1. Introduction

Microdamage in the skeleton forms under both normal and aberrant loading conditions, with accumulation of microdamage resulting in stress fractures and contributing to increased fragility and fracture risk (Frost, 1960). In young, healthy bone that can effectively remodel (i.e., repair) quickly, microdamage is of minimal concern (Burr, 2011). However, in the aging population, where activation frequency and thus the entire remodeling process slows with increasing age, microdamage formation can outpace repair and lead to compromised bone quality (Diab et al., 2005). Additionally, the use of bisphosphonate drugs to treat osteoporosis further impedes repair processes, which research suggests leads to further increased microdamage accumulation (Pazianas et al., 2014; Allen and Burr, 2011). Increased longevity in modern populations has seen a corresponding rise in fracture induced morbidity and mortality, particularly in the context of the thorax, where rib fractures in the elderly often have poor recovery outcomes (Sirmali et al., 2003). As such, studying patterns of microdamage variation is essential to unraveling microdamage’s contribution to fracture risk and bone health.

The rib serves as an ideal location for such a study. Unlike the long bones of the appendicular skeleton that undergo a wide range of dynamic loads, mechanical loading in human ribs is likely primarily cyclic in nature due to the regular demands of respiration (Agnew and Stout, 2012; Kondo et al., 2000). Additionally, the rib is highly susceptible to metabolic and physiological changes (Frost, 1969), making it a worthy location to examine the effects of slowed remodeling and decreased activation frequency with increased age.

The objective of this study is to identify variability in vivo microdamage accumulation in the ribs between and within elderly individuals. Such variation could elucidate a potential mechanism of differential fragility. Using en bloc staining and fluorescent microscopy techniques, we specifically considered whether significant differences in microcrack accumulation exist (1) between individuals, (2) between left and right bilateral ribs within individuals, and (3) between the pleural and cutaneous cortices within a rib.
2. Materials and methods

Ribs from post-mortem human subjects (PMHS) received at The Ohio State University’s Whole Body Donation Program were used for this research. The sample includes five males and five females aged from 76 to 92 years (mean = 84.7 years). The presence of preexisting thoracic trauma was the only exclusion criterion, therefore the sample utilized is random and assumed to be representative of a “typical” elderly population.

Bilateral sixth ribs were dissected, complete from head to sternal end. All soft tissue, including the periosteum, was carefully removed and two-centimeter transverse segments were cut at 50% of the rib length along its curvature with a Dremel grinding disc. Undecalciﬁed segments were rinsed of marrow and stained en bloc using 1% basic fuchsin hydrochloride (JT Baker) in a series of graded ethanol solutions (70, 80, 90, and 100%), a protocol derived from Burr and Condon (pers. comm.). This method allows staining of microcracks which existed in vivo and excludes those that may be processing artifacts (Burr and Stafford, 1990). Stained bone segments were subsequently embedded in methyl methacrylate and two transverse sections (~80 μm) per rib at the 50% location were sectioned and mounted with Eukitt mounting medium.

Utilizing an Olympus BX51 microscope, overlapping bright ﬁeld images were taken at 100 × magniﬁcation across the entire rib cross-section with a Diagnostic Instruments™ digital camera to form a composite cross-sectional image of each rib section. Cortical Area (Ct.Ar) and Porosity Area (Po.Ar) were manually traced on the composite with SPOT™ Advanced imaging software and a digital tablet, with veriﬁcation through live microscopy. Porosity was deﬁned as any vascular or resorption spaces within the cortex, excluding canaliculi and osteocytic lacunae, and was subtracted from the Ct.Ar to create a Bone Area (B.Ar) variable that represents only the absolute amount of cortical bone within a transverse section.

The MomentMacro plug-in (Ruff, no date) for ImageJ software (NIH) was utilized to deﬁne the centroid and principle bending axes, after which the primary axis was referenced to systematically separate the pleural from the cutaneous cortex for further data analysis (see Agnew and Stout, 2012).

All microcracks present in the cortical bone of each rib section were identiﬁed at 400 × magniﬁcation using a FITC ﬁlter and epifluorescent light (Fig. 1). The position of each was marked on the parent composite image to ensure every crack was located and none were counted twice. Microcrack variables were quantiﬁed using Microsuite digital software for each of the two sections and an average was subsequently used. Variables were deﬁned according to Burr and colleagues (Burr et al., 1998), with the exception of the use of B.Ar instead of Ct.Ar to calculate crack densities. Variables are:

1) Crack Number (Cr.N); numerical count of all linear microcracks
2) Crack Length (Cr.Le); mean length of all linear microcracks
3) Crack Density (Cr.Dn); calculated as Cr.N/B.Ar
4) Crack Surface Density (Cr.S.Dn); calculated as Cr.N × Cr.Le/B.Ar

A natural log transformation resulted in normally distributed data, for which subject and side were analyzed in a two-way mixed model per cortex using analysis of variance (ANOVA). A paired t-test was then used to further assess differences between cortices. Signiﬁcance was set at 0.05 for all statistical tests.

3. Results

Table 1 provides subject information and subject-level means for each variable. Cr.Le, Cr.Dn, and Cr.S.Dn were found to vary signiﬁcantly between subjects for each cortex (p < 0.05), while no differences were identiﬁed between right and left side paired ribs (p > 0.4; Table 2). Furthermore, no signiﬁcant diﬀerence were found in Cr.Le, Cr.Dn, or Cr.S.Dn between pleural and cutaneous cortices (paired t-test results, p = 0.88, p = 0.19, and p = 0.16, respectively). However, in all instances the cutaneous cortex values were higher than those of the pleural cortex. Cr.Le values for the cutaneous cortex are only slightly greater than those of the pleural cortex (39.48 and 39.26, respectively), while the difference in crack densities was greater. Mean Cr.Dn was 37.96 (±16.36) in the cutaneous cortex and 31.93 (±11.72) in the pleural, while mean Cr.S.Dn was 1536 (±742) in the cutaneous and 1265 (±565) in the pleural cortex.

4. Discussion

This study presents preliminary evidence for variation in microcrack accumulation in a sample of sixth rib pairs from 10 elderly individuals. The results show signiﬁcant differences in the presence of microcracks in size and areal density measures between individuals. Under the assumption that similar loading conditions are present for sixth ribs of all individuals, these inter-subject differences are likely attributable to differential crack initiation/propagation or related differences in repair rates (i.e., bone turnover). Furthermore, the lack of signiﬁcant results when comparing paired ribs within an individual supports the notion that systemic factors play a role in the accumulation of skeletal microdamage.

While some researchers have quantiﬁed in vivo microdamage in human long bones (Schaffer et al., 1995; Norman and Wang, 1997; Ziopoulos, 2001), to our knowledge, no direct attempt to quantitatively assess in vivo measures of linear microcracks in human ribs has been made. Of the few studies that do report in vivo microcrack data for the ribs, though they do so incidentally, most have found much lower amounts of linear microcracks, as well as shorter microcracks, than those presented here (Frost, 1960; Burr and Stafford, 1990). The higher values reported here are attributed in part to increased age of the
sample (mean age of 84.7 years) and the high magnification (400×) used for this study. The elderly age of our sample increases the likelihood of compromised repair mechanisms, potentially leading to increased microcrack accumulation. Additionally, higher magnification improves visual resolution. What appears as a long, singular microcrack under low magnification, often under high magnification reveals itself to be multiple, shorter cracks.

Another factor that likely influenced our results is the use of B.Ar rather than Ct.Ar when calculating areal density values. B.Ar is smaller than Ct.Ar, measuring the true bone in a cross-section by excluding the surface area of all porous spaces. A paired t-test between these variables for this sample showed the difference to be significant (p < 0.01), so it is sensible that using B.Ar to calculate density measures will result in inflated numbers when compared to previously published studies. Prior work has suggested utilizing B.Ar to calculate crack densities as it is a more biologically correct measure of altered bone quality, quantified in the context of Po.Ar, which has also shown significant variation between individuals and contributions to differential fragility (Agnew and Stout, 2012; Dominguez et al., 2016). Additionally, the lack of validated descriptions and criteria for counting and measuring microcracks may also contribute to quantitative differences between this and previous studies since there are currently no established standards in existence.

Lastly, we note that linear microcracks are neither the only type of microdamage to form in bone, nor the most prevalent. Other microdamage types, such as diffuse damage that can itself lead to linear microcracks, comprise a larger percentage of bone microdamage, but cannot be detected with light microscopy, requiring instead more powerful methods such as confocal analysis (Skedros et al., 2011). Despite the limitations imposed by considering only one type of microdamage, the results presented here are illuminating. Diffuse damage, another common form of microdamage, tends to occur at lower magnitudes and under tensile loads, rather than linear microcracks, which usually derive from compression and shear loading modes (Diab and Vashishth, 2005; Diab and Vashishth, 2007). As such, linear microcracks are thought more detrimental to bone than other damage types, resulting in compromised material properties and a greater propensity to fracture (Burr et al., 1998). Furthermore, examining the spatial distribution and differences in crack length may help inform our understanding of strain mode and/or magnitude experienced in human ribs (Boyce et al., 1998). The specific biomechanical loading environment of human ribs associated with breathing has yet to be established. Thus, studying linear microcracks can contribute to studies of mechanical adaptation and fracture etiology, though future work should also use confocal microscopy or similarly advanced techniques to fully assess all forms of microdamage in the human rib.

This study indicates that significant variability in microdamage accumulation between elderly individuals could contribute to differential fracture risk and merits further investigation, including expanding the study to include a wider age range to gauge the occurrence of in vivo microdamage across the lifespan. As a component of bone quality, a multi-faceted concept that considers the structural and material properties of bone to define bone health, understanding the role of microdamage and its developmental and repair mechanisms in bone holds great promise for improving our understanding of fracture risk (Seeman and Delmas, 2006). Illustrating this point, the subject in this study with the greatest amount of bone (Subject F), also has the highest Cr.Dn (Table 1), supporting the notion that future research should further investigate the role of bisphosphonates in preserving bone area/volume by suppressing remodeling and the potential impact of the resulting increased microdamage on rib fragility in humans. Additionally, since rib pairs were found to vary by subject but not side, future work may use corresponding bilateral pairs for experimental studies directly measuring bone strength, maintaining one side as a control.

**Author contributions**

A. Agnew designed the study and performed all data collection. V. Dominguez prepared the draft manuscript and provided interpretation of results. P. Sciulli was responsible for statistical analysis of the data. S. Stout provided crucial input on study design, methods, and interpretation. All authors contributed intellectual content and approved the final version of the paper. All authors agree to be held accountable for the study.

**Conflict of interest**

The authors declare that they have no conflict of interest.

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**Table 1**

| Subject | Age yrs | Sex | B.Ar mm² | Cr.N # | Cr.Le μm | Cr.Dn # mm⁻² | Cr.S.Dm μm mm⁻² |
|---------|---------|-----|----------|--------|----------|--------------|-----------------|
| A       | 88      | M   | 16.10    | 734.5  | 48.018   | 45.598       | 2189.524       |
| B       | 92      | F   | 11.25    | 366.5  | 36.414   | 32.559       | 1185.594       |
| C       | 77      | F   | 13.86    | 696.5  | 39.248   | 33.746       | 1105.259       |
| D       | 76      | M   | 14.72    | 370    | 40.618   | 25.131       | 1020.773       |
| E       | 91      | F   | 12.24    | 282.25 | 36.375   | 23.041       | 838.127        |
| F       | 83      | M   | 34.04    | 1829   | 44.357   | 32.467       | 2382.660       |
| G       | 90      | M   | 23.40    | 917.25 | 43.153   | 39.392       | 1691.218       |
| H       | 82      | M   | 19.24    | 545.25 | 37.705   | 32.372       | 1068.070       |
| I       | 88      | F   | 13.07    | 203.5  | 39.098   | 15.568       | 608.679        |
| J       | 80      | F   | 16.71    | 564    | 32.753   | 33.746       | 1105.259       |
| Mean    | 84.7    |     | 17.46    | 650.87 | 39.774   | 34.711       | 1406.132       |

* Data presented are means of right and left 6th ribs per subject.

**Table 2**

| Cr.N, Cr.Le, Cr.Dn, Cr.S.Dm | Cutaneous | Pleural |
|----------------------------|-----------|---------|
| DF                         | F-stat    | p-value | F-stat    | p-value  |
| Crack length (Cr.Le)       |           |         |           |         |
| Side                      | 1         | 0.310   | 0.676     | 0.021   | 0.909   |
| Subject                   | 8.884     | **0.001** | 4.804     | **0.014** |
| Interaction               | 0.668     | 0.721   | 1.458     | 0.291   |
| Crack density (Cr.Dn)     |           |         |           |         |
| Side                      | 1         | 0.166   | 0.753     | 1.457   | 0.440   |
| Subject                   | 4.445     | **0.018** | 3.549     | **0.036** |
| Interaction               | 2.693     | 0.078   | 1.655     | 0.232   |
| Crack surface density (Cr.S.Dm) |       |         |           |         |
| Side                      | 1         | 0.058   | 0.849     | 1.114   | 0.482   |
| Subject                   | 4.694     | **0.015** | 4.660     | **0.015** |
| Interaction               | 4.086     | **0.023** | 2.415     | 0.102   |

* Intra-individual variation is expressed as variation between each source “side” to assess directional differences. Inter-individual variation is expressed as variation between each source “subject.” The “interaction” refers to the effect of one independent source variable (i.e., side or subject) being influenced by the other. Bolded p-values indicate statistical significance at the α < 0.05 level.
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