Image-Based Radiodensity Profilometry Measures Early Remodeling at the Bone-Callus Interface in Sheep

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Abstract (150-250 words: currently 222)

Bone healing has been traditionally described as a four-phase process: inflammatory response, soft callus formation, hard callus development, and remodeling. The remodeling phase has been largely neglected in most numerical mechanoregulation models of fracture repair in favor of capturing early healing using a pre-defined callus domain. However, *in vivo* evidence suggests that remodeling occurs concurrently with repair and causes changes in cortical bone adjacent to callus that are typically neglected in numerical models of bone healing. The objective of this study was to use image processing techniques to quantify this early-stage remodeling in ovine osteotomies. To accomplish this, we developed a numerical method for radiodensity profilometry with optimization-based curve fitting to mathematically model the bone density gradients in the radial direction across the cortical wall and callus. After assessing data from 26 sheep, we defined a dimensionless density fitting function that revealed significant remodeling occurring in the cortical wall adjacent to callus during early healing, a 23% average reduction in density compared to intact. This fitting function is robust for modeling radial density gradients in both intact bone and fracture repair scenarios and can capture a wide variety of the healing responses.

30 The fitting function can also be scaled easily for comparison to numerical model predictions and may be useful for
31 validating future mechanoregulatory models of coupled fracture repair and remodeling.

32

33 Keywords: Remodeling, Bone fracture healing, Micro-computed tomography, Tibia, Ovine osteotomy

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41 **Conflicts of interest**

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44 Introduction

45 The fracture healing process is commonly described as a four-phase concept: inflammation phase, soft
46 callus formation phase, hard callus formation phase, and bone remodeling phase (Claes et al. 2012; Pivonka and
47 Dunstan 2012; Einhorn and Gerstenfeld 2015; Ghiasi et al. 2017). Numerical simulations have been used to predict
48 mechanobiological processes in the regions of fracture healing. Numerous finite-element (FE) models have been
49 developed to analyze local mechanical responses to varying loading conditions (Ament and Hofer 2000; Claes et
50 al. 2002; Lacroix and Prendergast 2002; Shefelbine et al. 2005; Simon et al. 2011; Steiner et al. 2013, 2014; Wilson
51 et al. 2017; Wang and Yang 2018; Pietsch et al. 2018; Ghiasi et al. 2019; Engelhardt et al. 2021). Such models have
52 been used to study different factors that impact the healing process, for example the mechanics of the fixation
53 hardware, or outcomes following inverse dynamization (Wehner et al. 2014; Wilson et al. 2017). Some studies
54 have also emphasized that the initial phase of healing is critical for modeling of the healing process (Ghiasi et al.
55 2019). Recently, we applied a mechanoregulatory model of fracture healing to show that the rate of healing
56 depends on the geometry of the fracture and that all fracture types experience a small healing delay with torsional
57 instability (Ren and Dailey 2020). We also showed that modifying a strain-based mechanoregulatory model of
58 fracture healing to include a geometric proximity function can successfully restrict callus formation to the region
59 near the fracture line and achieve simulation domain independence (Schwarzenberg et al. 2021).

60 These techniques hold promise to predict outcomes under different mechanical or biological conditions
61 and in response to new treatment strategies, but they all share a key limitation: the cortical wall is assigned static
62 mechanical properties and included in the mechanical loading simulations, but it is excluded from the
63 mechanoregulatory healing algorithms. These models neglect remodeling activity during fracture healing because
64 this last stage of fracture healing is often described conceptually as having limited overlap with the repair phase.
65 However, *in vivo* data suggests that remodeling of the cortical wall occurs concurrently with soft callus
66 development and mineralization. For example, previous reports generated from nanoindentation testing have
67 provided highly localized evidence of decreasing bone density in the radial direction through the cortical bone
68 toward the callus (Preininger et al. 2011). This evidence indicates the remodeling phase of secondary bone

69 fracture healing is associated with structural changes in both the callus and the adjacent cortical bone throughout
70 the healing process and that these effects should be included in early-stage fracture repair models.

71 The key barrier to developing new mechanoregulatory models that can consider coupled repair and
72 remodeling is the lack of available validation data. The validation strategy used for most recent
73 mechanoregulation models has focused on the global structural mechanics of the limb and qualitative comparison
74 of model predictions to histological analysis of tissue in the callus. For example, the assessment of a model's
75 performance in predicting the spatial and temporal development of tissues in the callus is usually based on only
76 the general trends of mechanical data (e.g., torsional rigidity) (Ren and Dailey 2020; Schwarzenberg et al. 2021)
77 or temporal data (e.g., bridging time, interfragmentary motion) (Lacroix and Prendergast 2002; Isaksson et al.
78 2006; Wehner et al. 2014; Pietsch et al. 2018). Ideally, the localized tissue composition and distribution should
79 also be investigated to assess whether tissue growth and differentiation criteria within the algorithms are
80 functioning correctly. The historical gold standard for assessing mechanical properties within tissue samples has
81 been nanoindentation testing. This method has been used to probe the micro-scale properties of bone and callus
82 and identify gradients in porosity and density (Manjubala et al. 2009; Preininger et al. 2011; Mora-macías et al.
83 2017). However, these physical measurement techniques involve time-consuming destructive postmortem testing
84 with specialized equipment and the resulting data cannot be obtained *in vivo*, or for large regions of tissue, or in
85 large numbers of samples. Regrettably, these limitations mean that generalizable quantitative data describing
86 remodeling across the cortical wall into callus does not currently exist.

87 Accordingly, the purpose of this study was to develop a numerical technique for noninvasively quantifying
88 structural remodeling adaptations at the bone-callus boundary in sheep by investigating the radiodensity data
89 from micro-computed tomography (μ CT) scans. We hypothesized that measurable cortical remodeling happens
90 early in the healing period, in conjunction with bridging of the external callus in secondary fracture healing.

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92

93 **Methods**

94 ***Animal Specimen Information***

95 Twenty-six adult female Swiss alpine sheep (2-3 years old, weighing 59 – 87 kg) were part of two previously
96 completed research studies with two different tibial osteotomy models stabilized by medial plating. Group 1
97 consisted of data from twelve animals with a 17 mm tibia defect augmented with autografts and stabilized with a
98 13-hole stainless steel plate (broad straight veterinary 3.5 mm LCP, 172 mm in length, with 3.5 mm bicortical
99 screws; DePuy Synthes®). Sheep in group 1 were sacrificed 12 weeks after surgery. Group 2 consisted of data from
100 fourteen animals with a 3 mm gap tibia defect stabilized with a 12-hole stainless steel plate (broad straight
101 veterinary 3.5 mm locking compression plate (LCP), 159 mm in length, with 3.5 mm bicortical screws; DePuy
102 Synthes®). Sheep in group 2 were sacrificed 9 weeks after surgery. All experiments were conducted at the
103 Musculoskeletal Research Unit in Zürich, Switzerland, according to the Swiss laws of animal protection and welfare
104 and authorized by the local governmental veterinary authorities (License No. ZH 183/17).

105 ***Micro-Computed Tomography (μ CT) Scanning***

106 After animal sacrifice, both intact and osteotomized tibiae were excised, stripped of soft tissue, and all
107 hardware was removed, taking care not to disrupt the callus. Samples were then wrapped in saline-soaked gauze,
108 and μ CT scanned using an XtremeCT II Micro-CT scanner (Scanco Medical AG, Bruettisellen, Switzerland) with an
109 X-ray voltage of 68 kVp and X-ray current of 1470 μ A. The resulting scans had an isotropic resolution of 60.7 μ m.

110 ***Image Segmentation***

111 A region of interest (ROI) was selected at three different locations of each operated limb diaphyseal
112 segment scan: 10 mm from the most proximal aspect of the tibia, 10 mm from the most distal aspect of the tibia,
113 and just proximal to the fracture line at the level of maximum callus radius (**Fig. 1**). The proximal and distal ROI
114 were above and below the most proximal and distal screw holes. Sampling slices from the intact limb were also
115 taken at the corresponding levels. At each sampling location (region of interest), a total of 20 slices were selected
116 for analysis.

117 An automated segmentation algorithm was developed in MATLAB 2021a (The MathWorks, Inc., Natick,
118 Massachusetts, USA) to define the outer boundary of the callus as well as the area enclosed by the pericortical
119 boundary of the preexisting cortical bone. The detailed workflow can be seen in **Fig. 2**. Post-segmentation data
120 processing was carried out on all 2D tomograms located within each ROI.

121 The process for segmentation of cortical bone and callus was based in part on previously reported
122 methods (Buie et al. 2007; Morgan E, Mason Z, Chien K, Pfeiffer A, Barnes G, Einhorn T et al. 2009; Bissinger et al.
123 2017). To complete this task, the algorithm required two initial estimates of the segmentation thresholds. The
124 selection of these thresholds affects the cortical bone and callus boundaries of the output mask and requires
125 subsequent refinement. First, the soft tissue region was excluded using connected-component labeling. Noise was
126 then reduced by applying a median filter. Next, the original dataset was re-thresholded and masked with the
127 detected boundaries of the bone-callus region. Dilation and erosion operations were then used to determine the
128 bone and callus boundary. The initial estimates of the segmentation thresholds were then refined by minimization
129 of spline energy to achieve smoothness of the detected tissue boundaries for each sheep. A locally convex
130 boundary criterion was also used to correct any boundary defects caused by small regions of lower-density cortical
131 bone. Finally, a mask of the callus, cortical bone, and non-bone regions was created by combining the output of
132 the previous steps.

133 The slice extraction and segmentation processes were repeated at all three levels of height in both the
134 intact and operated tibiae. No callus was identified in the intact limbs.

135 ***Radiodensity Data Sampling and Curve Fitting***

136 The process for radiodensity sampling is illustrated in **Fig. 3**. In each 2D tomogram within the region of
137 interest, the centroid of the cortical bone was calculated from the endocortical boundary (red boundary) in the
138 mask geometry. A polar array of sampling lines was created in 1-degree increments throughout the entire 360-
139 degree angle range, with each line having its origin at the centroid and extending through the cortical wall and
140 callus. The radiodensity values along the profile lines were sampled in 60 μm increments in the radial direction, a
141 distance equal to the in-plane resolution of the scan. Pixel data was retrieved from the grayscale image using a

142 bicubic interpolation technique. Data quality was checked for each profile line and individual profile lines were
143 excluded from the data set if the callus was not present or too thin (thickness smaller than 10mm, measured in
144 the radial direction along a profile sampling line). Radial sampling was repeated using the same procedure for all
145 20 slices in each ROI and pooled to create a point cloud that defined the radiodensity profile line for each ROI in
146 each animal (**Fig. 1c**).

147 In this animal model, medial plating produced asymmetric callus formation, as can be clearly seen in **Fig.**
148 **3c**. Cortical wall thickness was also not constant across all radial profile lines. To enable a combined curve-fitting
149 analysis of the profilometry samples from within each region of interest, a registration and scaling procedure was
150 defined to create a consistent dimensionless coordinate system for the cortical wall and for the callus, when
151 present. Each profile line was registered to set its linear coordinate system to zero at the bone-callus boundary,
152 or the pericortical boundary if intact. Negative linear coordinates corresponding to the cortical wall were
153 normalized to the local cortex thickness of each profile line (**Fig. 3c/d**), such that $r = -1$ was the endocortical
154 boundary and $r = 0$ was the pericortical boundary. For the positive linear coordinates, the normalization
155 procedure depended on whether callus was present. For the intact limbs, positive coordinates were normalized
156 to the local cortex thickness (**Fig. 5b**). For the operated limbs with callus, positive coordinates were normalized to
157 the doubled local callus thickness, producing $r = 0.5$ at the outer boundary of the callus mask and ensuring data
158 sampling beyond the callus into the soft tissue zone (**Fig. 5c**). The resulting average number of sampling points
159 per region of interest was 170,340.

160 To enable comparisons between the treated and untreated limbs of all animals, the sampled radiodensity
161 values were also normalized to the median cortical bone density of the intact diaphysis of each animal. Within
162 each animal, all local radiodensity values sampled from the profile lines were normalized to this baseline median
163 cortical bone density, regardless of limb (intact or operated) and ROI location (proximal, midshaft, and distal).

164 Curve fitting techniques were then used to mathematically model the collective radial density profile using
165 the sampling points collected at each ROI. To develop an appropriate fitting function for measuring local structural
166 changes, we started with the simplest case, an intact cortical wall fit with a double sigmoid function:

$$f(r) = \frac{1}{1 + e^{-a_1(r-b_1)}} * \frac{1}{1 + e^{-a_2(r-b_2)}} \quad (1)$$

where in general for a sigmoid function, the parameters a sets the width of the transition zone from a function value of 0 to a function value of 1, and the parameter b sets the center of this transition zone.

Next, a logical linear term and a constant were added to the double sigmoid function to account for the possibility of a nonzero density gradient in the cortical wall ($r < 0$) and nonzero background radiodensity:

$$f(r) = \frac{1}{1 + e^{-a_1(r-b_1)}} * \frac{1}{1 + e^{-a_2(r-b_2)}} + d + \begin{cases} cr & r < 0 \\ 0 & r \geq 0 \end{cases} \quad (2)$$

where c is the linear slope applied only within the cortical wall and d is the background density constant.

To adapt this method for profilometry samples with callus, an additional sigmoid term was added:

$$f(r) = \begin{cases} \frac{1}{1 + e^{-a_1(r-b_1)}} * (g_1 + \frac{(1-g_1)}{1 + e^{-a_2(r-b_2)}}) + cr + d_1, & r < 0 \\ \frac{g_2}{1 + e^{-a_3(r-b_3)}} + d_2, & r \geq 0 \end{cases} \quad (3)$$

where the constants g_1 and g_2 in the sigmoid terms allow for non-unity in the upper sigmoid plateau and the constants d_1 and d_2 allow for nonzero background radiodensity on both the internal and external bounds of the fitting function. In this piecewise definition, the cortical function ($r < 0$) is a generalized double logistic function, and the callus function is a single logistic function.

Parameter values for constants in Eq. 2-3 were fit using the nonlinear least-squares method via the MATLAB Curve Fitting Toolbox (R2021a). In addition to this standard curve-fitting technique, a multi-objective optimization method was introduced to fit the parameters of the function defined in Eq. 3 with additional constraints requiring position and slope continuity of the piecewise fit function at $r = 0$ as described in Eq. 4. This multi-objective optimization method can be formulated to find the coefficients of $f(r)$, minimizing the residuals (sum of the squares), of all the data points:

$$\begin{aligned}
& \min_{a,b,g,d} \sum_{i=1}^m (y_i - f_{r<0}(r_i))^2 \\
& \min_{a,b,g,d,c} \sum_{k=1}^n (y_k - f_{r>0}(r_k))^2 \\
& s. t. f_{r<0}(0) - f_{r>0}(0) = 0 \\
& \quad f_{r<0}'(0) - f_{r>0}'(0) = 0
\end{aligned} \tag{4}$$

187

188 where r_i and r_k are the set of data points in the cortical wall or external to the cortical wall, n and m are the total
189 number of data points of the cortical wall or the callus. The minimization problem was solved using the MATLAB
190 Optimization Toolbox (R2021a). After the fitting parameters were obtained for each sheep, the resulting fit
191 function, $f(r)$, was evaluated to calculate the slope at the bone-callus interface to quantify the extent to which
192 remodeling had produced a smooth functionally graded interface at the bone-callus boundary. For intact bone
193 not bounded by callus, an additional custom MATLAB script was also used to calculate the average density value
194 in the cortical wall plateau, which was reported as the mean cortical density of each fitted curve.

195 **Statistical Analysis**

196 All statistical analyses were performed using SPSS Statistics 27 (IBM Corp; Armonk, New York, USA). A
197 value of $P = 0.05$ was used as the threshold for statistical significance. A two-tailed paired-sample t-test was used
198 to analyze if the fitting function constants and the evaluated slope of the fitting function at the pericortical
199 boundary were significantly different between the operated and contralateral limbs.

200 **Results**

201 **Choice of Curve Fitting Function**

202 Several alternative methods were investigated and critically evaluated with respect to their ability to
203 capture the essential features of the bone density profile curves. The fitting results of the double sigmoid function
204 (Eq. 2) are shown in **Fig. 4a**. This function provided a reasonable approximation for the intact cortical wall, but it
205 was not able to capture the increasing slope of the density in the radial direction for intact cortical bone and it

missed both the cortical and callus plateaus in the data for the operated tibia. Adding a logical linear term and a constant produced a good fit with $R^2 \geq 0.93$ for the double sigmoid function (Eq. 3) with cortical bone, but this fitting function failed to capture the remodeling behavior evidenced by a decrease in density in the cortical wall at the bone callus boundary (**Fig. 4b**). The piecewise logistic function (Eq. 4) was then used to get a better fit when callus was present, this function provided a better fitting quality ($R^2 \geq 0.95$), but when fit using a nonlinear least squares minimization, the function was not continuous at the bone callus interface (**Fig. 4c**).

For both the intact and operated tibiae, the best fitting method was to use the multi-objective optimization method (**Fig. 4d**) to identify the parameters of the fitting function shown in Eq. 4. This produced continuous fit functions with goodness of fit statistics showing an overall $R^2 \geq 0.87$ for all intact tibiae and operated tibiae. In all samples, all six fitting parameters had 95% confidence intervals that did not include zero. The fitting parameters for all 26 animals at each of the six sampled ROIs can be seen in the supplementary digital content.

The performance of the final fitting function (Eq. 4 with multi-objective optimization) is shown for a different animal in **Fig. 5**. This single function successfully captured several observed characteristics of both the intact and operated limb. Specifically, in the intact tibia, it captured the observed increase in radiodensity in the radial direction from the endosteal to the pericortical boundary (**Fig. 5a**). For the operated limb, the fit curve captured the overall decrease in radiosity in the cortical wall and the localized remodeling at the pericortical boundary bounded by callus (**Fig. 5b**).

Each parameter of the fitting function was included to capture the observable features of the profilometry data in different regions of the fitted curve and at transitions between regions. The resulting constants can then be associated with physical features related to the bone-callus radial density function. The magnitudes of parameters a_1 and a_2 define the width of the region of density change at the periosteal and endocortical boundaries of cortical bone. The parameters b_1 and b_2 define the location of the center of the transition of the periosteal and endocortical boundaries of cortical bone. Parameter a_3 defines the width of the region of density change at the outer boundary of the callus, while parameter b_3 sets the location of the center of the transition

231 between callus and the surrounding soft tissue. The linear term c in the cortical bone zone ($r \leq 0$) defines the
232 increasing density of cortical bone from the endosteal to pericortical boundary. Finally, the constant d_1 captures
233 the average density of cortical bone and the term d_2 compensates for the noise of the data outside the callus.
234 Finally, g_1 and g_2 capture the cortical density at the pericortical boundary of the injured limb.

235

236

237 ***Cortical Tissue Alterations in Operated Limbs***

238 In addition to profilometry sampling at the level of the fracture, we also analyzed cortical bone at the
239 proximal and distal ends of the diaphysis of the intact and operated limbs. The resulting profilometry curves of all
240 animals combined are shown in **Fig. 6**. Comparing the operated group to their matched intact tibiae at both the
241 distal and proximal ends of the bones, there were several changes in the cortical walls of the operated tibiae, both
242 at the level of the fracture and at the proximal and distal ends of the bone. **Table 1** shows the comparisons
243 between the operated and intact limbs based on two summary parameters derived from the fitting functions—
244 density slope at the pericortical boundary and mean cortical wall density. Each parameter was evaluated at the
245 proximal, midshaft, and distal ROIs in both the intact and osteotomized limbs. Both parameters were significantly
246 different between operated and intact for all ROIs, although the cortical density decreases in the operated limb
247 were 10x larger at the midshaft than at the proximal and distal ends of the bone. Considering all animals, sampling
248 at the proximal and distal ends of the bone showed that the mean cortical density was 1.3% and 2.2% lower
249 respectively in the operated limbs compared to intact ($p < 0.001$).

250 All animals also showed evidence of remodeling in the cortical wall bounded by callus (**Fig. 6d**). The
251 preexisting increase in radiodensity toward the pericortical boundary that can be seen in the intact bones (**Fig. 6a**)
252 disappeared in the osteotomized bones (**Fig. 6c**). At the pericortical boundary bounded by callus, these changes
253 corresponded to a 23.4% drop on average from the peak density of the native cortical wall. Overall, comparing
254 the intact and operated groups at the midshaft, there were statistically significant differences in all obtained
255 radiodensity profile fitting function parameters except for g_1 ($p \leq 0.001$; see supplementary **Table S1**). The slope

256 of the fitted density profile curve at the pericortical boundary was also significantly reduced in operated limbs
257 compared to intact at all three ROIs ($p \leq 0.007$; see **Table S1**).

258 **Discussion**

259 This study presents a scalable mathematical method for non-invasive quantification of tissue
260 mineralization density of the cortical tissue matrix and callus within an ovine tibial shaft. The results show that
261 remodeling alterations occur not only in the callus tissue due to mineralization but also in the cortical tissue
262 adjacent to the repair zone throughout the healing process. The findings are consistent with expectations from
263 destructive physical testing and confirm the presence of a decreasing gradient in mineral density of the cortical
264 bone at the vicinity of a fracture during secondary healing. We previously showed that at 9 weeks, most of these
265 osteotomies had achieved a structural rigidity comparable to intact tibiae (Schwarzenberg, 2020). This
266 profilometry curve fitting data shows that in sheep, significant adaptive changes have occurred in cortical bone
267 bounded by callus, concurrent with structural bridging as early as 9 or 12 weeks post-op. It should be noted that
268 the remodeling of cortical bone was measurable not only near the fracture site, but also to a lesser extent at the
269 proximal and distal end of the operated limbs. The methods described herein could be used to measure
270 remodeling as a temporal *in vivo* outcome measure from imaging only. These measures could be useful as an
271 indicator of healing speed by establishing the status of a fracture on the continuum of mechanical and structural
272 adaptations that arise during healing.

273 The intended purpose of this investigation was to provide a useful set of validation data for the future
274 development of mechanoregulatory models that include the cortical wall and address coupled remodeling at the
275 cortical-callus boundary. The results confirm that significant changes do occur on the cortical side of the cortical-
276 callus boundary and that not addressing these changes is a critical limitation in the current computational models
277 of bone fracture healing. These models usually assume a time-invariant value for cortical bone stiffness, which
278 does not reflect the *in vivo* reality of a 23% drop in cortical radiodensity at the cortical-callus boundary. The
279 normalized generalizable fitting function we reported could be used to assess whether mechanoregulatory models

that include the cortical zone are capturing the appropriate magnitude of changes, and whether the distribution of predicted material properties at the boundary and within the callus region are valid.

Investigators who are interested in applying our normalized, dimensionless remodeling curve to validate their dimensional mechanoregulation models may wish to use the following scaling procedure. First, observe that the fitting curve cannot be used directly because the function has been normalized in both the radius coordinates (r) and radiodensity values (y axis). To dimensionalize the radial coordinates, recall that the radial coordinates were normalized to the cortical wall thickness for $r < 0$ and to the doubled callus thickness for $r > 0$. To scale this function appropriately in r , identify the cortical wall thickness, t , and callus thickness, t_c . The y coordinates in our fit curve were normalized to the median radiodensity [HU] of the intact limb for each animal. For the $N = 26$ sheep analysed in this study, the resulting average intact tibia radiodensity was 3164 ± 10 HU. The apparent radiodensity [HU] must be scaled to bone mineral density by means of a linear conversion derived from a phantom scan. This density value can then be converted to a Young's modulus using a species-specific scaling law. For ovine cortical bone, we recommend the equation derived by (Schwarzenberg et al. 2021), which produces an average Young's modulus, $E_{intact} = 12.9$ GPa for intact bone. After selecting E_{intact} and matching t and t_c to the chosen computational model, the following scaled remodeling function can be used to validate the model predictions:

$$E(r) = E_{intact} * \begin{cases} \left(\frac{1}{1 + e^{-a_1(\frac{r}{t} - b_1)}} * (g_1 + \frac{(1 - g_1)}{1 + e^{-a_2(\frac{r}{t} - b_2)}}) + \frac{rc}{t} + d_1 \right), & r < 0 \\ \left(\frac{g_2}{1 + e^{-a_3(\frac{r}{t_c} - b_3)}} + d_2 \right), & r \geq 0 \end{cases} \quad (5)$$

Where the fitting constants ($a_1, b_1, g_1, a_2, b_2, c, d_1, a_3, b_3, g_2$, and d_2) are drawn from **Table S1**. This dimensionalized remodeling curve will have radial coordinates r that are zeroed at the cortical-callus boundary, but are dimensionally scaled to match the physical size of the geometry in the computational model.

This study is not without limitations. First, the sheep imaging data were only available at the time of sacrifice. As a result, no information about the temporal variations of local mechanical properties of the cortical tissue is currently available for this study. However, due to the inclusion of both large-gap and small-gap healing models, the selected sheep groups represent a wide range of healing responses and callus sizes, suggesting that

302 the numerical methods and resulting fitting function we have proposed are robust. Another key limitation is that
303 we applied the segmentation algorithm to evaluate only a small fraction of the 2D tomograms that comprise the
304 entire cortical wall and focused on gradients in the radial direction. Remodeling effects in the cortical wall also
305 have an axial dependency on proximity to the fracture line, which has not been explored here. Additional work
306 would be required to achieve profilometry analysis of tissue density profiles in a more general 3D sense. Finally,
307 it is important to note that the resolution of the medical images themselves could also influence the curve fitting
308 quality. In this study, we have used high-resolution of micro-CT scans that were obtained post-mortem. Applying
309 these same techniques to *in vivo* computed tomography would likely require larger voxel sizes and may not
310 provide enough data points for curve fitting purpose in the area of transition especially at the cortical-callus
311 boundary. We recommend that future studies explore whether larger voxels are inferior for detection of
312 remodeling effects in the cortical wall.

313 **Conclusions**

314 This study showed that structural remodeling at the bone-callus interface can be detected in sheep using
315 radiodensity profilometry from micro-CT scans. It also showed that the density of cortical bone adjacent to callus,
316 which correlates with its elastic properties, is not constant during the early healing period and that this region
317 represents an integral part of the process of coupled fracture repair and remodeling. The scalable function
318 presented herein for mathematically representing density gradients in the cortical wall and callus may be useful
319 for validating future mechanoregulatory models of fracture repair.

320 **Figure Captions**

321 **Figure 1:** Fig. 1 – (a) Coronal slice view from a micro-CT scan of an ovine tibial osteotomy at 9 weeks post-op. In
322 this study, radiodensity gradients were sampled in the direction indicated by the red arrow. (b) Three regions of
323 interest (ROI) were identified for sampling in each intact and each operated limb: proximal, distal, and at the level
324 of the fracture. (c) Each ROI consisted of 20 axial slices that were analyzed to measure radial radiodensity profiles
325 in the cortical wall and callus region.

326

327 **Figure 2:** Regions of cortical bone and callus were identified using an automated segmentation method developed
328 in MATLAB 2021a. This method requires two global threshold inputs, which are used to initialize the splines that
329 define the endosteal, pericortical, and callus boundaries, and subsequently refined as shown.

330

331 **Figure 3:** (a) A polar array of radial profile lines was created from the cortical centroid through the cortical wall
332 and callus, if present, and radiodensity values were sampled from the underlying scan data. (b) For intact bones,
333 each profile line was registered to set its linear coordinate system to zero at the pericortical boundary and
334 sampling points were extended up to one cortical thickness into the surrounding soft tissue. (c) For samples with
335 callus, profile lines were registered to zero at the bone-callus boundary and sampling points were extended to
336 double the local callus thickness. (d) Resulting compiled profilometry sampling data for one animal at the level of
337 the callus in the osteotomized limb. All radiodensity values were normalized to the median cortical bone density
338 of the intact tibia for that animal.

339 **Figure 4:** Profilometry data sampled at the level of the fracture for intact tibiae (black, left) and operated tibiae
340 (red, right). Fit curves from the three tested fitting equations in panels (a)-(c), with all parameters obtained
341 following a least-squares minimization approach using the Curve Fitting Toolbox in MATLAB. Fit curves in panels
342 (c) and (d) both use Eq. 4, but additional continuity constraint at $r = 0$ was achieved in (d) using multi-objective
343 optimization.

344 **Figure 5:** Profilometry sampling points and resulting fitted curves using the method of Fig. 4(d) in a different animal.
345 The radial coordinate registration procedure allowed consistent referencing of key physical locations across
346 different animals: endocortical boundary, pericortical boundary, and callus outer boundary (if present). The
347 density normalization procedure eliminated baseline differences in bone properties between animals by scaling
348 all sampling data points to the median cortical bone density for the intact tibia in each animal.

349

350 **Figure 6:** Profilometry curves were fitted from data sampled at the same levels at the proximal end (a) or distal
351 end (b) end of intact tibiae (black curves) and operated tibiae (red curves). Profilometry curves were also fitted
352 from data sampled at the midshaft of all intact tibiae (c, black curves) and operated tibiae (d, red curves).

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354 **Table 1:** Comparison of profilometry curve fitting parameters between operated and contralateral groups.

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433

**Input
Raw Data**

Apply Gaussian
Smoothing Filter

Input
Threshold 1

Dilate with
Kernel Size = (5,5)

Apply Connectivity
to Identify Callus Region

Erode with
Kernel Size = (5,5)

Input
Threshold 2

Dilate with
Kernel Size = (5,5)

Apply Connectivity
to Identify Cortical Region

Erode with
Kernel Size = (5,5)

Require Convex
Cortical Boundary

**Output
Mask**

Candidate Fitting Function

Intact Tibia

Operated Tibia

(a) Double sigmoid function
(Eq. 2)

(b) Double sigmoid function
with logical linear term in
the cortical wall ($r < 0$)
and constant offset (Eq. 3)

(c) Piecewise defined double
sigmoid function in the
cortical wall ($r < 0$) and
single sigmoid function
external to the cortical
surface ($r > 0$) (Eq. 4)
with parameter fitting
using the Curve Fitting
Toolbox

(d) Same fitting function as
(c) above (Eq. 4) with
parameter fitting using
the Optimization Toolbox

Proximal Cortical Sampling

(a)

Distal Cortical Sampling

(b)

Intact Tibia – Midshaft

(c)

Operated Tibia – Midshaft

(d)