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Sealed osteons in animals and humans: low prevalence and lack of relationship with age

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Abstract

Sealed osteons are unusual variants of secondary osteons that have received little attention, especially in non-human bones. Sealed osteons are characterized by central canals that are plugged with bone tissue. As with other variants of secondary osteons (e.g. drifting, dumbbell, multi-canal), understanding how and why sealed osteons form can shed light on the mechanisms that regulate normal bone remodeling and how this process can be perturbed with aging and some diseases. In a recent microscopic evaluation of human tibiae obtained after traumatic amputations, 4–5% of the osteons were sealed. It is suggested that this high prevalence reflects occasional localized microscopic ischemia from normal osteonal remodeling; hence sealed osteons are implicated in human skeletal fragility. Therefore, osteon prevalence would be expected to correlate with the bone remodeling seen with aging; for example, showing positive relationships between sealed osteons and the population density of typical secondary osteons (OPD). We evaluated the prevalence of partially sealed (80–99% sealed) and fully sealed osteons with respect to age and variations in OPD in 10 adult human femora (34–71 years) and in various non-human appendicular bones of mature animals that were not of advanced age, including deer calcanei, equine radii and equine third metacarpals. An additional sample of 10 bilateral human femora with unilateral non-cemented total hip replacements (F,+HR) and non-implanted contralateral femora (F,−HR) were evaluated (10 patients; 52–94 years). In non-human bones, sealed + partially sealed osteons were rare (~0.1%) even when having relatively high OPD. When considering sealed + partially sealed osteons in femora from patients without any HR, results showed that 1.6% of the osteons were sealed or partially sealed, which was much lower than anticipated, but this is 10- to 20-fold more than in any of the non-human bones. Additionally, in all bones, sealed + partially sealed osteons were significantly smaller than typical secondary osteons (mean diameters: 125 vs. 272 μm; $P < 0.005$). In the patients with HR, the percentage of sealed + partially sealed osteons: (i) did not correlate with age, (ii) showed no significant difference between F,−HR and F,+HR (1.9 vs. 2.1%; $P = 0.2$), and (iii) was positively correlated with OPD ($r = 0.67$, $P = 0.001$), which differs from the very weak or lack of correlations in the non-human bones and the other human femur sample. The lack of an age-related relationship, in addition to the very low prevalence of sealed + partially sealed osteons are inconsistent with the idea that they contribute to reduced bone quality seen in aging humans. The small size of sealed and partially sealed osteons, regardless of species affiliation, suggests that they represent closing cones at the termini of some osteons. Available evidence suggests that osteons of primates might have a greater capacity for branching that is associated with closing cones, which might explain the 10–20 times higher prevalence of sealed + partially sealed osteons in the human bones examined in this study.

Key words: bone; bone microstructure; femur; Haversian systems; hip replacement; sealed osteons.

Introduction

Sealed osteons are unusual variants of secondary osteons that have received little attention, especially in non-human bones. Sealed osteons occur when the central canals of secondary osteons become filled or nearly completely filled with bone tissue (e.g. with one osteocyte lacuna remaining in the central canal) (Fig. 1). All osteons that are considered in the current study are secondary osteons (Haversian systems).

It is suggested that increases in the prevalence of sealed osteons might reflect reduced bone quality which can accompany ischemia that presumably accompanies progressive remodeling seen with age in humans (Congiu & Pazzaglia, 2011; Pazzaglia et al. 2015). The material that plugs

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sealed osteons is not the abnormal/hypermineralized tissue that occludes lacunae or central canals in some humans with advanced age or disease (e.g. micropetrosis) (Frost, 1960; Busse et al. 2010a; Congiu & Pazzaglia, 2011). Milovanovic et al. (2017) described the hypermineralized material seen in micropetrosis as 'nanospherites' comprising high levels of magnesium (i.e. not bone tissue) that accumulate in the lacuna-canalicular system following osteocyte death causing 'dysfunction of the bone's mechano-sensitive network'. They further described this process as the fusion of calcified nanospherites in osteon lacunae and canaliculi. However, this deleterious process differs from the phenomenon of sealed osteons, which involve the central canal rather than the lacunae and canaliculi.

In 1853, Tomes & De Morgan (1853) observed sealed osteons in human cortical bone. Their illustration of one is reproduced here in our Fig. 2. In 1958, Cohen & Harris (1958) described sealed osteons in their three-dimensional (3D) reconstructions of secondary osteons in canine femora. When they observed a blind end of a secondary osteon they called it a 'sealed, blind osteon' (Fig. 3). Within the past 10 years, several studies have described sealed osteons in secondary bone (Pazzaglia et al. 2010, 2013, 2015; Congiu & Pazzaglia, 2011; Maggiano et al. 2016), yet none has conclusively demonstrated their origin.

As with other variants of secondary osteons (e.g. drifting, dumbbell, multi-canal, double zonal) (Skedros et al. 2007), understanding how and why sealed osteons form can shed light on the mechanisms that regulate normal bone remodeling and how this process can be perturbed with aging and some diseases. In their study of the cortices of tibiae from three human males (amputated legs, ages 25, 28, 52 years), Congiu & Pazzaglia (2011) showed that, as a percentage of all secondary osteons examined, 4–5% were sealed. They theorized that sealed osteons occur because

> 'changes in flow dynamics in the system may cause some branches to become excluded from blood flow, with subsequent collapse and degeneration of the capillary vessel. If this occurs, the apposition process can be resumed by the resting osteoblasts on the inner surface of the central canal. According to this hypothesis the sealed osteons represent evidence of the plasticity of the canal system, and their ubiquitous distribution (documented by this study) suggests they are not related to the ageing [*sic*] of cortical bone, but rather to an adaptation of intracortical blood flow dynamics' (Congiu & Pazzaglia, 2011; p. 198).

In the present study, we indirectly evaluated this and other possibilities by quantifying the prevalence of sealed osteons with respect to age and variations in the population density of all typical secondary osteons (OPD) in a broad spectrum of adult appendicular bones. The aim of this study was to determine whether the prevalence of sealed osteons correlates with age, the amount of remodeled bone and/or with other circumstances where ischemia of bone might have occurred, e.g. comparing femora with and without an implanted femoral component of a total hip replacement (HR) (Rhinelander et al. 1979; Bridgeman & Brookes, 1996; Pazzaglia et al. 1997; Hupel et al. 2000). We first tested the hypothesis that a similar prevalence of sealed osteons reported by Congiu & Pazzaglia in human adult tibiae (4–

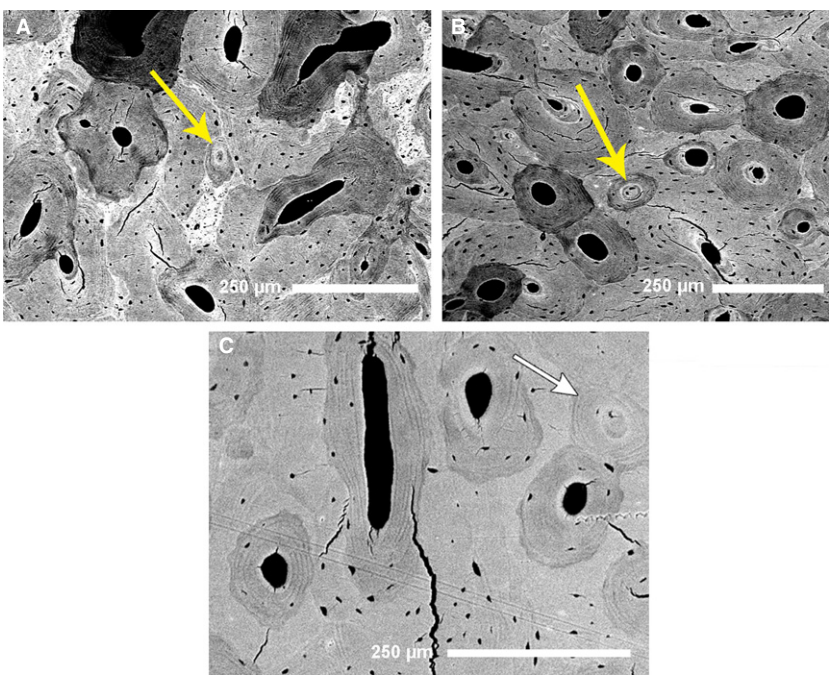

Fig. 1 Backscattered electron images showing sealed osteons found in this study. The widths of the images are 850 µm (A,B) and 587 µm (C).

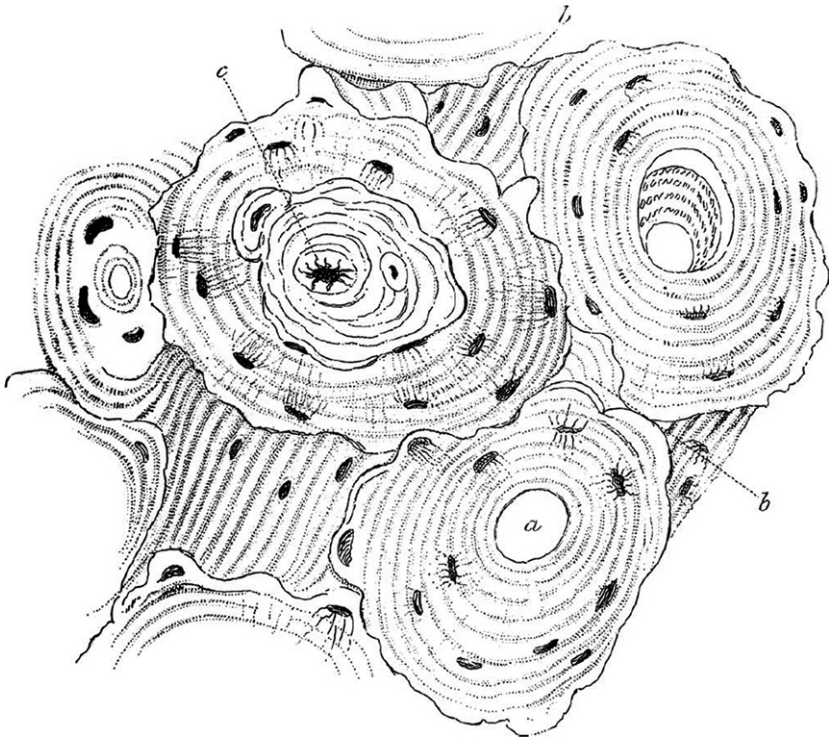

Fig. 2 Drawing reproduced from Tomes & De Morgan (1853) illustrating a sealed osteon, which is indicated with the original label 'c' at the upper left. In the present study, sealed and partially sealed osteons were always much smaller than *neighboring* secondary osteons, unlike the relatively large sealed osteon shown in this figure. Additionally, sealed and partially sealed osteons detected in the present study are, in over 90% of cases, smaller than the smallest of the non-sealed osteons that we measured. The original text of the legend for the label 'c' is: 'A new Haversian system within an older one, the Haversian canal obliterated by the development of a lacunal cell.' [Reproduced with permission of John Wiley & Sons, Inc.].

5%) would resemble that found in our samples of human (with and without femoral implants) and non-human bones when they had comparable ages (humans) and OPDs (all bones examined).

The second objective of the present study was to determine the prevalence of sealed osteons in sections of 10 femora with non-cemented HR as compared with the contralateral femora, which did not have HR, of the same individual. We hypothesized that the prevalence of sealed osteons would increase with patient age, the presence of a femoral component and the amount of time since the HR.

Materials and methods

The specimens of bones used in the current study were obtained from prior studies, and included 10 adult human femora (Boyce & Bloebaum, 1993; Vajda & Bloebaum, 1999) and adult non-human bones that included adult deer calcanei and equine radii and third metacarpals (Skedros et al. 1994, 1996, 2007; Mason et al. 1995). An additional sample of 10 bilateral femora ($n = 20$ femora) from adult humans with unilateral HRs and contralateral femora without HRs were evaluated from a prior study (Rosenbaum Chou et al. 2008). Thus, 30 human femora were examined. Additional details for each specific sample (e.g. ages, imaging locations) are described below.

All individuals in all human and non-human samples were healthy prior to death, had no evidence of musculoskeletal disease, and had not taken medications that could alter bone metabolism or remodeling activity. Ethical approval from our institutional review boards (University of Utah and Department of Veterans Affairs Hospital) for the use of these human and non-human tissues had been granted for our ongoing histomorphological studies.

As described in these previous studies, all of these human and non-human bones were obtained in a fresh-frozen state, thawed and manually cleaned of soft tissue and periosteum, fixed in 70% ethanol, and embedded in polymethyl methacrylate (PMMA) using published methods (Emmanual et al. 1987). All images that were analyzed were backscattered electron (BSE) images and were obtained in cortical quadrants of transversely cut sections. As shown in our above-mentioned studies, we typically analyzed at least 80% of the middle of the central cortex in order to account sufficiently for local variation (Iwaniec et al. 1998). Additionally, the specimens studied herein were obtained from previous studies that had sufficient sample sizes to discern statistically significant regional variations in several histomorphological characteristics, including OPD, predominant collagen fiber orientation, and porosity. In the statistical analysis section below, we describe in more detail the statistical power analyses that we used for detecting differences in sealed osteons.

Imaging: all bones

For all of the bones used in this study, two observers, who worked independently, identified the numbers of completely sealed and partially ('sealed + partially') sealed osteons. Each observer examined the BSE images that had been obtained in these bones at magnifications ranging from 50 to 200×. The sealed + partially sealed osteons were selected using the criteria described by Congiu & Pazzaglia (2011) with additional details as described below. Partially sealed osteons were those that were > 80% sealed but less than fully sealed.

The non-sealed secondary osteons were selected if they: (1) demonstrated a clear cement line (seen as gray level differences in the BSE images) that surrounded the entire osteon, (2) lacked a Volkmann's canal connection, (3) were reasonably circular or ellipsoid, (4) were refilled completely, or nearly completely, and (5)

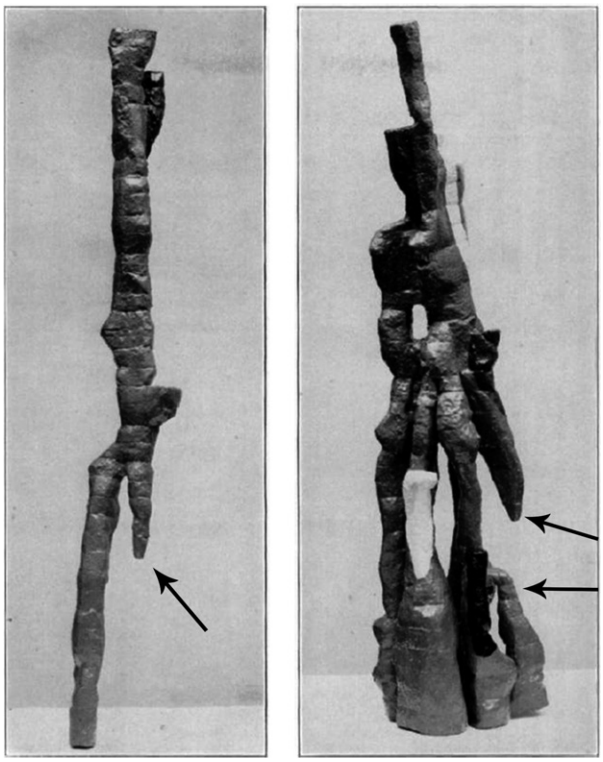

Fig. 3 Images of models of the 3D reconstructed osteons (Haversian systems) from Cohen & Harris (1958). Their models demonstrate closing cones (arrows). [Images reproduced with permission of *British Journal of Bone and Joint Surgery*]. [Colour figure can be viewed at wileyonlinelibrary.com]

contained a non-sealed central (Haversian) canal (Skedros et al. 2007, 2009). Criteria 1–4 were also used to identify sealed or partially sealed osteons, with 'partially sealed' being at least 80% central canal plugging as stated above. Neither osteon size and shape, nor osteon canal size and shape, were considered in the selection process of sealed osteons.

The prevalence of sealed osteons is described as a percentage of the total number of non-sealed secondary osteons per bone section or individual. When measurements of OPD are performed, they are presented as sealed + partially sealed osteons within each image (n mm^{-2}).

In each sample, we measured the prevalence of sealed + partially sealed osteons and cross-sectional areas of all sealed + partially sealed osteons vs. randomly selected typical secondary osteons. The randomly selected non-sealed secondary osteons that were obtained within each image were identified with the use of a grid with 16 equally sized rectangular regions. The grid was randomly placed on each image. The osteon in the center of the image (center of the grid) was selected, as well as one osteon from the center of each quadrant of the grid. Thus, five typical (non-sealed) secondary osteons were selected using this method and subsequently measured in each image. The total numbers of sealed osteons, partially sealed osteons, and non-sealed secondary osteons were also quantified for each image.

The principal investigator (J.G.S.) independently examined all selected sealed + partially sealed osteons and verified that they did not represent packets of interstitial bone or remnants of previous osteons without central canals.

Sealed + partially sealed osteons were analyzed together in all analyses conducted in the present study. We deemed this especially important in view of the fact that serial sections were not obtained in the present study, and focusing on only fully sealed osteons would further bias the data by precluding some sealed/sealing osteons that otherwise would have been detected had serial sectioning been done.

The population density of secondary (non-sealed) osteons (OPD) was calculated for each image by dividing the total number of osteons by the area of the image, which is expressed in mm^2. The abbreviation 'OPD', which can also be expressed as On.N/T.Ar, refers to the number of secondary osteons (On.N) divided by the total area of the image (T.Ar), which included primary osteon canals, secondary osteon canals (i.e. central canals), and osteocyte lacunae, but excluded larger porosities (such as Volkmann's canals).

Using Adobe PHOTOSHOP (version 10.0.1, Adobe Systems Incorporated, San Jose, CA, USA), all sealed + partially sealed osteons were manually traced. The five randomly sampled, non-sealed secondary osteons from each image were also traced. The tracings were used to determine osteon area and mean diameter in the ImageJ program (v. 1.43, National Institutes of Health, USA; Rasband, 1997–2016).

Bilateral femora from patients with unilateral hip replacements (HR)

Twenty human femora were obtained from 10 postmortem donors (52–94 years; mean 82 years; male : female = 3:7). Each donor had undergone unilateral non-cemented total HR for osteoarthritis. This sample of paired bones allowed for the comparison between implanted femurs and non-implanted femurs. All hip implants were of the Alloclassic® Zimmer® brand (Warsaw, Indiana, USA) and were stable and performing well at the time of the patient's death. The BSE images of these bones were obtained from a prior study from our laboratory (Rosenbaum-Chou et al. 2008).

As was also described in that prior study, the entire proximal portion of each of the 20 femora from the patients with HRs (10 of which were implanted) was first embedded and then cut transversely into ~5-mm segments at equivalent transverse locations. Using a diamond-blade saw, sections were taken at 25, 45, 65, and 85% of the distance along the femoral component length from the proximal apex of greater trochanter portion to distal end of the stem portion of the implant. Therefore, the 85% location is closest to the distal end. The section levels are shown in Fig. 4. Sections at the same percentage length were obtained from the contralateral, non-implanted femora. The distance between two adjacent section locations was approximately 3 cm.

The imaged locations included two 200× images from each of the endosteal, middle, and periosteal regions of the cortical bone along the geometrical axes (i.e. Imax and Imin, which are about 10° from the anterior, posterior, medial and lateral anatomical axes) for a total of 24 images per section level.

The measurements of OPD, diameters of individual osteons, and sealed + partially sealed osteon prevalence were averaged over the four section levels for each of the 20 femora from the patients who had an HR.

Femora from patients without hip replacements

The femora from 10 patients without HRs (34–71 years; mean: 55 years; male : female = 8:2) had been used in prior studies (Boyce

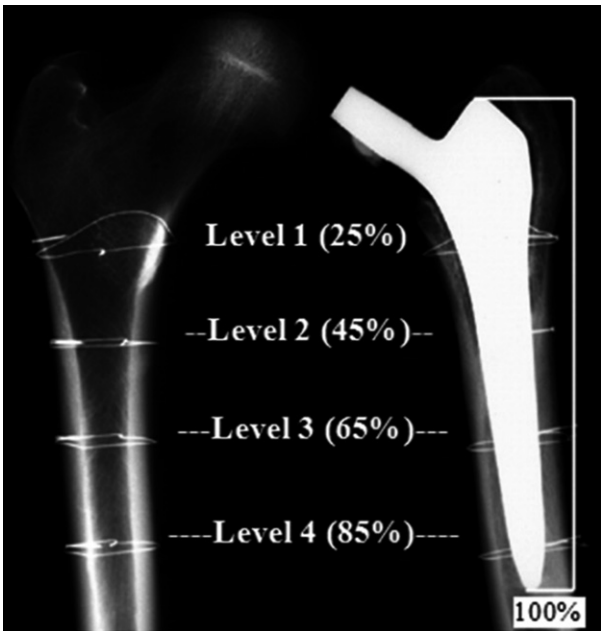

Fig. 4 Radiograph showing one pair of femora from a patient with a unilateral femoral component of a total hip replacement (the acetabular component not shown). The numbers represent percentages of the length of the femoral implant shown as '100%' in the image on the right. For the purposes of the present study, the measurements of OPD, diameters of individual osteons, and sealed + partially sealed osteon prevalence were averaged over the four section levels for each bone. [Reproduced from Rosenbaum-Chou et al. (2008) with permission of the authors].

& Bloebaum, 1993; Vajda & Bloebaum, 1999) but the 50% diaphyseal locations were not examined previously.

As in the other samples, BSE images were obtained of the cross-sections. The imaged locations included the mid-cortex regions of the anterior, posterior, medial and lateral cortices. Two 50× images were obtained from each cortical region.

Non-human bones

The non-human bones were from adult animals and included calcanei of wild mule deer (*Odocoileus hemionus hemionus*, $n = 15$), and equine radii ($n = 18$) and third metacarpals (MC3s) ($n = 10$). The equine bones were from non-racing animals. Of these animals, the oldest was 9 years old (a horse). The 50× and 100× BSE images of these bones were obtained from our prior studies (Skedros et al. 1994, 1996, 2007; Mason et al. 1995). The imaged locations included mid-cortical regions in anterior, posterior, medial, and lateral cortices from mid-diaphyseal segments of these non-human bones.

Statistical analysis

Statistical analyses were done in the NCSS program (Hintze, 2015). Comparisons of the prevalence of sealed + partially sealed osteons with the other parameters were made after first obtaining a mean for each bone. Differences between section levels in the sample of femora from patients with HR were not assessed. Data were then examined for statistical significance ($P < 0.05$) using a one-way ANOVA with Fisher's PLSP test, or Kruskal–Wallis tests for data that are not normally distributed. Pearson or Spearman correlation coefficients were determined for various comparisons. Correlation coefficients were classified according to the following method: 0–0.30 (0 to −0.30), little if any correlation; 0.30–0.50 (−0.30 to −0.50), low positive (negative) correlation; 0.50–0.70 (−0.50 to −0.70), moderate positive (negative) correlation; 0.70–0.90 (−0.70 to −0.90), high positive (negative) correlation; 0.90–1.00 (−0.90 to −1.00), very high positive (negative) correlation (Hinkle et al. 2003).

Our statistical power and sample analyses were done in two steps. The first step used data from Table 2 of Congiu & Pazzaglia (2011) (Table 1 in the present study). We used these data because they are the only suitable published data that we could find for estimating the sample size needed to detect a significant difference in sealed osteon population density. As shown in Table 1, they reported a statistically significant difference between the endosteal and periosteal regions, which we then used to determine the sample size needed to detect an effect at least this large when employing the paired comparison approach used herein. Because the standard deviations for the sealed osteon data were not provided by Congiu & Pazzaglia (2011), they were estimated from the standard deviations in their OPD data using the methods shown in the footnote of Table 1. It was then determined that our sample size of 10 in each human group achieved > 99% power ($\alpha = 0.05$) to detect a population density difference of 1.26 vs. 0.40 mm^{-2} in sealed osteons and also with similar power for the partially sealed osteon data shown in Table 1.

The second step of the statistical power analysis was done using the OPD data from Rosenbaum-Chou et al. (2008) from their 10 patients with and without HRs. For the implanted group, the mean OPD was 10.6 ± 2.0 and was 8.4 ± 2.4 for the non-implanted group. These data were provided prospectively by our colleague T. Rosenbaum-Chou (they were not reported in that published study). Using an alpha level of 0.05 and their sample size of 10 in each group resulted in 72% statistical power. We assumed that statistically significant differences in sealed osteon population densities shown in Table 1 could also then be detected with statistical power ≥70% in the human comparisons made in the present study. This logic also applies to the anticipated sealed osteon data from our non-human samples because the non-human OPD data exhibited regional differences that were typically much larger than those stated above for our human femora.

Results

Implanted femora (F) from patients with hip replacements (F, +HR) ($n = 10$)

The prevalence of sealed + partially sealed osteons in this sample was 2.1%. Sealed + partially sealed osteons are also significantly smaller than typical secondary osteons (mean diameters: 114 vs. 163 μm; $P = 0.03$) (Fig. 5). Analysis of sealed + partially sealed osteons revealed no correlation with age ($r = -0.36$, $P = 0.3$) or implant duration (Fig. 6), and a high positive correlation with OPD ($r = 0.78$, $P = 0.008$). There was a high negative correlation between age and OPD ($r = -0.79$, $P = 0.007$). Mean OPD was 8.4 osteons mm^{-2} (range: 4.1–12.2, SD 2.4).

Table 1 Data for statistical power and sample size analyses. (A) Congiu & Pazzaglia (2011) data at left, estimated data at right. (B) Human femur sample size analysis (data from T. Rosenbaum-Chou).

					Mean OPD and estimated SD			
			% Osteon type		Completely sealed		Partially sealed	
	Mean OPD, all osteons (n mm^{-2})	Regular	Completely sealed	Partially sealed	Mean OPD, completely SO(n mm^{-2})	SD	Mean OPD, partially SO (n mm^{-2})	SD
A.								
Periosteal	20.34 ± 3.39*	91.8	6.2*	2.0	1.26†	0.21‡	0.41†	0.07‡
Endosteal	18.17 ± 3.01*	96.4	2.2*	1.4	0.40†	0.07‡	0.25†	0.04‡

B.	Mean OPD (n mm^{-2})	SD	Sample size	Alpha	Statistical power
F, +HR	10.6	2.0	10	0.05	72%
F, −HR	8.4	2.4	10		

F,+HR, femur with hip replacement; F,−HR, femur without hip replacement;OPD, osteon population density, SO, sealed osteons, SD, standard deviation.
*Statistically significant periosteal vs. endosteal differences.
†Calculated by the following: (mean OPD, all osteons) (% osteon type).
‡Calculated by the following: (mean OPD, all osteons SD) (% osteon type).

Non-implanted femora from patients with hip replacements (F, −HR) (n = 10)

The prevalence of sealed + partially sealed osteons in this sample was 1.9%. Sealed + partially sealed osteons are significantly smaller than typical secondary osteons (mean diameters: 134 vs. 201 μm; $P = 0.001$) (Fig. 5). Analysis of sealed + partially sealed osteons revealed no correlation with age ($r = -0.40$, $P = 0.25$) and a high positive correlation with OPD ($r = 0.71$, $P = 0.02$). There was a high negative correlation between age and OPD ($r = -0.74$, $P = 0.01$). Mean OPD was 10.6 osteons mm^{-2} (range: 7.9–14.1, SD 2.0).

Femora from patients without any hip replacements (n = 10)

In this sample of 10 femora, a total of 1.6% osteons were either sealed or partially sealed. Sealed + partially sealed osteons are also significantly smaller than typical secondary osteons (mean diameters: 125 vs. 272 μm; $P < 0.001$) (Fig. 5). Analysis of sealed + partially sealed osteons revealed no correlation with age ($r = -0.03$, $P = 0.9$) and no correlation with OPD ($r = 0.15$, $P = 0.7$). There was also no correlation between age and OPD ($r = -0.05$, $P = 0.9$). Mean OPD was 16.8 osteons mm^{-2} (range: 13.3 to 23.7, SD 3.2).

All non-implanted femora (n = 20)

The prevalence of sealed + partially sealed osteons in all non-implanted femora (n = 20) was 1.7% (range: 0.5–4.4%, SD 1.0%). Sealed osteons are also significantly smaller than typical secondary osteons (mean diameters: 127 vs. 250 μm; $P < 0.0001$) (Fig. 5). Analysis of sealed + partially sealed osteons showed no correlation with age ($r = -0.14$, $P = 0.5$) and a high positive correlation with OPD ($r = 0.71$, $P < 0.001$). Mean OPD was 13.7 osteons mm^{-2} (range: 7.9–23.7, SD 4.1).

All femora (n = 30)

The prevalence of sealed + partially sealed osteons in all femora (n = 30) was 1.8% (range: 0–4.4%, SD 1.1%). Sealed + partially sealed osteons were also significantly smaller than typical secondary osteons (mean diameters: 125 vs. 236 μm; $P < 0.0001$). Sealed + partially sealed osteons showed no correlation with age ($r = -0.14$, $P = 0.47$) (Fig. 7) and a high positive correlation with OPD ($r = 0.77$, $P < 0.0001$) (Fig. 8). Mean OPD was 11.9 osteons mm^{-2} (range: 4.1–23.7, SD 4.4).

Non-human bones

Of approximately 160 000 total osteons in the non-human bones, we found 185 sealed + partially sealed osteons (0.12%). The percent prevalence was similar in each non-human species (deer calcanei, 0.11%; equine radii, 0.10%; equine MC3s, 0.17%) (Fig. 5). The number of sealed + partially sealed osteons with OPD in the non-human bones was compared: (i) deer calcanei showed no correlation ($r = -0.02$, $P = 0.7$), (ii) equine MC3 showed a trend towards a weak negative correlation

A Human Bones

		S+PSO*	Mean OPD (number of osteons/mm^2)	Sealed Osteon Mean Diameter (μm)	Secondary Osteon Mean Diameter (μm)	Mean Age (years)
F,+HR (n = 10)		2.06%	8.41	114.15	163.49	82.60
	SD	1.47%	2.40	35.64	67.09	12.20
	range	0 - 4.2%	4.1 - 12.2	46 - 167	76 - 278	52 - 94
F,–HR (n = 10)		1.87%	10.58	133.64	201.13	82.60
	SD	1.15%	1.97	55.46	76.49	12.20
	range	0.49 - 4.4%	7.9 - 14.1	47 - 225	85 - 460	52 - 94
Femora from patients without HR (n = 10)		1.62%	16.76	124.59	271.92	55.90
	SD	0.83%	3.18	49.37	56.25	13.20
	range	0.6 - 2.9%	13.3 - 23.7	46-292	162-432	34 - 71
All nonimplanted (n = 20)		1.69%	13.67	126.94	250.26	69.25
	SD	0.98%	4.08	50.78	70.86	18.46
	range	0.5 - 4.4%	7.9 - 23.7	46-292	85-460	34 - 94
All femora (n = 30)		1.75%	11.92	125.14	236.16	73.70
	SD	1.14%	4.37	48.96	77.06	17.62
	range	0 - 4.4%	4.1 - 23.7	46-292	76-460	34 - 94

B Non-human Bones

		S+PSO*	Mean OPD (number of osteons/mm^2)	Sealed Osteon Mean Diameter (μm)	Secondary Osteon Mean Diameter (μm)	Mean Age (years)
All non-human bones		0.12%	15.85	130.81	193.30	NA
	SD	0.46%	12.91	41.56	49.26	
	range	0 - 4.0%	0 - 73.3	57 - 213	103 - 307	
Deer Calcanei (n = 15)		0.11%	21.04	116.00	170.08	NA
	SD	0.47%	17.79	36.09	45.21	
	range	0 - 2.2%	0 - 73.3	72 - 174	103 - 291	
Horse Radii (n = 18)		0.10%	11.62	162.60	213.21	NA
	SD	0.39%	6.89	30.40	48.73	
	range	0 - 2.7%	0 - 68.0	112 - 213	112 - 307	
Horse MC3s (n = 10)		0.17%	15.14	117.63	219.61	NA
	SD	0.50%	7.02	41.16	35.05	
	range	0 - 4.0%	0.8 - 35.9	57 - 180	150 - 304	

*** S+PSO = sealed plus partially sealed osteons; F = femora; -HR = no hip replacement; +HR = with hip replacement; SD = standard deviation**

Fig. 5 Summary of sealed + partially sealed osteon prevalence, OPD (*n* mm^{-2} of tissue area), osteon diameters, and patient ages. A = human data; B = non-human data. The relatively small sample of human femora in patients without hip replacements likely accounts for the seemingly paradoxical finding that OPD in that group is considerably higher than in the F, −HR group even though the mean age of this latter group is 27 years older (i.e. older bones should have higher OPD) (Britz et al. 2009). Of note, it is also accurate that in all groups of human and all non-human bones when considered separately and together, the mean value of sealed + partially sealed osteons seems very low compared with the relatively large ranges of these values. These apparent anomalies reflect the fact that the ranges are large because many images had one or no sealed osteons, and there were a few instances where a single image had multiple sealed osteons. In this latter case these images had a high percentage of sealed + partially sealed osteons, even though overall the percentage of these osteons was very low. This influenced the seemingly errant ranges and standard deviations that are in fact accurately reported.

($r = -0.13$, $P = 0.06$), (iii) equine radii showed a significant, but weak, positive correlation ($r = 0.13$, $P = 0.007$), and (iv) all non-human bones combined ($n = 1309$ images) showed no correlation ($r = 0.002$, $P = 0.9$). The mean OPD in each species was: (i) 21.0 osteons mm^{-2} in the deer calcanei (range: 0–73.3, SD 17.8), (ii) 11.6 osteons mm^{-2} in the equine radii (range: 0–68, SD 6.9), (iii) 15.1 osteons mm^{-2} in the equine MC3 (range: 0.8–35.9, SD 7.0), and (iv) 15.9 osteons mm^{-2} in all non-human bones (range: 0–73.3, SD 12.9).

Sealed + partially sealed osteons were also significantly smaller than typical secondary osteons (mean diameters using data from all species: 131 vs. 193 μm; $P < 0.0001$). The data for sealed and non-sealed osteon diameters for each species are listed in Fig. 5.

Discussion

Results of this study showing no association of sealed + partially sealed osteons with age, in addition to a generally low prevalence of sealed + partially sealed osteons, are inconsistent with the idea that they contribute to reduced bone quality seen with aging in humans. Our results, however, do support the conclusion that osteon sealing may be associated with the process of bone remodeling. This conclusion is based on our data showing that the percentage of sealed + partially sealed osteons increases with increased secondary osteon population density (OPD) (all 30 femora analyzed, $r = 0.77$, $P < 0.0001$). However, this relationship was not found in the non-human bones.

Sealed + partially sealed osteons were much less frequent in the non-human bones (< 0.1 vs. 1.8% in human femora, $n = 30$) despite comparatively equivalent and often higher OPD compared with the human bones we examined. Congiu & Pazzaglia (2011) reported that the percentage of sealed and partially sealed osteons from amputated tibiae from three patients (ages 25, 28, and 52 years) was 4.2 and 1.7%, respectively. However, in a later study of tibial and femoral mid-diaphyses from four healthy young males (ages 25, 28, 30, and 32) who underwent amputations because of traumatic limb injuries, they found that only 2.2% of the total osteons were sealed (Pazzaglia et al. 2013). Potential

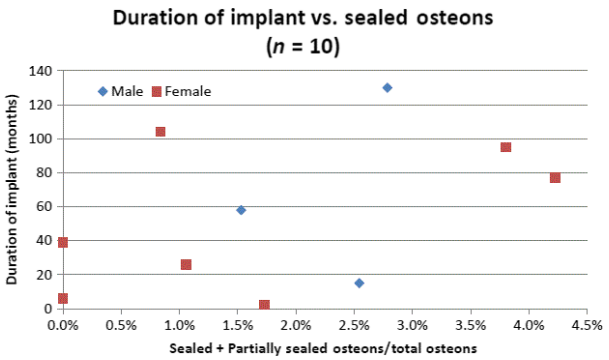

Fig. 6 Scatter plot of the duration of implantation of the hip replacement at the time of patient's death (n = 10 femora with femoral components). No apparent relationship is revealed by these data. [Colour figure can be viewed at wileyonlinelibrary.com]

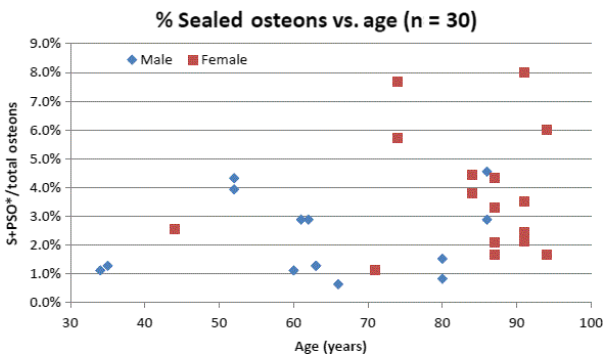

Fig. 7 Scatter plot of the percentage of sealed osteons vs. age (n = 30 human femora). There are two data points that are overlapping (i.e. two 63 year-old males have 1.28% sealed osteons). No apparent relationship is revealed by these data. [Colour figure can be viewed at wileyonlinelibrary.com]

reasons for the discrepancy between their studies are discussed further below.

Results of this study also show that the percentage of sealed + partially sealed osteons does not increase after the implantation of a femoral component of a total HR. This conclusion is based on our data from bones that were obtained at a minimum of 2.5 months after implantation (one of our bones was obtained at 2.5 months and one at 6 months after implantation of the HR, all others were at ≥15 months). It is well known that reaming and/or broaching the medullary canal during the implantation of a femoral component creates a region of ischemia (Rhinelander et al. 1979; Bridgeman & Brookes, 1996; Pazzaglia et al. 1997; Hupel et al. 2000). However, this ischemia is transient in most cases, and in studies conducted in dogs it has been shown that approximately 6 months is required to restore the blood flow to the endosteal cortex (direct data in humans are not available) (Rhinelander et al. 1979). If osteon sealing increases during this time of ischemia, then bones within a few weeks of implantation would be required to detect this phenomenon.

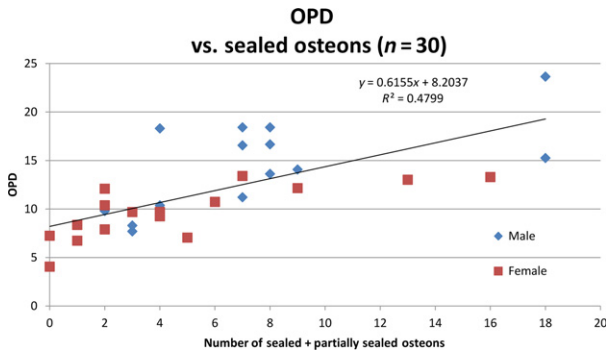

Fig. 8 Regression plot with least-squares best-fit line of OPD vs. number of sealed + partially sealed osteons (n = 30 femora). Note that the R^2 value shown above is the least-squares regression coefficient. The r value reported in the text is Spearman's correlation coefficient. A high positive correlation is revealed by these data.

Does ischemia in cortical bone actually result in increased bone formation?

The idea that some osteons seal because their blood supply is diminished during normal osteonal remodeling seems counterintuitive. In other words, the quote by Congiu & Pazzaglia (2011) in the Introduction section above seems unlikely at first glance because bone formation (at least normal bone tissue) seems unlikely during a time of reduced oxygen and nutrient supply that characterizes ischemia. But observations that seem to support this idea have been reported. For example, data from cultured osteoblastic cells show that hypoxia can result in decreased sclerostin production (Genetos et al. 2010), which increases osteoblast activity. However, Genetos et al. (2010) did not attempt to measure bone formation in their study. Wang et al. (2007) provided compelling evidence that the hypoxia-inducible factor alpha (HIFα) couples angiogenesis and osteogenesis with hypoxia. In their model, new osteoblasts on the nascent bone surface sense low oxygen levels and upregulate HIFα, thereby upregulating vascular endothelial growth factor (VEGF), which stimulates new blood vessel formation and introduces more osteoblast progenitors. These develop into mature osteoblasts, which create new bone. However, this model involves new blood vessel formation and subsequent blood vessel invasion into bone during skeletal development, which would not be expected in the formation of a sealed osteon during normal remodeling in an adult and especially not in cases where bone ischemia is caused by trauma.

The bulk of studies, however, reject the idea that ischemia results in bone formation. For example, data from osteoblastic rat cells (2 days old) show that reducing oxygen tension (pO_2) from atmospheric pressure (20%) to 5% results in a net 1.7-fold decrease in formation of mineralized bone nodules (Utting et al. 2006). Further reduction to 2% pO_2 results in an 11-fold *decrease* in the formation of

mineralized bone (Utting et al. 2006). The mechanisms at play in this decreased bone formation include: decreased osteoblast proliferation, reduced alkaline phosphatase activity, and induction of a 'reversible state of quiescence' in osteoblasts (Utting et al. 2006). In addition to diminished osteoblast activity, Arnett et al. (2003) found that increased bone resorption (up to 21-fold) occurs at 2% oxygen through increased osteoclast stimulation in mouse marrow cells (8-week-old animals). There are other studies that support the notion that hypoxia/anoxia decreases factors involved in bone mineralization and formation (Park et al. 2002; Ontiveros et al. 2004; Salim et al. 2004). For example, Park et al. (2002) found that hypoxia reduces Runx2 (a transcription factor necessary for osteoblast differentiation), type I collagen formation, osteocalcin, and alkaline phosphatase activity. Although the overall effect of ischemia/hypoxia on osteoblast activity and bone formation is complex and involves various pathways, available evidence suggests that bone formation, such as osteon sealing, is not the ultimate consequence of ischemia/hypoxia. Our data indicate a decrease in OPD when a femoral component is implanted (10.6 vs. 8.4 osteons mm^{-2}, $P = 0.04$), suggesting decreased osteon activity, which perhaps is correlated with ischemia resulting from 'trauma' caused by the reaming/broaching during implantation of the femoral component. This relationship is consistent with Busse et al. (2010b), who reported a significant decrease in OPD in the periosteal region of femora from patients with total HRs ($n = 8$, age range: 7th to 9th decade) compared with a control group ($n = 23$, age range: 1st to 9th decade).

Nevertheless, if sealed osteons do, in some unexpected way, result from ischemia that occurs during the normal osteonal remodeling process, then we hypothesized that we would find a similar prevalence of sealed + partially sealed osteons in highly remodeled bones regardless of whether they are from humans or non-humans. However, compared with the 4–5% frequency of sealed + partially sealed osteons reported by Congiu & Pazzaglia (2011) and the 2.2% reported in their subsequent study (Pazzaglia et al. 2013), we found that far fewer (~ 0.1%) osteons in our three groups of non-human bones were sealed or partially sealed.

Through personal communication with us, and with regard to the three patients' tibiae examined by Congiu & Pazzaglia (2011), U. Pazzaglia also considered the amputation-related ischemia to be an unlikely explanation for the high prevalence in their sample. He stated that the 'decision to carry out amputation was taken in an interval of time varying from few hours to a maximum of 20 days' (U. Pazzaglia, pers. comm.). Even if trauma-related ischemia was the main factor that incited the formation of sealed osteons, U. Pazzaglia still feels that even 20 days is too short a period of time to increase the number of sealed osteons. He feels that the discrepancy between their two studies (4–5% sealed osteons vs. 2.2%) might be better explained by the age differences between the two populations. The ages in their 2011 study were 25, 28, and 52 (mean age = 35 years) (all tibiae) and the ages in their 2013 study were 25, 28, 30, and 32 (mean age = 28 years) (four tibial and four femoral sections were obtained from each).

Two of the patients (ages 25 and 28 years) were the same in both studies (U. Pazzaglia, pers. comm.). The OPD reported in their 2011 study is as follows: (i) anteromedial cortex: mean = 18.09 osteons mm^{-2}, SD = 3.53, (ii) lateral cortex: mean = 19.67 osteons mm^{-2}, SD = 3.27, and (iii) posterior cortex: mean = 20 osteons mm^{-2}, SD = 3.23. They did not report the OPD for each patient. However, the relatively small standard deviations suggest that there is no substantial increase between their younger patients (ages 25 and 28 years) and their oldest patient (age 52 years). The only difference between the two studies regarding the patient population was that the 2013 study demonstrated a more homogeneous (and younger) age range. It is possible that the much higher prevalence of sealed osteons reported by Congiu & Pazzaglia (2011) might reflect increased OPD of the three patients' limbs that they used, or it could be due to vagaries of a small sample size.

Sealed osteons are not the result of micropetrosis

Age-related hypermineralization (i.e. micropetrosis) of osteocyte lacunae and canaliculi is related to ischemia in bone. In 1960, Frost published a paper entitled 'Micropetrosis' (Frost, 1960). In that study he described micropetrosis as the filling of canaliculae and osteocyte lacunae with mineralized tissue. Congiu & Pazzaglia (2011) also performed elemental analysis and showed that the material of sealed + partially sealed osteons has Ca : P ratios consistent with hydroxyapatite found in bones, providing evidence that sealed osteons are not the result of age-related hypermineralization. We did not perform a similar compositional analysis. However, we did not see evidence of central canals plugged with the degree of hypermineralization seen in micropetrosis (Busse et al. 2010a; Milovanovic et al. 2017). Nevertheless, this limitation of our study could lead to an over-count of sealed osteons if some of them were actually a consequence of micropetrosis.

Busse et al. (2010a) showed that the endosteal cortex is more obviously affected by hypermineralization than is the periosteal compartment. The endosteal region of the human femur is known to become ischemic with age as a result of medullary atherosclerosis (Bridgeman & Brookes, 1996). In turn, as the medullary blood supply to the cortex diminishes, the periosteal blood supply becomes increasingly important for the perfusion of the entire femoral diaphyseal cortex in old age (Bridgeman & Brookes, 1996). Endosteal ischemia is likely even greater in cases where a femoral stem of an HR has been implanted (Bridgeman & Brookes, 1996; Pazzaglia et al. 1997). If sealed osteons are related to this regional ischemia (as seen with

micropetrosis), then there should be an increased prevalence of sealed osteons in the endosteal cortex. However, Pazzaglia et al. (2013) showed that sealed osteons were not significantly different between the endosteal and periosteal regions. Similarly, we did not find this relationship in the samples where we examined bone close to the endosteum vs. toward the periosteum (i.e. which we could evaluate in our non-human bones and the 10 femora from patients with unilateral HR). Consequently, sealed + partially sealed osteons that we identified do not appear to be the result of, or associated with, age-related micropetrotic/hypermineralization changes in bone in general or in specific regions of the cortex.

Closing cone hypothesis

The smaller size of sealed + partially sealed osteons in all of our samples leads us to hypothesize that they represent the closing cone region of the Haversian system. The basic multicellular unit (BMU) is a temporary structure for bone remodeling, made up of osteoclasts and osteoblasts (Parfitt, 1994). During bone remodeling, the BMU propagates, with a cutting cone (osteoclasts) leading the BMU and a closing cone (osteoblasts) in its wake. We hypothesize that the majority of sealed + partially sealed osteons likely represent the closing cone region that is known to occur in some regions of secondary osteons (Cohen & Harris, 1958; Cooper et al. 2006; Maggiano et al. 2016) and therefore are not a pathological or otherwise ischemia-related infilling of the Haversian canal with bone tissue. Recent 3D studies suggest the possibility that sealed osteons represent the closing cones of normal secondary osteons.

Maggiano et al. (2016) reconstructed 3D models of Haversian systems in human cortical bone using synchrotron radiation-based micro-computed tomography (CT) scans. They described blind (sealed) osteons that, in three dimensions, gradually become narrower before the Haversian system ends. Subsequent to their publication, these investigators created videos using their serial Z-stack images (i.e. transverse sections) of various locations. C. Maggiano showed these to one of us (J.G.S.) at the April 2017 meeting of the American Association of Physical Anthropologists in New Orleans, LA, USA. In all cases where SOs were found ($n = 5$), it was clear that SOs were at the termini of typical secondary osteons.

In another micro-CT study of BMUs, Cooper et al. (2006) described significant variation among closing cones in human femora (ages 18–92, mean 54 years), with some resorption spaces demonstrating a closing cone closely trailing a cutting cone, whereas other resorption spaces showed 'little or no evidence of a closing cone along many millimeters'. This lag between the cutting cone and closing cone has been described previously (Johnson, 1964; Parfitt, 1983). Furthermore, Cooper et al. (2006) found that cutting cone morphology varied considerably, with some cutting cones following the standard definition and others forming interconnecting networks that overlapped with existing Haversian systems. This overlap can be caused by drifting osteons, which have continuous bone formation on one side and resorption on the other; thus they can propagate obliquely/transversely and longitudinally through the cortex (Robling & Stout, 1999). An increased prevalence of drifting osteons in human bone compared with non-human bone has been reported (Knese et al. 1954; Knese & Titschak, 1962; Skedros et al. 2007). For example, Knese & Titschak (1962) found that < 1% of all osteons in the third and fourth metacarpals of pigs (6 months to 4 years; $n = 33$) and wild boars (8 months to 7 years; $n = 25$) are drifting osteons. In a study of a 43-year-old male patient's femur, tibia, fibula, and radius, Knese et al. (1954) found that the prevalence of drifting osteons is far higher in human bones (~ 10%). The greater prevalence of drifting osteons found in primates might reflect a greater capacity for plasticity of interconnections in primate secondary osteons. This could provide an explanation for the 10–20 times higher prevalence of sealed osteons in the human bones examined in this study.

Limitations

One limitation of this study is that the images obtained were not from multiple serial sections. Consequently, it is almost entirely random that our two-dimensional (2D) sections captured a completely sealed or partially sealed osteon. We are not aware of large 3D studies that have focused on establishing 'normal' closing cone patterns and prevalence, and did not find published data regarding how far through a bone section a sealed osteon courses. However, it is well known that microscopic 2D regions analyzed in single thin planar sections of bone can be used for stereological sampling of 3D entities such as osteons. This is based on Delesse's principle that shows that analysis of 2D entities such as osteons can be used to express the data in 3D as long as there is no significant heterogeneity in the distribution within the local volume of the tissue (Weibel & Gomez, 1962; Mayhew & Cruz Olive, 1974). In other words, to extrapolate data from multiple regions within one planar section to a 3D material (e.g. a 0.5–1.0 cm segment of a bone diaphysis such as used in the present study), two criteria must be met: (i) the plane of the section must be typical (i.e. the material is completely homogeneous) and (ii) the plane of the section must be random. In this context our data dealing with the prevalence and size of sealed + partially sealed osteons should be representative and unbiased by our sampling methods (single planar sections and analysis within them in anatomical quadrants) because sealed + partially sealed osteons would be expected to be homogeneously distributed in the bone regions that we analyzed, as also indicated by data of various other secondary osteon variants in many different bones and species (Skedros et al. 2007; Keenan et al. 2017). If, however, there

is some unrecognized reason that there is in fact inhomogeneity in the distribution of sealed + partially sealed osteons, then rigorous proof of our assumption that there is no bias can only be obtained in future studies that employ multiple serial sections.

Conclusion

Results of this study showing no association of sealed + partially sealed osteons with age and an overall low prevalence of sealed + partially sealed osteons, contradicts the idea that sealed + partially sealed osteons contribute to reduced bone quality seen with aging in humans. Our data also show that the percentage of sealed + partially sealed osteons does not increase after the implantation of a femoral component of a total HR. However, there could be a transient increase in sealed + partially sealed osteons within weeks of the implantation that could not be detected in our sample. This lack of association between sealed osteons and the presence of a femoral component, or the amount of time since the HR, opposes the idea that sealed osteons are the result of ischemic changes in bone.

The small size of sealed + partially sealed osteons, regardless of species affiliation, suggests that they represent closing cones at the termini of some osteons. Available evidence suggests that osteons of primates might have a greater capacity for branching that is associated with closing cones. This could provide an explanation for the 10–20 times higher prevalence of sealed + partially sealed osteons in the human bones examined in this study even though these bones had an OPD generally similar to that seen in the non-human bones.

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Author contributions

All authors contributed to the concept/design of the project, data analysis/interpretation, and approval of the article. T.R.H. and M.S.D. were primarily involved in acquisition of data, and J.G.S., R.D.B., and T.R.H. drafted and critically revised the manuscript.

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