Anabolic action of parathyroid hormone (PTH) does not compromise bone matrix mineral composition or maturation

Christina Vrahnas^{1,2}, Thomas A Pearson^{1,2}, Athena R Brunt³, Mark R Forwood³, Keith R Bambery⁴, Mark J Tobin⁴, T John Martin^{1,2} & Natalie A Sims^{1,2}

¹St. Vincent's Institute of Medical Research, Fitzroy, Victoria, Australia

²The University of Melbourne, Department of Medicine at St. Vincent's Hospital, Fitzroy, Victoria, Australia

³School of Medical Science, Griffith University, Gold Coast, Queensland, Australia

⁴Australian Synchrotron, Clayton, Victoria, Australia

Corresponding author: Natalie A Sims

9 Princes St, Fitzroy, Victoria 3065, Australia

Email: nsims@svi.edu.au

Phone: +613-9288-2555

Fax: +613-9416 2676

Abstract

Intermittent administration of parathyroid hormone (PTH) is used to stimulate bone formation in patients with osteoporosis. A reduction in the degree of matrix mineralisation has been reported during treatment, which may reflect either production of undermineralised matrix or a greater proportion of new matrix within the bone samples assessed. To explore these alternatives, high resolution synchrotron-based Fourier Transform Infrared Microspectroscopy (sFTIRM) coupled with calcein labelling was used in a region of non-remodelling cortical bone to determine bone composition during anabolic PTH treatment compared with region-matched samples from controls.

8 week old male C57BL/6 mice were treated with vehicle or 50µg/kg PTH, 5 times/week for 4 weeks (n = 7-9/ group). Histomorphometry confirmed greater trabecular and periosteal bone formation and 3-point bending tests confirmed greater femoral strength in PTH-treated mice. Dual calcein labels were used to match bone regions by time since mineralisation (bone age) and composition was measured by sFTIRM in six 15µm² regions at increasing depth perpendicular to the most immature bone on the medial periosteal edge; this allowed *in situ* measurement of progressive changes in bone matrix during maturation.

The sFTIRM method was validated in vehicle-treated bones where the expected progressive increases in mineral:matrix ratio and collagen crosslink type ratio were detected with increasing bone maturity. We also observed a gradual increase in carbonate content that strongly correlated with an increase in longitudinal stretch of the collagen triple helix. PTH treatment did not alter the progressive changes in any of these parameters from the periosteal edge through to the more mature bone.

These data provide new information about how the bone matrix matures *in situ* and confirm that bone deposited during PTH treatment undergoes normal collagen maturation and normal mineral accrual.

Introduction

Parathyroid hormone (PTH) is the only current clinically available pharmacological agent that increases bone mass in patients with osteoporosis ⁽¹⁾. This is achieved by direct actions on the osteoblast lineage: promoting precursor differentiation ⁽²⁾, preventing mature osteoblast apoptosis ⁽³⁾, activating lining cells ⁽²⁾, and inhibiting sclerostin production by osteocytes ^(4,5). Bone formation involves two sequential events: deposition of Type I collagen-rich organic matrix (osteoid), and mineralisation by hydroxyapatite crystal formation within that matrix ⁽⁶⁾. While PTH treatment increases osteoid production, lower mineral levels in bone samples from PTH-treated rats, monkeys and humans have raised concerns that the mineralisation process may be compromised in the presence of PTH ⁽⁷⁻⁹⁾.

Early studies in ovariectomised rats reported a reduction in ash weight in PTH-treated samples which seemed paradoxical ⁽⁷⁾. Later work using backscattered electron microscopy indicated a lower average degree of bone tissue mineralisation in samples from rats treated with PTH ⁽⁸⁾. Cortical bone of PTH-treated monkeys showed a lower degree of mineralisation, crystallinity and collagen crosslinking than untreated controls on both periosteal and endosteal surfaces ⁽⁹⁾. Biopsies from patients with osteoporosis treated with PTH showed significantly less matrix mineral, mineral crystallinity and collagen crosslinking in both cortical and trabecular bone compared to biopsies from placebo-treated patients ⁽¹⁰⁾. Low mineralisation density was also observed in trabecular bone from PTH-treated patients ⁽¹¹⁾, and mice also showed reduced crystallinity in the cortical and trabecular matrix deposited during PTH treatment ⁽¹²⁾.

The mineralisation process has two phases. Within ~5-10 days, osteoid undergoes primary mineralisation with initiation being controlled by mature osteoblasts ⁽¹³⁾. Over subsequent weeks, months and years, secondary mineralisation occurs with mineral continuing to accumulate at a slower rate ⁽¹⁴⁾. Low mineral content in PTH treated bone may relate to a slower progression of primary or secondary mineralisation. Alternatively, a higher rate of remodelling may not allow sufficient time for secondary mineralisation. More recently, it has been reported that regions with lower mineralisation density in biopsies from PTH-treated patients exist primarily at sites of new bone tissue formation ⁽¹¹⁾. This provides support for the concept that the lower

mineral levels in PTH-treated bone result from a greater proportion of newly formed (less mineralized) bone in each sample, rather than a change in matrix composition *per se*, since analysis regions used in the above studies were not corrected for a greater proportion of new bone.

We tested whether the progression of bone matrix mineralisation is altered by PTH and accounted for differences in the proportion of newly formed bone by assessing regions matched for bone age. To do this, we combined *in vivo* calcein-labelling (to identify bone commencing mineralisation at specific times) with synchrotron-based Fourier-transform infrared microspectroscopy (sFTIRM). The sFTIRM method allows *in situ* measurement of mineral composition, and collagen structure without protein denaturation or bone maceration^(15,16). The highly focused infrared beam provided by the synchrotron light source⁽¹⁷⁾ allows us to collect high signal-to-noise IR spectra from smaller regions than those measured by laboratory based instruments. By also coupling this to a fluorescence microscope, we are able to measure at sites identified by calcein labels. The calcein labels identify bone that has recently undergone primary mineralisation, thereby allowing us to compare bone at the same level of mineral maturity (bone age) between PTH-treated mice and controls. We have measured bone maturation in murine cortical bone; since rodent cortical bone lacks Haversian remodelling⁽¹⁸⁾, at the tibial diaphyseal periosteum, a site that lacks osteoclasts⁽¹⁹⁾. This allows measurement of continually maturing bone matrix without changes caused by recent osteoclast activity or remodelling cycles, making it possible to measure uninterrupted primary and secondary mineralisation.

Materials and Methods

Animals

8-week old male mice (26.8 ± 0.7 g) on a C57BL/6 background were randomized to two treatment groups; 9 mice were administered vehicle (2% heat inactivated (HI) mouse serum + saline), and 10 mice administered PTH (1-34) ($50 \mu\text{g/kg}$) (Bachem, Bubendorf, Switzerland) by intraperitoneal injection 5 days a week for 4 weeks, as previously described^(20,21). Mice were kept in an SPF facility, weighed daily and injection volumes adjusted according to weight changes over the 4-week treatment period; all injections were carried out in the morning. Animals were housed in a 12-hour light and dark cycle with standard food and water provided *ad libitum*. Calcein (20mg/kg) was administered by intraperitoneal (IP) injection 7 and 2 days before tissue collection. After the treatment protocol, mice were fasted for 12 hours and one hour after the last injection mice were anesthetized with ketamine/xylazine and killed by cervical dislocation. At tissue collection, all samples were randomized and labelled with non-identifiable numbers so that all subsequent observations were blinded to the treatment group; treatment groups were revealed after all laboratory-based analyses were complete. All animal procedures were conducted with approval from the St. Vincent's Health Melbourne Animal Ethics Committee and complied with the Australian Code for the Care and Use of Animals for Scientific Purposes. No adverse events were observed.

Micro-computed tomography (micro-CT)

Ex vivo micro-CT was performed on femora using the SkyScan 1076 system (Bruker-microCT, Kontich, Belgium); one PTH-treated femur was broken during collection and was excluded. Images were acquired using the following settings: $9 \mu\text{m}$ voxel size, 0.5mm aluminium filter, 50 kV voltage, and $100 \mu\text{A}$ current, 2600 ms exposure time, rotation 0.5 degrees, frame averaging =1. Images were reconstructed and analysed using SkyScan software programs NRecon (version 1.6.9.8), Dataviewer (version 1.4.4) and CT Analyser (version 1.11.8.0). Cortical analyses were performed in a region that was 15% of total bone length commencing 30% above the distal femoral end toward the femoral mid-shaft. Cortical bone was analysed using global thresholding (81-255) in CT Analyzer.

3-point bending

After microCT analysis, anteroposterior (A-P) and mediolateral (M-L) femoral widths were measured with digital calipers at the mid-shaft. Structural and material properties were identified by three-point bending tests, as previously described ⁽²²⁾. Data from one vehicle-treated femur was excluded due to an abnormal loading curve resulting from rotation of the bone during testing. Load was applied to the femoral mid-shaft anterior surface between two supports located 6.0 mm apart. Load-displacement curves were recorded at 1.0 mm/s crosshead speed using an Instron 5565A dual column material testing system, and Bluehill 2 software (Instron, Norwood, MA, USA). Ultimate force (N), ultimate deformation (mm) and energy absorbed to failure (mJ) were measured from the load-displacement curves. Combining the geometric calculations and the biomechanical test results, material properties were calculated for each bone as described by Schriefer and colleagues ⁽²³⁾ to obtain ultimate stress (MPa), elastic modulus (MPa), and toughness (MPa).

Histomorphometry

Tibiae were fixed by immersion in cold 4% paraformaldehyde overnight and embedded in methyl methacrylate resin as described previously⁽²⁴⁾. Periosteal parameters (cortical thickness (Ct.Th), periosteal mineral apposition rate (Ps.MAR), periosteal mineralising surface/bone surface (Ps.MS/BS), periosteal bone formation rate/bone surface (Ps.BFR/BS)) were measured on sagittal tibial sections commencing 1500 μm distal to the growth plate hypertrophic zone, as previously described ⁽²⁵⁾. Trabecular parameters (mineral apposition rate (MAR), mineralising surface/bone surface (MS/BS), bone formation rate/bone surface (BFR/BS), osteoid surface/bone surface (OS/BS), osteoid thickness (O.Th), osteoblast surface/bone surface (ObS/BS) and osteoclast surface/bone surface (OcS/BS)) were measured on the same tibial sections in the proximal secondary spongiosa, commencing 370 μm below the growth plate, in a 1110 μm^2 region, as previously described ⁽²⁵⁾ (Osteomeasure; Osteometrics, Atlanta, GA, USA).

Synchrotron-based Fourier Transform Infrared Microspectroscopy (sFTIRM)

sFTIRM data were collected using a Bruker Hyperion 2000 IR microscope coupled to a V80v FTIR spectrometer located at the IR Microspectroscopy beamline at the Australian

Synchrotron. The Hyperion microscope was equipped with an epifluorescence accessory suitable for calcein visualisation (excitation/emission $\sim 490\text{nm}/\sim 515\text{nm}$). Thin $3\text{ }\mu\text{m}$ sagittal sections were taken from the same methyl methacrylate (MMA)-embedded tibial blocks used for histomorphometry. Sections were placed on 22 mm diameter $\times 0.5\text{ mm}$ polished barium fluoride (BaF_2) windows (Crystan Limited, UK) for sFTIRM analysis. IR absorbance spectra were recorded in transmission mode and collected in the mid-IR region from 750 to 3850 cm^{-1} using a mercury cadmium telluride detector, at 8 cm^{-1} spectral resolution and 128 co-added scans/pixel spectral resolution. All data acquisition was undertaken with OPUS version 6.5 and data analysis completed with OPUS version 7.2 (Bruker Optik, Germany).

For each tibial section, the microscope video camera was used to image the cortical diaphysis ($1500\text{ }\mu\text{m}$ from the base of the growth plate hypertrophic zone), the same location used for histomorphometric periosteal apposition measurements (Figure 1B). We have previously observed that this is a site of bone modelling, with continuous periosteal apposition, from at least 6 weeks of age⁽¹⁹⁾. Spectra were collected from 3 regions matched for bone age by superimposing regions for measurement on the calcein labels incorporated during mineralisation at 2 days (region 1), and 7 days before tissue collection, respectively, on each sample. An intermediate zone equally spaced between the two calcein labels was also selected for analysis (region 2). Additional measurement regions then progressed, evenly spaced, into the bone matrix (regions 4 – 6) (Figure 1C). Based on the known periosteal appositional rate, we termed these zones primary mineralisation (regions 1-3) and secondary mineralisation (regions 4-6). sFTIRM mapping was performed with the synchrotron source, using a $15 \times 15\text{ }\mu\text{m}$ aperture. For each treatment group, marked regions were spaced according to distance between calcein labels. Data were collected in transmission mode, with a background spectrum collected through clear BaF_2 . For each sample an additional MMA reference spectrum was collected from within the embedding material.

After acquisition, raw spectra for each region and sample were baseline corrected using a 3-point baseline at 3 wavenumbers: 1800 , 800 , and the closest minimum to 1200 cm^{-1} using OPUS 7.2. Residual MMA absorbance peaks were then subtracted using the relevant MMA reference spectrum for each sample by iterative manual subtraction in

OPUS 7.2. A residual 1730 cm^{-1} MMA band remained after MMA subtraction that was excluded from analysis. Spectroscopic parameters calculated were integrated peak areas of the following peaks: Phosphate (mineral) (1180-916 cm^{-1}), amide I (matrix) (1588-1712 cm^{-1}), amide II (1600-1500 cm^{-1}) and ν_2 carbonate (890-852 cm^{-1}). Ratios of these integrated peak areas were calculated as follows: mineral:matrix ratio⁽²⁶⁾ (1180-916 cm^{-1} /588-1712 cm^{-1}), carbonate:mineral ratio⁽²⁷⁾ (1180-916 cm^{-1} /890-852 cm^{-1}) and amide I:II ratio⁽²⁸⁾ (1588-1712 cm^{-1} /1600-1500 cm^{-1}). Sub-bands from the amide I, amide II and phosphate peaks were analysed by spectral curve fitting using Grams /AI (Version 9.2, Thermo Scientific, USA). Briefly, individual spectra were base corrected according to wavenumber limits of the sub-bands of interest (amide I and II peaks for 1660 cm^{-1} and 1690 cm^{-1} sub-bands and phosphate peaks for 1030 cm^{-1} , 1020 cm^{-1} , 1127 cm^{-1} and 1096 cm^{-1} sub-bands. The second-derivative of each peak was used to estimate subpeak positions that were then curve-fitted using 500 maximum iterations with an auto limit fix function. Subpeak integrated areas were reported as collagen crosslinking (1660:1690 cm^{-1})⁽²⁹⁾, crystallinity (1030/1020 cm^{-1})⁽³⁰⁾ and acid phosphate content (1127/1096 cm^{-1})⁽³¹⁾ ratios.

Statistics

All graphs show mean $\pm$ SEM; sample number (n) is reported in the figure legends; one section was analysed per mouse. Statistical significance was determined by unpaired t-tests for histomorphometric and microCT analysis, and two-way ANOVA with Fisher's LSD test for sFTIRM-derived data (GraphPad Prism 7 (version 7.0a)). $p < 0.05$ was considered statistically significant. To relate indices of collagen crosslinking and stretch to mineral accrual, data from vehicle-treated animals, including all regions of analysis was tested with a straight line-of-best fit least-squares model and GraphPad Prism 7 (version 7.0a).

Results

Confirmation of model: PTH treatment increased osteoblast activity, bone size and bone strength

Histomorphometric trabecular bone analysis in the secondary spongiosa confirmed that 8 week old male mice treated with PTH at 50 $\mu\text{g/kg/day}$ for 4 weeks showed

significantly greater trabecular mineral apposition rate (MAR) and bone formation rate (BFR/BS) (Figure 2A, C) than vehicle treated age- and sex-matched controls as previously reported ^(13,20,21,25). PTH treatment was also associated with a greater osteoblast surface (ObS/BS), thicker osteoid (O.Th) and greater osteoid surface (OS/BS) within the trabecular bone than in vehicle-treated controls (Figure 2D-F). Osteoclast surface (OcS/BS) was also significantly greater in PTH treated mice (Figure 2G).

Cortical thickness (Ct.Th), area (Ct.Ar) and anteroposterior (A-P) widths were also greater in PTH-treated mice compared to vehicle-treated controls (Figure 3A-D). The greater cortical dimensions in PTH-treated mice were reflected in a greater moment of inertia (Figure 3E) and significantly stronger bones, indicated by a greater ultimate force than vehicle-treated controls by 3-point bending tests (Figure 3F-G). However, material properties normalized for bone size such as ultimate stress (Figure 3H), ultimate strain and toughness were not different in PTH treated samples compared to vehicle-treated controls (Table 1).

Periosteal mineral apposition rate (Ps.MAR, mineralizing surface/bone surface (Ps.MS/BS) and bone formation rate/bone surface (Ps.BFR/BS) measured by histomorphometry on the medial tibial mid-shaft were all significantly greater with PTH treatment (Figure 3I-K). Tibial cortical thickness was significantly greater in PTH-treated mice compared to controls (Mean $\pm$ SEM, w/w: $136.01 \mu\text{m} \pm 5.01$, f/f: $155.61 \mu\text{m} \pm 6.54^*$; $*p < 0.05$). This confirmed that the PTH treatment protocol increased bone formation at the periosteum.

Mineralized bone matrix maturation in vehicle- and PTH-treated periosteum

Representative IR absorbance spectra taken at increasing depths from the periosteum of a vehicle-treated mouse demonstrated a gradual increase in phosphate peak height with increasing bone maturity (Figure 4A).

Phosphate (mineral) and amide I (protein content) peak areas indicated that the mineral:matrix ratio increased rapidly for the first 4 regions from the commencement of mineralisation at the first calcein label, then progressed more slowly from regions 4 to 6; mineral:matrix ratio was approximately doubled from regions 1 to 6 (Figure 4B). When matched for bone age, similar changes were observed in maturing bone of PTH-

treated mice to the observations in vehicle-treated mice; there were no significant effects of PTH treatment on any FTIRM-derived markers of bone matrix composition. As observed in vehicle-treated mice, periosteal bone from PTH-treated mice demonstrated a significant and gradual increase in the mineral:matrix ratio (Figure 4B) that approximately doubled between regions 1 and 6.

A similar observation was made in the carbonate:mineral ratio, which reached a plateau within the more mature bone from region 4 onwards in samples from both vehicle- and PTH-treated mice (Figure 4C). The increase in carbonate:mineral ratio between the most immature and most mature bone did not differ significantly between PTH- and vehicle-treated mice.

Crystallinity, which represents the relative amount of crystal size and perfection^(32,33), was highly variable during bone maturation in both treatment groups. Vehicle-treated mice showed a gradual increase (from regions 2-4) then a decrease (from regions 4-5) in the mature regions of bone (Figure 4D). In contrast to the decreased crystallinity between regions 4 and 5 in vehicle-treated mice, the mature regions of bone (region 5) in PTH-treated mice demonstrated increased crystallinity compared to the immature regions of bone (regions 1 and 3). This crossover in the curves between regions 4 and 5 was significant, as indicated by a significant interaction between region and treatment. A lower level of crystallinity in bones from PTH-treated mice was detected in region 4 compared to vehicle (Figure 4D). Acid phosphate content was not significantly modified by bone age or by PTH treatment (Figure 4E).

The amide I:II ratio significantly reduced with increasing depth from the periosteal edge (Figure 5A,B). This was observed in bones from both vehicle- and PTH-treated mice; there was no significant effect of treatment. From region 4 onwards, collagen crosslinking was significantly greater than at the periosteal edge (Figure 5C); again, this was observed in bones from both vehicle- and PTH-treated mice and there was no significant effect of treatment.

Relationships between mineral accrual and collagen fibre orientation

The correlation matrix of data obtained from vehicle-treated samples confirmed a significant relationship between the magnitudes of the amide I:II, mineral:matrix,

carbonate:mineral ratios and collagen crosslinking and the regions of bone analysed (Table 2). In addition to significant relationships that exist between parameters derived from the same peaks (e.g. amide I:II and mineral:matrix both contain data from the amide I peak), we observed a novel highly significant negative correlation between the amide I:II and carbonate:mineral ratios, and between the amide I:II ratio and acid phosphate content (Figure 6). The relationship between amide I:II and carbonate:mineral ratios was significant, not only when the full spectrum of regions was included in the analysis, but also when analysis was restricted to regions 2, 4 and 6 (Region 2: $p = 0.034$, $R = -0.746$; Region 4: $p = 0.004$, $R = -0.875$; Region 6: $p = 0.045$, $R = -0.717$).

Discussion

Anabolic PTH treatment does not alter the progression of bone matrix maturation. Neither the rate of mineral accumulation, nor mineral composition, collagen crosslinking or collagen fibre alignment was modified in bones from PTH treated mice compared to bone age matched vehicle-treated samples. Three point-bending tests also indicated that the greater bone strength induced by PTH in this model could not be detected after normalizing for the altered bone geometry, and was therefore fully explained by the increase in cortical dimensions.

We used timed calcein labels and high resolution synchrotron-light based FTIRM to identify newly mineralised bone on the periosteal surface, and to measure bone composition while controlling for the greater amount of bone deposited during PTH treatment. In addition, assessment of this process in murine cortical bone, which does not undergo Haversian remodelling, has allowed us to assess the continual process of matrix maturation, including both primary and secondary mineralisation processes. When accounting for bone age, we observed that PTH did not change the normal progression as bone matrix matures from the periosteal edge through to the more mature cortical bone, and that earlier reports of lower bone mineral content, crystallinity and collagen crosslinking can be explained by the greater proportion of immature bone in PTH-treated samples.

The use of high resolution synchrotron light allows quantification of bone composition in small regions ($15\ \mu\text{m}^2$). This has allowed us to study the normal progression of bone matrix maturation *in situ*, and define the significant changes in both bone mineral and collagen fibre orientation that occur as bone matures. With respect to bone mineral, we observed a gradual increase in mineral:matrix ratio and in the proportion of carbonate to mineral. The increase in mineral:matrix ratio, indicating mineral incorporation into the bone matrix, has previously been described in rabbit, rat, and baboon cortical bone (14,34,35), and within human osteonal bone (32,36). The lower slope of this parameter in the more mature regions of bone is consistent with initial rapid primary mineralisation followed by slower accumulation of mineral. The rise in carbonate:mineral ratio with increasing distance from the periosteum in both treatment groups suggests that carbonate substitution occurs in hydroxyapatite as cortical bone ages. This is consistent

with early observations that carbonate content is higher in bone samples from older animals, noted in chicken ⁽³⁷⁾, rat and bovine cortical bone ^(38,39). Earlier studies of *in vitro* crystallisation also showed increased carbonate substitution with increasing maturity of apatite crystals ⁽³⁷⁾. Our data provides the first *in situ* evidence that carbonate substitution occurs during the normal process of bone matrix maturation in healthy young bone. In contrast, in human osteonal bone, a lowering in the carbonate:mineral ratio has been observed as mineral:matrix ratio increases from the centre of an osteon to the periphery ⁽³⁶⁾. The reason for this difference is not yet clear. It may relate to differences in the type of bone: the present study measured bone in an area of modelling, without prior resorption, while the human osteon data could reflect changes in bone that has undergone remodelling, and has therefore been resorbed and replaced by less mature matrix.

As bone matrix became mineralised, we also observed changes in the collagen matrix: a decrease in amide I:II ratio in samples from both PTH and vehicle treated mice. This novel observation indicates that collagen fibre orientation changes as bone matrix becomes more heavily mineralised. While the amide I peak reflects the vibration of C=O bonds running perpendicular to the collagen helical axis, the amide II peak reflects vibration of the C-N bond along the collagen triple helix axis ⁽⁴⁰⁾. Sensitivity of peak intensities to functional group orientation requires the use of polarised infrared radiation, or placement of an infrared polariser between the sample and detector ⁽⁴⁰⁾. The observation of collagen orientation reported here arises from the inherent polarisation of the synchrotron IR beam, whereby the electric vector at the microscope stage is aligned approximately perpendicular to the orientation of the femur sections, (left to right in Figure 1). The amide I:II ratio therefore reflects the proportional change in perpendicular (amide I) to parallel (amide II) stretch of the collagen triple helix (See Figure 5B)⁽⁴⁰⁾. This suggests that collagen fibres stretch along their longitudinal orientation during the process of mineral accrual, even after mineralisation is initiated. The robust correlation of carbonate:mineral ratio with amide I:II is strongly supportive of this concept (it should be noted that although the correlation of mineral:matrix and amide I:II is even stronger, this is largely because the amide I peak is represented in both parameters). Acid phosphate content was also significantly correlated with amide I:II ratio, showing a significant increase with increasing collagen stretch. Whether the

collagen fibres stretch in response to hydroxyapatite crystal growth, or their stretching determines crystal size is not known.

The present study demonstrates that with increasing matrix maturity, in addition to longitudinal collagen stretching, there is an increase in collagen crosslinking. This is consistent with earlier work in remodelling human trabecular bone where normal individuals showed a gradual increase across a similar distance to our measurements here in non-remodelling cortical bone ⁽⁴¹⁾. Collagen's intermolecular crosslinking pattern contributes to bone's tensile strength and elasticity ⁽²⁹⁾, but studying collagen crosslinking during bone maturation has been challenging because of the need to extract protein for mass spectroscopic analysis. By quantifying two specific crosslinks *in situ*, pyridinoline (Pyr) and dehydro-dihydroxylysinoxaline (deH-DHLNL)⁽²⁹⁾, Pyr:deH-DHLNL (1660:1690 cm⁻¹) reflects collagen crosslinking level within the bone matrix ⁽²⁹⁾. In contrast to our results and earlier work in human trabecular bone, biopsies from high turnover osteoporosis patients showed a high 1660:1690 ratio at the mineralised bone edge that declined rapidly, and then increased again ⁽⁴¹⁾. Our observations are similar to that of healthy remodelling human bone ⁽⁴¹⁾. This indicates that regardless of whether it is remodelling or modelling bone, or in the presence or absence of PTH, collagen crosslinking increases with matrix maturity in healthy bone.

The normal composition of bone that we have detected in PTH-treated samples contrasts with previous assessments of bone samples from PTH-treated monkeys ⁽⁹⁾ and humans ⁽¹⁰⁾. These earlier studies, although of high spatial resolution (6-7 µm) quantified FTIR-derived indices of bone composition averaged over a 400 µm² area. Such a large area would include a high proportion of less mature, and therefore, less mineralised bone. Indeed, the authors of those papers suggested that this may have been the explanation for the low proportion of mineralized bone in PTH-treated samples. Our analysis, in much smaller regions of 15 µm², coupled with calcein labelling has allowed us to measure immature and mature bone separately, and to correct for the higher proportion of new bone that exists with PTH treatment. With such correction, there is no difference in the matrix composition in bone from PTH- treated and untreated mice.

To account for the significant increase in tibial cortical thickness induced by PTH treatment, our first three regions of analysis began on the calcein labels which labelled 2 and 7-day old bone on the periosteal surface. Regions 4-6 aligned perpendicularly to the 3rd region. Based on the known mineral appositional rate, bone in regions 5 and 6 is likely to have been formed prior to the intervention of PTH or vehicle treatment, which is a limitation to this study. To restrict the measurements to only the bone formed during the treatment protocol, in future studies, calcein (or a differently coloured label) should be administered prior to the commencement of PTH and vehicle treatment.

In human biopsies, trabecular bone histomorphometry showed new bone formation induced by PTH on both modelling and remodelling surfaces, with a predominant effect on remodelling surfaces ⁽⁴²⁾ accounting perhaps for >70% of new bone ⁽⁴³⁾. Modelling, the process that we have assessed here, also contributes to PTH action, both in humans ⁽⁴³⁾ and in rats and mice ^(2,44,45). Whether bone deposited on modelling surfaces differs in composition from that deposited on remodelling surfaces is not yet known. Since calcein labels are short-lived in mouse trabecular bone, which has very low mineralisation levels, replicating the present study in remodelling murine trabecular bone would be technically challenging. Therefore, it would be useful to attempt a similar study in larger species to measure bone maturation in the presence and absence of PTH in osteonal cortical bone, and in more highly mineralised trabecular matrix. While we observed no change in bone matrix deposited in the presence of PTH in young healthy male animals, the question of whether the quality of bone laid down in the presence of PTH is of the same quality as compromised bone matrix deposited in aged estrogen deficient animals, or aged patients remains unanswered. This too would be a useful follow-up study.

In summary, this study quantified changes in bone composition during bone matrix maturation in normal growth and during PTH treatment in modelling murine bone. We conclude that mineralisation levels, collagen crosslinking and collagen fibre longitudinal stretch all increase during matrix maturation on a modelling surface, and their progression is not altered by PTH treatment. These data confirm that lower mineralisation levels previously observed in PTH-treated bone reflect a higher proportion of newly formed bone, and that bone deposited on the murine periosteal

surface during PTH treatment is not inadequately mineralised but instead undergoes a normal collagen maturation process and normal mineral accrual as vehicle-treated bones.

Author contributions:

Study design: NAS. Study conduct: CV, TAP, ARB, KRB, MJT, and NAS. Data collection: CV, ARB, and NAS. Data analysis: CV, TAP, KRB, and NAS. Data interpretation: CV, MRF, KRB, LPP, MJT, TJM, and NAS. Drafting manuscript: CV, NAS. Revising manuscript content: CV, KRB, MRF, KRB, MJT, TJM, and NAS. All authors approved the final version of the manuscript. NAS takes responsibility for the integrity of the data analysis.

Acknowledgements:

The authors gratefully acknowledge Mrs Ingrid Poulton for expert technical assistance, Dr Michael Gorman for advice on collagen structure, Dr Eleftherios P Paschalis for help with spectral curvefitting, Drs Adele Boskey and Ljudmila Spevak for initial FTIR advice. This work was supported by National Health and Medical Research Council (Australia) Project Grant 1042129. N.A.S. is supported by a National Health and Medical Research Council (Australia) Senior Research Fellowship. St. Vincent's Institute is supported by the Victorian Government's Operational Infrastructure Support Programme. sFTIRM data collection was undertaken on the infrared microspectroscopy (IRM) beamline at the Australian Synchrotron, Victoria, Australia.

Table 1. Additional material strength properties determined by 3-point bending test were unchanged after 8-week old male mice were treated with PTH (50µg/kg/day) for 4 weeks. Mean $\pm$ SEM, n = 7-9/group. No significant differences were observed.

	Vehicle	PTH	P-values
Ultimate Strain (%)	3.05 $\pm$ 0.23	2.58 $\pm$ 0.23	0.1768
Toughness (mJ/mm³)	0.34 $\pm$ 0.03	0.35 $\pm$ 0.05	0.8495
Energy absorbed to failure (mJ)	1.04 $\pm$ 0.11	1.27 $\pm$ 0.20	0.3465

Table 2: Correlation matrix for sFTIRM-derived indices from vehicle-treated mice. Significant differences are noted in bold.

	Mineral: Matrix	Carbonate: Mineral	Collagen crosslinking	Crystallinity	Acid phosphate content	Region
Amide I:II	r²= -0.875 p= 4.4 x 10⁻¹⁶	r²= -0.632 p= 1.418 x 10⁻⁶	r²= -0.665 p= 2.465 x 10⁻⁷	r ² = -0.051 p= 0.732	r²= 0.483 p= 0.001	r²= -0.718 p= 9.231 x 10⁻⁹
Mineral:Matrix		r²= 0.587 p= 1.164 x 10⁻⁵	r²= 0.471 p= 0.001	r ² = -0.064 p= 0.664	r²= -0.424 p= 0.004	r²= 0.721 p= 7.285 x 10⁻⁹
Carbonate:Mineral			r ² = 0.251 p= 0.085	r ² = -0.027 p= 0.854	r²= -0.306 p= 0.043	r²= 0.428 p= 0.002
Collagen crosslinking				r ² = -0.081 p= 0.582	r ² = -0.181 p= 0.241	r²= 0.620 p= 2.602 x 10⁻⁶
Crystallinity					r ² = -0.149 p= 0.334	r ² = -0.190 p= 0.195
Acid phosphate content						r ² = -0.169 p= 0.273

Figure legends

Figure 1. sFTIRM measurement regions on thin tibial sections. **A:** Representative microCT image of tibia indicating the depth at which tibial sections were taken for analysis (black dashed line). **B:** Transmitted visible light micrograph showing the region 1500 μm below the tibial growth plate on the medial side at which FTIR spectra were collected. Scale bar = 250 μm . **C:** Representative fluorescence micrographs showing the six 15 x 15 μm square regions from which spectra were collected on tibial samples from vehicle and parathyroid hormone (PTH) treated animals. Measurement regions commenced at the periosteal edge on the most recent calcein label indicating 2-day old mineralised bone (region 1), between the two labels (region 2), on the calcein label indicating 7-day old mineralised bone (region 3), then progressing into increasing depths of more mature cortical bone (regions 4-6). A methylmethacrylate (MMA) reading was taken in a blank area of section to subtract from bone spectra. Scale bar = 20 μm . Note the greater distance between labels in the PTH-treated sample, and subsequent wider spacing of the measurement regions.

Figure 2. Trabecular bone formation was stimulated by PTH treatment. **A:** Trabecular Mineral Apposition rate (MAR), **B:** Mineralising Surface/Bone Surface (MS/BS), **C:** Bone Formation Rate/Bone Surface (BFR/BS), **D:** Osteoid Surface/Bone Surface (OS/BS), **E:** Osteoid Thickness (O.Th), **F:** Osteoblast Surface/Bone Surface (ObS/BS) and **G:** Osteoclast Surface/Bone Surface (OcS/BS) measured in the secondary spongiosa of tibiae from 8-week old male mice treated with vehicle or PTH (50 $\mu\text{g/kg/day}$) for 4 weeks. N = 7-9/group. Mean + SEM, ** $p < 0.01$, *** $p < 0.001$ vs. vehicle-treated controls.

Figure 3. Effect of PTH treatment on cortical structure, strength and periosteal bone formation. **A,B:** MicroCT-derived measurements of **(A)** femoral cortical thickness (Ct.Th) and **(B)** cortical area (Ct.Ar), **(C-E)** caliper measurements of femoral mid-shaft anteroposterior (A-P) and mediolateral (M-L) widths and moment of inertia. **F:** Mean Load-Deformation curves from 3-point bending tests on femora from 8-week old male mice treated with vehicle (white circles) or PTH (black squares) (50 $\mu\text{g/kg/day}$) for 4 weeks, and derived ultimate force **(G)** and ultimate deformation **(H)** data. **I-K:** Histomorphometric measures of **(I)** Periosteal Mineral Apposition Rate (Ps.MAR), **(J)**

Periosteal Mineralising Surface/Bone Surface (Ps.MS/BS) and (K) Periosteal Bone Formation Rate/Bone Surface (Ps.BFR/BS) from the medial tibial midshaft $n = 8-9/\text{group}$. Mean + SEM, * $p < 0.05$, ** $p < 0.01$ vs. vehicle-treated controls.

Figure 4. Changes in mineral composition with bone age and PTH treatment. A: Representative IR absorbance spectra from one vehicle-treated mouse showing changes in peak height in bone age-specific regions within the tibial cortex (bone age 1=immature to 6=mature, refer to Figure 1C). **B-E:** sFTIRM-derived indices relating to mineral composition, B: Mineral:Matrix ratio, C: Carbonate:Mineral ratio, D: Crystallinity, and E: acid phosphate content in regions 1-6 from 8-week old male mice treated with vehicle (dashed lines) or PTH (solid lines) ($50 \mu\text{g/kg/day}$) for 4 weeks. Wavenumbers are shown in y-axis labels. Values are mean $\pm$ SEM, $n = 7-8/\text{group}$. * $p < 0.05$, ** $p < 0.01$ vs. younger bone age region in vehicle-treated mice. + $p < 0.05$, ++ $p < 0.01$, +++ $p < 0.001$ vs. younger bone age region in PTH-treated mice; Φ , $p < 0.05$ vs vehicle treated. Also shown are p values derived from two-way ANOVA.

Figure 5. Effect of bone age and PTH treatment on collagen orientation and collagen crosslinking within the tibial cortex. sFTIRM indices derived from averaged integrated absorbance peak areas of (A) Amide I:II ratio, (B) Diagram of Amide I and Amide II bonds within a collagen molecule and the direction of stretch detected by sFTIRM, (C) Collagen crosslinking derived from amide I sub peaks. Data from tibial sections of 8-week old male mice treated with vehicle (dotted lines) or PTH (solid lines) ($50 \mu\text{g/kg/day}$) for 4 weeks. Values are mean $\pm$ SEM, $n = 7-9/\text{group}$. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. younger bone age region in vehicle-treated mice. + $p < 0.05$, ++ $p < 0.01$, +++ $p < 0.001$ vs. younger bone age region in PTH-treated mice. Also shown are p values derived from two-way ANOVA.

Figure 6. Relationship between collagen stretch with mineral accumulation and carbonate substitution. Scatterplots of individual data points from vehicle-treated mice showing the relationship between (A) Carbonate:Mineral ratio and Amide I:II ratio and (B) Acid phosphate content and Amide I:II ratio. Shades of grey indicate the region of analysis from which data is derived, with the lightest colour indicating the most immature bone. Black line indicates the line of best fit with a linear regression model,

and dashed lines indicate 95% confidence intervals. R^2 and p values are shown within each graph.

ACCEPTED MANUSCRIPT

References

1. Neer RM, Arnaud CD, Zanchetta JR, et al. Effect of parathyroid hormone (1-34) on fractures and bone mineral density in postmenopausal women with osteoporosis. *The New England journal of medicine*. 2001;344(19):1434-41.
2. Dobnig H, Turner RT. Evidence that intermittent treatment with parathyroid hormone increases bone formation in adult rats by activation of bone lining cells. *Endocrinology*. 1995;136(8):3632-8.
3. Jilka RL, Weinstein RS, Bellido T, Roberson P, Parfitt AM, Manolagas SC. Increased bone formation by prevention of osteoblast apoptosis with parathyroid hormone. *The Journal of clinical investigation*. 1999;104(4):439-46.
4. Bellido T, Ali AA, Gubrij I, et al. Chronic elevation of parathyroid hormone in mice reduces expression of sclerostin by osteocytes: a novel mechanism for hormonal control of osteoblastogenesis. *Endocrinology*. 2005;146(11):4577-83.
5. Keller H, Kneissel M. SOST is a target gene for PTH in bone. *Bone*. 2005;37(2):148-58.
6. Tonna S, Sims NA. Talking among ourselves: paracrine control of bone formation within the osteoblast lineage. *Calcif Tissue Int*. 2014;94(1):35-45.
7. Ejersted C, Andreassen TT, Nilsson MH, Oxlund H. Human parathyroid hormone(1-34) increases bone formation and strength of cortical bone in aged rats. *Eur J Endocrinol*. 1994;130(2):201-7.
8. Kneissel M, Boyde A, Gasser JA. Bone tissue and its mineralization in aged estrogen-depleted rats after long-term intermittent treatment with parathyroid hormone (PTH) analog SDZ PTS 893 or human PTH(1-34). *Bone*. 2001;28(3):237-50.
9. Paschalis EP, Burr DB, Mendelsohn R, Hock JM, Boskey AL. Bone mineral and collagen quality in humeri of ovariectomized cynomolgus monkeys given rhPTH(1-34) for 18 months. *J Bone Miner Res*. 2003;18(4):769-75.
10. Paschalis EP, Glass EV, Donley DW, Eriksen EF. Bone mineral and collagen quality in iliac crest biopsies of patients given teriparatide: new results from the fracture prevention trial. *J Clin Endocrinol Metab*. 2005;90(8):4644-9.
11. Misof BM, Roschger P, Cosman F, et al. Effects of intermittent parathyroid hormone administration on bone mineralization density in iliac crest biopsies from patients with osteoporosis: a paired study before and after treatment. *J Clin Endocrinol Metab*. 2003;88(3):1150-6.
12. Ross RD, Mashiatulla M, Robling AG, Miller LM, Sumner DR. Bone Matrix Composition Following PTH Treatment is Not Dependent on Sclerostin Status. *Calcified tissue international*. 2016;98(2):149-57.
13. Tonna S, Takyar FM, Vrahnas C, et al. EphrinB2 signaling in osteoblasts promotes bone mineralization by preventing apoptosis. *FASEB J*. 2014;28(10):4482-96.
14. Fuchs RK, Allen MR, Ruppel ME, et al. In situ examination of the time-course for secondary mineralization of Haversian bone using synchrotron Fourier transform infrared microspectroscopy. *Matrix Biol*. 2008;27(1):34-41.
15. Miller LM, Vairavamurthy V, Chance MR, et al. In situ analysis of mineral content and crystallinity in bone using infrared micro-spectroscopy of the nu(4) PO(4)(3-) vibration. *Biochimica et biophysica acta*. 2001;1527(1-2):11-9.
16. Huang RY, Miller LM, Carlson CS, Chance MR. In situ chemistry of osteoporosis revealed by synchrotron infrared microspectroscopy. *Bone*. 2003;33(4):514-21.
17. Miller LM, Dumas P. Chemical imaging of biological tissue with synchrotron infrared light. *Biochimica et biophysica acta*. 2006;1758(7):846-57.
18. Baron R, Tross R, Vignery A. Evidence of sequential remodeling in rat trabecular bone: morphology, dynamic histomorphometry, and changes during skeletal maturation. *Anat Rec*. 1984;208(1):137-45.

19. Johnson RW, McGregor NE, Brennan HJ, et al. Glycoprotein130 (Gp130)/interleukin-6 (IL-6) signalling in osteoclasts promotes bone formation in periosteal and trabecular bone. *Bone*. 2015;81:343-51.
20. Walker EC, Poulton IJ, McGregor NE, et al. Sustained RANKL response to parathyroid hormone in oncostatin M receptor-deficient osteoblasts converts anabolic treatment to a catabolic effect in vivo. *J Bone Miner Res*. 2012;27(4):902-12.
21. Takyar FM, Tonna S, Ho PW, et al. EphrinB2/EphB4 inhibition in the osteoblast lineage modifies the anabolic response to parathyroid hormone. *J Bone Miner Res*. 2013;28(4):912-25.
22. Johnson RW, Brennan HJ, Vrahnas C, et al. The primary function of gp130 signaling in osteoblasts is to maintain bone formation and strength, rather than promote osteoclast formation. *J Bone Miner Res*. 2014;29(6):1492-505.
23. Schriefer JL, Robling AG, Warden SJ, Fournier AJ, Mason JJ, Turner CH. A comparison of mechanical properties derived from multiple skeletal sites in mice. *J Biomech*. 2005;38(3):467-75.
24. Sims NA, Clement-Lacroix P, Da Ponte F, et al. Bone homeostasis in growth hormone receptor-null mice is restored by IGF-I but independent of Stat5. *J Clin Invest*. 2000;106(9):1095-103.
25. Standal T, Johnson RW, McGregor NE, et al. gp130 in late osteoblasts and osteocytes is required for PTH-induced osteoblast differentiation. *J Endocrinol*. 2014;223(2):181-90.
26. Faibish D, Gomes A, Boivin G, Binderman I, Boskey A. Infrared imaging of calcified tissue in bone biopsies from adults with osteomalacia. *Bone*. 2005;36(1):6-12.
27. Ou-Yang H, Paschalis EP, Mayo WE, Boskey AL, Mendelsohn R. Infrared microscopic imaging of bone: spatial distribution of CO₃(2-). *Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research*. 2001;16(5):893-900.
28. Camacho NP, West P, Torzilli PA, Mendelsohn R. FTIR microscopic imaging of collagen and proteoglycan in bovine cartilage. *Biopolymers*. 2001;62(1):1-8.
29. Paschalis EP, Verdelis K, Doty SB, Boskey AL, Mendelsohn R, Yamauchi M. Spectroscopic characterization of collagen cross-links in bone. *Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research*. 2001;16(10):1821-8.
30. Paschalis EP, Betts F, DiCarlo E, Mendelsohn R, Boskey AL. FTIR microspectroscopic analysis of human iliac crest biopsies from untreated osteoporotic bone. *Calcified tissue international*. 1997;61(6):487-92.
31. Spevak L, Flach CR, Hunter T, Mendelsohn R, Boskey A. Fourier transform infrared spectroscopic imaging parameters describing acid phosphate substitution in biologic hydroxyapatite. *Calcified tissue international*. 2013;92(5):418-28.
32. Paschalis EP, Betts F, DiCarlo E, Mendelsohn R, Boskey AL. FTIR microspectroscopic analysis of normal human cortical and trabecular bone. *Calcified tissue international*. 1997;61(6):480-6.
33. Hanschin RG, Stern WB. X-ray diffraction studies on the lattice perfection of human bone apatite (Crista iliaca). *Bone*. 1995;16(4 Suppl):355S-63S.
34. Donnelly E, Boskey AL, Baker SP, van der Meulen MC. Effects of tissue age on bone tissue material composition and nanomechanical properties in the rat cortex. *Journal of biomedical materials research Part A*. 2010;92(3):1048-56.
35. Gourion-Arsiquaud S, Burket JC, Havill LM, et al. Spatial variation in osteonal bone properties relative to tissue and animal age. *Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research*. 2009;24(7):1271-81.

36. Paschalis EP, DiCarlo E, Betts F, Sherman P, Mendelsohn R, Boskey AL. FTIR microspectroscopic analysis of human osteonal bone. *Calcified tissue international*. 1996;59(6):480-7.
37. Rey C, Renugopalakrishnan V, Collins B, Glimcher MJ. Fourier transform infrared spectroscopic study of the carbonate ions in bone mineral during aging. *Calcified tissue international*. 1991;49(4):251-8.
38. Legros R, Balmain N, Bonel G. Age-related changes in mineral of rat and bovine cortical bone. *Calcified tissue international*. 1987;41(3):137-44.
39. Akkus O, Adar F, Schaffler MB. Age-related changes in physicochemical properties of mineral crystals are related to impaired mechanical function of cortical bone. *Bone*. 2004;34(3):443-53.
40. Gadaleta SJ, Landis WJ, Boskey AL, Mendelsohn R. Polarized FT-IR microscopy of calcified turkey leg tendon. *Connective tissue research*. 1996;34(3):203-11.
41. Paschalis EP, Shane E, Lyritis G, Skarantavos G, Mendelsohn R, Boskey AL. Bone fragility and collagen cross-links. *Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research*. 2004;19(12):2000-4.
42. Ma YL, Zeng Q, Donley DW, et al. Teriparatide increases bone formation in modeling and remodeling osteons and enhances IGF-II immunoreactivity in postmenopausal women with osteoporosis. *J Bone Miner Res*. 2006;21(6):855-64.
43. Lindsay R, Cosman F, Zhou H, et al. A novel tetracycline labeling schedule for longitudinal evaluation of the short-term effects of anabolic therapy with a single iliac crest bone biopsy: early actions of teriparatide. *Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research*. 2006;21(3):366-73.
44. Kim SW, Pajevic PD, Selig M, et al. Intermittent parathyroid hormone administration converts quiescent lining cells to active osteoblasts. *Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research*. 2012;27(10):2075-84.
45. Demiralp B, Chen HL, Koh AJ, Keller ET, McCauley LK. Anabolic actions of parathyroid hormone during bone growth are dependent on c-fos. *Endocrinology*. 2002;143(10):4038-47.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Highlights:

- Synchrotron-based Fourier-transform infrared microscopy (sFTIRM) was directed to specific ages of bone using fluorescent labelling on non-remodeling periosteal bone
- This method allowed measurement of changes in bone matrix composition and collagen conformation during the process of bone mineralization
- We also identified that intermittent parathyroid hormone (PTH) treatment does not alter mineral accrual during bone formation
- Low bone mineralization levels with PTH observed in larger regions may relate simply to a greater proportion of new bone

Accepted Manuscript

Anabolic action of parathyroid hormone (PTH) does not compromise bone matrix mineral composition or maturation

Christina Vrahnas, Thomas A Pearson, Athena R Brunt, Mark R Forwood, Keith R Bambery, Mark J Tobin, T John Martin, Natalie A Sims

PII: S8756-3282(16)30277-0
DOI: doi:[10.1016/j.bone.2016.09.022](https://doi.org/10.1016/j.bone.2016.09.022)
Reference: BON 11140

To appear in: *Bone*

Received date: 10 August 2016
Revised date: 23 September 2016
Accepted date: 25 September 2016

Please cite this article as: Vrahnas Christina, Pearson Thomas A, Brunt Athena R, Forwood Mark R, Bambery Keith R, Tobin Mark J, Martin T John, Sims Natalie A, Anabolic action of parathyroid hormone (PTH) does not compromise bone matrix mineral composition or maturation, *Bone* (2016), doi:[10.1016/j.bone.2016.09.022](https://doi.org/10.1016/j.bone.2016.09.022)

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.