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Osteolytic and mixed cancer metastasis modulates collagen and mineral parameters within rat vertebral bone matrix

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Mikhail Burke – Primary Author. Responsible for co-ordinating resources, study design, sample preparation prior to analysis, data and statistical analysis and drafting the paper.

Ayelet Atkins – Responsible for Raman spectrographic study design and data acquisition and review of paper.

Margarete Akens – Contributed to study design. Responsible for tumour cell line maintenance and inoculation of rats with tumour cells to optimize vertebral metastases growth and review of paper.

Tom Willett – Contributed to study design. Facilitated HPLC data acquisition and helped to critically revise paper draft.

Cari Whyne – Senior Author. Developed study design. Provided direct links to study resources, guidance on data analysis, critically revised paper draft.

All authors have read and approved the final submitted manuscript.
Abstract

Metastatic involvement in vertebral bone diminishes the mechanical integrity of the spine, however, minimal data exists on the potential impact of metastases on the intrinsic material characteristics of the bone matrix. Thirty-four (34) female athymic rats were inoculated with HeLa (N=17) or Ace-1 (N=17) cancer cells lines producing osteolytic or mixed (osteolytic & osteoblastic) metastases respectively. A maximum of 21 days was allowed between inoculation and rat sacrifice for vertebrae extraction. High performance liquid chromatography (HPLC) was utilized to determine modifications in collagen-I parameters such as proline hydroxylation and the formation of specific enzymatic and non-enzymatic (pentosidine) cross-links. Raman spectroscopy was used to determine relative changes in mineral crystallinity, mineral carbonation, mineral:collagen matrix ratio, collagen quality ratio and proline hydroxylation. HPLC results showed significant increase in the formation of pentosidine and decrease in the formation of the enzymatic cross-link deoxy-pyridinoline within osteolytic bone compared to mixed bone. Raman results showed decreased crystallinity, increased carbonation and collagen quality(aka 1660/1690 sub-band) ratio with osteolytic bone compared to mixed bone and healthy controls along with an observed increase in proline hydroxylation with metastatic involvement. The mineral:matrix ratio decreased in both osteolytic and mixed bone compared to healthy controls. Quantifying modifications within the intrinsic characteristics of bone tissue will provide a foundation to assess the impact of current therapies on the material behaviour of bone tissue in the metastatic spine and highlight targets for the development of new therapeutics and approaches for treatment.
**Introduction**

Metastatic involvement in vertebral bone occurs in up to 1/3 of cancer patients\(^1\) and can manifest as bone-resorbing (osteolytic), bone forming (osteoblastic) or a mixture of the two. Vertebral metastasis has been linked with diminished mechanical integrity of the spine with bone metastases leading to a high incidence (~66\%) of skeletal related events (SREs), which can significantly impact quality of life and physical function\(^2\).

Previous work has focused on structural, mechanical and mineral density changes in metastatically involved bone with respect to the risk and pattern of fracture\(^3\). Significant correlations between specific CT-based stereological parameters and mechanical behaviour have also been established\(^4\). However, these detected modifications in total bone structure or mass may not fully account for observed SREs due to modified mechanical behaviour. The intrinsic material characteristics of bone has been shown to be impacted by pathological conditions such as type-I diabetes\(^5\) and should be accounted for in metastatic involvement.

Bone is a complex two-phase composite material whose material properties are dependent on both its organic (collagen) and mineral (hydroxyapatite) components and their combined ultrastructure. In its organic phase, the rectilinear array of collagen type-I fibrils provides bone with enhanced ductility increasing the tissue's toughness\(^6\). This observed fibrillar structure, into which the helical collagen-I molecules aggregate, is maintained and stabilized in part by the formation of covalent cross-links between these molecules. These cross-links can be formed through enzymatic pathways connecting the non-helical telopeptides of adjacent collagen molecules\(^7\) to form immature divalent cross-links or mature trivalent cross-links or through non-enzymatic pathways connecting the helical peptides\(^8\). A variety of enzymatic and non-enzymatic
cross-links exist within bone but studies tend to focus on the analysis of the trivalent mature enzymatic pyridinium cross-links: pyridinoline (pyr) and deoxy-pyridinoline (dpyr); and the non-enzymatic crosslink: pentosidine (pen). This focus is due to the natural fluorescence of these cross-links making them easily identifiable markers in high performance liquid chromatography (HPLC). Decreases in pyridinium cross-links has been associated with a reduction in bending strength and the elastic modulus of bone. Modifications in the ratio between immature cross-links and mature pyridinium cross-link levels within bone have been associated with changes in observed bone strength or toughness. Increase in non-enzymatic crosslinks have been shown to negatively impact bone ductility, toughness and post-yield properties. While no data exists for metastatically involved bone, pathologic bone secondary to diabetes and osteoporosis has demonstrated increases in pen levels suggesting pen level’s potential as a biomarker to pathologic bone tissue. Note, these studies utilized bone samples for direct and absolute measurement of cross-link concentration. While this allows for assessment of cross-link changes due to both bone turnover and pathologically formed bone, pen levels can be inferred by concentration detected in urine or serum due to turnover.

Formation of these cross-links could be impacted by numerous factors. An imbalance between produced reactive oxygen species (ROS) and the local system's ability to counteract with sufficient anti-oxidising mechanisms and repair potential damage (known as oxidative stress) could provide an environment conducive to oxidative reactions associated with advanced glycation endproduct (AGE) formation. Additionally, hydroxylation of residues such as lysine in the collagen molecule are critical in dictating the pathways of enzymatic cross-link formation. Modifications in collagen cross-links and hydroxylation of residues are detectible via techniques such as high powered liquid chromatography.
Bone's mineral component primarily exists as hydroxyapatite crystals, which nucleate and grow within the spacing between collagen fibrils. This mineral presence within bone is critical in providing bone its strength and stiffness. While it has been theorized that mineral dimensions would impact bone stiffness, studies have shown conflicting data between modified mineral size and mechanical behaviour\textsuperscript{19}. Modified chemistries of the calcium mineral can occur due to ionic substitution of phosphate ion within the environment; the most common of those being with the carbonate ion, which make up roughly 7wt\% of the apatite lattice\textsuperscript{20}. This type-B carbonate substitution is suspected to be driven by the presence of non-collageneous proteins in bone and the presence of carbonate ions has been linked to modifications of metabolic activity within bone's localized biosystem such as buffering acidity potentially connected with bone resorption\textsuperscript{21} and stimulating bone growth\textsuperscript{22}. Carbonate within the apatite structure has also been associated with a modification in crystal structure with observed decreases in crystal dimensions or increased lattice strain\textsuperscript{23}. Some work has started to study the impact of metastasis on the mineral crystal structure through Raman spectroscopy showing patterns of decreased crystallinity, crystal size and increased carbonation\textsuperscript{24}. This impact could also be cyclic in nature as factors such as crystallinity and crystal size have been shown to impact metastatic cell behaviour\textsuperscript{25, 26}. Raman spectroscopy can also be utilized to assess the mineral/collagen ratio within bone tissue indicative of tissue mineral density instead of observing total bone mass.

To understand the material behaviour of metastatically involved bone requires evaluation of changes which occur in both organic and mineral phases. As such, this study aims to evaluate collagen cross-linkage and mineral crystal structure in osteolytic and mixed metastatically involved vertebral bone using well established \textit{in vivo} rat models\textsuperscript{27, 28}. It is hypothesized that due to the associated increase of reactive oxygen species (ROS) production with cancer cells \textsuperscript{29}, the
increase in the oxidative stress within the bone microenvironment due to metastatic involvement would lead to increase oxidation reactions and hence an increase in the formation of the non-enzymatic pentosidene. Furthermore, an increase in the hydroxylation of specific collagen amino residues is hypothesized to occur within metastatically involved bone as cancer cells have been associated with an increase in the expression of hydroxylation enzymes such as and prolyl hydroxylase (PH) and lysyl hydroxylase (LH). Hydroxylation impacts enzymatic cross-linkage pathways as the cross-linkage formed differs by whether substrates/intermediates were hydroxylated. Additionally, previous work utilizing a murine metastatic lung-cancer model has linked metastatic involvement to increased expression of LH2 and subsequent modification in enzymatic collagen cross-link concentrations. Factors such as hypoxia-inducible factor–1(HIF-1) and signal transducer and activator of transcription 3 (STAT3), which were linked to the observed increase in LH2, are also associated with the development or presence of bone metastases. This, combined with the fact that the coexpression of LH and lysyl oxidase (LO), an oxidizing enzyme key in the formation enzymatic crosslink intermediates, can occur because both genes are targets for HIF-1 binding and are upregulated by extracellular signals associated with metastatic behaviour, and increases the likelihood of observed changes in the quantity of various enzymatic cross-links formed. Finally, we predict decreased crystallinity, collagen mineralization, crystal size and increased carbonation with metastatic involvement based on trends observed previously.

Material/Methods

Animal model and metastatic inoculation: Well established rat models for studying spinal metastases were utilized. These models use systemic inoculation of tumour cells to simulate the physiologic development of vertebral metastases. The animal use protocol was
approved by the Ontario Cancer Institute prior to performing the study. Randomly assigned 5–6 week old athymic female rnu/rnu rats were inoculated with human HeLa cervical cancer cells (previously misidentified as MT-1 cells) to create osteolytic metastases or canine Ace-1 prostate cancer cells to produce mixed (osteolytic/osteoblastic) metastases (N=17 per group). An additional 12 rats were used as healthy controls. The HeLa and Ace-1 cell lines were cultured in RPMI and DMEM/F-12 media respectively at 37°C with 5% CO₂ and stably transfected with the luciferase gene to enable bioluminescent image monitoring of tumor growth within the rat via subcutaneous injection of luciferin. Cultured cell viability was determined utilizing a trypan blue exclusion. Rats were anesthetised using nose-cone inhalation of a 2% halothane/air mixture. Intracardiac injections containing ~1.5×10⁶ cells (in 0.2mL of media) were injected into the left heart ventricle. After 14 and 21 days, bioluminescence imaging (Xenogen) was performed for detection and semi-qualitative assessment of the degree of metastatic growth within the rat, and particularly in the spine. The rats were euthanized 21 days after initial inoculation via CO₂ asphyxiation, their vertebrae harvested and wrapped in saline dampened gauze and stored in -20°C freezer until testing. In cases of premature rat death the vertebrae were removed immediately and noted accordingly.

**High Performance Liquid Chromatography:** HPLC was performed to quantify crosslinks using a previously published protocol\textsuperscript{11,35,36}. Briefly, soft tissue was removed from extracted T12-T13 vertebrae via papain digestion. Samples were defatted and then hydrolyzed in 11 M HCl at 110°C for 24 h. Samples were diluted and added to a sample buffer containing 10% acetonitrile, 1% HFBA and water plus an internal standard (pyridoxine). Pyridinoline, deoxy-pyridinoline and pentosidine were quantified against standards of pentosidine (PolyPeptide Group, Strasbourg, France) and pyridinoline (Qiagen, Hilden, Germany). Agilent Zorbax Eclipse XDB-
C18 Reversed-Phase C18 HPLC columns were used (150 × 4.6 mm, 5 μm particle size, 80 Å pore size, end-capped; Agilent Technologies, Mississauga, Canada). The mobile phase for crosslink quantification incorporated A: 26% methanol+0.1% heptafluorobutyric acid (HFBA), B: 85% acetonitrile+0.1% HFBA and C: 38% methanol +0.08% (HFBA) at different elution times (A: 0-18 min, 40-50 min, B: 18-30 min, C: 30-40 min). A separate HPLC method utilizing the same type of column was used to measure collagen content via hydroxyproline quantification of the same samples using both hydroxyproline and amino acid standards (Sigma-Aldrich). Samples were diluted with borate buffer and homoarginine (internal standard) and underwent derivatization via the cycled addition of fluorenylmethyloxycarbonyl chloride (FMOC-Cl) + acetone, pentane and extraction of waste top layer. For each sample run, an elution profile measuring fluorescence vs. elution time was obtained. The areas under the peaks were measured and compared to a standard curve in order to calculate the concentration of each crosslink, hydroxyproline and proline. Cross-link concentration was then normalized to collagen content. The degree of proline hydroxylation was also determined by hydroxyproline/proline ratio.

**Raman Spectroscopy:** Extracted T8 vertebrae were cut in the sagittal plane with a diamond blade (Buehler, Illinois, USA) then polished with grit papers (600, 1200, 2400, 4000 and diamond paste 3um and 1um). The bone specimens were thawed in a 4°C refrigerator 24 hours prior to Raman spectra acquisition. Acquisition was done with an inVia Confocal Raman Microscope (Renishaw, Gloucestershire, UK) which includes a Renishaw spectrometer coupled into a DMI6000 epifluorescence microscope (Leica, Wetzar, Germany). A 785 nm laser light was used to focus on the vertebral body through a Leica HC PLAN APO 20x/0.70NA (laser power 3 mW, spot size 4um in the y direction and 0.5um in the x direction). The bone sample was kept wet with PBS, each sample had 3-5 locations tested and 4-6 spectra were acquired from each location.
(Figure 1a), consisting of two accumulations with an exposure time of 15s. The number of testing points chosen reflected that which was utilized in previous studies and point locations were chosen on trabecular bone adjacent to abnormal voids in trabecular structure attributed to metastatic involvement. Custom script written in Matlab was used to correct the baseline of each spectrum. Peak intensities of phosphate ν1 (959cm\(^{-1}\)), carbonate (1070cm\(^{-1}\)), proline (855cm\(^{-1}\)), hydroxyproline (876cm\(^{-1}\)) and amide-1 (1655-1670cm\(^{-1}\)), were determined for the mineral and collagen properties of the bone (Figure 1b). For peak analysis, background signal noise was removed by subtracting a baseline polynomial curve that best fit the data. To verify the results, a second derivative of the spectrum was taken to attenuate the low-frequency components. Three sub-bands (1650-1660cm\(^{-1}\), 1665-1670cm\(^{-1}\), 1680-1690cm\(^{-1}\)) in the amide-1 region (1650-1690cm\(^{-1}\)) were identified and were Gaussian in nature.

From these intensities, a number of ratios were calculated for analysis. The mineral/matrix ratio (phosphate ν1/proline+hydroxyproline) and carbonate/matrix ratio (carbonate/proline+hydroxyproline) illuminate the degree of mineralization of the bone tissue. Proline+hydroxyproline were chosen as the matrix factors to utilize in the mineral/matrix and carbonate/matrix ratios due to those factors having a distinct tie to collagen-I\(^{37}\). Mineral crystallinity (1/FWHM of phosphate ν1 peak), carbonate/phosphate ν1 ratio (indicative of mineral carbonation), hydroxyproline/proline ratio (indicative of proline hydroxylation), and the collagen quality parameter (mature/immature enzymatic cross-link ratio) were also calculated and compared between sample groups.

**Statistical data analysis:** For each data set, the normality of the data was evaluated utilizing the Shapiro-Wilk test. If the normality assumption held, then a test for the homogeneity of variance between groups via Levene's test was performed. In data sets without equal variance, Welsh
followed by Tamhane post hoc tests for multiple comparisons were performed to test for differences between sample groups. When equal variance and normality could be assumed, analysis of variance followed by the Tukey post hoc test for multiple comparisons between samples groups were used. In cases where the normality assumption could not be held, the Kruskal-Wallis H test followed by the Dunn-Bonferroni post-hoc approach for multiple comparisons between sample groups were used. All data are presented as mean±standard deviation, with a significance level of p<0.05 (SPSS v23, SPSS, Chicago, IL, USA).

Results

Metastatic Involvement: Bioluminescence analysis found that 14/17 rats injected with HeLa cells (~82% success rate) and 12/17 rats injected with Ace-1 cells (~71% success rate) displayed metastatic tumour growth in the spine (including the approximate location of the T8-T13 vertebrae as displayed in Figure 2), as well as in the heart, lungs and femurs. The degree of metastatic growth in each rat was random. A total of 25 rats (HeLa: N=9; Ace-1: N=7; Healthy: N=9) were utilized for HPLC analysis. For Raman, 25 samples were used (HeLa: N=10; Ace-1: N=9; Healthy: N=6).

Cross-link and Proline Hydroxylation Quantification via HPLC: The non-enzymatic cross-link pentosidine showed a large statistically significant increase (2.4x, p<0.05) in observed pen levels in osteolytic vertebrae compared to healthy controls (Figure 3a). Although total pyridinium cross-links content showed no significant difference between healthy and metastatic involved bone, a trend (p = 0.087) was observed between osteolytic and mixed bone samples (Figure 3b). Deoxy-pyridinoline levels were significantly lower (p<0.05) in the osteolytic compared to the mixed vertebrae (Figure 3c). There were no significant differences in pyridinoline levels between
the 3 groups (Figure 3d). There was a significant increase in hydroxyproline formation (~16%, p<0.05) in osteolytic bone compared to healthy controls (Figure 4a) with no statistically significant changes in proline concentration (Figure 4b). Osteolytic bone samples demonstrated a trend toward an increase (~6%) in the hydroxyproline/proline ratio (Figure 4c) compared to mixed bone and healthy controls (p = 0.053, p = 0.077, respectively).

*Modifications in Mineralization, Collagen Quality and Proline Hydroxylation Ratios assessed via Raman:* Modifications in both crystallinity and crystal structure were found due to metastatic involvement. The 1/FWHM value of ν₁Phosphate peak showed a small but statistically significant decrease (~3%) in the osteolytic group compared to both the mixed model and healthy controls, indicative of decreased crystallinity (Figure 5a). The carbonate/phosphate ratio in the osteolytic samples was elevated (~6%) compared to the other groups, implying increased carbonate ion substitution in the crystal lattice (Figure 5b).

The mineral/matrix ratio showed a statistically significant decrease in the presence of the phosphate component of hydroxyapatite relative to the proline/hydroxyproline residues specific to collagen-I for both osteolytic (~15%) and mixed groups (~11%) versus healthy controls (Figure 5c). There was also a decrease in the carbonate/matrix ratio in bone with metastatic involvement (Figure 5d).

The collagen quality parameter, thought to be indicative of the level of the ratio between mature and immature cross-links, was moderately reduced (5%) in osteolytic samples compared to both mixed and healthy controls (Figure 5e). Additionally, there were observed differences in the hydroxyproline/proline ratio between all three samples groups with osteolytic samples exhibiting the highest ratio (~10% increase) (Figure 5f).
Discussion

Both the organic and mineral phases of metastatically involved vertebrae demonstrated changes as compared to healthy controls, suggesting that tumour induced changes impact the remaining bone at a material level. The most striking differences were seen in pentosidine (pen) levels. Pen is an AGE crosslink between lysine and arginine found in the helical domains of adjacent collagen molecules. Pathological conditions such as Type 1 and Type 2 diabetes considered to negatively impact bone quality have been shown to modify the quantity of collagen cross-link formed within bone tissue\textsuperscript{5,9}. In each case, pen levels were shown to increase. This, combined with increased pen levels being associated with diminished bone mechanical properties\textsuperscript{13,14}, implies an importance of pen as a potential biomarker for poor bone quality and a risk factor for fracture. In our study, osteolytic metastatic involvement within the vertebrae (HeLa) was shown to greatly increase the formation of pen in the bone. In general, an increase in ROS species concentration associated with the presence of cancer cells\textsuperscript{29} could facilitate the oxidation of open chain aldehyde metabolic intermediates leading to the production of AGEs such as pen. However, Ace-1 metastatic involvement did not impact pen levels in a statistically significant manner. This contrast to the HeLa involvement could be due to variability in tumour burden among samples. An increase in pen has been linked to diminished bone quality and may act as a marker for oxidative stress and AGE production which can impact bone remodelling\textsuperscript{13,14}. Amplified concentrations of ROS can damage the extracellular matrix (collagen) and impact cellular metabolic reactions (osteoblasts and osteoclasts remediated bone remodelling)\textsuperscript{38}. Collagen degradation, both in terms of fragmentation and susceptibility to enzymatic degradation, has been shown to be facilitated by ROS presence\textsuperscript{39,40}. All these factors combined with other AGE presence may have an impact on bone's mechanical behaviour.
Pyridinoline (pyr) and deoxypyridinoline (dpyr) are formed via a hydroxyallysine dominant (ahyl) pathway. Since ahyl versus allysine (alys) formation is dependent upon the availability of hydroxylysine (hyl), both the action of lysyl hydroxylases (LH) and lysyl oxidases (LO) on lysine (lys) are important in the formation of these cross-links. The level of Lys hydroxylation has been shown to play an important role in the tissue-specific pattern of enzymatic cross-links and defining the functionality of type I collagen in tissue\textsuperscript{18}. Metastatic involvement in other systems has been linked to increased LH and LO levels within the microenvironment\textsuperscript{31}. Additionally, ROS presence could also induce non-enzymatic hydroxylation of amino residues\textsuperscript{41}. Therefore, one might anticipate an increase in the formation of ahyl dominant enzymatic cross-links with metastatic involvement. However, this was not the case for the observed mature enzymatic cross-links with no significant difference between osteolytic or mixed bone compared to healthy bone. The significant relative decrease in dpyr formation in osteolytic compared to mixed bone however, does suggest that collagen cross-link formation pathways are impacted differently by the type of cancer involved. Although such modifications in enzymatic cross-linking (both mature and immature) have been shown to impact bone mechanical behaviour\textsuperscript{12, 42}, the presence or directionality of the change in pyridinium cross-link levels in bone can differ under different pathological conditions\textsuperscript{5, 9, 10, 43}. Note, post-hoc power analysis showed that the comparison of dpyr differences was underpowered (power=0.48). This may have impacted the ability to detect a statistically significant difference between osteolytic and healthy bone (p=0.114). This general decrease in pyridinium cross-link formation is likely due to competitive utilization of lys and hlys residues in AGE formation creating a deficiency in the formation of enzymatic cross-links. The emphasis on dpyr decrease compared to pyr might be due to the potential increase in the hydroxylation of lysine, limiting the available of helical lys which is
required in dpyr formation but not for pyr formation. Unfortunately, we did not measure lysine hydroxylation in this study.

Hydroxylation of proline in collagen is mediated by the enzyme prolyl hydroxylase. The presence of hydroxyproline in collagen is critical for the stability of the collagen triple helix via hydrogen bonding interactions. Additionally, the polar interactions between protruding hydroxyl groups of collagen molecules within collagen fibrils could impact both the chemical and observed physiological properties of the fibril. For example, it has been shown that hydroxylation is favourable for HA nucleation and growth\textsuperscript{44}. The expression of prolyl hydroxylase has been linked to cancer metastases in primary tumours often associated with bone metastasis, likely to facilitate increased extracellular matrix production to providing structure for tumour cell adhesion, growth and taxis\textsuperscript{30,45}. Additionally, ROS species associated with metastatic involvement could potentially lead to non-enzymatic hydroxylation of amino residues\textsuperscript{41}. Therefore metastases could facilitate an increase in non-enzymatic hydroxylation and the prolyl hydroxylase levels in the bone micro-environment promoting increased hydroxyproline expression and the observed increase in the hydroxyproline/proline ratio in metastatic bone groups compared to healthy controls. Note that in this study, the modifications in hydroxyproline/proline ratio observed among sample groups was different between HPLC and Raman. HPLC provides absolute values on the quantity of proline and hydroxyproline residue within all bone in the vertebrae whereas Raman analysis does not provide absolute values (just the ratio), is focal in nature, and locations of analysis were chosen by their likely proximity to tumour. This difference in data collection methodology could account for Raman’s perceived increased sensitivity in detecting differences in the hydroproline/proline ratio in metastatically involved bone and suggests that tumour impact on bone collagen and bone is proximal in nature.
Note, although hydroxyproline levels may be modified with metastatic involvement, its expression as the gold standard in measuring collagen content was utilized.

Proline and hydroxyproline were chosen as the matrix factors to utilize in the mineral/matrix and carbonate/matrix ratios in Raman due to those factors having a distinct tie to collagen-I\textsuperscript{37}. The observed decrease in the mineral/matrix ratio for both the osteolytic and mixed bone samples agrees with decreases in tissue mineral density associated with metastatic involvement observed via other modalities (i.e. μCT scanning + segmentation, pycnometry + mass measurements)\textsuperscript{4,27,34}.

Metastatic involvement has been previously shown to both influence and be influenced by hydroxyapatite (HA) crystallinity and crystal size\textsuperscript{25,26}. Our results revealed a decrease in crystallinity in osteolytic samples compared to healthy controls (p<0.05) with a similar pattern (p<0.05) in the mixed metastasis groups. This could be attributed to the observed increase in carbonate/phosphate ratio, implying increased carbonate substitution crystal defects or may be associated with changes in HA crystal dimensions. The observed increase in the relative carbonation of the HA mineral could be indicative of the increased acidity of the local bone biosystem due to both increased overall osteolytic activity associated with tumour involvement in both metastatic models and the acidic extra-cellular fluid of the tumour tissue\textsuperscript{46}. It has been shown that acidic microenvironments favor the dissolution of phosphate compared to carbonate in the cross-sectional areas of bone\textsuperscript{47}.

In Raman spectroscopic analysis, the 1660 cm\textsuperscript{-1} and the 1690 cm\textsuperscript{-1} peaks within the amide-I region (1650-1690 cm\textsuperscript{-1}) are associated with the presence of mature pyr and immature deH-DHLNL respectively. Although there is contention on the direct link of the 1660/1690 to the
ratio of mature/immature crosslinks in the bone, it is still commonly used in Raman to provide information on bone age and the quality of the collagen (as immature cross-links mature over time). HPLC analysis showed no significant changes in pyr formation between the groups. However, a decreasing 1660/1690 ratio in the osteolytic samples implies that there is a change in the amount of immature cross-links formed in the collagen. This potential modification in the ratio between mature and immature cross-links could impact bone’s material behaviour.

While the results of this study show clear changes in the formation of collagen cross-links and modifications in mineralization of the metastatically involved bone matrix, some limitations must be noted with respect to the approach utilized. Although the presence of tumour within the spinal area of inoculated rats was established via bioluminescence imaging, tumour burden was not volumetrically quantified within each tested vertebrae. Therefore the potential impact of the quantity of tumour on collagen-cross-link formation and mineralization was not evaluated. As well, the tumour cell lines utilized were from different animal and tissue sources, however, the immune compromised nature of the rats should minimize any impact of cell species differences in the study. Additionally, potential sub differences between existing bone/new matrix and osteolytic/osteoblastic bone tissue within the Ace-1 mixed metastatic model were not evaluated. Raman provided qualitative, comparative assessment of bone quality between samples groups, yet it does not provide absolute value assessment for specific phase characteristics (such as the dimensional changes in the HA crystals). Finally, while pen is an AGE product and a relationship between pen fluorescence and bulk fluorescence of all AGEs has been shown in other studies, no clear relationship between pen formation and the formation of other non-enzymatic cross-links has been proven.
Conclusion

This study demonstrates that metastatic involvement has a clear impact on the formation of specific non-enzymatic and mature enzymatic cross-links in vertebral bone. Future work will need to link these cross-link modifications to potential changes in collagen microstructure, bone mineralization and the overall material behaviour of the pathologic bone matrix. Additionally, specific elucidation of potential differences between osteolytic and osteoblastic tissue will be important in characterising focal impact and distinguishing sub-differences within mixed metastatic involvement. Also, studying the relative impact of these tissue level changes to the mechanical behaviour of vertebral bone is important for future analysis. Ultimately, by quantifying the influence of cancer on bone tissue, a fundamental basis for understanding the mechanics of structural changes can be established; which will be key in developing new integrative strategies to mechanically model metastatically involved vertebral bone and highlight areas of focus in the evaluation of new and existing treatment options.

Acknowledgements

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Figure Legend

Figure 1 - a) Photograph of cross section of representative rnu/rnu rat T8 vertebrae. White arrows point to Raman spectra collection points. Points were chosen on trabeculae adjacent to seemingly larger abnormal voids in trabecular structure. b) Example of Raman spectrograph from each sample group (osteolytic, mixed and healthy bone). Selective bands were marked with biochemical assignments.

Figure 2 – Representative bioluminescent images of rats inoculated with HeLa (a) and Ace-1 (b) 21 days post-inoculation. Metastatic growth was observed in various regions of the rat anatomy with the region of interest being the spinal column area (indicated by oval overlay). The dotted square overlay indicates the approximate location of T8-T13 vertebrae.

Figure 3 - Quantitative assessment of a) pentosidine b) total pyridinium c) deoxypyridinoline and d) pyridinoline collagen crosslinks concentrations from HPLC analysis of rat vertebral bone within each sample group. Osteolytic bone showed increases in expression of the non-enzymatic cross-link pentosidene and decreases in the mature enzymatic cross-link deoxypyridinoline compared to healthy and mixed bone respectively. A total of N=25 rats (HeLa: N=9; Ace-1: N=7; Healthy: N=9) were assessed.

Figure 4 - Quantitative assessment of a) hydroxyproline and b) proline collagen residue concentrations from HPLC analysis and the calculated c) hydroxyproline/proline ratio of rat vertebral bone within each sample group. Osteolytic bone showed a statistical trend towards higher hydroproline/proline ratios compared to healthy and mixed bone. A total of N=25 rats (HeLa: N=9; Ace-1: N=7; Healthy: N=9) were assessed.
Figure 5 - Raman spectrograph-derived parameters of bone composition between sample groups. Characteristics analyzed included a) mineral crystallinity, b) carbonate/phosphate, c) mineral/matrix, d) carbonate/matrix ratios, e) the collagen quality parameter and f) hydroxyproline/proline ratio. All parameters showed statistical difference between healthy and metastatic groups. A total of N=25 rats (HeLa: N=10; Ace-1: N=9; Healthy: N=6) were assessed.
References


Figure 1

a) Image showing a close-up view of bone structure with arrows indicating different mineral components.

b) Raman spectroscopy graph showing peaks for Phosphate $v_1$, Proline, Hydroxyproline, Carbonate, and Amide-1. The x-axis represents Raman Shift (cm$^{-1}$) and the y-axis represents Raman Intensity.
Figure 2

a) 

b) 

Figure 2
Figure 3

a) Pentosidine Concentration

- Healthy: p < 0.05
- Osteolytic
- Mixed

b) P = 0.087

c) Total Pyridinium Cross-link Concentration
   (Deoxypyridinoline + Pyridinoline)

- Healthy
- Osteolytic
- Mixed

Deoxypyridinoline Concentration

- Healthy
- Osteolytic
- Mixed
Figure 4

a) Hydroxyproline Concentration

b) Proline Concentration

c) Hydroxyproline/Proline Ratio (HPLC)