle of the top plate aligned with the middle cross-section of the specimen. Initially, the jaws of the loading stage were fully open and two consecutive XCT (Xradia 510 Versa, Carl Zeiss Microscopy, USA) tomograms were taken prior to the *in situ* step-wise indentation to allow for DVC error analysis. After each indentation step the tip was retracted, the specimen allowed to relax for 20 minutes and then a tomogram was acquired. The XCT operated at 110 kV, 9 W with exposure time set at 3 s/projection (1600 projections), resulting in a voxel size of 4.2 μm . The field of view (FOV) included all the specimen diameter and approximately half of its height (4.2 x 4.2 mm).

The loading stage was set in depth control and the displacement rate was 0.1 mm/min. The specimens were divided in three different groups of four to accommodate for three different loading conditions (Table 1). There were two axial and two transverse specimens in each group ($A_{i,j}$ and $T_{i,j}$, where i represents the specimen number and j the loading condition for axial and transversal, respectively). In the first group, loading condition 1 ($A_{1-2,1}$ and $T_{1-2,1}$), the specimens were indented once to a final displacement of 0.4 mm. The second group, loading condition 2 ($A_{3-4,2}$ and $T_{3-4,2}$), involved two indentation steps; first reaching 0.2 mm and then increasing the depth to a final 0.4 mm in the same location. The third group loading condition 3 ($A_{5-6,3}$ and $T_{5-6,3}$), included three steps at 0.2 mm, 0.3 mm,

and final 0.4 mm displacement. These indentation steps are annotated as l_0 , l_2 , l_3 , l_4 for 0 mm, 0.2 mm, 0.3 mm and 0.4 mm displacement, respectively.

The loading stage used here is not a conventional set up for indentation and does not automatically recognise contact with the surface of the specimen. Therefore, the initial contact of the tip with the specimen was identified as the point where an increase in the force (>0.3 N) occurred and the loading continued until it reached the desired displacement. Hence, the measurements mentioned above are not the plastic depth of indentation, rather the displacement of the loading stage actuator.

This resulted in a total experimental time of 4 hrs and 15 mins, 6hrs and 10 mins and 7 hrs and 55 mins for the first, second and third loading condition respectively.

Table 1: Loading conditions and direction of extraction for all the specimens.

	Axial	Transverse
Loading condition 1 (0.4 mm displacement)	$A_{1,1}$	$T_{1,1}$
	$A_{2,1}$	$T_{2,1}$
Loading condition 2 (0.2 and 0.4 mm displacement)	$A_{3,2}$	$T_{3,2}$
	$A_{4,2}$	$T_{4,2}$
Loading condition 3 (0.2, 0.3 and 0.4 mm displacement)	$A_{5,3}$	$T_{5,3}$
	$A_{6,3}$	$T_{6,3}$

Image post-processing

The 3D datasets obtained from the tomograms after reconstruction (16-bit) and all the imaging post-processing was performed using FIJI (ImageJ, USA). The images were first converted to 8-bit and then rigidly registered to the first scan prior to indentation (Karali et al., 2020). Datasets containing the whole specimen were cropped discarding images with zero grayscale intensity at the top of the tomographic dataset, to be used for subsequent DVC analysis. The artifacts and noise in the images

were reduced by creating a dataset specific mask. Using a plug-in (BoneJ), a purifying cycle was run in the binary images of each dataset (Doubé et al., 2010). The mask was finally applied by implementing an arithmetic and logical operation between the original and purified dataset (Peña-Fernández et al., 2018b).

Bone morphometry

A set of parallelepipeds in the middle of the bone volume underneath the indent were cropped using FIJI (ImageJ, USA). The size of the parallelepipeds varied among the specimens due to the discrepancies in the borders of their volume (i.e. uneven edges, coarse surfaces etc.), the mean volume was $13 \pm 2.27 \text{ mm}^3$. The binary images of the datasets were then imported into Avizo 9.7.0 (ThermoFischer Scientific, USA) where non-bone volume was segmented using the wand function to determine the intracortical porosity, pore and canal network. The canal network identifies the Haversian and Volkmann's canals, whereas the porosity represents the canal network volume combined with that of the pores (connected pores, i.e. osteocyte lacunae; isolated pores, i.e. resorption spaces) (Cooper et al., 2016). In the different loading stages, the canal and pore assessment incorporate the crack formation and distortion in the network. Properties such as the number of canals (N.Ca), canal diameter (Ca.Dm), number of pores (N.Po) and pore diameter (Po.Dm) were obtained to evaluate how incremental indentation relates to changes in cortical bone morphology using pore segmentation and separation process to visualise and quantify the Pore Network Model. Statistical indicators in the mean Ca.Dm and mean Po.Dm between the *in situ* XCT indentation stages in each specimen individually were estimated using a non-parametric independent Kruskal-Wallis test (SPSS Statistics 25, IBM, US), with level of significance $\alpha = 0.05$.

Digital Volume Correlation

A local DVC approach was used in the cropped parallelepipeds (Davis 10.0.3, LaVision, UK) and further details on the operating principles of the algorithm are detailed elsewhere (Peña-Fernández et al., 2018a). Briefly, the reference and deformed images are matched via a direct cross-correlation (DC)

function of the grey-levels, which is preceded by a Fast Fourier Transform (FFT)-based correlation to initialise the computation. A multi-step processing scheme from 128 (FFT, initial) to 48 (DC, final) voxels/subvolume using sum of differential with a 0% overlap was employed for the strain computation of the first and third principal full-field strains (ϵ_{p1} and ϵ_{p3} respectively) at the different indentation steps/group (Dall'Ara et al., 2017; Karali et al., 2020). In order to determine the DVC settings, two consecutive tomograms were obtained prior to indentation and used to calculate the zero-strain error uncertainties from DVC (Liu and Morgan, 2007; Tozzi et al., 2017) in the form of accuracy (or mean absolute error – MAER) and precision (or standard deviation of the error – SDER) for different subvolume sizes of the multi-step analysis (Fig.S2); and identifying a suitable trade-off between level of strain uncertainty and spatial resolution of the measurement (i.e. final subvolume size by the voxel size).

Results

Microcracks were formed in four of the axial specimens ($A_{1,1}$, $A_{2,1}$, $A_{3,2}$, and $A_{5,3}$). The transverse samples did not show any large crack formation. In fact, in all the specimens with microcrack development, this was observed at the end of the indentation pile-up. $A_{1,1}$ and $A_{2,1}$ were indented in one step (I_4), whereas $A_{4,2}$ and $A_{5,3}$ went through two (I_2 , I_4) and three (I_2 , I_3 , I_4) indentation steps, respectively. In all specimens, pile-up of the tissue was visible and it was more dominant in the ones that showed crack formation. Microcracks were localised at the ends of the pile-up in correspondence of the Haversian canal, propagating further and following the side of the pyramidal-shaped imprint.

The effective depth of indentation was measured from the 3D images (Fig.1). The minimum effective depth was observed in $T_{1,1}$ and $T_{2,1}$ at 0.07 mm. The largest imprint displacement occurred in $A_{2,1}$, at 0.19 mm, followed by $A_{1,1}$ at 0.18 mm. Furthermore, $A_{3,2}$ and $A_{5,3}$ achieved 0.15 mm final depth. From the segmentation of the reversed volume in the area underneath the imprint, loss of the original canals was observed (Fig.1). In the specimens where fracture occurred, the microcrack was visible at the side of the imprint as well as distortion of the original network. Distortion was also present in

those that did not crack. Specifically, as shown in Fig. 1, the network in T_{6,3} does not illustrate any canal loss, instead the load had a compressive effect in the horizontal canals reducing the distance between them (Fig. 1).

The global porosity was calculated after every indentation stage. It varied among the specimens and it was observed that none of the specimens with the highest porosity values developed cracks. The highest porosity was found for T_{6,3} (9%), followed by T_{3,2}, A_{6,3}, T_{1,1} and T_{4,2} (7.6%, 5.1%, 4.8% and 3.72%, respectively). The four specimens that formed microcracks had a mean porosity value of 1.46%. The porosity in the rest of the samples ranged between 1.53% and 1.68%. A_{1,1}, A_{2,1}, A_{5,3} showed higher porosity values after microcrack formation. However, in A_{3,2} despite the fact that a microcrack developed at I₄, the porosity was reduced. The remaining samples displayed a general decrease, with the exception of A_{6,3} and T_{2,1}. The porosity in A_{6,3} increased by 0.83% after I₂ and in T_{2,1} by 0.41% after I₄.

Statistical significance in the mean Ca.Dm and Po.Dm of each sample within their indentation stages was found in all the specimens that developed microcracks (Fig.2). In all the cases with fracture, both mean Ca.Dm and Po.Dm increased. Significant difference ($p < 0.0001$) in the mean Ca.Dm was also found in T_{6,3}. The comparison between the mean Po.Dm, however, showed no significance. Conversely, statistical significance in the mean Po.Dm, but not in the mean Ca.Dm, was observed in T_{5,3}.

The distortion and microcracks were also visible in the canal network for the rest of the volume (Fig. 3-6). In A_{1,1} the canal network appeared to be thicker and more connections between the canals were created due to the cracking. This was also observed in the N.Ca that increased from 365 to 1531, from I₀ to I₄. The N.Po also increased in this particular sample by 445. Similarly, the porosity rise in T_{2,1} occurred due to the increase of the N.Ca by 50. In A_{3,2} the N.Ca is halved, after the first step of indentation, with major N.Ca reduction at the borders of the specimen (from 696 to 320) (Fig. 4). In A_{5,3}, there is both loss of canals (Fig. 5) and porosity increase after microcrack formation. The N.Ca in

$A_{5,3}$ was reduced from 403 to 395 after reaching 0.4 mm tip displacement; however, the N.Po in the final stage was twice as much (183 to 458). In addition, in $A_{6,3}$ the larger porosity at I_2 was the result of both the N.Ca increasing by 50 and the Ca.Dm widening; in an individual canal, it was seen that the diameter increased from 242 to 256 μm .

Specimen	Indentation Depth (mm)	Imprint Region at I_0	Imprint Region at I_4
$A_{1,1}$	0.18
$A_{3,2}$	0.15
$A_{5,3}$	0.15
$T_{6,3}$	0.10

Figure 1: Representation of the canal network underneath the indentation area, before (I_0) and after the defect (I_4), showing the effective indentation depth [($A_{1,1}$, $A_{3,2}$, $A_{5,3}$) depth (brown font) identifying the cracked axial specimens; ($T_{6,3}$): transverse specimen]. Red arrows are showing the microcracks.

Figure 2: Graphical analysis of the mean canal network diameter (Ca.Dm) of all the specimens in the different groups. Blue bar: mean Ca.Dm at l_0 prior to defect; grey bar: mean Ca.Dm at l_2 after 0.2 mm tip displacement; yellow bar: mean Ca.Dm at l_3 after 0.3 mm tip displacement; orange bar: mean Ca.Dm at l_4 after 0.4 mm tip displacement. The significant difference between the indentation stages was estimated with a Kruskal-Wallis test ($\alpha = 0.05$). A schematic representation of the progressive indentation steps for each group is also reported (right).

The imprint left on the bone surface after the last indentation step was approximately $\frac{1}{4}$ of the top surface area, resulting in a global compression effect on the specimens, as shown in the strain maps (Fig.3-6). The ϵ_{p1} and ϵ_{p3} computed using DVC showed a combination of tension and compression. Specifically, ϵ_{p1} displayed the same trend in all the specimens. High strain values were distributed in the imprint region (maximum value $\sim 27000 \mu\epsilon$) following the shape of the imprint. In $A_{1,1}$ (Fig. 3) and $A_{2,1}$ however, the strain continued to the distal part of the volume decreasing to $\sim 16000 \mu\epsilon$. $A_{3,2}$ presented high tensile strain values at I_2 in the region of the imprint ($\sim 30000 \mu\epsilon$) (Fig. 4). At I_4 , however, the strain decreased and crack appeared adjacent to the imprint. Tensile strain, of the same magnitude ($\sim 28000 \mu\epsilon$) also appeared in the distal part of $A_{3,2}$ opposite to the crack in both I_2 and I_4 (Fig. 4). $A_{4,2}$, at I_2 , showed a uniform strain map following the shape of the indent (pyramidal distribution) with peak value in the middle of the imprint ($\sim 10000 \mu\epsilon$). The strain increased to $\sim 27000 \mu\epsilon$ in I_4 maintaining the same trend. $T_{3,2}$ exhibited the lowest tensile strain in all the specimens and remained at $\sim 3000 \mu\epsilon$ overall in all stages of indentation. $T_{4,2}$ experienced the same trend as $A_{4,2}$, where tension was present at the imprint region rising in I_4 ; however, the distribution here showed a circular trend around the imprint. In the last group, the tensile strain varied across the specimens. $A_{5,3}$ experienced concentration of strain only around the imprint (Fig. 5). Specifically, the strain increased with the depth until microcrack formation in I_4 , where it attained $\sim 30000 \mu\epsilon$. $A_{6,3}$ displayed maximum ϵ_{p1} strain of $\sim 6000 \mu\epsilon$ at I_2 in the indent region. At I_3 the strain was uniformly distributed around the volume at $\sim 8000 \mu\epsilon$. It increased during the last stage in the indented area at one side of the specimen reaching $\sim 23000 \mu\epsilon$. I_2 and I_3 appeared similar in the last two specimens. Specifically, tensile strain concentration was present in one side of the specimens (~ 20000 and $\sim 26000 \mu\epsilon$ for $T_{5,3}$ and $T_{6,3}$, respectively). Furthermore, an increase in the strain was observed for $T_{6,3}$ at I_3 around the imprint ($\sim 23000 \mu\epsilon$) (Fig. 6). For I_4 , $T_{5,3}$ the tensile strain expanded towards the centre of the volume and the indentation region ($\sim 27000 \mu\epsilon$). In $T_{6,3}$, however, the distribution remained similar to the previous two stages with a slight increase of strain in the opposite border as well.

In the first group, ϵ_{p3} illustrated a reduced compressive strain in the region of the imprint. The specimens extracted in the axial direction ($A_{1,1}$, $A_{2,1}$) experienced strain reaching $\sim -26000 \mu\epsilon$ under and on the side of the pile-up. In the region of the microcrack formation for both these specimens the strain was locally $\sim -14000 \mu\epsilon$ (Fig. 3). In $T_{1,1}$, compressive strain was concentrated under the indent in the middle of the volume ($\sim -20000 \mu\epsilon$) and in the rest of the specimens remained under $-4000 \mu\epsilon$. $T_{2,1}$ presented high compressive strains on the borders of the specimen reaching $\sim -26000 \mu\epsilon$; however, no microcracks were observed. In the second group only one of the two axial specimens fractured under the indentation load ($A_{3,2}$) and only after I_4 (Fig. 4). In the first indentation (I_2), high compressive strain concentration was observed on one side of the imprint ($\sim -26000 \mu\epsilon$). At I_4 , a microcrack formed in the opposite side attaining $\sim -14000 \mu\epsilon$. The rest of the specimens in the second group ($A_{4,2}$, $T_{3-4,2}$) experienced similar values of ϵ_{p3} at I_2 not exceeding $-4000 \mu\epsilon$ overall, with some compressive strain concentration in the borders of their volume. At I_4 , however, the strain in the axial specimen ($A_{4,2}$) increased; compressive strain reached $\sim -28000 \mu\epsilon$ around the pile-up region. In the third group only one of the axial specimens failed ($A_{5,3}$) (Fig. 5). Both axial specimens showed similar behaviour at I_2 not surpassing overall $-5000 \mu\epsilon$. The compressive strain in $A_{5,3}$ however, started to increase from one side in I_3 , causing microcrack formation in the opposite side after the last indent repetition (Fig. 5). $A_{6,3}$ exhibited high compressive strain concentration at I_4 especially in the imprint region reaching $\sim -18000 \mu\epsilon$. The last two specimens, that were from the transverse direction, did not display any crack formation. In $T_{5,3}$ ϵ_{p3} was increasing locally in multiple regions with the highest strain occurring in the borders ($\sim -20000 \mu\epsilon$) during I_3 . In fact, at I_4 it followed the same rising pattern with values adjacent to the indent of approximately $-26000 \mu\epsilon$. $T_{6,3}$ showed the same strain pattern in all three indentation stages (Fig. 6). There were high compressive values laterally on the one side of the specimen achieving

$\sim 29000 \mu\epsilon$. At I_3 and I_4 there was an isolated strain concentration in the other side of the specimen and in the region of the imprint.

Figure 3: Visualisation of (a) Canal and (b) Pore network distribution underneath the defect area of $A_{1,1}$ (axial specimen of the first group). Alteration of the canal and pore network was observed due to the microcrack formation at I_4 ; (c) DVC analysis, illustrating ϵ_{p1} and ϵ_{p3} full-field strain maps. Black arrows are pointing at the microcrack formation after indentation.

Figure 4: Visualisation of (a) Canal and (b) Pore network distribution underneath the defect area of $A_{3,2}$ (axial specimen of the second group). Alteration in the network was observed due to the microcrack formation at I_4 ; (c) DVC analysis, illustrating ϵ_{p1} and ϵ_{p3} full-field strain maps. Black arrows are pointing at the microcrack formation after indentation.

Figure 5: Visualisation of (a) Canal and (b) Pore network distribution underneath the defect area of $A_{5,3}$ (axial specimen of the third group). Alteration of the canal and pore network was observed due to the microcrack formation at I_3 which increased further at I_4 ; (c) DVC analysis, illustrating ϵ_{p1} and ϵ_{p3} full-field strain maps. Black arrows are pointing at the microcrack formation after indentation.

Figure 6: Visualisation of (a) Canal and (b) Pore network distribution underneath the defect area of $T_{6,3}$ (transverse specimen of the third group). Minimum alterations were observed in the canal and pore network in all the loading conditions, no microcrack formation was observed; (c) DVC analysis, illustrating ϵ_{p1} and ϵ_{p3} full-field strain maps.

Discussion

The aim of this study was to investigate the behaviour of cortical bone microstructure after plastic deformation achieved by *in situ* XCT indentation in the axial and transverse orientation, in relation to the strain distribution in the region.

In all the specimens, the canal network directly under the imprint was compressed. The pile-up observed in the tomograms was localised around the indent and resulted in the deformation of the volume and thus strain concentration. There was no cracking observed in axial specimens that had porosity greater than 1.6%. In the specimens with intermediate levels of porosity, 3.5-7.5 %, (i.e. T_{3,2}, T_{4,2} and A_{6,3}), the distribution of the strain in the overall volume was uniform and less in magnitude compared to the remaining specimens (up to -4000 $\mu\epsilon$). This phenomenon is dominant in the transverse samples. Specifically, in T_{3,2} where the porosity was 7.6 % and maximum canal diameter 295.5 μm at I₀. The porosity decreased slightly by 0.08 and 0.25 % at I₂ and I₄, respectively and there was Ca.Dm widening. The combination of the two helped in maintaining bone integrity and is in accordance with findings from Zebaze et al. (2019), where it was found that high porosity levels in cortical bone did not necessarily lead to fracture.

Additionally, the orientation of the network has been deemed a major factor in the formation and/or prevention of microcracks (Nalla et al., 2005). In the current study, A_{1,1} and A_{2,1} both experienced microcrack formation. These specimens were extracted in the axial direction and indentation was performed in one step with a large deformation introduced in the structure, without allowing relaxation to occur. However, this procedure was also completed for T_{1,1} and T_{2,1}, where cracks did not develop (Fig. S1). The formation of cracks in this loading condition was dependent on the extraction direction relative to the long axis of the femur; axial extraction resulted in cracking, whereas transverse specimens did not display cracking (Basillais et al., 2007). Observing the morphometry in A_{2,1}, it was noticed that a Haversian canal (Ca.Dm = ~200 μm) was adjacent to the borders of the imprint longitudinally, creating a strain concentration point, which in turn promoted fracture. In Group

2 only one of the axial specimens failed under applied load ($A_{3,2}$) in the last indentation stage, I_4 . The morphometry in $A_{3,2}$ and $A_{4,2}$ was very similar. In fact, at I_0 the N.Ca was 696 and 659, and N.Po. was 263 and 230 for $A_{3,2}$ and $A_{4,2}$, respectively. The mean Ca.Dm was also similar (Fig. 2), at approximately 23 μm for both. The principal difference however, was the canal network distribution under the indentation area. In $A_{3,2}$, there was a canal located under the edges of the imprint. It can be assumed that with the large strain concentration in this region, due to the indentation pile-up, the canal created a weakness point leading to crack formation (Fischer-Cripps, 2006). A similar behaviour occurred in $A_{5,3}$, where microcrack was observed at I_3 , propagating further at I_4 (Fig. 5). A canal was located under the surface and, when the imprint periphery size increased, microcrack formation occurred and then expanded towards the inferior part of the volume.

In the case where a Haversian canal was directly under the indent tip at contact, failure did not occur ($A_{6,3}$). This could be considered as a mechanism that prevents crack propagation. In fact, it is known that bone plasticity ahead of a crack dissipates energy and reduces the strain locally (Ritchie et al., 2009). Although the image resolution achieved in this study did not allow optimal visualisation of the osteons, they are known to deflect crack formation (Ritchie et al., 2009) which here could explain the crack prevention in the two axial samples ($A_{4,2}$ and $A_{6,3}$). Furthermore, in the transverse direction the Haversian canals perpendicular to the indent assisted in maintaining the structural integrity, hence no crack formation occurred. The lack of fracture in the transverse specimens was better understood when observing the morphology variation along with the mechanical behaviour. The change of the N.Ca and N.Po in the specimens throughout the indentation stages was representative of strain distribution in the transverse samples. Specifically, in regions where the N.Ca was reduced, high compressive strain concentration was present, while in regions where the N.Ca was decreased there was high tensile strain concentration.

The effect of the Canal Network on the strain distribution was more obvious in the case of $T_{6,3}$, where at I_0 the N.Ca was 1285. The N.Ca was reduced at I_2 to 918, increased to 1000 at I_3 and finally reached

897 in the last stage. These values were in accordance to the compressive strain, reaching $\sim 29000 \mu\epsilon$ at I_2 in the border of the specimen where most of the loss of the N.Ca occurred (Fig. 6). At I_3 , tensile strain increased in the opposite side ($\sim 23000 \mu\epsilon$), leading to believe that there was an opening of the canals augmenting their number. In the last stage, the strain distribution expanded throughout the volume with a combination of tensile and compressive states. The effect of the load in the structure is clear from the N.Ca and pore network distribution. Specifically, presence of larger pores is dominant in regions of high strain concentration (either tensile or compressive). The strain distribution in the pore network followed that of the canals. In fact, this observation produced evidence that the defect location with respect to the canal and pore network was a major determinant in fracture formation. As seen from $T_{6,3}$, large pores and canals surrounding the indentation region (defect) were allowing uniform deformation in the structure that prevented large crack formation. Instead, strain was concentrated in such areas and not directly in the defect region. The network distortion accompanied by strain concentration at the borders of the specimens can be attributed to the indentation edge effect due to the size of the imprint (Jakes and Stone, 2011).

The canal network influence in the mechanical behaviour has been previously investigated with regards to the axial compressive loading in mice femur (Voide et al., 2009). Specifically, a transverse notch was artificially introduced in the diaphysis of the bone, *in situ* SR-XCT micro-compression was applied to the specimens and morphometric properties such as canal volume (Ca.V) and thickness (Ca.Th) were estimated. It was observed that the cracks were initiated at the canal surface, propagating towards the endosteal and periosteal regions. In a different study with similar mice models under micro-compression, DVC was computed for regions of microcrack formation (Christen et al., 2012). In both cases it was observed that canals act as strain raisers and encourage crack formation axially. However, in both these studies (Christen et al., 2012; Voide et al., 2009) the notch produced in the bone had most probably induced a residual deformation to the canal network prior to testing. In the present study the network was intact prior to plastic deformation. Furthermore, both axial and transverse orientation were examined.

In situ XCT Indentation has been previously employed for the evaluation of plastic deformation in elephant tusk (Lu et al., 2019). In that study the elephant dentin tubules were running parallel to the length of the tusk and three different tubules orientation with respect to the indentation axis were examined. Consistent to the results found in the present study, tubules parallel to the indentation tip created a weakness point for the structure leading to larger crack formation. For tubules at 70°, crack propagation was prevented and microcracks due to the contact with the tip deflected horizontally, in the axial direction. The orientation of the bone canal network is more complex than that of the tubules, however; the canals seemed to behave in the same manner as dentin tubules regarding the axial and transverse alignment.

This study has a number of limitations. A small number of specimens was tested and in order to generalise the findings and the conditions at which fracture occurs, the protocol should be implemented in a larger group. The presented statistical analysis is only meant to provide an indication on the correlation between changes occurring in canal network and plastic deformation for each specimen individually. However, the nature of DVC based on high-resolution XCT is to qualitatively assess local mechanics in great detail to highlight the interplay between microstructure and damage development, as showcased in this study. It must be noted that the ability of DVC in detecting strain changes due to microcrack formation is clearly dependant on image contrast and spatial resolution of the measurement (~200 µm in this study); thus, the crack initiation phases may be difficult to identify, as well as the role of osteons in microcrack deflection. In spite of this, DVC has already shown its reliability to interpret the smallest changes in bone greyscale texture and architecture as strain gradients, in correspondence of which microcracks would appear with progressive loading (Peña Fernández et al., 2020; Tozzi et al., 2016). Furthermore, although the relation between the Canal and Pore Network to the strain distribution is clearly shown here, the relation between indent and bone surface could also affect the strain due to the edge effect (Jakes and Stone, 2011). Therefore, the morphometry and mechanical behaviour of the microstructure should also be investigated in whole bone specimens. Resistance of bone to microcracks highly depends on metabolic diseases and aging

(Cai et al., 2019; Mori et al., 1997; Zimmermann et al., 2015, 2011). Thus, it is vital to assess these phenotypes in diseased tissue.

Conclusion

This paper presented the first study combining *in situ* XCT indentation and DVC to correlate the plastic strain accumulation and canal network distortion in cortical bone. Fracture development as a result of plastic deformation was evaluated in the axial and transverse orientation of the intracortical microstructure. It was observed that crack formation was predominant in the axial direction, whereas when the Haversian canals were perpendicular to the vertical axis of the tip they acted in preventing fracture. Furthermore, the indentation testing caused deformation and hence strain distribution in the whole volume of the specimen affecting the canal and pore network of the structure underneath the indent. The importance of the canal network regarding the deformation was confirmed by the strain accumulation patterns. In areas of canal loss, compressive strain was observed and widening of the canals was accompanied by tensile strain. Tensile strain was also present in the indentation imprint.

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Supplementary Material

Figure S7: Visualisation of transverse specimens of the first group ($T_{1,1}$, $T_{2,1}$). Minimum alterations were observed in the canal network after I_4 , no microcrack formation was observed. Red arrows are showing the indentation site.