

**Oxidative damage of bone collagen as a cause of reduced fracture
resistance in human cortical bone**

by

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Author's Declaration

This thesis consists of material all of which I authored or co-authored (see Statement of Contributions included in the thesis). This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

I understand that my thesis may be made electronically available to the public.

Statement of Contributions

This thesis was written under the supervision and mentorship of Prof. Thomas L. Willett who reviewed and edited this manuscript.

I, Daniel Dapaah was the sole author for Chapters 1, 2, 3 and 6 and were not written for publication.

Chapter 4 contains a large part of a published manuscript co-authored with and conducted under the supervision of Prof. Thomas L. Willett who assisted with conceptualization, methodology development, revision and editing of the manuscript as well as funding the research.

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Chapter 5 contains research conducted under the supervision of Prof. Thomas L. Willett in collaboration with Dr. Shoutaro Arakawa and Dr. Mitsuru Saito both in the Department of Orthopaedic Surgery, Jikei University School of Medicine in Tokyo, Japan. In addition, there were various contributors to the work conducted in chapter 5 and this is outlined below:

Zulaikha Zakiullah, assisted with the writing of the MATLAB code for computing crack extensions from the fracture test described in section 5.2.2 of chapter 5.

Gwenyth Alexandra Carroll under the supervision of Prof. Stewart McLachlin assisted in the development of the micro-computed tomography protocol described in section 5.2.5 of chapter 5

Dr. Shoutaro Arakawa performed the liquid chromatography mass spectrometry analyses and provided the data to Daniel Dapaah. Dr. Arakawa also wrote the original draft of section 5.2.7 of chapter 5.

Jindra Tupy spearheaded the development of the trypsin and pepsin digestion, the hydroxyproline and carbonyl assays as well as the sodium dodecyl sulfate polyacrylamide gel electrophoresis protocols described in sections 5.2.8 and 5.2.9 of chapter 5.

Dr. Yiran Wang provided guidance and assisted by providing preliminary R codes for the multiple linear regression models developed as described in section 5.2.10.

As the lead author of these studies, I was involved in all aspects of conceptualization, study design, data collection and analysis as well as writing and revising of draft manuscripts presented in this thesis.

Abstract

Bone fragility and fracture remain a prominent issue in the populace especially for the older adult population with associated significant health, social and economic implications. The X-ray based clinical tools used to assess bone fracture risk only “see” the mineral and are unable to predict fracture risk accurately in certain individuals especially for those with type 2 diabetes (T2D) and chronic kidney diseases (CKD). This suggest that factors not measured by these X-ray based clinical tools may be important contributors to predicting bone fracture risk. One such factor is bone collagen which forms about 41% by volume of the bone tissue and has been shown to be vital to the fracture resistance of bone and possibly an important determinant for bone fracture risk assessment. Ex vivo human bone studies have shown that bone collagen network connectivity degrades with age, and this is associated with a reduction in the fracture resistance of cortical bone. Collagen network connectivity is a term used to describe how well linked together the collagen molecules are in the collagen network of the bone tissue. However, the exact mechanisms that lead to this degradation of connectivity remain inconclusive. Interestingly, elevated levels of oxidative stress has been implicated in the development and progression of osteoporosis, T2D and CKD, diseases with higher incidences of fracture. Oxidative stress is known to cause damage to macromolecules, referred to as oxidative damage, which includes long lived proteins such as collagen. Consequently, the overarching goal of this thesis was to investigate oxidative damage as a cause of collagen network connectivity degradation leading to reduced fracture resistance in cortical bone.

To achieve this goal, this thesis work consisted of two large studies. The first study sought to establish the most accurate and precise way to measure cortical bone fracture toughness. Therefore, current methods for cortical bone fracture testing were critically evaluated. Specifically, the unloading compliance (UC) method developed for metals that has been widely used in cortical bone J-integral resistance curve fracture testing was assessed for its accuracy and precision. The UC method employs multiple progressive load partial unload cycles and uses the unloading compliance of the unloading curve to estimate the current crack extension. Fracture toughness measures from the UC method were compared to those generated using a continuously monotonic load and crack extension measured by an optical method. Results showed that the UC method on average underestimated crack extension by 73% and this consequently resulted in inaccurate and less precise fracture toughness measures from the UC method. This indicates optical based approaches provide more accurate and precise results and highlights a need for rigorous and standardized methods for cortical bone fracture resistance testing.

Results from this study were used as a guide to conduct fracture toughness testing of cortical bone specimens in the second study.

In the second study, oxidative damage was investigated as a cause of collagen network degradation associated with reduced human cortical fracture toughness. For this, fracture toughness testing of ex vivo human femoral cortical bone specimens from a large heterogenous group of 81 donors with and without a history of T2D and/or CKD were performed. In addition, various biomarkers of oxidative damage alongside collagen network quality measures, cortical porosity as well as mineralization profiles were measured in these specimens. Relationships between the oxidative damage biomarkers, collagen network quality measures and cortical bone fracture toughness measures were investigated using statistical methods. In addition, differences between these measures for the donors with T2D and/or CKD and those without (controls) were also investigated. Results show that advanced glycation end-products (AGEs), one of the biomarkers of oxidative damage were higher in quantity in the T2D and/or CKD donor group compared to controls. However, fracture toughness measures were not different between the two groups suggesting the impact of oxidative damage on bone collagen happens on a spectrum and is general to both groups. Further, in general, the oxidative damage biomarkers correlated with measures of immature crosslinking and collagen chain fragmentation which in turn correlated with other measures of collagen network quality. Multiple linear regression models that were developed showed that interaction terms between collagen network connectivity and immature crosslinking, collagen chain fragmentation as well as the lysine-based AGE adducts were important to explaining fracture toughness measures. From all this, it was proposed that oxidative damage indirectly impacts collagen network connectivity through disruption of immature crosslink formation and collagen chain fragmentation. This in turns negatively impacts the collagen network connectivity leading to reduced fracture resistance of human cortical bone.

This work provides a better understanding of how oxidative stress may contribute to loss of fracture resistance in human cortical bone and this new knowledge could be critical towards the development of better means to detect, prevent and treat bone fragility.

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Dedication

This thesis is dedicated to my parents, Harrison and Joyce, my wife, Caroline and my little boy, Jeremy. I am who I am and where I am today because of you all.

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List of Abbreviations

Abbreviation	Meaning
aBMD	areal bone mineral density
AGE(s)	advanced glycation end product(s)
ALE	advanced lipoxidation end-product
avBMD	apparent volumetric bone mineral density
BAPH	β -aminopropionitrile
BMD	bone mineral density
BMI	body mass index
CEL	carboxyl-ethyl-lysine
CKD	chronic kidney disease
CML	carboxyl-methyl-lysine
CNC	computer numerical control
DHLNL	dihydroxylysine norleucine
DIC	digital image correlation
DMAB	dimethylaminobenzaldehyde
DMSO	dimethyl sulfoxide
DNP	dinitrophenyl
DNPH	2,4-dinitrophenylhydrazine
DPD	deoxypyridinoline
DPL	deoxypyrrrololine
DSC	differential scanning calorimetry
EDTA	ethylenediaminetetraacetic acid
EFPM	elastic-plastic fracture mechanics

ELISA	enzyme-linked immunosorbent assay
F-CHP	fluorescently labelled collagen hybridizing peptides
Fmoc	9-fluorenylmethylchloroformate
FTIR	Fourier transform infrared spectroscopy
FTC	fluorescein-5-thiosemicarbazide
FWHM	full width at half maximum
GFR	glomerular filtration rate
Gly	glycine
ΔH	enthalpy of denaturation
HCl	hydrochloric acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIT	hydrothermal isometric tension
HLNL	hydroxylysionorleucine
HOAc	acetic acid
HPLC	high performance liquid chromatography
Hyp	hydroxyproline
IR	infrared
ISTD	isotope-labelled internal standard
J_{IC}	crack initiation J-integral fracture toughness
J-int	crack instability J-integral fracture toughness
J-R	J-integral resistance
LC-MS	liquid chromatography-mass spectrometry
LDS	lithium dodecyl sulfate
LEFM	linear elastic fracture mechanics

LH	lysyl hydroxylase
LNL	lysionorleucine
LOX	lysyl oxidase
MAPE	mean absolute percentage error
Max.Slope	maximum slope
MDPZ	micro-damage process zone
MeCN	acetonitrile
MG-H1	5-hydroxymethyl-imidazolone
micro-CT	micro-computed tomography
MLR	multiple linear regression
MOLD	methylglyoxal-lysine dimer
M _{peak}	mineralization peak
NaOH	sodium hydroxide
NCPs	non collagenous proteins
NEG	non enzymatic glycation
OH-pro	hydroxyproline
OM	optical microscope
OM-C	optically measured compliance
OPG	osteoprotegerin
PBS	phosphate buffered saline
PEN	pentosidine
PITC	phenylisothiocyanate
PYD	pyridinoline
PYL	pyrrololine

RANKL	receptor activator of nuclear factor-kappa b ligand
RMSE	root-mean square error
RNS	reactive nitrogen species
ROS	reactive oxygen species
RPM	revolutions per minutes
RTC	rat tail collagen
RTD	resistive temperature device
SD	standard deviation
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SENB	single edged notched bend
SENT	single edged notched tensile
T2D	type 2 diabetes
T_d	denaturation temperature for HIT test
TGA	thermogravimetric analysis
TMD	tissue mineral density
T_{onset}	onset of denaturation temperature
T_{peak}	peak heat flow difference temperature
T_R	tearing modulus
UC	unloading compliance
vBMD	volumetric bone mineral density
VOI	volume of interest
W_{fx_n}	normalized work to fracture

List of Symbols

Symbol	Meaning
R	crack growth resistance
K	stress intensity factor
G	energy release rate
J	J-integral fracture toughness
J_{el}	elastic J-integral
J_{pl}	plastic (inelastic) J-integral
B	thickness of SENB specimen
B_N	net thickness of SENB specimen
B_e	effective thickness of SENB specimen
W	width of SENB specimen
a	crack length
C	unloading compliance
α	Alpha
β	beta or standardized beta coefficient
γ	gamma
l_o	initial unbroken ligament length
l_i	current unbroken ligament length
ΔH	enthalpy of denaturation

Chapter 1: Introduction

In 2019, there were an estimated 178 million cases related to bone fragility and fracture worldwide highlighting their prevalence [1]. Associated with these are the loss of independence and morbidity severely impacting quality of life. Even more devastating is that these also lead to increased mortality rates [2,3]. Further, there is a wider socio-economic impact on the populace. For instance, hip fractures are reported to be the third most common type of fracture that occur [4]. In Canada, hip fractures have been estimated to cost \$ 1.1 billion annually [2]. Worryingly, this is projected to increase to \$ 2.4 billion by 2041 [5]. A big reason for the increased cost is Canada's aging population, with projections predicting 25% of Canadians will be 65 years or older by 2030 [6]. This age group has the highest incidence of fractures with roughly 87% to 96% of all hip fractures occurring in this age group [7]. This underlines the need for effective strategies in mitigating bone fragility and fracture.

One approach to minimize the impact of bone fragility and fracture is to be able to assess an individual's risk of fracture and if beyond a clinically proven threshold, initiate early interventions to prevent a fracture or even a recurrence of a fracture. Currently, the clinical tools used for fracture risk assessment are predominantly X-ray based, for example, dual X-ray absorptiometry (DEXA) and these measure the areal bone mineral density (aBMD) [8]. aBMD is a two-dimensional measure of bone mineral density (BMD) and is the amount of bone crystals present for a unit volume of bone tissue divided by the scanned cross-sectional area. A score of at least 2.5 standard deviations below the bone mass for a healthy young adult of the same sex is considered high fracture risk and the associated disease with this is known as osteoporosis. aBMD is used as the standard for fracture risk assessment due to its strong significant correlation with ex vivo hip bone strength [9].

However, in recent years, these X-ray based tools have been shown to be unable to adequately assess fracture in certain individuals [10]. Almost half of all non-vertebral fractures for people above 55 years occur in individuals with normal BMD [11]. In fact, fracture risk has been shown to increase four-fold every 20 years in normal aging, *independent of aBMD* [12]. That is, a 75-year-old will be 4 times more likely to have a fracture compared to a 55-year-old if both have the same aBMD. In addition, age matched studies have shown type 2 diabetics to have higher incidences of fractures compared to controls (non-diabetics) despite reporting clinically normal or, in most studies, higher BMD levels than controls [13–15].

This suggests factors associated with aging and other bone affecting diseases such as type 2 diabetes (T2D) that are not visible to X-ray-based tools contribute to the fracture risk of an individual. While fall rates are known to increase with age and in T2D patients, this does not fully explain the increased fracture risk [16]. Consequently, there has been a shift to investigate compositional and microstructural changes in the bone tissue that affect its ability to resist fracture. From these, various studies have shown the organic phase of bone, which is basically proteins and not quantified by the clinical X-ray tools, to be vital to the fracture resistance of bone [17]. This strongly implies the need to develop better clinical tools that account for the organic phase and other key determinants of bone fracture resistance in assessing fracture risk, that is, measuring tissue-level bone quality (bone tissue quality) instead of only measuring bone mineral density. Bone tissue quality or bone quality for short is broadly defined as aspects of the bone such as its composition, microstructure and mechanical properties independent of BMD that are important to its ability to resist fracture [18].

Type I collagen forms about 90% by volume of the organic phase in bone [17] and its specific importance to the fracture resistance of bone has been shown in various studies. Zioupos et al. [19] showed that with age, the collagen network connectivity degrades, and this correlated with a decreased work to fracture, a measure of bone's fracture resistance. Collagen network connectivity is a measure of how well linked together the collagen molecules are in the bone structure and is a function of crosslinking and collagen polypeptide chain lengths [20–22]. Interestingly, they also showed that the amount of mature enzymatic cross-links stayed fairly constant with age and thus assumed crosslinking did not explain the collagen network connectivity degradation. Wang and others [23] also showed that the deterioration of the collagen network quality with age highlighted by its reduced strength, stiffness and toughness as well as greater existing denatured collagen correlated strongly with reduced fracture resistance. In addition, a more recent study which encompassed a more general and heterogeneous population of donors also showed a moderate to strong positive correlation between collagen network connectivity and the fracture toughness of bone [24]. Further, they showed that the change in collagen network connectivity helped explain about 49% of the variation observed in the fracture resistance of the bone samples [24]. Although the importance of bone collagen to its fracture resistance is well established, the underlying mechanisms of collagen network degradation leading to bone fragility remains inconclusive.

Bone collagen, like many proteins, undergoes various post-translational modifications after its synthesis [25]. One of these modifications is the formation of advanced glycation end-products (AGEs).

The accumulation of AGEs, whether in bone, urine or serum, is widely regarded as a biomarker of poor bone quality [26,27]. AGEs are classically formed from non-enzymatic glycation (NEG) reactions that involve the aldehyde of a reducing sugar reacting with an amino acid group on a protein; in this case, the bone collagen molecules [28]. Interestingly, AGEs have been found to accumulate with age and in diseases that negatively affect the bone such as osteoporosis, type 2 diabetes (T2D) and chronic kidney disease (CKD) [23,29–32]. In addition, using in vitro models, it has been shown that the accumulation of AGEs lead to reduced toughness and fracture resistance of bone [26,28]. These AGEs can form as non-enzymatic crosslinks in the collagen network therefore it is widely thought that over-crosslinking of the collagen network by the AGEs reduces its ductility hence leading to reduced toughness and thereby fracture resistance in bone [26,28].

The caveat with this idea of “over-crosslinking” by AGEs is that these non-enzymatic AGEs crosslinks have been shown to lead to increased stiffness, strength and connectivity of the bone collagen network [28,33]. This is contrary to findings of the earlier mentioned ex vivo studies where degradation in collagen strength, connectivity and quality with age and in bone affecting diseases were associated with reduced fracture resistance [19,23,24]. In addition, there are two main types of AGEs that form: (1) crosslinks, that form covalent bonds between two neighbouring collagen molecules and (2) adducts, that form as modifications to the side groups of the collagen molecules. Recent studies have shown that the AGE adducts are 40 to 200 times more abundant than the AGE crosslinks in bone [34,35]. However, the mechanisms by which the AGE adducts may affect the collagen network quality and in turn fracture resistance are not completely understood.

This suggests that, although AGE crosslinks may be present in older bones and bones affected with bone degrading diseases, they may not be causative of the loss of fracture resistance (bone fragility) with aging and these diseases. The notable question then is, what causes the degradation of the collagen network quality and connectivity if over-crosslinking by AGEs does not explain this effect?

Interestingly, AGEs can be formed as a by-product of a pathological condition known as oxidative stress [36]. Oxidative stress is a condition where there is a greater number of free radicals usually reactive oxygen species (ROS) compared to antioxidants in the body [37]. Elevated oxidative stress is recognized as a fundamental feature in the aging process as well as in several acute and chronic diseases including osteoporosis, T2D and CKD [38]. Oxidative stress is known to cause oxidation of macromolecules such as proteins in the body which leads to harmful changes in their bonding and

structure, and this is termed as oxidative damage [39]. For proteins, oxidative damage is known to lead to polypeptide chain fragmentation or scission, modification of side chain groups, formation of crosslinks as well as denaturation [40,41]. Oxidative stress can lead to the formation of AGEs through glycoxidation reactions where sugars are first oxidized forming highly reactive carbonyl compounds that react with the amino acid groups of proteins [40,41]. As such, it is possible the accumulation of AGE crosslinks in bone may be indicative of elevated oxidative stress levels that cause oxidative damage to the bone collagen. To support this idea, the consequences of protein oxidative damage aligns with potential causes of collagen network quality and connectivity degradation observed with reduced fracture resistance *ex vivo*. This situates oxidative damage as a possible mechanism of collagen network quality and connectivity degradation leading to reduced fracture resistance in bone. Although, oxidative stress has been shown to affect the function of the bone cells particularly in relation to bone remodeling (making of new bone) [42] and shown to cause damage to collagen in other tissues [43], the idea of oxidative damage causing degradation of the collagen network connectivity leading to reduced fracture resistance in bone has received little attention.

Therefore, the goal of this thesis work was to investigate the overarching role of oxidative damage including the formation of AGE adducts as a means of collagen network connectivity degradation associated with reduced fracture resistance in bone that occurs with aging and in bone degrading diseases such as T2D and CKD. This work sought to answer questions such as: (1) is oxidative damage in general, a better predictor of fracture resistance in cortical bone beyond just AGEs?, (2) is oxidative damage elevated in T2D and CKD and does this cause a difference in fracture resistance compared to controls without the disease?, (3) are AGE adducts better predictors of reduced fracture resistance compared to AGE crosslinks?, and if so (4) what are the potential mechanisms by which AGE adducts impact the collagen network quality especially its connectivity?.

This study provides a better understanding of potential mechanisms by which collagen network quality and connectivity degradation may occur and how this influences cortical bone fracture resistance. Such an understanding will help identify potential biomarkers for collagen network quality which will be vital towards the development of clinical tools for better detection, prevention and treatment of bone fragility and fracture. For this work, the focus was on cortical bone fragility. This was because in long bones such as the femur, which is of particular interest to our group, it bears a major portion of the applied load. In addition, it has been showed that the cortical bone component of the femoral neck plays

the most critical role for fracture to occur [44,45]. The femoral neck is the most common fracture site for the femur, for people above the age of 50 [46].

Chapter 2: Background and relevant literature review

2.1 Mammalian bone structure and composition

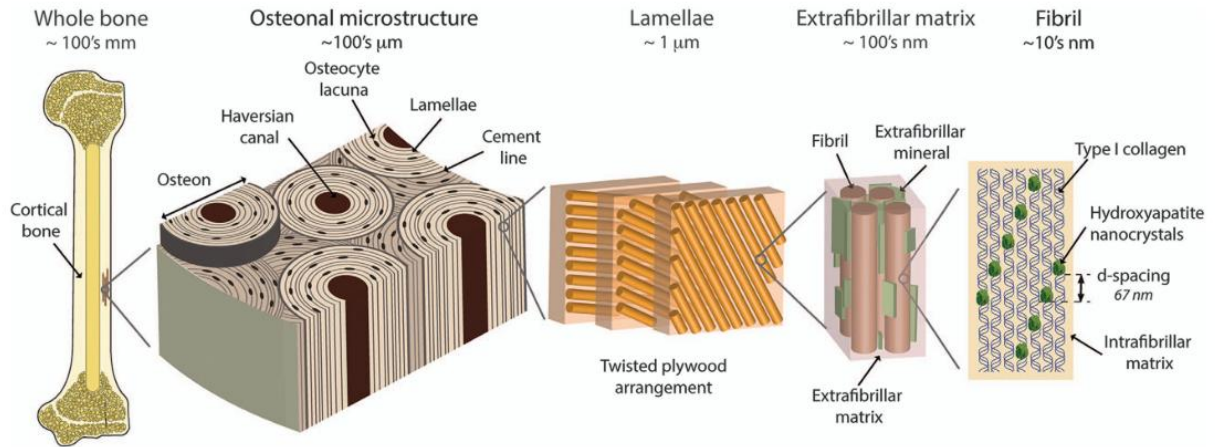


Figure 2.1: Hierarchical structure of mature human cortical bone showing different structures at different length scales. Image adapted from Zimmermann et al. 2015 [47].

Bone is a natural nanocomposite comprised of three phases: a mineral phase, an organic matrix and water [48]. These form approximately 33-43% , 32-44% and 15-25% by volume of the bone tissue respectively [49]. The mineral phase is made up of poorly crystalline, calcium deficient, carbonated hydroxyapatite (bone crystals) that are thin (thickness ~ 1.5 nm to 4 nm) and plate-like shaped [50,51]. The organic phase consists of type I collagen which makes up about 90% by volume of the organic phase with the rest being non-collagenous proteins (NCPs). The NCPs found in bone include phosphoproteins such as osteopontin, sialoprotein and osteonectin as well as proteoglycans such as biglycan and decorin [51,52]. These constituents of bone are arranged in a hierarchical manner. That is, there is a defined organization at different length scales and this spans from the nanoscale up to the macroscale of bone (See Fig. 2.1). Bone's complex hierarchical structure is central to its mechanical performance and properties [52–54].

The primary constituents of bone: hydroxyapatite, collagen molecules, NCPs and water are found at the nanoscale. The collagen molecules align parallel to one another in a staggered pattern with a periodic offset of 67 nm (see Fig. 2.2A). In addition, there are gaps of about 35-40 nm from one collagen molecule to the next along the long axes of the collagen molecules [52]. These gaps serve as nucleation sites for the bone crystals, which are thought to eventually grow out around the staggered collagen

molecules [54]. The long axes (c axes) of the bone crystals align roughly along the long axes of the collagen molecules [48,55]. This staggered arrangement of the collagen molecules with embedded bone crystals is called the mineralized collagen fibril and is the basic unit of bone's nanostructure. These mineralized collagen fibrils are oval shaped with diameters of approximately 80 nm to 120 nm [56].

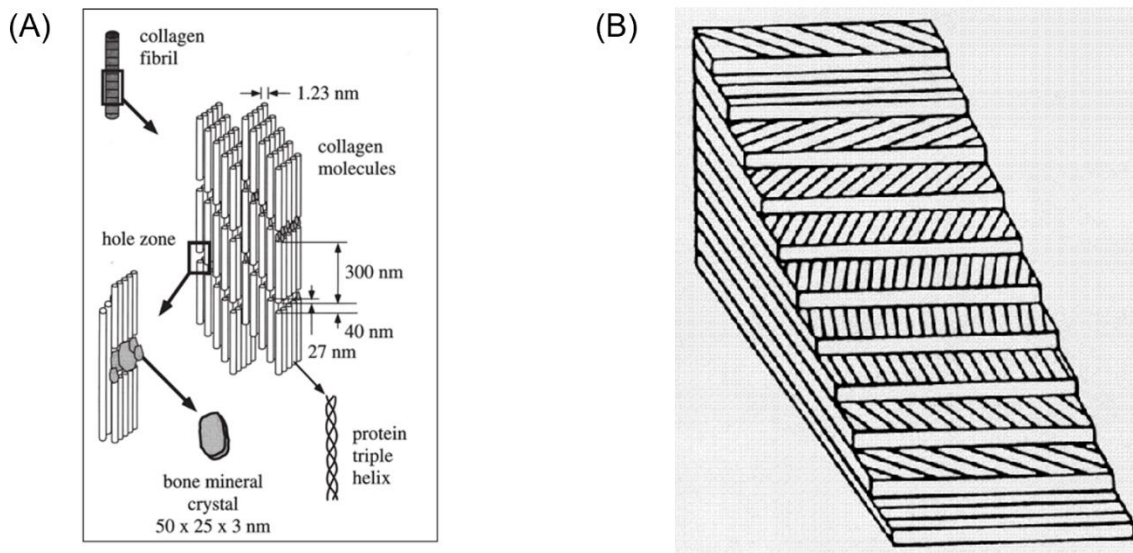


Figure 2.2: Illustration of: (A) the staggered arrangement of the collagen molecules interspersed with bone crystals which together form the mineralized collagen fibril, (B) the twisted plywood architecture characteristic of lamellar bone. Images adapted from Rho et al., 1998 and Martin et al., 2015 [52,57].

NCPs are postulated to be vital to the mineralization process in bone, aiding in bone mineral nucleation and growth [58]. However, the exact locations of the various NCPs are not precisely known but some are thought to line the bone crystal-collagen interfaces [51]. The last major constituent, water has been found to be bound to the hydroxyapatite as well as between and within the collagen molecules [59–61]. In addition, a layer of water is thought to exist between the bone crystal-collagen interface as well as unbound in the canaliculi, lacunae, and blood vessels [60].

The mineralized collagen fibrils collate in a regular array to form cylindrically shaped collagen fibers with diameters varying from a few to several microns [48,54]. Surrounding the collection of the mineralized collagen fibrils is a disordered phase of bone termed fibrillar matrix. This contains less ordered individual collagen fibrils together with non-collagenous proteins and aggregates of polycrystalline bone crystals [62–64] (see Fig. 2.1). Further, the mature bone cells (osteocytes) which

are housed in the lacunae and connected by the canaliculi are predominately found in the fibrillar matrix [64]. These collagen fibers are then arranged in one of two ways. Either they cluster in an irregular pattern subsequently forming what is known as woven bone or they arrange in a well-organized planar pattern called lamellae which make up lamellar or fibro-lamellar (plexiform) bone [52]. Fibro-lamellar and lamellar bone are more predominant in cortical bone than woven bone [65]. Usually, the lamellae have a thickness between 3-7 μm [56]. Two distinct patterns have been identified for how the collagen fibers align in the lamellae even for the same bone type: unidirectional and fanning (twisted plywood) [66]. In the unidirectional pattern, all the collagen fibers align in the direction of the long bone axis when moving from one lamellae layer to the next. However, for the fanning or twisted plywood pattern, the orientation of the aligned collagen fibers changes gradually at a uniform rate moving from one lamellae layer to the next (see Fig. 2.2B).

Fibro-lamellar or plexiform bone is characterized by a “brick like” structure where hyper-mineralized non-lamellae layers are sandwiched between lamellae layers with predominately unidirectional collagen fiber pattern arrangement. This type of bone is common among rapid developing animals such as pigs and cows [51,53]. On the other hand, lamellar bone is formed from lamellae exhibiting both types of fiber patterns: unidirectional and twisted plywood. Lamellar bone forms the basis of an osteon which are characterized by 3 to 8 concentric lamellae layers wrapped around a canal which houses blood vessels and nerves [52,53]. Osteons can be categorised into two groups: primary or secondary. Primary osteons connote freshly formed bone whereas secondary osteons are formed during bone remodeling. Bone remodeling is the removal of old bone and the making of new bone. Secondary osteons are differentiated from primary osteons by the presence of a cement line. Cement lines are highly mineralized outer layers around osteons that form after bone resorption stops [67]. Primary osteons might be present in some fibro-lamellar bone types. Secondary osteons are also referred to as the Haversian canal or system and have diameters ranging between 150 μm and 200 μm . The Haversian system is the most abundant bone type in the matured skeleton of most large animals including humans and is considered the basic unit at the microscale level [52,53].

The macroscale level is characterized by cortical or compact bone and trabecular or cancellous bone. The cortical bone structure is predominantly defined by the Haversian system discussed above. However, the trabecular bone is an interconnected array of collagen fiber rods that form a cell foam matrix and longitudinally arranged lamellae plates [68]. Trabecular bone exhibits far greater porosity reaching values of 80% [68] as compared to cortical bone which is typically less than 6% [54]. In

addition, trabecular bone is typically less mineralized as compared to compact bone [69]. Due to this, compact bone usually forms the outer covering and shield of bone with the trabecular bone found in the inner portions. In some cases, the entire inner part is filled with trabecular bone such as the vertebrae and for long bones, such as the femur, trabecular bone is found predominately at the ends (epiphyses) [51].

2.1.1 Collagen molecule structure

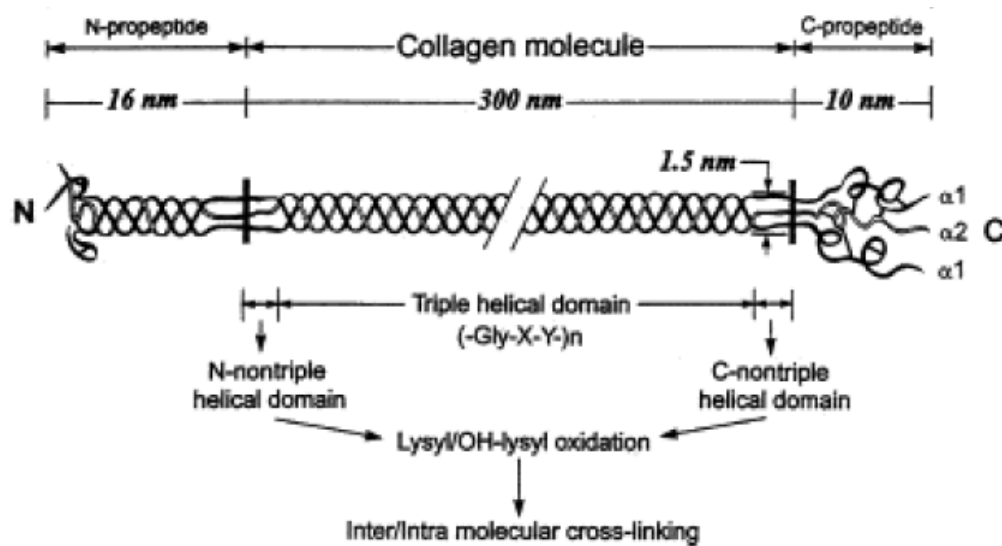


Figure 2.3: Helical structure of type I collagen molecule. Image adapted from Nyman et al., 2005 [70]

The collagen molecule is made up of three polypeptide chains referred to as procollagens which are tightly bound together to form a triple helix [71] (see Fig. 2.3). The three procollagens include two identical alpha-1 ($\alpha 1$) helices and one alpha-2 ($\alpha 2$) helix, and each have 1014 amino acids which adds up to 3042 amino acids in total [70,72]. The triple helix, also known as tropocollagen, exhibits a characteristic repetitive amino acid pattern of Gly-X-Y, where Gly represents glycine, the simplest amino acid and forms about 30% of the total amino acid content [73]. Proline and hydroxyproline typically occupy the X and Y position of the pattern but other amino acids such as lysine and arginine may be present at these positions [70]. This pattern enables the glycine amino acid to occupy the central core of the triple helix allowing for closer packing resulting in the formation of strong hydrogen bonds between the procollagens [71]. This in turn provides greater stability to the collagen molecule, that is,

it allows the collagen molecule to maintain its triple helix structure. Although the collagen molecule is characterized by its helical structure, its ends are denoted by non-helical structures known as telopeptides (See Fig. 2.3). One end of the telopeptide has an amine group ($-\text{NH}_2$) and is denoted as the N terminus and the other end has a carboxyl group ($-\text{COOH}$) denoted as the C-terminus [74]. The C-terminus serves as the starting site for the formation of the triple helix whereas the N-terminus regulates the diameter of the primary fibril [74]. These telopeptides are also important because they provide sites for covalent intermolecular crosslinking which results in even greater stability for the collagen molecules. Crosslinking occurs during post translational modification of the collagen matrix. There are two broadly defined pathways for crosslink formation: enzymatic and non-enzymatic. The enzymatic pathway occurs by the action of the enzyme lysyl oxidase (LOX) [75]. For this pathway, the amine groups on either the lysyl or more predominately the hydroxylysyl residues in bone present in the telopeptide region are converted to more reactive aldehyde forms known as allysine and hydroxyallysine, respectively. These then condense with a helical lysine or hydroxylysine residue on a neighboring collagen molecule [75,76]. Hydroxylysine forms from the post translational modification of lysine by the action of the enzyme lysyl hydroxylase (LH) during collagen biosynthesis [77]. The condensation reaction forms a divalent intermolecular covalent crosslink also known as an “immature” crosslink. There are three types of immature crosslinks that are formed (see Fig 2.4): (1) lysinonorleucine (LNL) by the reaction of allysine with a helical lysine residue, (2) hydroxylysinonorleucine (HLNL) by the reaction of allysine with helical hydroxylysine residue or hydroxyallysine with a helical lysine residue, and (3) dihydroxylysinonorleucine (DHLNL) by the reaction of hydroxyallysine with a helical hydroxylysine [75,78]. These divalent bonds may further spontaneously react with another allysine or hydroxyallysine on the telopeptide of a neighbouring collagen molecule to form a trivalent intermolecular crosslink, also called a “mature” crosslink [75]. When DHLNL and HLNL react with hydroxyallysine, it gives rise to pyridinoline (PYD) and deoxypyridinoline (DPD) crosslinks respectively (see Fig. 2.4). When DHLNL and HLNL react with allysine, it gives rise to the pyrrole crosslinks; pyrrololine (PYL) and dexopyrrololine (DPL) respectively [78]. Pyrrole crosslinks as well some of the immature crosslinks are unstable during acid hydrolysis hence are difficult to quantify [79]. Since the formation of the immature and mature crosslinks is regulated by the LOX enzyme, they are often referred to as LOX-mediated crosslinks or LOX crosslinks for short.

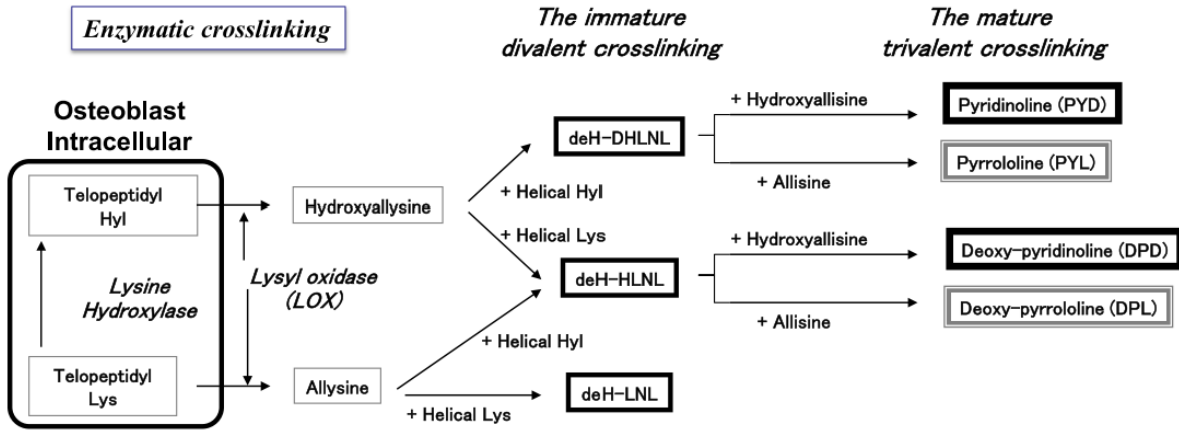


Figure 2.4: Reaction pathways for the formation of immature and mature crosslinking in type I collagen. Lys represents lysine and Hyl represents hydroxylysine. Image adapted from Saito et al, 2015 [79].

The alternative pathway for crosslinking does not involve enzymes and happens more spontaneously. These cross-links are known as non-enzymatic cross-links and their formation is speculated to be non-specific to the telopeptide regions [39,47]. These types of crosslinks can form through a number of pathways involving oxidation, nitration, deamidation and/or glycation reactions. These non-enzymatic crosslinks are widely postulated to be detrimental to the mechanical competence of collagen network [70,80]. Conversely, the enzymatic cross-links are considered to improve the mechanical competence of the bone collagen consequently conferring strength to the bone tissue [81].

2.2 Mechanical behavior of cortical bone at the apparent level

Due to its composition as a naturally occurring nano-composite and its hierarchical structure, bone tissue blends stiffness, strength and toughness to achieve its function in contrast to general engineering materials where one property is often optimized over the other [82]. The stiffness as well as the uniaxial and flexural strength of the bone tissue are primarily attributed to the mineral phase whereas the organic phase and water are considered as the major driving forces for its toughness and “plasticity” [83,84]. In addition, enzymatic crosslinking of the bone collagen network is thought to contribute significantly to bone strength and stiffness [50,85,86].

A key property of the bone tissue is its anisotropic nature, that is, its mechanical properties are orientation or direction dependent. For cortical bone, the strength in the longitudinal direction is greater than in the circumferential and radial directions. The longitudinal direction is taken along the long axis

of long bones. Additionally, there is minimal difference between the properties along the circumferential and radial directions as such cortical bone is often assumed to be transversely isotropic with little error [87]. Further, the bone tissue is asymmetric in strength, being stronger when loaded in compression than in tension [87]. Further, cortical bone is quasi-brittle meaning it has an initial linear elastic/viscoelastic deformation response to loading followed by a non-linear response up to fracture, due to microdamage formation. Microdamage are very fine sub-micron sized cracks (in the order of tens to a few hundred of microns) that form in bone. Typically, the non-linear behaviour of cortical bone has been characterized using yielding parameters such as the yield stress which is calculated using the conventional 0.2% offset method. However, the transition between linear (elastic) to non-linear (inelastic) is not definite and not entirely due to yielding but more so the onset of microdamage formation [69]. In addition to the above, cortical bone is viscoelastic, making its response to loading, rate dependent. Consequently, during cyclic loading of cortical bone, hysteresis occurs. Hysteresis becomes more significant upon the onset of micro-damage formation [88]. Furthermore, with increasing strain rate, cortical bone is stiffer and stronger but less tough (post yield strain decreases) [89].

Whereas stiffness of cortical bone remains fairly constant with age, the other mechanical properties of cortical bone have been showed to deteriorate [90–94]. Cortical bone strength, that is, its tensile, compressive and flexural strengths all decrease with age with about a 30-35% decline over the six decades following the age of 20. A more significant decline is seen in the post yield properties of cortical bone with ultimate strain decreasing by roughly 80% and toughness declining over 40% during the same time span [94–96].

A material's ability to undergo permanent (inelastic) deformation without failure allows for greater energy dissipation which contributes to its overall resistance to fracture. Consequently, the sharper decline in post yield properties for cortical bone suggest that measures beyond cortical bone tissue strength and whole bone strength are necessary to assess fracture risk. In the next section, fracture toughness, a relevant mechanical property to understanding bone's overall fracture resistance, is reviewed. Lastly, since the organic phase is associated with the post yield properties of cortical bone, the decline in post yield properties demonstrate that there are age-related changes to the organic phase that may contribute to reduced bone fracture resistance and thereby bone fragility [50].

2.3 Cortical bone fracture: overview, mechanisms and mechanics

2.3.1 Introduction to fracture mechanics and cortical bone fracture

Fracture mechanics is a specialized branch of mechanics that characterizes the behaviour of materials during the onset and propagation of cracks to catastrophic failure where there is complete separation in the material [97]. This characterizes localized failure due to cracks arising from flaws and defects in a material. By applying fracture mechanics principles, the material property known as fracture toughness can be measured or computed which gives a measure of a material's ability to resist the initiation of crack growth as well as the growth of a crack to catastrophic fracture.

Flaws and defects in materials act as stress concentrators serving as initiation sites for cracks that can propagate. For fracture to occur, there must be enough energy available whether from the material's stored energy or external work done on the material to supply the surface energies needed to create new surfaces as the material undergoes fracture [98]. The energetic cost to create these new surfaces needed for the crack to propagate is termed crack growth resistance (R) [98]. In brittle materials like most ceramics, the crack growth resistance remains constant throughout the fracture process, hence once exceeded, the crack grows rapidly to catastrophic failure. This is termed as unstable crack growth or unstable fracture. In many materials, especially biological materials, the crack growth resistance increases as the crack propagates leading to the scenario where crack growth to catastrophic failure is gradual. This is termed as stable crack growth or stable fracture [97] (see Fig 2.5).

It is important to note that fracture toughness is different from the idea of traditional strength-based measures. In fracture toughness computation, the size of the pre-existing crack or flaw is considered along with the externally applied stress [97]. Whereas strength deals with how a material will resist permanent deformation in response to an applied stress, fracture toughness deals with the localized event of how a material will resist the growth of a pre-existing crack in the presence of an applied stress/load. Cortical bone contains numerous flaws and defects such as linear microcracks, microdamage and microstructure related pores (osteons, lacunae and canaliculi) which makes it highly susceptible to the initiation of cracks that can lead to fracture. Linear microcracks are cracks with length of a few hundred microns and accumulate mostly in interstitial bone and in older adults [99,100]. Traditionally, bone fracture risk has been primarily assessed using the whole bone strength paradigm. Strength typically denotes the force or stress needed to permanently deform or break a material however fracture toughness extends this by dealing with the localized event of crack initiating and propagating

until the material or structure fails [101] . In other words, fracture toughness accounts for the presence of flaws and cracks in determining failure in a material especially important in quasi brittle materials such as bone. Consequently, bone fracture risk may not just be a function of bone strength, but fracture toughness or resistance may well be a critical component to determining overall bone fracture risk and fragility [101].

Interestingly, cortical bone has multiple mechanisms during deformation and fracture which act to dissipate mechanical energy, reducing the energy available to drive crack growth [102]. These mechanisms are known as toughening mechanisms, and they promote stable crack growth in cortical bone giving cortical bone its characteristic rising R curve discussed in section 2.3.3.

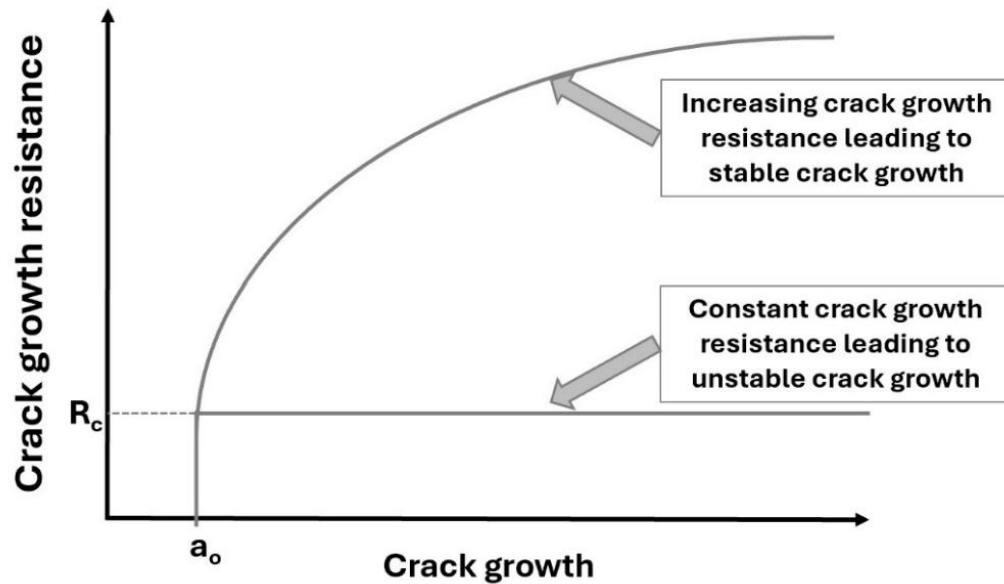


Figure 2.5: Idealized plot of crack growth resistance versus crack growth illustrating how crack growth resistance influences whether crack growth is stable or unstable. a_0 represents the initial crack length before the crack begins to grow. R_c represents the associated critical crack growth resistance at which crack growth initiates. If the crack growth resistance remains constant, then the crack grows instantaneously to catastrophic failure. However, if the crack growth resistance increases then crack growth is gradual as more energy is needed to keep the crack growing.

2.3.2 Cortical bone fracture toughening mechanisms

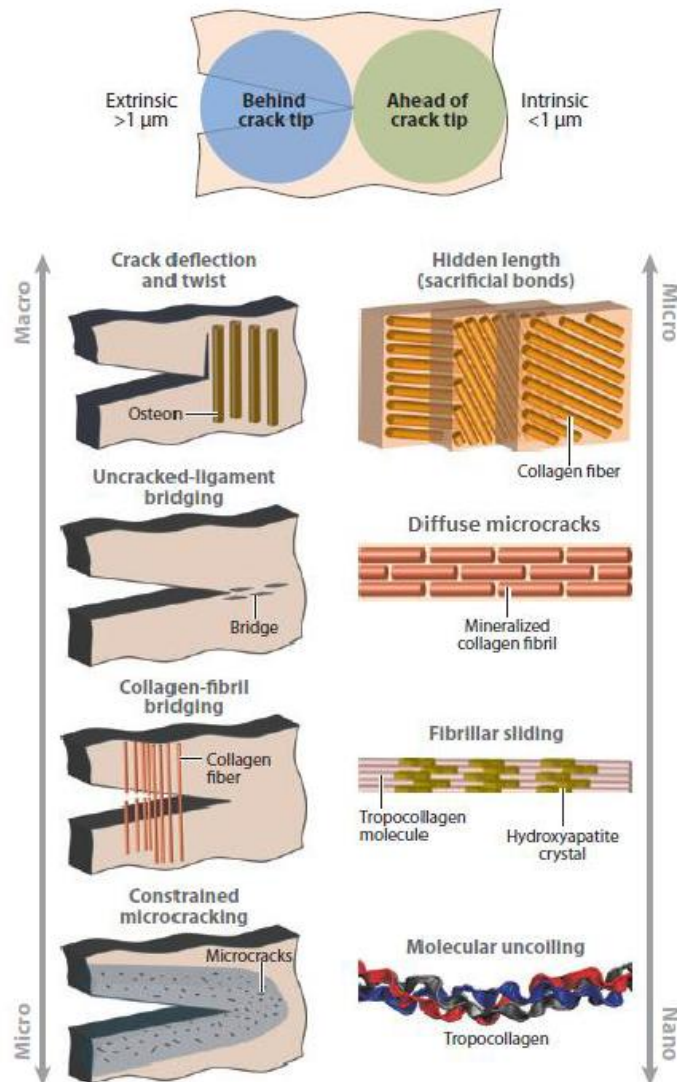


Figure 2.6: Intrinsic and extrinsic toughening mechanisms of cortical bone. Image adapted from Launey et al., 2010 [17].

To diminish the amount of energy available for crack growth, bone employs various toughening mechanisms which can be categorized into two groups: intrinsic and extrinsic (see Fig. 2.6) [17]. Intrinsic toughening mechanisms seek to obstruct the initiation of crack growth, that is, there are mechanisms that occur before the onset of crack growth. As such, they are mechanisms that occur ahead of the crack tip. The intrinsic toughening mechanisms are irreversible deformation processes arising from engagement with the bone structure below the micro-scale level which dissipate energy [17,103].

These irreversible deformation processes may include: the uncoiling and stretching of individual collagen molecules [104–106], sliding and/or breaking of cross-links between neighboring collagen molecules [102] as well as slipping and sliding at the collagen-hydroxyapatite interfaces mediated by non-collagenous proteins [105,107]. Individual collagen molecules (procollagens) have been found to achieve tensile strains close to 50 % and ultimate tensile stresses in the range of 10-20 GPa before failure [104,108]. These properties are critical to its ability to unwind and stretch out, dissipating energy hence acting as a toughening mechanism. Gautieri and co-workers [104] showed using molecular dynamics simulations that at low displacement rates (< 0.5 m/s), the collagen molecules initially stretch up to a strain of 10 %. This is followed by uncoiling of the collagen molecules up to a strain of 25 %, with the stretching of the collagen molecules resuming afterwards. To add to this, a recent experimental study using fluorescently labelled collagen hybridizing peptides (F-CHP) showed empirically that collagen denatures during stable transverse directed fracture in cortical bone [106]. Further, they showed that the amount of denatured collagen correlated strongly with the work to fracture, that is, the total energy dissipated to grow the crack to fracture. These highlight the important role collagen plays in these toughening mechanisms vital to cortical bone's fracture resistance.

Another toughening mechanism which traverses both toughening categories is diffuse microcracking or micro-damage process zone (MDPZ) formation [17]. These are voids, thought to initiate at the sub-micron scale due to the sliding processes at the collagen-bone crystal interfaces, hence an intrinsic toughening mechanism [107,109]. However, they continue to form even after the onset of crack growth, probably with the micron sized micro-damage cracks growing larger hence also an extrinsic toughening mechanism [99,110]. The extensive formation of the micro-damage process zone ahead of a growing crack, blunts the stress field at the crack tip lowering the stored potential energy available to drive continued crack growth. This thereby considerably delays and slows down crack growth [99,111,112]. Recent studies have shown this mechanism to be rate dependent highlighting the importance of the organic phase to this mechanism [112,113]. In addition, it is thought collagen molecules stabilise the bone material during microdamage formation due to their ductile nature further highlighting collagen's importance to bone's fracture resistance. [111].

Extrinsic toughening mechanisms occur after the onset of crack growth and therefore act predominantly behind the advancing crack tip. These mechanisms typically engage the osteons, the predominant structure at the microscale level [17,103]. These include microdamage formation, as mentioned earlier, crack bridging and crack deflection. Microdamage formation is thought to be a precursor for the two

latter mechanisms [114]. Crack bridging involves either unbroken ligaments of the bone tissue lining the path of crack growth or collagen fibrils spanning and connecting the fracture surfaces as the crack grows [88,114,115]. The high stiffness and ductility of the collagen molecules allow the collagen fibrils to bear large stresses as they span the fracture surfaces. This is the predominant mechanism in longitudinally directed fracture [17]. Crack bridging may also occur during the transition from crack growth initiation to propagation. The predominant extrinsic mechanism during transversely directed fracture is crack twisting or deflection. This is a result of osteons lying perpendicular to the crack path with the outer cement lines offering weaker paths for crack growth [114,116]. This shifts the crack tip roughly perpendicular to the direction of the maximum crack growth driving force, slowing down crack growth [116,117]. Due to the considerable crack twisting or deflection in transverse directed fracture, it is comparatively easier to split bone along the longitudinal direction than it is to break bone across the transverse direction [116]. To better understand the impact of these toughening mechanisms on cortical bone fracture, it is important to be able to quantify the resistance they offer against fracture.

2.3.3 Quantification of cortical bone fracture resistance

Early work and some more recent studies have used the idea of work-to-fracture to quantify cortical bone's fracture resistance [90,118]. Fracture as used here occurs at the apparent level and can be defined as the initiation and propagation of a macroscopic crack up until the point of separation in the bone material. Work to fracture is the total energy needed to grow a crack to fracture and basically is the area under the load deflection curve for a fracture test normalized to the area of crack growth. The downside of this measurement are that the size of the starter notch as well as possible geometric differences between samples is not accounted for. This makes it difficult to compare between studies that have different pre-crack sizes and sample geometries. The use of fracture mechanics alleviates these concerns and provides a more comprehensive evaluation of the fracture resistance of cortical bone.

Fracture mechanics approaches have been applied to cortical bone dating back to early work by Bonfield and Datta [119] over four decades ago. Earlier work by investigators used the linear elastic fracture mechanics (LEFM) framework to evaluate cortical bone fracture resistance [23,120–125]. Generally, in this framework, there are two approaches that can be used: (1) stress-based approach and (2) energy-based approach [126]. In the stress-based approach, the stress and displacement fields around the region of the crack tip are used to compute a parameter known as the stress intensity factor (K). This gives a measure of the intensity of the stress ahead of the crack tip. In the energy-based

approach, the energy dissipated to propagate the crack is quantified and used to compute a parameter known as energy release rate (G). Mathematically, they are given respectively as [126]:

$$K = Y\sigma\sqrt{\pi a} \quad (2.1)$$

where Y is known as the geometric factor, σ is the applied nominal stress and a is half the length of the pre-existing (initial) crack.

$$G = -\frac{dU}{da} \quad (2.2)$$

where dU represents the incremental energy released and da represents the incremental crack extension. The negative sign indicates energy must be dissipated to create new crack surfaces.

A key requirement for the LEFM framework is that the material must behave purely elastic until fracture, that is, there should be no or negligible plasticity/inelasticity at the crack tip [97]. Consequently, most materials used in the LEFM framework undergo unstable crack growth. When the value of K or G is found just after crack growth initiation, these are called the critical K or G . When these critical values are found with tension as the mode of failure (Mode I failure), these are then denoted by K_{Ic} or G_{Ic} respectively. These are intrinsic material properties and give a measure of the crack initiation fracture toughness of a material. Typical ranges found for K_{Ic} and G_{Ic} of various mammalian cortical bone species are 2 to 7 MPa $\sqrt{m}$ and 0.15 to 3.25 kJ/m² respectively [115]. The wide variance in the data is a result of the orientation dependence (transverse directed fracture yields almost twice that of longitudinal directed fracture), the different testing approaches and geometric configurations as well as the innate biological variation even for cortical bone from the same species [115].

Subsequent studies however found that the fracture toughness of cortical bone increases with crack growth resulting in stable crack growth [127–131]. This is a consequence of the extrinsic toughening mechanisms discussed in section 2.3.2. As such, a rising resistance (R) curve based on the LEFM framework was used to characterize cortical bone's fracture toughness instead of a single value parameter (see Fig. 2.7). The resistance curve was developed by evaluating K or G at multiple crack extensions after crack growth initiation and plotting these against a function of the corresponding crack extensions. Generally, they showed that the fracture resistance of cortical bone rose linearly with crack extension. The slope of a line of best fit through the data points provided a measure of the crack propagation fracture toughness of cortical bone (see Fig. 2.7).

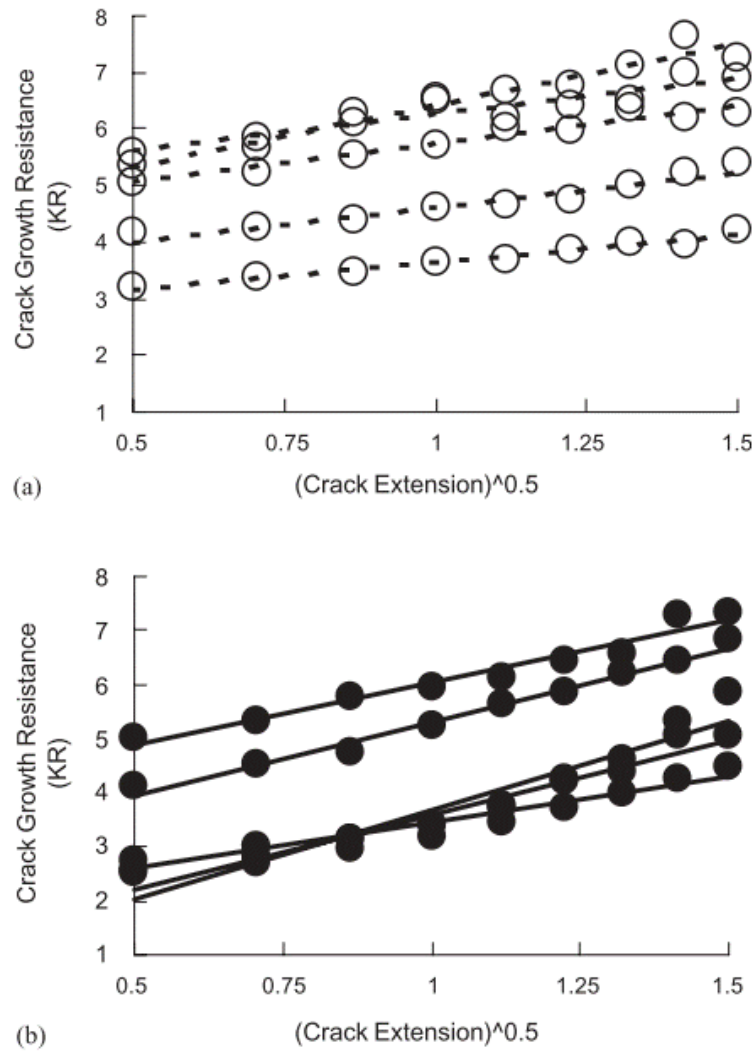


Figure 2.7: Representative LEFM-based rising resistance curve plot for (a) bovine bone and (b) antler bone. This shows a plot of K_R which represents the stress intensity factor computed at a specific crack extension versus the square root of the specific crack extension. Image adapted from Vashishth, 2004 [131].

However, in 2007, Yan and co-workers [132] proposed that an elastic-plastic fracture mechanics (EPFM) approach should be used in quantifying fracture toughness of bone. EPFM is an example of a non-linear fracture mechanics approach. In fact, two earlier studies [133,134] had argued for the use of non-linear fracture mechanics approaches for bone as it undergoes considerable non-linear behaviour before crack propagation. This violates LEFM's assumption of negligible non-linear behaviour. EPFM uses an energy-based approach to quantify fracture toughness by assessing the energy dissipated in both

the linear and non-linear processes. This is typically done by using a contour J-integral to capture both elastic and inelastic energy dissipated around the region of the crack tip during fracture. Mathematically this is presented as [97]:

$$J = \oint_{\Gamma} W dy - T_i \frac{\partial u}{\partial x} ds \quad (2.3)$$

where W is the strain energy density, u is the displacement vector, T is the tractions along the contour and Γ represents the contour path. The first term, $W dy$ captures the decrease in stored strain energy per unit crack extension while the second term captures the added work done due to external forces per unit crack extensions.

This J integral approximates to the energy release rate, G , for a purely elastic material [126]. Eq. 2.3 can be further simplified to make use of experimentally derived load-deflection curves from fracture bending test as [126]:

$$J = \frac{\eta U}{Bb} \quad (2.4)$$

where U is the area under the load-deflection curve corresponding to the specific amount of crack growth, B is the thickness of the specimen, b is the length of the unbroken ligament and η is a plastic configuration factor (dependent on test specimen configuration and dimensions).

Like LEFM, a critical J value can be found just at crack growth initiation, and this is denoted as J_{IC} . Furthermore, the concept of resistance curves can be applied to generate J integral resistance (J-R) curves. Currently, the J-R curve is the most widely used approach to quantifying the fracture toughness of cortical bone [22,24,116,135–142]. From this curve, the crack initiation fracture toughness (J_{IC}) and crack propagation fracture toughness or tearing modulus (dJ_R/da) can be found (See Fig 2.8A). The tearing modulus is the slope of the J-R curve beyond the J_{IC} point on the curve and quantifies the ability of cortical bone tissue to engage extrinsic toughening mechanisms. J_{IC} quantifies the ability of the cortical bone tissue to employ intrinsic toughening mechanisms. Another parameter that has been computed from the J-R curve is the J integral at the point of instability often termed as J-int [24,135]. This corresponds to the last valid point on the J-R curves and occurs roughly at the point the J-R curve begins to plateau (See Fig 2.8A). This is the peak energy release rate and gives a measure of the overall energy dissipated before unstable crack growth is experienced.

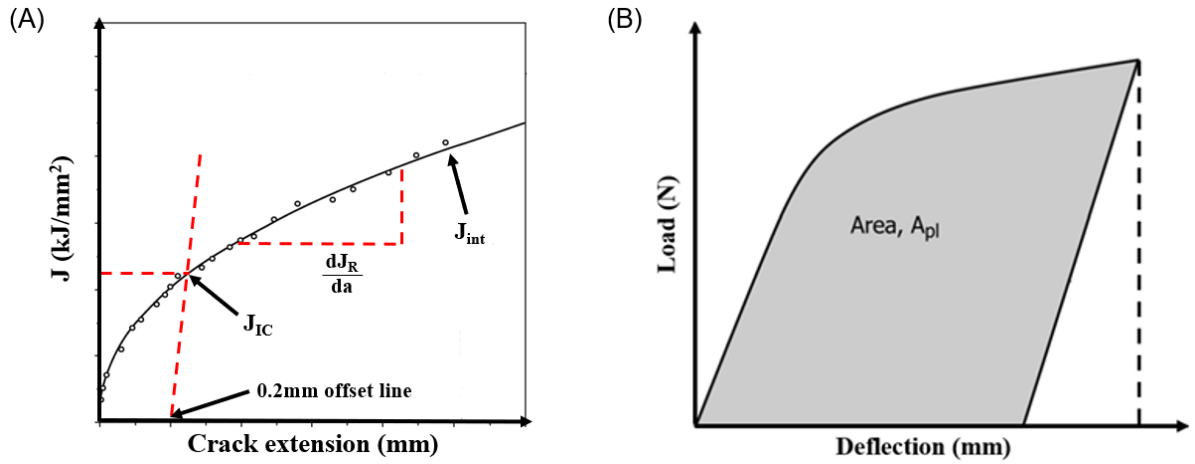


Figure 2.8: (A) A typical J-R curve showing the crack initiation fracture toughness (J_{1c}), crack propagation fracture toughness (dJ_R/da) and crack instability fracture toughness (J_{int}) (B) A typical load deflection curve where the shaded region represents the area under the load-plastic deflection region (A_{pl}). These are not representative of the behaviour of cortical bone. Image adapted from ASTM E1820 standard [143].

2.3.4 Determination of cortical bone J-R curve

Traditionally to develop a J-R curve for a specific material, multiple identical test specimens were required. A J-R curve basically plots the J-integral at multiple crack extensions against those crack extensions (Fig. 2.8A). Hence, in this multi-specimen approach, identical specimens were used to quantify the J-integral at different crack extensions. These specimens were not tested to catastrophic failure but were loaded to pre-determined loads after which they were broken open and the fracture surfaces imaged to determine the crack extension from their topographical information. This approach, however, was tedious and led to significant errors, as it was difficult to prepare multiple specimens with the same dimensions. In addition, biological tissues have innate variability between the specimens also leading to errors. Further, this approach required a lot of material which meant increased cost and for biological materials, there is limited availability.

Consequently, a single specimen approach for J-R curve determination was developed and is outlined in the ASTM E1820 standard [143]. This standard was specifically designed for metallic materials but has been widely adopted for assessing J-R curves in cortical bone. This approach estimates the crack extension within a single specimen in conjunction with recording the load versus displacement/deflection curve from the fracture test. From these, the J-R curve can be computed.

Adjustments must be made to Eq. 2.4 to account for a continuously growing crack. As such, the J-integral is split into elastic (J_{el}) and plastic (inelastic) components J_{pl} [143]:

$$J = J_{el} + J_{pl} \quad (2.5)$$

The elastic component is updated for a crack extension-load pair measurement as [143]:

$$J_{el} = \frac{K_{(i)}^2 (1 - \nu^2)}{E} \quad (2.6)$$

and

$$K_{(i)} = \frac{P_i S}{B W^{3/2}} f\left(\frac{a_i}{W}\right) \quad (2.7)$$

where $K_{(i)}$ represents the updated stress intensity factor, ν is the Poisson ratio, E is the elastic modulus, S is the span of the fracture test, B is the thickness of the specimen, W is the width of the specimen, P_i is the current load value, a_i is the current crack length (initial crack length plus crack extension) and $f\left(\frac{a_i}{W}\right)$ is the updated geometric factor accounting for the type of specimen used for the fracture test.

In the case of the plastic component, however, an incremental approach is used to update it [143]:

$$J_{pl} = \left[J_{pl(i-1)} + \left(\frac{\eta_{pl}}{b_{(i-1)}} \right) \left(\frac{A_{pl(i)} - A_{pl(i-1)}}{B} \right) \right] \left[1 - \gamma_{pl} \left(\frac{a_{(i)} - a_{(i-1)}}{b_{(i-1)}} \right) \right] \quad (2.8)$$

where the subscript $(i - 1)$ represents the previous measurement of the denoted parameter, b is the unbroken ligament length, $A_{pl(i)}$ is the current area under the load versus plastic (inelastic) deflection curve (see Fig. 2.6B, shaded region), η_{pl} is a configuration plastic constant and γ_{pl} is a geometric factor related to η_{pl} .

The practical challenge with the single specimen approach is how to accurately measure or estimate the crack extension throughout the fracture test. A common method used to estimate crack extension for metals is the unloading compliance (UC) method [144]. In this method, a series of partial unload and reload cycles are applied at regular intervals during the fracture test and the compliance of the unloading cycles are used to estimate the current crack length or extension. Empirically derived standard calibration equations are used to relate the unloading compliance to the crack length. These standard calibration equations were specifically derived for metals and have some major assumptions which are: (1) the change in unloading compliance is only due to crack growth, (2) there is no or negligible energy

loss during the unload-reload cycles and (3) the crack front grows in a relatively straight line [97]. Although, this UC method has been widely adopted for cortical bone, cortical bone violates these assumptions. Microdamage formation causes a change in unloading compliance while there is no true crack growth [69]. In addition, the viscoelastic nature of cortical bone causes energy to be lost in the form of hysteresis especially when microdamage formation begins [69,145]. Significant crack deflection and twisting is observed especially for transverse directed fracture in cortical bone if side grooves are not used [146]. Further, metals are isotropic materials, therefore, the empirically derived standard calibration equations are tailored to isotropic materials. A recent study showed that when these isotropic based standard calibration equations are adopted for orthotropic materials, significant errors arise in the fracture toughness measures [147]. Meanwhile, cortical bone is assumed to be a transversely isotropic material, a variant of an orthotropic material. Therefore, it is unsurprising that the standard calibrations equations were found to produce inaccurate crack extensions for equine cortical bone and that new empirically derived equations are required for various species of cortical bone for accurate crack extension estimation [148,149].

A better approach would be to optically track the growing crack and measure this directly rather than estimating the crack extension using the UC method [141]. Surprisingly, how these limitations of the UC method impact fracture toughness measurement of human cortical bone has received little attention.

2.3.5 Human cortical bone fracture toughness studies and determinants of fracture resistance

As mentioned earlier, cortical bone is known to be transversely isotropic and as such, there is an orientation dependence with fracture toughness. Cortical bone has a higher fracture toughness in transverse directed fracture compared to longitudinal directed fracture [116,122]. In addition, fracture toughness of cortical bone is also rate dependent. It becomes more brittle as strain rate increases. J based measurements have shown a 30-40% decrease in fracture toughness at fall-like rates compared to quasi-static rates [136]. The reduction in fracture toughness has been associated with a decrease in micro-damage process zone (MDPZ) formation as strain rate increases in cortical bone [150,151]. That is cortical bone reaches unstable crack growth faster with increasing strain rates. In fact, for most cortical bone types, a transition from stable to unstable crack growth at a certain strain rate can be found and this is termed a ductile-to-brittle transition. Interestingly, a recent study [118] found that this ductile-to-brittle transition happens at lower strain rates, 10^{-3} s^{-1} , in older human bone and this is

consistent with the physiological rates of walking and running, compared to younger bone that occur at much higher rates (10^{-2} s^{-1}).

Beyond the idea of fracture toughness being orientation and rate dependent, one of the key findings of fracture toughness studies is that fracture toughness of cortical bone decreases with aging [23,90,135,146,152]. There is a reduction in the crack initiation fracture toughness (intrinsic toughness) but a more prominent reduction in crack propagation fracture toughness (extrinsic toughness) [128,146]. This suggests there are compositional, morphological and/or microstructural changes that occur in the cortical bone tissue with aging leading to reduced fracture toughness. Therefore, the notable question is “what are these changes at the microstructural level and below that affect the fracture resistance of cortical bone?” This will help identify key determinants of fracture toughness in cortical bone that may be relevant to the development of better clinical fracture risk assessment tools. As such, various studies over the years have sought to find these various key determinants that relate with cortical bone fracture toughness.

From a microarchitecture standpoint, increased cortical bone porosity has been found to correlate positively with age and is associated with a reduction in fracture toughness [125,135,152,153] (see Fig. 2.9) Conversely, osteonal density which represents the number of osteons per unit area of bone tissue is positively correlated with bone fracture toughness and has been found to decrease with age [152,153]. Changes in these two parameters with age are thought to be the effect of continuous bone remodeling. Bone remodeling is thought to result in more but smaller osteons being formed which increases the number of cement lines allowing for greater crack deflection thereby enhancing transverse directed fracture toughness of the bone. Increased porosity provides more stress concentration sites for easier initiation and propagation of cracks. In addition, the ratio of the interstitial bone to osteonal bone increases with age and this has also been associated with a reduction in fracture toughness as total osteonal bone area correlates positively with cortical bone fracture toughness [153] (see Fig. 2.9).

For the mineral phase, it is thought that an increase in crystallinity (crystal size and lattice perfection) and heterogeneity (spatial variation of bone crystals across the bone tissue) with age correlates with loss of fracture toughness [90,154]. However, this remains inconclusive as other studies have reported opposing results [50,80]. The amount of mineralization has also been found to increase with age which is thought to promote bone strength but has been shown to potentially decrease fracture toughness [50]. The amount of mineralization is typically reported as mineral-to-matrix ratio.

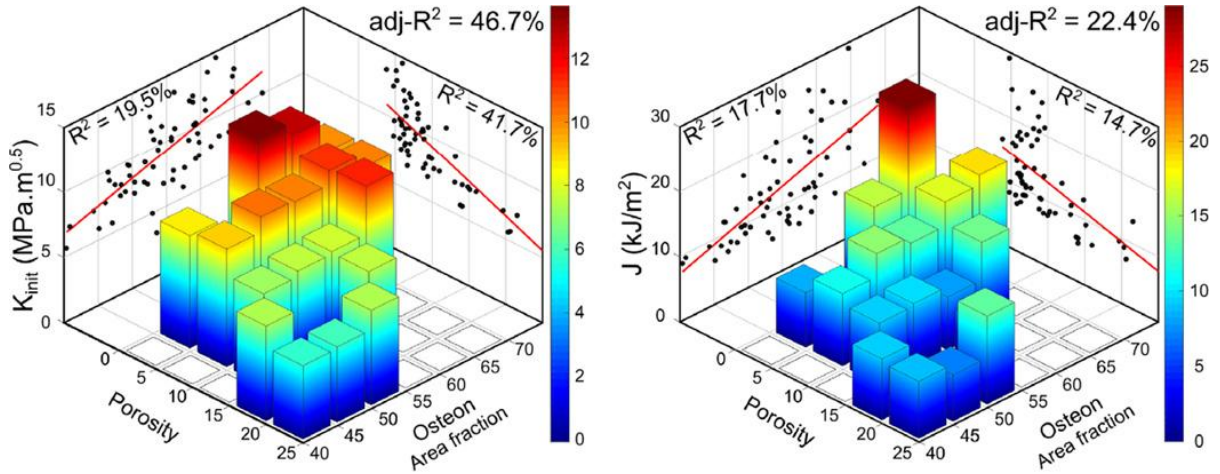


Figure 2.9: 3D bar graphs depicting the relationship between cortical porosity and osteon area fraction with crack initiation fracture toughness (K_{int} on the left) and crack instability fracture toughness (J on the right). Image adapted from Granke et al., 2016 [153]

For the organic phase, the amount of collagen present as well as the nature and extent of the intermolecular collagen crosslinking (pyridinolines and pyrroles) have been related to fracture toughness [50]. The pyridinolines and pyrroles are thought to provide more stability and connectivity for the collagen network which in turn boost the toughness of the bone [70]. Whereas the pyridinolines are thought to remain constant after puberty, the pyrroles are thought to reduce as bone mass is lost [155]. In contrast to the enzymatic mature cross-links, non-enzymatic crosslinks are thought to reduce fracture toughness as they inhibit sliding of the collagen fibrils [146]. Pentosidine, an example of the non enzymatic crosslink, has been found to accumulate in bone with age and in bone affecting diseases therefore is postulated to be a key factor in the loss of fracture toughness observed [50]. However, the role of pentosidine in fracture toughness loss remains inconclusive as they are produced in relatively low quantities compared to the enzymatic crosslinks in bone [156]. Further, a few studies have shown that collagen network connectivity and organisation degrade with age and in bone affecting diseases which correlates strongly with the loss of fracture toughness observed [19,23,24]. Collagen network connectivity is an abstract term used to denote the degree to which the various collagen molecules are linked or connected together. It is measured using a technique known as hydrothermal isometric tension test which is discussed in section 2.4. Collagen network connectivity is considered to be a function of the intermolecular crosslinking as well as the polypeptide chain length of the tropocollagen. This has been shown through irradiation and ribose incubation studies [21,22,33]. In a in-vitro model, where

bovine bone samples were incubated in ribose, it was found that the collagen network connectivity increased as pentosidine, a non enzymatic crosslink increased in the ribated samples [33]. Interestingly, for the ribated samples, collagen network connectivity was positively associated with energy absorption capability measured using a 3-point bending test. In another study where bone specimens were irradiated to mimic how allografts are sterilized before implantation; the collagen network connectivity was found to be severely degraded [21]. This was because of fragmentation or scission of the collagen molecules, a consequence from the irradiation.

Zioupos et al. [19] measured collagen network connectivity in specimens taken from the femoral mid diaphysis of 10 male donors between 35 to 92 years of age. They showed that collagen network connectivity correlated strongly with age as well as various measures of fracture resistance including J-based fracture toughness and work to fracture. A more recent study, using a larger and more heterogenous donor group of 62 donors between 21 and 101 years also found collagen network connectivity to correlate with measures of fracture toughness [24] (see Fig. 2.10). This donor group included those with a history of cancer, type 1 and 2 diabetes and osteoporosis with specimens taken from the lateral femoral mid-shaft. In addition, using general linear models, this study found that the combination of age, volumetric BMD and collagen network connectivity explained 49% of the variation seen in crack initiation fracture toughness. The combination of age and collagen network connectivity explained 36% of the variation observed in crack instability fracture toughness with a standardized beta coefficient of 0.52 for collagen network connectivity [24]. Lastly, Wang and others [23] showed that the mechanical integrity of collagen network decreased with age highlighted by greater collagen denaturation or disorganisation. This correlated with reduced work to fracture as well as fracture toughness of cortical bone. They used samples from 30 human cadaveric femurs from donors between the ages of 19 and 89 years.

Despite these findings from ex vivo human bone studies, mechanisms that could potentially degrade the collagen network's connectivity and organization with aging and in bone affecting diseases have received little attention. Knowledge of these mechanisms could reveal important biomarkers of collagen network connectivity and organization degradation which will be vital to the development of better bone fracture risk assessment tools. This highlights an important gap that needs to be addressed.

Non-collagenous proteins (NCPs) are considered important to bone's fracture resistance because they facilitate adhesive and viscoelastic energy dissipation mechanisms at the bone crystal-collagen

interface [107,157]. However, there are but a few studies that have investigated their impact on cortical bone fracture toughness. An animal model study with rats showed that an increase in NCPs resulted in increased fracture toughness [158].

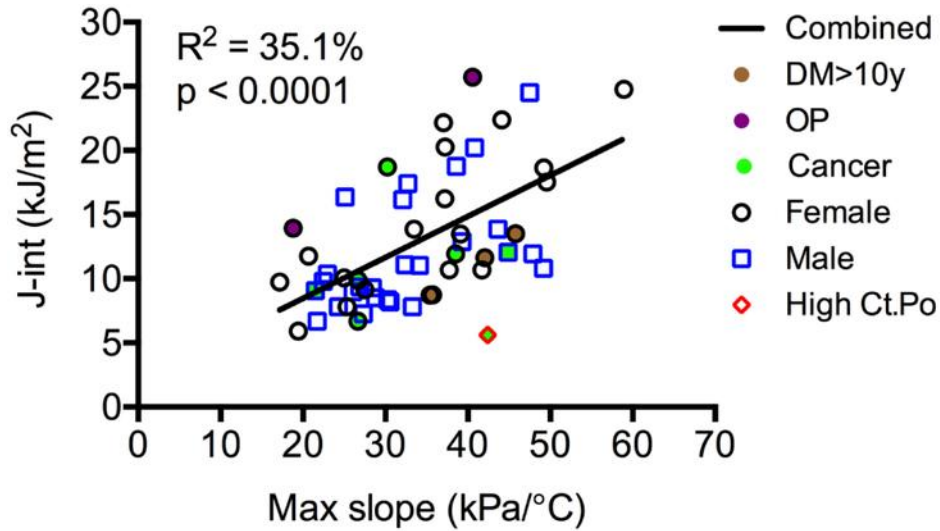


Figure 2.10: Linear regression plot of J-integral fracture toughness at instability versus Max slope, a measure of the collagen network connectivity. Imaged adapted from Willett et al., 2019 [24]

Water exists in bone as pore or free water within the Haversian canals, canaliculi and lacunae or as bound water; an integral part of the bone crystal lattice or attached to the collagen molecules or hydrogen bonded at the collagen-mineral interface [159]. There is evidence that bound water may contribute to the fracture toughness of bone [24,159,160], probably because of its close inter-relationship with the matrix of the bone [80]. Pore water, on the other hand, has been found to correlate negatively with fracture toughness [135]. In addition, it strongly correlated with cortical bone porosity hence can be thought of as a surrogate measure for porosity [80].

While a lot of progress has been made using cortical bone fracture mechanics to better understand mechanisms that lead to reduced bone fracture resistance, much remains to be learned and understood. For instance, while degradation of the bone collagen network is implicated in reduced fracture resistance, the mechanisms by which this occurs are poorly understood. Such an understanding is important on two fronts: (1) towards developing better tools for fracture risk assessment as well as (2) better preventative and therapeutics measures against bone fragility.

2.4 Assessment of the quality of the cortical bone collagen network

It has been established that the organic phase is critical to the “post yield” properties as well as the fracture resistance of cortical bone. However as noted, current clinical fracture risk assessment tools do not assess the quality of the organic phase. With type I collagen forming 90% by volume of the organic phase, it is considered to be vital to the above-mentioned properties of cortical bone. Therefore, the ability to assess the quality of the collagen network is beneficial to understanding how it affects the fracture resistance of cortical bone.

Collagen content and the LOX crosslinks can be measured using various biochemical analyses or assays. Collagen content in a sample/tissue is typically determined by measuring the amount of hydroxyproline. Hydroxyproline forms from the post translational hydroxylation of proline [161]. Hydroxyproline is rarely found in other proteins besides collagen explaining why it is used as a marker for collagen content. Further, hydroxyproline forms about 14 % of the dry weight of collagen in most mammalian tissues [162]. Hydroxyproline does not fluorescence therefore colorimetric based methods are most widely employed to measure hydroxyproline [163]. This method involves the oxidation reaction of hydroxyproline with chloramine-T to form a pyrrole. This is then subsequently reacted with dimethylaminobenzaldehyde (DMAB) usually dissolved in a solution of perchloric acid and propanol commonly known as Ehrlich’s reagent [164]. This reaction gives rise to red chromophores from which their absorbance at 560 nm can be measured using a spectrometer or plate reader and related back to hydroxyproline concentration through standard curves. Alternatively, hydroxyproline can be measured using high performance liquid chromatography (HPLC) individually or coupled with mass spectrometry [161,165–167] but these are not commonly used in cortical bone related studies. HPLC works by introducing a mobile phase which contains the sample of interest in solution by means of a pump or manual injector into a column with a stationary phase. The interaction between the stationary phase and the various components of the mobile phase causes the various components to move through the column at different speeds. That is, the various components in the mobile phase will travel through the column at different times known as retention time causing the components to separate from each other. The components once out of the column are then directed to a detector where a physical property such as absorbance or fluorescence is measured [168]. This helps give a measure of quantity and the retention time helps denote what type of molecule/substance it is by comparing to standards. Generally, for HPLC, the hydroxyproline undergoes a double derivatization before being fed through the column to be separated from the rest of the collagen. In general terms, derivatization is a chemical reaction that

changes a functional group in a compound to a product called the derivative, which has similar structure but differing and more stable physicochemical properties [169]. The derivatization therefore allows the hydroxyproline to be detected either by absorbance or fluorescence based on the derivatizing agents. Derivatizing agents that have been used include o-phthaldialdehyde, 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl) , phenylisothiocyanate (PITC), 4-dimethylaminoazobenzene-4'-sulfonyl chloride and 9-fluorenylmethylchloroformate (Fmoc) [170,171].

Concerning the crosslinks, the mature LOX-mediated crosslinks fluoresce hence can be measured directly using HPLC with an excitation-emission regime of 295 nm and 395 nm respectively [172]. The immature LOX mediated crosslinks, similar to the hydroxyproline, must be derivatized. This is typically done using O-phthaldialdehyde and the fluorescence of the derivative is measured using an excitation-emission regime of 350 nm and 450 nm respectively [172]. In addition, mass spectrometry can be coupled with HPLC to measure the LOX crosslinks. In all cases, the collagen tissue must be acid hydrolyzed and the concentration of the hydroxyproline or the LOX mediated crosslinks is found by comparing to standard curves.

Another method that has been used extensively to assess bone collagen quality is differential scanning calorimetry (DSC). DSC is a widely used thermo-analytical tool capable of measuring characteristic phase transitions such as the glass transition, melting and crystallization temperatures as well as the heat capacity of macromolecules and materials [173]. For bone collagen, it can be used to investigate the thermal properties of the collagen network including its stability, nativity, disorder and enthalpy of denaturation [21,174]. Differential scanning calorimetry works by measuring the heat flow difference between a sample or specimen and a reference over time or temperature undergoing a controlled heating programme [175]. Typically, the sample is kept in a sealed pan whereas the reference is an empty sealed pan. When the heat flow difference versus time or temperature is plotted, an endotherm develops if the sample absorbs more heat compared to the reference. Conversely, an exotherm develops if the sample dissipates more heat compared to the reference [173]. When type I collagen and for that matter, bone collagen is heated, at a certain temperature (or temperature range) it undergoes melting or denaturation. Denaturation is characterized by the breaking of the hydrogen bonds between the individual collagen polypeptide chains (procollagens) that make it a triple helix structure [176]. This leads to the triple helix structure unravelling, isolating the procollagens. Since energy is required to break the bonds, the denaturation process is characterized by heat absorption. Consequently, DSC gives rise to an endothermic heat flow versus temperature curve for bone collagen. From this endotherm, various

thermal properties of the specific bone collagen can be determined (see Fig. 2.11A) [21]. These includes the temperature at which denaturation starts (T_{onset}) and the peak temperature (T_{peak}). These are measures of the thermal stability of the collagen network. The denaturation temperature is measured as the intersection between the steepest tangent of the initial slope of the endotherm and the baseline heat flow. The peak temperature is the maximum heat flow difference recorded. In addition, the full width at half height (FWHM) can be determined which gives a measure of the heterogeneity (disorder) of the collagen thermal stabilities in the network [177]. Finally, the enthalpy of denaturation (ΔH) which is the energy required to completely denature the collagen network can also be determined and this is a function of the molecular structure of the collagen [21]. The enthalpy of denaturation is calculated as the total area under the endotherm and is normally normalized to the mass of the sample.

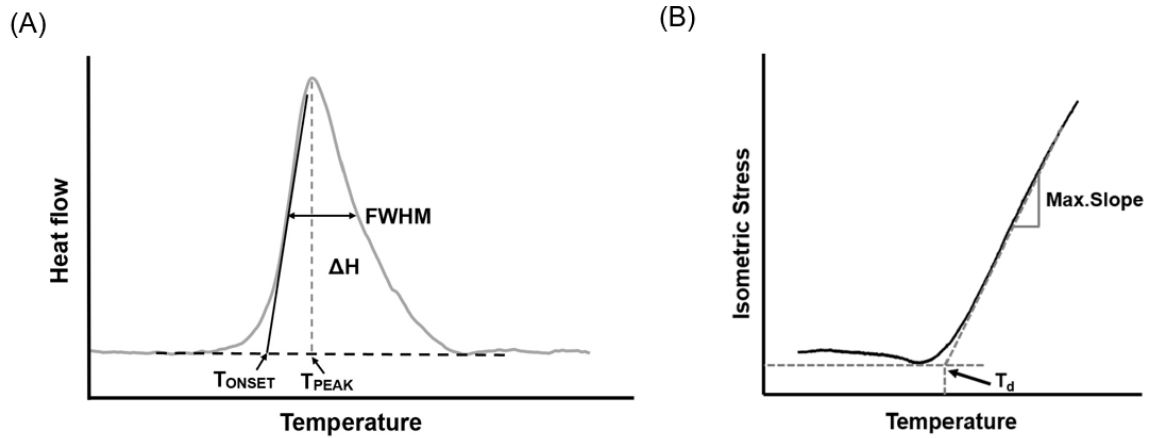


Figure 2.11: Representative (A) DSC curve and (B) HIT curves for human cortical bone collagen showing various parameters that can be computed from the curves. For (A) T_{ONSET} represents the temperature at which denaturation of the collagen starts, T_{PEAK} represents the temperature at which peak heat flow occurs, FWHM is the full width at half maximum of the endotherm and ΔH represents the enthalpy of denaturation, the total energy absorbed to denature the collagen. For (B) T_d represents the denaturation temperature where denaturation starts and Max.Slope represent the maximum rate of isometric stress generated after denaturation a measure of collagen network connectivity.

Hydrothermal isometric tension (HIT) testing is another technique that has been employed to study collagen network quality in bone [19,24]. HIT works by heating the collagenous material in a water bath while keeping it under isometric constraint [20,178] (see Fig 2.12). As already discussed, when collagen is heated, it undergoes denaturation. During the denaturation process, the triple helix structure of the collagen unravels resulting in a random amorphous arrangement of the procollagens which causes

the collagen network to shrink. However, in HIT because the collagenous material is under isometric constraint, it generates an increasing isometric force. This isometric force is typically converted to isometric stress by dividing the force by the cross-sectional area of the collagenous sample to account for geometric differences. The isometric force is measured using a load cell while the temperature of the water bath is recorded using a temperature detector or thermometer. Using these, the HIT test curve is obtained by plotting the generated isometric stress against temperature (see Fig. 2.11B).

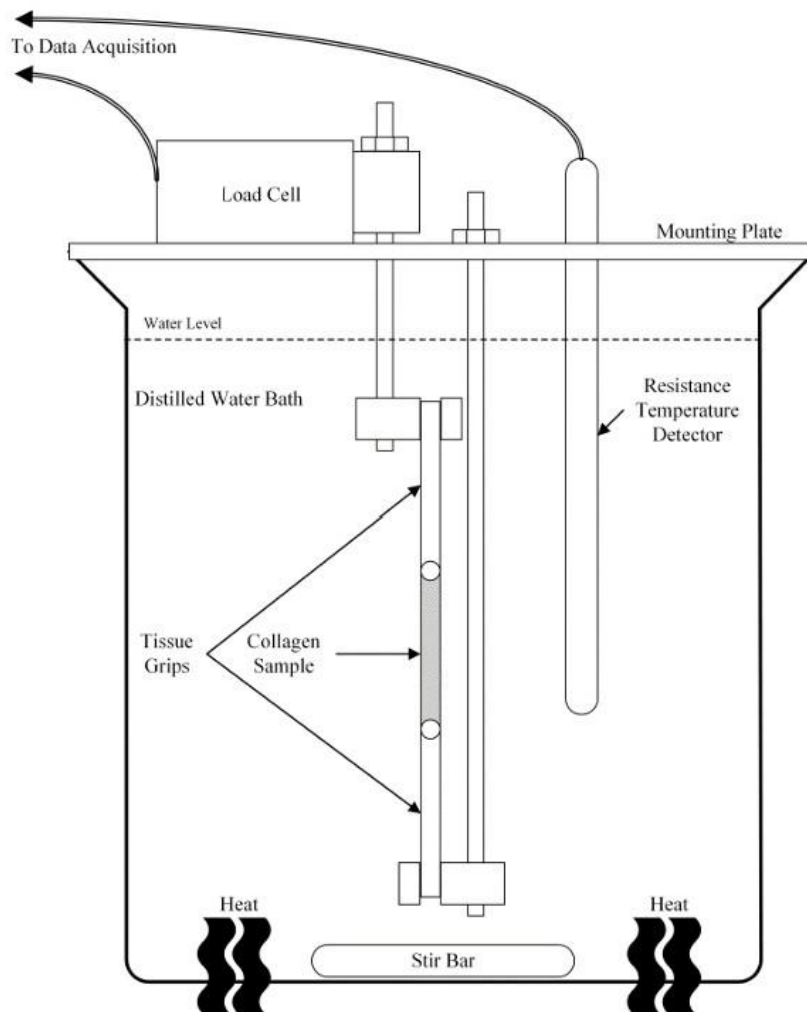


Figure 2.12: HIT test system set-up. Image adapted from Woodside, 2015 [179]

From the test curve, it is observed there is an initial relaxation period due to the viscoelastic nature of the collagen molecules followed by the onset of denaturation characterized by the generation of an increasing isometric stress afterwards (see Figure 2.11B). From the HIT curve, the denaturation temperature, a measure of stability of the collagen network can be determined as well as the maximum rate of isometric force/stress generation known as maximum slope (Max.Slope). Max.Slope is a measure of the collagen network's connectivity and is a function of the intermolecular crosslinking and collagen's polypeptide chain lengths [21,141]. Other studies have also measured the half-life of load decay using the HIT test [180]. This is done by heating the collagenous sample to 90°C and holding it at an isotherm for about five hours. The load decays over time at the isotherm. This is as a result of procollagen hydrolysis where the water molecules cause the peptide bonds within the chains to break [181]. A maxwell decay model is typically fitted to the decaying curve during the isotherm. The half time of the fitted model is reflective of the rate at which the load decays and can be calculated. This half time of load decay is thought to be a measure of the intermolecular network integrity.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is another technique that can be used to assess the collagen network quality. It can evaluate the purity as well as the fragmentation profile of bone collagen. It has been used in irradiation studies to assess the damage caused to bone collagen in terms of fragmentation of the $\alpha 1$ and $\alpha 2$ polypeptide chains [182]. SDS-PAGE works by using an electric field to separate proteins or protein fragments based on their molecular weight [183]. Sodium dodecyl sulfate (SDS) which is a detergent unravels the tertiary structure and binds to all the polypeptides within the protein providing a uniform negative surface charge. The protein is then injected into wells of an polyacrylamide gel across which an electric field is applied. Due to the negative charge on the proteins, the protein components will migrate according to their mass to charge ratio towards the positive electrode which is at the base of the gel. However, since the SDS gives all the proteins a uniform negative surface charge, the protein components are separated based on their mass, with the lowest molecular weight moving the fastest through the gel. The separated proteins can be visualized as bands by using a staining reagent such as Coomassie blue which binds specifically to proteins [184]. These bands are then imaged using a gel imaging system or gel scanner [185]. The intensity of the bands from the images gives a measure of the number of the specific protein polypeptide associated with that band. The intensity of the band is quantified by integrating the area under the histogram derived from the band using various imaging processing software such as ImageJ or Bio-Rad's Image Lab [186,187]. The molecular weight associated with these bands can be calibrated using

protein standards with polypeptides of known molecular weight [188]. Native type I collagen typically has four main distinct bands that form [182] (see Fig 2.13). The topmost band represents three α chains crosslinked together known as the gamma (γ) band and has the highest molecular weight. The next band below it represents two α chains crosslinked together known as the beta (β) band. The last two bands represent the individual α -I and α -II chains with the band of the α -I chain occurring at a slightly higher molecular weight (see Fig 2.13) [189]. The α chains have a molecular weight between 100 and 150 kDa [189]. In addition, the α -I band is generally brighter or more intense as the triple helix of type I collagen consist of two α -I chains and one α -II chain. Any bands or smearing stains below these represent fragmentation of the bone collagen molecules [182].

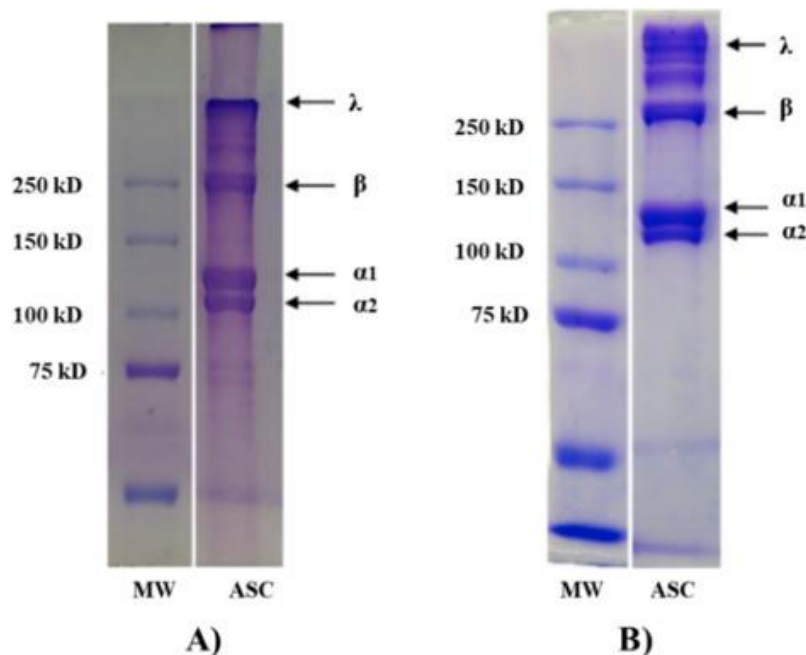


Figure 2.13: Representative SDS-PAGE pattern for (A) salmon collagen obtained by acid soluble collagen (ASC) extraction and (B) codfish collagen obtained by ASC extraction. The pattern on the left side of each figure represents the molecular weight (MW) bands from a protein standard. Image adapted from Alves et al., 2017 [190]

In addition to the above-mentioned methods, two common imaging modalities that have been used to assess collagen network quality are Fourier transform infrared spectroscopy (FTIR) and Raman spectroscopy. These approaches have the advantage of being non-destructive modalities but the disadvantage of low signal to noise ratio as well as not being a direct measurement of the collagen tissue. Both modalities make use of molecular bond vibrations generated by excitation from incident

light. The various molecular bonds have different characteristic vibrational frequencies hence these can be recorded and used to assess the compositional make-up of a sample. In bone, these two modalities provide compositional information on the mineral and organic phases thereby can be used to assess collagen network quality [191,192].

In FTIR, an intense infrared (IR) light is shone onto the material of interest where a range of wavelengths are absorbed, and these are monitored to produce an absorption IR spectrum. The range of wavelengths that are absorbed is proportional to the characteristic vibration frequencies of the bonds that make up the material. The absorption IR spectrum is a plot of absorbance versus wavelength (Fig. 2.14A). Each wavelength corresponds to a particular molecular or chemical bond while the level of absorbance at a particular wavelength corresponds to the number of the associated bond present in the material. In the case of Raman, a monochromatic light is introduced into the material which interacts and excites the molecules resulting in vibrational motion. This vibrational motion causes inelastic scattering, where a small portion of the incident light loses energy and is scattered at longer wavelengths than the incident light based on the molecular bonds present. In other words, the different wavelengths at which the incident light is scattered are characteristic of the composition and bonding present in the material. The different wavelengths of the scattered light are recorded, labelled as wavenumber units. From these, a Raman spectrum can be obtained as a plot of the intensity of each scattered light wavelength which is proportional to the number of the associated bond present in the material versus wavenumber units (see Fig 2.14B).

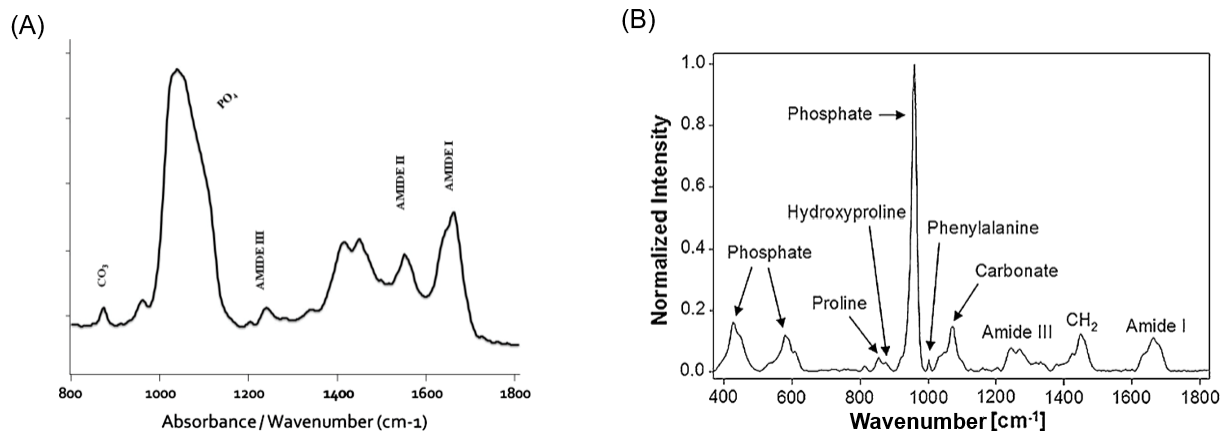


Figure 2.14: Representative test curves for (A) FTIR absorption spectra and (B) Raman spectra for mammalian bone. Images taken from Paschalis et al., 2011 and Morris et al., 2011 [191,192]

Fig. 2.14 provides a typical example of an FTIR and Raman spectra for mammalian bone. It may be noticed that the absorption or intensity peaks are similar with strong molecular bond vibration in the absorption IR spectrum corresponding to weak bond vibrations in the Raman spectrum and vice versa [193]. In terms of collagen network quality, these can provide information on the collagen molecular conformation and network organisation as well as collagen maturation (ratio of mature to immature LOX crosslinks) [193,194].

In recent years, Raman has gained prominence in bone fragility related studies [195–199] as it has a better spatial resolution than FTIR and can be used on bone when in the hydrated state unlike FTIR [193]. These studies have shown Raman as a promising tool for fracture risk assessment. One such study found the amide I sub-peak ratio (I_{1670}/I_{1640}) which is related to collagen organisation correlated significantly ($R^2 = 0.5$) with fracture toughness measured using the J-integral approach [160]. Further, using structural bone allografts, it was shown that parameters from Raman spectra accounted for 60% of the variance observed in their fatigue life cycle with the amide I sub-peak ratio (I_{1670}/I_{1640}) alone explaining 44% of the variance observed [198].

As already noted, it has been shown that collagen network quality and connectivity degrade with age and is associated with reduced fracture resistance of cortical bone. These were found employing some of the techniques discussed above specifically DSC and HIT. However, the mechanisms by which this occurs remains inconclusive which leads us to the idea of oxidative stress and damage of proteins.

2.5 Oxidative stress, protein oxidation and its implications in bone

Increased amounts of oxidized proteins have been associated with normal aging as well as in several acute and chronic human diseases such as osteoporosis, cancers, cardiovascular diseases, diabetes, osteoarthritis and chronic renal diseases [40,200,201]. This results from a condition termed oxidative stress (also referred to nitroxidative stress in some instances). In this condition, there is an abundance of free radicals such as reactive oxygen species which oxidize proteins and other macromolecules such as lipids and nucleic acids in the body. Free radicals are molecular entities that exist on their own but have one or more unpaired valence electron(s) [37]. These free radicals are produced from normal daily metabolic activities in the cells (mainly in the mitochondria) and the response of white blood cells to infections or injury [37,40]. This oxidative stress phenomenon is postulated to be a significant factor in damage to cells, macromolecules and tissues which accompanies aging and disease. This theory was first proposed by Denham Harman in 1954, and he termed it “the free radical theory of aging” [202].

Under normal physiological conditions, the body contains and generates various antioxidants that scavenge these free radicals minimizing their potential damaging effect on the macromolecules in the body [203]. However, with aging and most diseases, the production rate and/or effectiveness of antioxidants declines, or the production rate of these free radicals increase, or both leading to an imbalance between the free radicals and antioxidants leading to oxidative stress [37,203,204]. The exact cause of the imbalance remains inconclusive. These free radicals include reactive oxygen species (ROS) such as the singlet oxygen (O_2), superoxide (O_2^-), hydroxyl radical ($OH^\cdot$), hydrogen peroxide (H_2O_2) and hypochlorous acid ($HOCl$) as well as reactive nitrogen species (RNS) such as nitric oxide and peroxynitrite with the ROS being more prominent [205] .

2.5.1 Protein oxidation pathways and detection

As noted earlier, the presence of reactive oxygen species (ROS) in the body may result in the oxidation of biomolecules such as nucleic acids, proteins and lipids, especially under oxidative stress [39]. For proteins, these oxidation reactions can cause polypeptide chain fragmentation, modify the amino acid side chains, lead to the formation of non enzymatic crosslinks or even damage the secondary or tertiary structure of the protein (denaturation), and these modifications are termed as oxidative damage [39]. Further, these modifications affect the biological function of the protein [39]. There are a number of pathways by which proteins can become oxidized by ROS or RNS. These can be classified into two main groups: (1) the direct pathway and (2) the indirect pathway of protein oxidation.

2.5.1.1 Direct pathway of protein oxidation by ROS

In the direct pathway of protein oxidation, oxidation occurs by the ROS directly oxidizing the protein polypeptide chain backbone or the amino acid side chain groups [39,41]. The direct oxidation of the protein backbone may lead to the fragmentation of the protein chain and/or the crosslinking of two protein molecules [41]. The protein backbone is categorized by a ‘central’ carbon atom linked to amide ($-NH_2$) and carboxyl ($-COOH$) function groups, a hydrogen atom, and a side chain group (R), which determines the type of amino acid. This ‘central’ carbon atom is called the alpha-carbon atom (C_α -atom). In the protein backbone oxidation pathway (See Fig. 2.15), oxidation is initiated by a hydroxyl radical, which abstracts the H atom attached to C_α -atom creating a carbon centered radical [39,41]. If there is no oxygen present, this carbon centered radical may react with another carbon centered radical to form a protein-protein crosslink.

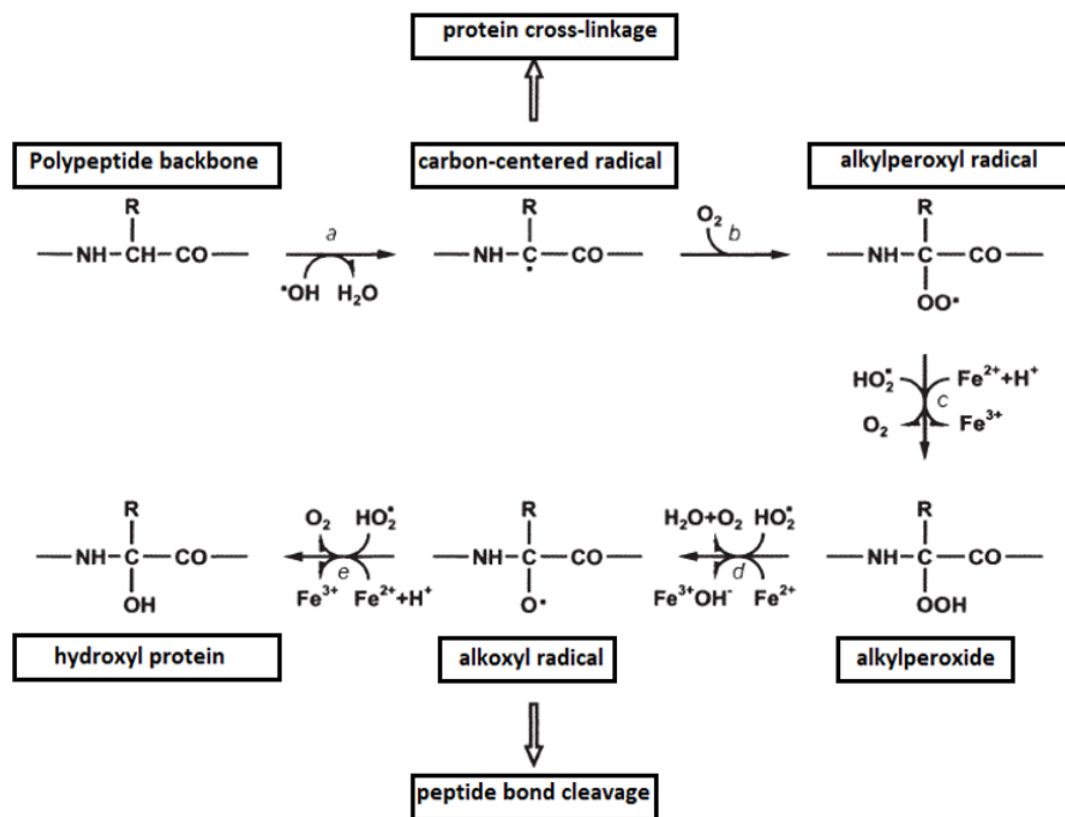


Figure 2.15: Pathway for oxidation of protein backbone potentially leading to protein fragmentation and/or crosslinking. Image was adapted from Sitte et al., 2003 [39]

However, in the presence of free oxygen, this carbon centered radical reacts spontaneously with the oxygen to form an alkylperoxyl radical intermediate which converts to an alkylperoxide derivative. This derivative then forms an alkoxy radical which can finally give rise to a hydroxyl protein derivative. The formation of the alkyl peroxyl radical, alkyl peroxide, alkoxy radical and hydroxyl protein derivatives are mediated by either the hydroperoxyl radical ($\text{HOO}^\cdot$), copper ions (Cu^+) or iron (II) ions (Fe^{2+}). Further, the alkyl, alkyl peroxide and alkoxy radical intermediates may react with other amino acid residues of different or the same proteins to form a new carbon centered radical in other amino acids (See Fig. 2.16). This newly formed carbon centered radical can undergo the same reactions described above [39,41]. The protein alkoxy derivative may also undergo peptide fragmentation (bond cleavage). This can occur in two different ways: by the diamide or α -amidation pathways (Fig. 2.17) [39]. Both pathways give rise to substrates that contain carbonyl derivatives which is not the case in normal proteolysis. Carbonyls are characterized by an oxygen-carbon double bond (C=O).

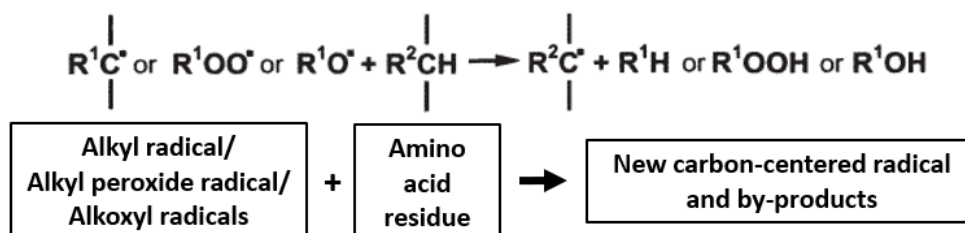


Figure 2.16: Reaction of radical intermediates, obtained from the direct oxidation of the protein backbone, with other amino acid residues continues the cascade of protein oxidation. Image adapted from Sitte et al., 2003 [39].

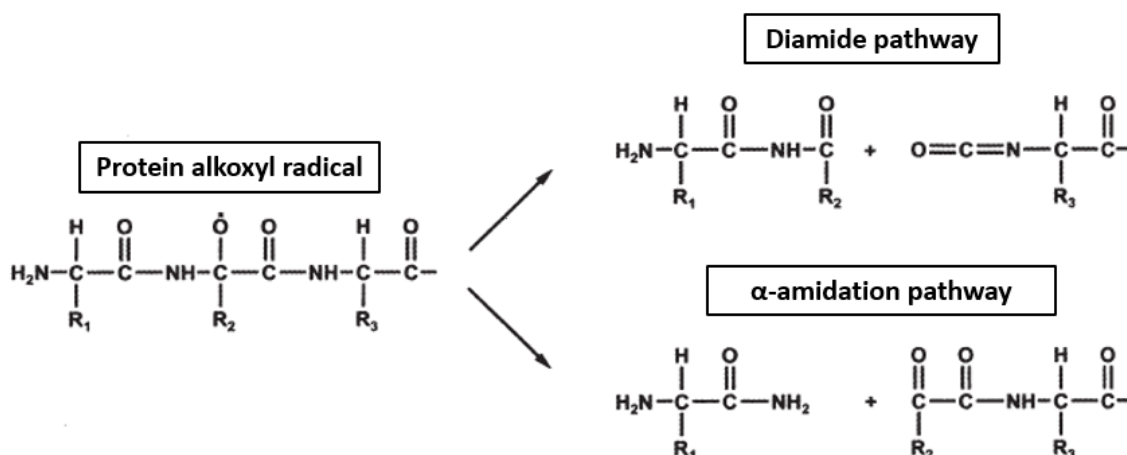


Figure 2.17: The two different pathways for fragmentation due to protein backbone oxidation. Image adapted from Sitte et al., 2003 [39].

In the second direct pathway, oxidation of proteins occurs by direct oxidation of the amino acid side chains that form part of the protein molecule. In the presence of the OH radical, all amino acid side chains are prone to oxidation, however, only some of the products of these oxidation reactions have been fully studied and defined [39,41]. Table 2.1 provides a summary of some of these oxidation products for different amino acid groups [39]. These products of amino acid side chain oxidation are important as they can serve as markers of protein oxidation.

For lysine, arginine, threonine and proline, the side chain oxidation reactions can be initiated by metal catalysts such as copper and iron ions [200]. Oxidation of these amino acids give rise to carbonyl containing groups, that is, aldehydes and ketones (see Table 2.1) [39]. An interesting note is the abundance of proline and to a lesser extent, lysine and arginine in bone collagen as described in section

2.2.1. Oxidation of proline side chains, however, may produce residues that do not contain carbonyls, that is, 4 or 5 hydroxyproline [39,206].

Table 2.1: Summary of products of oxidation of some amino acids side groups. Taken from Sitte et al., 2003 [39].

Amino acid	Products
arginine	Glutamic semialdehyde
cysteine	Disulfides (Cys-S-S-Cys, Cys-S-S-R); CySOH; CySOOH; CySO ₂ H
glutamic acid	oxalic acid; pyruvate adducts
histidine	2-oxohistidine; aspartic acid; asparagine
leucine	3-, 4-, and 5-OH-leucine
lysine	α -amino adipic semialdehyde
methionine	methionine sulfoxide; methionine sulfone
phenylalanine	2-, 3-, and 4-OH-phenylalanine; 2,3-dihydroxy-phenylalanine
proline	glutamylsemialdehyde; 2-pyrrolidone; 4- and 5-OH-proline; pyroglutamic acid
threonine	2-amino-3-ketobutyric acid
tryptophan	2-, 4-, 5-, 6-, and 7-OH-tryptophan; formylkynurenine, 3-OH-kynurenine; nitrotryptophan
tyrosine	3,4-dihydroxyphenylalanine; Tyr-Tyr cross-links; 3-nitrotyrosine
valine	3-hydroxyvaline

2.5.1.2 Indirect pathways of protein oxidation by ROS

There are two main ways ROS can indirectly oxidize proteins. The first means is by the ROS initially oxidizing lipids and carbohydrates [207]. For example, ROS often oxidize polyunsaturated fatty acids due to the presence of numerous double bonds in the fatty acid structure. The oxidation reaction leads to a breakdown of the fatty acids in a process known as peroxidation. This peroxidation leads to products bearing aldehyde function groups that can react and become attached to proteins mainly through their side chain groups [207]. The product of this reaction is referred to advanced lipoxidation end-product (ALE).

The second means is by the action of a reducing sugar or their oxidized products [200,208]. A reducing sugar like glucose can react with the amine group of proteins to form an aldimine which undergoes a molecular re-arrangement into a ketoamide attaching the sugar to the protein [206]. This process is called glycation of proteins and is non-enzymatic. The glycated protein, containing a double bond, can be further oxidized to form what is known as an advanced glycation end-product (AGE) [206]. Alternatively, the sugar molecule can be directly oxidized by ROS to give rise to highly reactive

carbonyl compounds such as glyoxal, glycolaldehyde and methylglyoxal [36]. These compounds will in turn react with the amino acid groups on the protein and give rise to AGEs. This process is typically termed as glycoxidation [36]. AGEs can be grouped into two main types: AGE crosslinks and AGE adducts. AGE crosslinks are AGEs that form covalent bonds between two neighbouring protein molecules whereas AGE adducts are AGEs that form as modifications to the side groups of the collagen molecules [156]. AGE crosslinks are the non-enzymatic crosslinks that have been found to accumulate in bone and thought to be detrimental to the mechanical competence of cortical bone and bone collagen network as discussed in sections 2.1.1 and 2.3.5. AGEs as it relates to cortical bone and bone collagen are further discussed in section 2.5.3.

As noted earlier with the direct protein oxidation pathway, the reactions in many cases give rise to ketones and aldehydes which are carbonyl containing compounds. Carbonyls are chemically very stable and hence are suitable compounds for detection. Consequently, protein carbonylation is widely used as a biomarker to assess protein oxidation and oxidative damage during aging and in diseases involving oxidative stress [38,200,201]. It is important to note that, glycation and glycoxidation reactions give rise typically to compounds containing a carboxylic functional group which contains a carbonyl. However, most methods for protein carbonylation are specific to ketones and aldehydes hence quantify the oxidative damage due to the direct protein oxidation pathway.

2.5.1.3 Detection and Quantification of Protein Carbonyls

Protein-bound carbonyls do not exhibit fluorescence or absorbance. Hence, they cannot be detected or quantified directly [206]. Therefore, protein carbonyls must be derivatized before they can be quantified. In protein carbonylation, the most widely used reagent to derivatize is 2,4-dinitrophenylhydrazine (DNPH) [209,210]. It reacts with the carbonyl group to form a stable 2,4-dinitrophenyl (DNP) hydrazone product [201]. This acid hydrazone absorbs ultra-violet light (~370 nm) therefore using a spectrophotometric assay, the total amount of carbonyls in a protein can be measured by a spectrophotometer. However, this quantification is not specific, that is, if there is a mixture of proteins, the different proteins cannot be differentiated. Further, it is thought that a portion of unreacted DNPH remains in the solution due to its low solubility artificially increasing the measured carbonyls [211]. In addition, this method suffers from inherently low sensitivity [211] hence large quantities are needed making it unsuitable for some biological tissues. To overcome these drawbacks,

the spectrophotometric quantification can be carried out using high-performance liquid chromatography (HPLC) which provides specificity and greater sensitivity [201,212].

HPLC splits up the different proteins based on their molecular weights therefore each can be quantified separately. Further, the higher sensitivity of the HPLC spectrophotometric detectors means lesser amount of the protein samples are needed for the quantification. Due to the ability of some HPLC systems to obtain a full spectrum, both the carbonyl content and individual protein content can be measured allowing the carbonyl content to be presented as a ratio of the protein content. This provides a more detailed picture of oxidative damage [201].

An alternate derivatization agent that has been used is fluorescein-5-thiosemicarbazide (FTC) [213–215]. FTC is a highly fluorescent compound that binds specifically to protein carbonyl containing compounds (ketones and aldehydes) but not to lipid carbonyls [215]. FTC provides better sensitivity than DNPH and unreacted FTC can be successfully washed off using acetone [215]. After FTC derivatization, the proteins can be separated using gel electrophoresis and the isolated protein bands measured using fluorescence at an excitation wavelength of 485/488 nm and emission wavelength of 520/535 nm [213,214]. Alternatively, Mohanty and co-workers processed the FTC-derivatized protein and used a fluorescence-based microplate assay to measure the carbonyls in the derivatized protein [215].

Another way to quantify protein carbonyls after derivatization is to use immunochemistry. The most common is the enzyme-linked immunosorbent assay (ELISA) method [216]. This basically makes use of anti-dinitrophenyl (anti-DNP) antibodies to measure how the derivatized protein carbonyls are absorbed in an ELISA plate. Absorbance is measured by relating it to a standard curve derived using colorimetric means [201]. The ELISA method is highly sensitivity, reproducible and easy to use but requires specialized equipment and can be non-specific.

Another immunochemical approach widely used to quantify protein carbonyls is the use of Western blot [217]. Like ELISA, this technique uses anti-DNP antibodies. However, the derivatized protein are first separated by one (1D) or two-dimensional (2D) SDS-PAGE. The difference in 1D and 2D SDS PAGE is in their ability to resolve protein mixtures with 2D having a higher resolution. The proteins are separated by their molecular weights using SDS PAGE hence the relative molecular mass of the type of protein associated with the carbonyls can be found using this technique. The separated derivatized proteins are either stained directly with anti-DNP antibodies or transferred to an absorbent

porous membrane on which are the anti-DNP antibodies, allowing for the quantification of protein carbonyls. Western blot immunoassay is extremely sensitive and provides more information on the extent of protein carbonylation as compared to ELISA.

In addition to the above, Raman micro-spectroscopy has been used recently to measure protein carbonylation. This novel technique also utilises DNPH as a derivatizing agent [218]. It was shown that derivatizing the protein carbonyls with DNPH and then imaging with Raman spectroscopy resulted in a significant shift at the 1340 cm^{-1} peaks which indicated the amount of protein carbonyls present. This was validated against the more traditional spectrophotometric assay technique. This approach is highly sensitive and accurate. It also provides additional information on the composition of the proteins [218].

Lastly, mass spectrometry in combination with other modalities such as the Western blot is an alternative to quantifying protein carbonyls [201,206]. Mass spectrometry provides the added benefit of not only identifying the type of protein that was modified but also provides information on the site and type of modification that occurred. However, it is quite expensive, requires specialized skill to use and the above-mentioned derivatization techniques before use [206].

2.5.2 Oxidative stress and disease pathologies associated with increased bone fracture risk

As previously mentioned, oxidative stress is implicated in many acute and chronic diseases. Among these are diseases that are specifically detrimental to bone. Osteoporosis is the most common bone disease pathology characterised by a loss in bone mass. Type 2 diabetes (T2D) and chronic kidney disease (CKD) are other diseases of particular interest as they report an increased bone fracture risk compared to controls, however current clinical tools do not do a good job of fracture risk prediction in these diseases [219–221]. Recent studies have therefore highlighted how oxidative stress may play a role in these three bone-related diseases.

Osteoporosis is a metabolic bone disease in which there is significant loss of bone mass or quantity leading to a reduction in bone strength [222]. One in 5 men and one in 3 women above the age of 50 years are estimated to suffer at least one osteoporotic fracture in their lifetime [223,224]. Osteoporosis occurs when there is an imbalance in the actions of the osteoblasts and osteoclasts. The osteoblasts are the bone making cells whereas the osteoclasts are the bone resorbing cells [225]. The osteoclasts break down old bone for the osteoblasts to lay down new bone. This process is termed bone remodeling. When more bone is resorbed than made then osteoporosis occurs. Osteoporosis most commonly occurs

among postmenopausal women, and this is attributed to the decline in estrogen levels [226]. Estrogen is an important hormone in the regulation of the bone remodeling process in women [226]. In addition, recent studies have implicated oxidative stress as an independent risk factor for osteoporosis [222]. Case control studies have shown that a significant higher amount of oxidative stress markers were measured in the urine, serum or plasma of osteoporotic patients as compared to control without osteoporosis highlighting its possible role in osteoporosis [222]. These biomarkers of oxidative stress measured include advanced oxidized protein products (includes AGEs), serum 8-iso-prostaglandin, serum lipid hydroperoxides, plasma enzyme activity of glutathione peroxidase and superoxide dismutase and downstream cytokines such as IL-6 and TNF-alpha [222]. The presence of ROS is thought to upregulate osteoclastogenic activity activators such as receptor activator of nuclear factor-kappa b ligand (RANKL) while downregulating osteoclastogenic activity inhibitor such as osteoprotegerin (OPG) [227]. RANKL is released by osteocytes, the matured bone cells, to initiate the bone remodeling process [227]. Therefore, oxidative stress is proposed to affect bone mass by impacting the metabolic activities of the bone cells. Specifically, it is thought to cause an increase in the conversion rate of osteoblasts into osteoclasts known as osteoclastogenesis, a decrease in osteoprogenitor cell differentiation that form osteoblasts, a decrease in osteoblast activity as well as an increase in osteoblast and osteocytes apoptosis [222].

Diabetes as defined by the World Health Organisation (WHO) is a “metabolic disorder of multiple etiology characterised by chronic hyperglycemia with disturbance of carbohydrate, fat, and protein metabolism resulting from defects in insulin secretion, insulin action, or both” [228]. The most common form of diabetes is type 2 diabetes (T2D) reportedly accounting for roughly 90% of all diabetes cases [229]. It was estimated there were about 382 million T2D cases worldwide in 2013 and this number is predicted to rise to an estimated 590 million people by 2035 [230]. Due to its prevalence worldwide, diabetes is considered a global pandemic [231]. T2D is characterized by reduced secretion of insulin by the beta cells in the pancreas, increased secretion of glucagon by the pancreas alpha cells which raises blood glucose levels as well as increased resistance to insulin by the muscle, fat and liver cells [232]. All these result in elevated blood sugar levels referred to as hyperglycemia associated with T2D. The major risk factor associated with type 2 diabetes is obesity, with up to a 90-fold increase in the risk of developing T2D [233]. Other risk factors include genetic make up, little to no physical activity, high fat diet intake, and hypertension [229]. T2D can lead to other health complications such as cardiovascular disease, blindness, renal or kidney diseases as well as neuropathy [234]. Oxidative stress

has been proposed to play a role in T2D development and progression. In fact, hyperglycemia is thought to exacerbate the production of ROS already being produced from mitochondrial dysfunction and other processes [235]. This has been proposed to occur through multiple pathways such as the activation of enhanced protein kinase C, the polyol pathway, autooxidation of glucose potentially leading to AGEs formation or hyperactivation of the hexosamine pathways [234,236]. This heightened oxidative stress in turn affects insulin and intercellular signaling thereby resulting in insulin resistance and pancreas beta cell dysfunction [237]. In addition, the ROS cause damage to blood vessels and impair endothelial cell functions contributing to the development of earlier mentioned complications associated with T2D [237]. An interesting note about T2D is though patients typically report clinically normal to high BMD, they have an increased bone fracture risk [238]. A relative bone fracture risk of 1.2 to 3 depending on the extent of T2D progression and skeletal site has been found for T2D patients compared to controls without diabetes [231]. For hip fracture, the fracture risk has been found to increase by 2 to 3 times compared to controls [231]. The accumulation of AGEs, a consequence of oxidative stress is highlighted as one of the main factors explaining this paradox between normal to high BMD but increased fracture risk [239,240].

Chronic kidney disease (CKD) is defined as the presence of persistent (greater than 3 months) abnormalities in the function and structure of the kidney that has a negative impact on health [241]. CKD is characterized by a lower glomerular filtration rate (GFR) of less than 60 ml/min/1.73 m², urine albumin of greater than 30 mg for a 24-hour period and/or kidney disease markers and structural abnormalities such as blood in urine and growth of cyst around kidney [241]. In 2022, it was estimated that approximately 850 million people suffered from CKD [242,243]. The occurrence of CKD is mainly associated with diabetes and hypertension but can be caused by other factors such as infections and environmental pollutants [244]. In addition, genetic risk factors have also been implicated in CKD [244,245]. The adverse clinical outcomes of CKD are end stage renal disease, cardiovascular diseases and increased mortality [246]. CKD has five stages of disease progression defined mainly based on GFR [241]. Oxidative stress is thought to be closely associated to the progression of CKD through its five stages [247]. Oxidative stress in CKD is thought to result from impaired mitochondrial function and enhanced mitochondrial ROS in the kidneys. Various oxidative stress biomarkers measured in plasma have been implicated in CKD [247–250]. Generally, these oxidative stress markers correlate with renal function and as they increase there was a decrease in antioxidant biomarkers [247,248]. Further, it was found these biomarkers related more to uremia than the use of dialysis [249]. Uremia is

the excessive build up of waste products in the blood associated with end stage renal disease. These waste products are thought to promote inflammation leading to elevated oxidative stress [250]. In fact, biomarkers of inflammation have been found to increase with increasing oxidative stress markers [248]. Consequently, oxidative stress is thought to be both a cause and a consequence of CKD [250].

Although oxidative stress is implicated in these chronic bone affecting diseases, it is unclear the mechanisms by which these oxidative processes may lead to deterioration in bone tissue quality and consequently its mechanical properties especially its fracture resistance.

2.5.3 Advanced glycation end products in bone

Advanced glycation end-products (AGEs), one of the by-products from oxidative stress, have elicited the most interest over the past two decades in terms of understanding how oxidative stress could damage bone tissue quality. Note these AGEs are non-enzymatic post translational modifications that occur in the bone collagen network. They form by one of the indirect pathways of protein oxidation discussed in section 2.5.1.2 through glycation and glycooxidation reactions. These reactions involve a reactive aldehyde of a reducing sugar or its oxidized products reacting with the amine group on the collagen molecule and through a series of re-arrangements and oxidation reaction form AGEs [156]. These AGEs can form as crosslinks (covalent bond between two neighbouring collagen molecules) or adducts (modification to the side chain of the collagen molecule).

Early work around understanding how AGE accumulation affected bone mechanical competence and fragility focused on using in vitro models. In these models, human and animal long bone pieces predominantly cortical bone pieces were used. These bone pieces were incubated in sugar solutions using glucose or ribose which cause AGEs to be formed through non-enzymatic glycation (NEG) reactions within the organic matrix [26,28,33,251–253]. Typically, ribose was used opposed to glucose as it favored more pentosidine formation [29]. Pentosidine is a fluorescent AGE crosslink and one of the first AGEs to be isolated and measured accurately using HPLC. Consequently, it was used as a biomarker for the other cumulative AGEs that formed in bone in these in vitro studies. Other experimental studies have shown that pentosidine levels increase (up to five-fold) with age in cortical bone [23,30,172,254,255] and occurs in greater quantities in urine, serum and/or other tissues such as cartilage, tendons and skin of T2D [31,256], osteoporotic [257,258] as well as CKD patients [32,259] when compared to controls without those diseases. Another biomarker for AGEs that has been widely used is total fluorescence AGEs (tfAGE). This basically uses a fluorometric-microplate based assay

usually with a 370 nm excitation, 440 nm emission regimen to capture all crosslinking and non-crosslinking AGE that fluoresce in this range. It is thought to capture a wide variety of AGEs including pentosidine, crossline and vesperlysine. The drawbacks of this measurement are that not all AGEs fluoresce and those that do in this range are not completely known. Therefore, their relative contribution to the total fluorescence measured is unknown. Further, fluorescence from other components of the bone for instance, the enzymatic crosslinks, may interfere with this fluorescence measurement. A couple of studies have reported weak correlations between pentosidine, and total fluorescent AGEs measured from the same samples [30,34].

Despite these shortcomings, these in vitro models showed that the accumulation of the afore-mentioned AGE biomarkers resulted in a stronger, stiffer, more connected but less ductile collagen network due to increased crosslinking [26,33,251,253]. Generally associated with these findings was an observed reduction in the post yield capabilities of the bone, failing at lower post yield strains for uniaxial tensile and bending tests [26,33,251,254] as well as reduced toughness and fracture resistance [23,28,33,254]. These in-vitro model results are supported by animal (rodent) model studies where an increase in pentosidine has been related to a decrease in toughness [260,261]. However, there were a couple of studies that found no correlation between pentosidine accumulation and reduction in the post yield properties and toughness of bone [262,263]. These inconsistencies may be attributed to the non-standardized procedures used across the in-vitro studies with differing type and location of bone pieces utilized, differing sugar (ribose) concentrations, differing incubation times and temperatures, different mechanical testing regimens and so forth.

The afore-mentioned biomechanical property changes associated with AGE accumulation (reduced post yield strain, toughness and fracture resistance) are consistent with changes observed in age-related bone fragility [146]. Because of this, pentosidine and AGE crosslinks, in general, are regarded as possible determinants of bone fragility. It is widely thought AGEs lead to over-crosslinking of the collagen network making them stiff and less ductile which ultimately results in the bone's reduced ability to resist fracture. However, the accumulation of pentosidine and AGE crosslinks have been showed to result in a stiffer, stronger and more connected collagen network [33]. This is opposite to findings from ex vivo human bone studies where a less strong, less stiff, less connected as well as a more denatured collagen network has been found to correlate with reduced post yield properties, toughness and fracture resistance associated with age-related bone fragility [19,23,24,264]. Therefore,

there is a contradiction between collagen network properties measured from the in vitro model and what is measured in human bone ex vivo.

In addition to this contradiction, recent studies have highlighted that AGE adducts such as carboxyl-methyl-lysine (CML), carboxyl-ethyl-lysine (CEL) and 5-hydroxymethyl-imidazolone (MG-H1) which are mostly non-fluorescent and non-crosslinking are much more abundant (20 to 200 times) than pentosidine in bone including cancellous bone [34,35] (see Fig. 2.18). These adducts result in modifications to the side chain groups of the collagen molecule. For instance, CML forms through a glycoxidative reaction between glyoxal or glycolaldehyde, reactive carbonyls and the ϵ -amine of a lysine resulting in a carboxyl group being added to the side chain leading to an overall charge change from positive to negative [156,265]. It has been shown that CML also increases with donor age and is negatively correlated to measures of fracture toughness [35,266]. CML has also been implicated as a risk factor for hip fractures [267] and its content in the serum of adults with T2D is associated with a higher incidence of fracture risk [268]. In addition, T2D patients have a significantly higher amount of CML in their serum than non-diabetics [268]. Using cancellous bone, Arakawa and co-workers found CML and other AGE adducts, CEL and MG-H1 to all strongly correlate ($r > 0.88$) with pentosidine ex vivo. Further, the relative abundance of AGE adducts (same order of magnitude as enzymatic LOX crosslinks [34,172]) suggest their effect on bone's mechanical properties will be much more prominent than pentosidine which is about two orders of magnitude lower in quantity (see Fig. 2.18). This demonstrates that pentosidine might be an indicator for the formation of other AGEs, specifically, AGE adducts and not a causative factor in bone fragility. It is important to note though, that there are other AGE crosslinks beside pentosidine including glucosepane and methylglyoxal/glyoxal lysine dimer (MOLD/GOLD), but these have not been isolated and detected in bone yet. Glucosepane is known to be more abundant (~70 times greater) than pentosidine in skin [269].

In addition, the abundance of AGE adducts compared to AGE crosslinks raises caution on how results from the in vitro AGE accumulation models should be interpreted. The NEG reactions that result from incubating bone pieces in a reducing sugar, most likely produce multiple AGEs including crosslinks and adducts. However, in these in vitro studies, PEN is predominantly measured as it is difficult to measure the other AGEs that form. The mechanisms by which AGE adducts may impact bone mechanical properties are different from pentosidine or other AGE crosslinks. However, since pentosidine is used as a biomarker, the effect of AGE adducts on bone mechanical properties is all lumped together with pentosidine and attributed to the “over-crosslinking” of the collagen network.

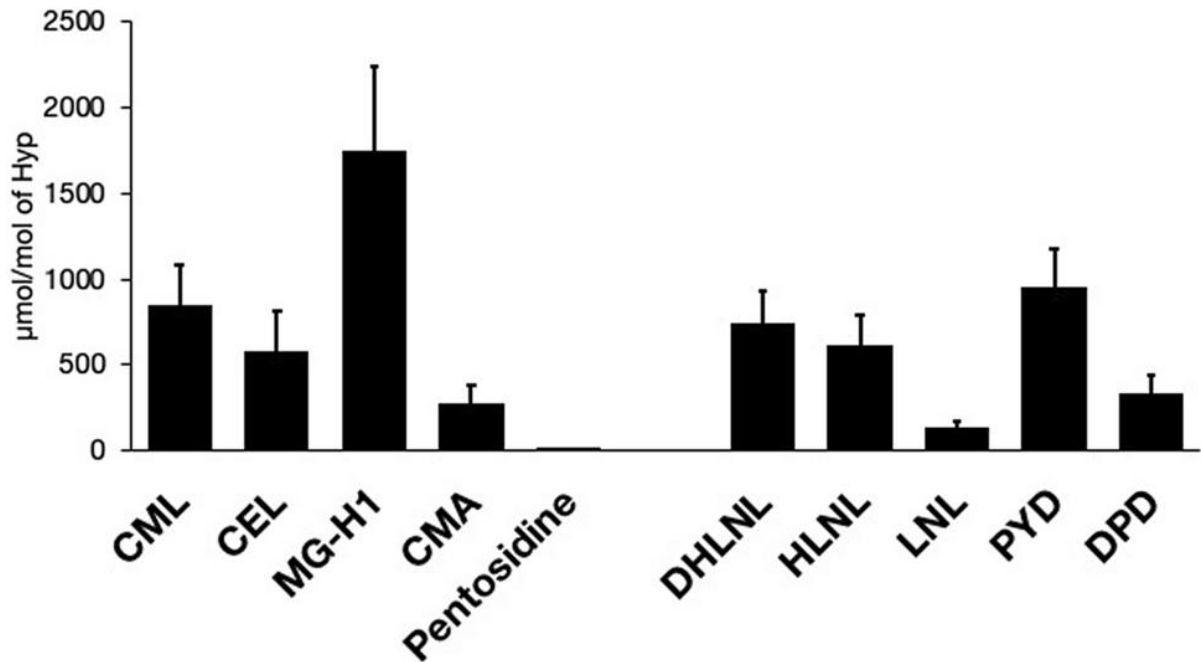


Figure 2.18: Quantification of the AGE crosslink, pentosidine, AGE adducts, carboxyl-methyl-lysine (CML), carboxyl-ethyl-lysine (CEL), 5-hydroxymethyl-imidazolone (MG-H1) and carboxy-methyl-arginine (CMA) and the enzymatic crosslinks, dihydroxylysinoxonorleucine (DHLNL); hydroxylysinoxonorleucine (HLNL); lysinoxonorleucine (LNL) pyridinolone (PYD), deoxypyridinolone (DPD) in human cancellous bone. The filled bars reflect mean values, and the error bars reflect one standard deviation. Their quantities are normalized with hydroxyproline (Hyp) content. Image adapted from Arakawa et al., 2020 [34].

Beyond the idea of “over crosslinking”, AGEs have been linked to reduction in the quantity and changed morphology of microdamage formation in bone leading to less energy dissipation as evidenced by reduced toughness and fracture toughness [28,270]. Also, one study found that increase in pentosidine concentration correlated with increased existing denatured collagen molecules in decalcified cortical bone samples [271]. They postulated that the accumulation of AGE crosslinks was as a result of defective collagen formation during bone re-modelling. In addition, AGE adducts, in particular, CML, is thought to change the overall charge of the collagen molecule leading to altered collagen configuration ultimately leading to lower energy dissipation mechanisms of the collagen network [272]. Further, in vitro and animal models have shown a relationship between AGEs predominantly pentosidine and altered bone mineralization, but results remain inconsistent [273,274]. Also, an in vitro ribation study found that an increase in pentosidine was associated with a decrease in

collagen bound water mainly because there was a change in the secondary structure of the collagen [275]. Lastly, a recent study found AGEs to affect non collagenous proteins, specifically osteocalcin [276]. Since non-collagenous proteins are important to the interaction between collagen, mineral particles and water as well as the overall nanoscale structure of bone, these might be negatively affected leading to reduced mechanical properties in bone.

Another interesting finding from Arakawa and colleagues' study [34] was that AGE adducts negatively correlated with immature LOX crosslinks albeit cancellous bone was used in their study [34]. They proposed that AGE adducts may competitively inhibit the formation of enzymatic crosslinks. In addition, an earlier study using a diabetic rat model showed an increased in the amount of pentosidine correlated negatively with the number of immature LOX crosslinks. They postulated that the reduction in immature LOX crosslinks was a result of vitamin B6 deficiency due to hyperglycemia from diabetes which suppressed the action of the LOX enzyme . This is of particular interest, as animal models have shown LOX crosslinking to be important to bone strength and fracture toughness through osteo-lathyrism models [277]. Osteo-lathyrism is an experimentally induced generalized disorder of connective tissues resulting from the ingestion of the seeds of common sweet pea [278,279]. The seeds of the common sweet peas contain a toxin, β -aminopropionitrile (BAPH), that was demonstrated to cause this disorder [279]. Interestingly, BAPH has been showed to inhibit the action of enzyme lysyl oxidase (LOX) important to the formation of enzymatic crosslinking in collagen. Consequently, BAPH has been used in animal models to investigate the importance of crosslinking to bone mechanical properties. From these models, it has been shown a loss of LOX crosslinking corresponds to reduced strength as well as fracture toughness in vivo [86,280,281]. Consequently, if AGE adducts competitively inhibit crosslink formation or suppress the action of LOX this could lead to reduced fracture toughness using the analogy of osteo-lathyrism models.

While the accumulation of AGEs has been linked to increased fracture risk especially in the T2D group, there remains inconsistent or inconclusive understanding of the mechanisms by which AGEs impact bone fragility. This highlights the need for more studies to elucidate these mechanisms which will ultimately be beneficial in developing better fracture risk assessment tools.

Chapter 3: Objectives and Hypotheses

3.1 Main objective

As already highlighted in Chapter 2, although bone collagen is widely regarded as critical to cortical bone's fracture resistance, the exact mechanisms by which the quality and connectivity of the bone collagen network becomes degraded remain inconclusive. The popular idea of “over-crosslinking” by advanced glycation end products which leads to increased collagen network connectivity is not supported by ex vivo measures of human cortical bone collagen which shows degradation of the collagen network connectivity is associated with reduced fracture resistance. Consequently, the over-arching aim of this research work was to investigate oxidative damage as a cause of collagen network connectivity degradation leading to reduced fracture resistance in cortical bone.

3.2 Sub-objectives and hypotheses

To accomplish the over-arching objective of this work, the following sub-objectives with their hypotheses and associated tasks are discussed below:

3.2.1 Sub-objective 1: A critical evaluation of cortical bone fracture toughness testing

As discussed in section 2.3.4, cortical bone violates the assumptions of the unloading compliance (UC) method widely used to estimate crack extension in cortical bone fracture studies. Therefore, the shortcomings of the UC method in relation to cortical bone could have an impact on the accuracy and precision of cortical bone J-R curve measurement. Consequently, this sub-objective sought to critically evaluate current methods used in quantifying fracture resistance of cortical bone using J-R curves.

To complete this sub-objective, the following tasks were performed:

- (I) Fracture toughness testing of ten paired specimens from bovine tibiae. One sample from each pair was assigned to a cyclic loading group, where a series of load unload cycles relevant to the UC method were used in the fracture test. The other sample was assigned to a monotonic loading group where it underwent continuous loading until fracture. Bovine bone was used opposed to human bone to limit inter-specimen variability.
- (II) Crack extension was calculated using an optical method for the monotonic loading group whereas for the cyclic loading group, both the UC method and optical method were used. Fracture toughness parameters were computed from the generated J-R curves.

- (III) Paired t-tests as well as F-tests were used to assess the accuracy and precision of the fracture toughness parameters derived from the J-R curves generated using the various methods.

It was hypothesized that the use of the UC method leads to an overestimation of the crack extension which will result in an underestimation of cortical bone's fracture toughness.

3.2.2 Sub-objective 2: Oxidative damage as a cause of collagen network connectivity degradation leading to reduced human cortical bone fracture resistance

As discussed in section 2.3.5, there is inconclusive knowledge of the mechanisms that lead to the degradation of the collagen network connectivity and quality. Furthermore, recent studies show an abundance of measurable AGE adducts compared to measurable AGE crosslinks in bone as highlighted in section 2.5.3. However, the mechanisms by which AGE adducts affects the collagen network and thereby fracture resistance is not fully known. Consequently, this sub-objective sought to investigate oxidative damage resulting from elevated oxidative stress as a cause of collagen network connectivity degradation already shown to relate with reduced cortical bone fracture toughness [19,24]. In addition, it also sought to investigate the role of advanced glycation end-product (AGE) adducts, a by-product of oxidative stress resulting from glycation and glycoxidative processes on the fracture resistance of human cortical bone as opposed to AGE crosslinks.

Various ex vivo human bone studies have reported degraded collagen network connectivity and organisation as discussed in section 2.3.5, as such for this study, the use of human cadaveric bones was chosen. This was to ensure physiologically relevant data was collected to depict what is happening ex vivo. In addition, this data could be used as a validating tool in the development of in vitro models to better understand oxidative stress related mechanisms in cortical bone. Further, animal bone or animal in vivo models were not used as the most common animals used (bovine and rats) do not have Haversian bone with osteonal microstructure which human bone does.

To complete this sub-objective, the following tasks were performed:

- (I) Measurement of fracture toughness parameters guided by results from sub-objective 1, collagen network properties as well as other key determinants of bone fragility identified in literature for a considerable number (~80) of cadaveric cortical bone specimens. The cadaveric cortical bone specimens were taken from a wide age range of heterogenous donors, with and without a history of type 2 diabetes (T2D) and/or chronic kidney diseases (CKD), both bone affecting diseases.

- (II) For the same cadaveric cortical bone samples from (I), carbonyl content and advanced glycation end products (AGEs) content (adducts and crosslinks) in the collagen network, both biomarkers for the extent of oxidative damage were measured.
- (III) For the same set of specimens again, sodium dodecyl sulfate gel electrophoresis (SDS-PAGE) to assess collagen fragmentation were conducted.
- (IV) Conducted analysis of correlations between the measured carbonyl content, collagen fragmentation as well as cortical bone fracture toughness measures and collagen network properties especially the measure of collagen network connectivity, Max.Slope.
- (V) Developed statistical models using measured carbonyl content, AGEs, collagen fragmentation, collagen network properties as well as other key determinants of bone fragility measured to explain the variation observed in cortical bone fracture toughness measures.

It was hypothesized that the biomarkers of oxidative damage; carbonyl and AGE content were significantly higher in the T2D and/or CKD donor specimens compared to the controls without T2D and/or CKD. Therefore, fracture toughness measures were significantly lower in the T2D and/or CKD donor specimens compared to the controls. Furthermore, it was hypothesized that the measures of oxidative damage would correlate strongly and negatively with collagen network connectivity, which in turn would strongly explain, through statistical models, variations observed in cortical bone fracture toughness measures.

The two sub-objectives were addressed by implementing them in two studies. Study 1 titled “a critical evaluation of cortical bone fracture toughness testing methods” focused on addressing sub-objective 1 and study 2 titled “oxidative damage as a means of collagen network connectivity degradation associated with reduced human cortical bone fracture resistance” focused on addressing sub-objective 2.

Chapter 4: Study 1

A critical evaluation of cortical bone fracture toughness testing methods

4.1 Introduction

The study of cortical bone fracture mechanics is and has been important to gaining a better understanding of skeletal fragility and fracture. Cortical bone fracture mechanics deals with the quantification of the resistance of the cortical bone material to the propagation of a crack which constitutes fracture. The ability to quantify the fracture resistance allows investigations into what material level changes in the cortical bone tissue relate to bone fragility and fracture. Such an understanding is imperative towards finding key determinants of bone fragility and fracture which will aid in the development of better fracture risk detection and preventive clinical tools. In understanding bone fragility, studies on cortical bone have taken pre-eminence compared to trabecular bone as it carries a greater portion of the load, specifically, in long bones such as the femur. There is also evidence of the greater role cortical bone plays in femoral neck fractures for instance [44]. Consequently, many studies over the last few decades applied cortical bone fracture mechanics to investigate potential key determinants of skeletal fragility and fracture [24,27,47,125,135,137,152,153,282,283].

In the early evaluations of cortical bone fracture toughness, a linear elastic fracture mechanics (LEFM) framework was adopted [23,120–125]. In this framework, the stress and displacement field distributions around the crack tip are assessed and this is characterized by a parameter known as the stress intensity factor (K). A critical K known as K_{IC} is defined at the point where the crack begins to propagate, and this is often considered a material property. Alternatively, in LEFM, the fracture resistance or more technically fracture toughness can be quantified as the incremental energy required to propagate a crack. This is known as the energy release rate (G) and when quantified at the initiation of crack propagation, a critical G_{IC} is determined [97].

However, single point critical fracture toughness values do not fully characterize cortical bone's fracture behaviour. Subsequent studies on cortical bone's fracture toughness have shown that there is an increase in fracture resistance as the crack propagates [127–131]. The increase in fracture resistance as the crack grows is attributed to the presence of extrinsic toughening mechanisms such as crack bridging that allows for stable crack growth before unstable fracture [17,114,115,131]. To account for

this, resistance curves have been employed to fully characterize cortical bone's fracture toughness. The resistance curves were developed by measuring the stress intensity factor parameter (K) at regular intervals of crack extension and plotting against a function of the corresponding crack extension. These showed a rising resistance curve behaviour for cortical bone and the slope of a line fitted through these points provided an additional measure of crack propagation resistance.

Despite resistance curves providing a better characterisation of cortical bone fracture resistance, the use of LEFM was later found to be inappropriate. The underlying assumption in LEFM is that there is negligible or small scale yielding at the crack tip of the material. In other words, the material must be linear elastic or brittle. However, cortical bone demonstrates significant non-linearity during its fracture. Consequently, Yang and others [133] argued non-linear fracture mechanics approaches are necessary to quantify fracture toughness of cortical bone. Later, Yan and co-authors [132] employed the J-integral, an elastic plastic fracture mechanics (EPFM) framework to quantify the fracture toughness of cortical bone. The J-integral quantifies both the elastic and inelastic contributions of the energy dissipated to propagate a crack [97]. Similar to how a K -based resistance curve is developed, a J-integral based resistance curve (J-R curve) can also be developed. However, a power law is usually used to fit the data points in a J-R curve which defines the fracture behaviour of a material. From this curve, various fracture toughness measurements can be computed that detail the stages of the material's fracture process. Currently, this is the most widely used approach in fracture toughness measurement of cortical bone. ASTM E1820 which was developed for metals is the most frequently followed standard in performing the J-integral fracture toughness measurement of cortical bone [143].

Traditionally, to develop a resistance curve for a material, multiple specimens of similar geometry but different machined initial crack lengths were required [284]. This approach required a lot of material and considerable specimen preparation time leading to high costs. Additionally, in the case of biological materials, inter-specimen variability affects the accuracy and precision of the resistance curve. Alternatively, a single specimen could be used but specialised equipment such as a travelling optical microscope was needed to keep track of the growing crack. Crack extensions measured at various points of the fracture test is a necessary component to construct a resistance curve. However, the need for specialized equipment increased the cost of conducting fracture toughness testing. Consequently, a new method known as the unloading compliance (UC) method was developed eliminating the need for a travelling microscope [144]. This single specimen method employs a series of partial unload-reload cycles during the fracture test and uses the unloading compliances calculated from the partial unloading

stages to estimate the crack extension at that point. The crack extension is estimated from the unloading compliance using an empirically derived standard calibration equation. It is important to note that this method makes some key assumptions which are: (1) there is negligible energy lost during the unload-reload cycles, (2) changes in the unloading compliance only occur due to crack extension and (3) the crack front should propagate in a relatively straight line [97]. In recent years, other methods such as the electric potential drop and normalization methods have also been developed for crack extension estimation however the UC method remains the most popular approach for J-R curves determination [284,285].

The UC method has been widely adopted to estimate crack extensions when quantifying J-R curves for cortical bone. However, cortical bone violates the assumptions of this method. Cortical bone is viscoelastic therefore energy is dissipated during the unload-reload cycles. In addition, the J-integral was originally developed for elastic and non-linear elastic materials and was found to approximate the energy release rate for elastic plastic materials where there is no plastic unloading [286]. Consequently, unloading during the fracture test violates the underlying theory used in calculating the J-integral. However, to combat this and account for errors from the growing crack, incremental approaches to calculating J-integral fracture toughness have been developed [285]. Further, cortical bone undergoes micro-damage formation during the fracture process should lead to a change in unloading compliance when there is no true crack extension [69]. In addition, micro-damage formation makes the tissue even more viscoelastic leading to greater energy dissipation during unload and reload cycles [145]. Apart from the violation of these assumptions, the standard calibration equations from literature that are utilised for crack extension estimation of cortical bone raise concerns. These standard equations are derived from isotropic metallic materials while cortical bone is an anisotropic biological material. Unsurprisingly, it has been found these standard calibration equations produced inaccurate crack extension estimation for equine cortical bone [148,149]. Moreso, recently, it has been shown that use of these isotropy-based standard calibration equations leads to errors in fracture toughness measurements for orthotropic materials [147].

Therefore, in this study, the effect of using the unloading compliance method for J-R curve determination of bovine cortical bone was examined. Specifically, fracture toughness measures from J-R curves generated using the UC method were compared to those obtained using an approach in which crack extension is measured by means of an optical microscope. In addition, a monotonic loading regime was used opposed to the repeated unload-reload cycles used in the UC method. This helped

ascertain the impact of the repeated unload-reload cycles as well as the crack extensions computed by the UC method on the accuracy and precision of fracture toughness measures of cortical bone. It was hypothesized that the formation of micro-damage during the fracture process would lead to an overestimation of the crack extension measurements by the UC method which would result in lower fracture toughness measurements.

4.2 Preliminary studies on method development

To ascertain the best way to conduct the fracture toughness testing of the cortical bone specimens for this study, two preliminary studies were conducted on trial single edged notched bend (SENB) specimens. The first study investigated the use of side grooves. The second study investigated how to match displacement rates between the two loading regimes employed in this study to generate comparable load versus load-line deflection curves.

4.2.1 Preliminary study 1: Use of side grooves

Human cortical bone is known to undergo significant crack deflections especially during transverse directed fracture [116,141,287] (also see Fig. 4.1). However, a requirement for a Mode I fracture test (fracture under tension) is that crack growth must occur along a straight path. Therefore, to drive crack growth along a straight path in cortical bone, grooves on both sides of the crack front have been previously used [111,130,142,148]. Because crack extension was to be measured using an optical microscope, the first preliminary study investigated how to conduct this optical means of crack extension measurement while having side grooves needed to drive straight crack growth in the SENB specimens.

4.2.1.1 Trial single edged notched bend specimen preparation

The trial SENB specimens were prepared from the posterior diaphysis of the tibiae of two young steers (1.5-2 years) which was sourced fresh frozen from a local farm and stored dry in a -20 °C freezer (see Fig. 4.6a). This was carried out consistent with prior work from Willett's group [111,112,141] with a few modifications and briefly described as follows. The bovine tibiae were rid of any soft tissue using a knife and scalpels. Afterward, the posterior diaphysis of the tibiae was isolated using a wood band saw (14" Craftex CX104, Busy Bee tools, Canada) and the bone marrow was removed. A rectangular block was then cut out using the combination of a pathology band saw with an irrigated diamond blade (IMEB, California, USA) and a mini-computer numerical control (CNC) vertical mill (Sherline,

California, USA). From this block, beams with dimensions of width ~5 mm, thickness ~5 mm and length ~50 mm were created using the pathology band saw. The thickness and width of these beams were then polished down to ~4 mm using a series of diamond discs and polycrystalline diamond suspension (MetaDi Supreme, Buehler, IL, USA) with a final finishing grade of 0.5 μm . Finally, a starter notch with a sharpened razor tip of length 2 mm was created at the mid-section of the beam along the width using a low-speed metallurgical saw mounted with a diamond wafering blade (Isomet, Buehler, IL, USA) as well as an ultra-sharp razor blade (American Line, Extra Keen single edge blades). The width of the specimens was directed along the circumferential direction of the bone. Finally, side grooves were created on the specimens. Initially, specimens were prepared with side grooves of depth 0.5 mm on both sides of the uncracked ligament region along the width. Later, specimens were prepared with a side groove of depth 1 mm on only one side of the uncracked ligament region along the width. The side groove on only one side of the uncracked ligament was done to allow for better optical crack extension measurement on the other side without a groove. The prepared specimens were kept in saline-soaked gauze at -20 °C until testing.

4.2.1.2 Fracture testing for preliminary study 1

The prepared trial SENB specimens underwent a displacement controlled three-point SENB fracture test. This was conducted using a micromechanical test system (μTS , Psylotech, IL, USA). The span for the fracture tests was 40 mm. The fracture tests were conducted at a constant displacement rate of 0.3 mm/min. During each test, a digital camera (Point Grey, 5MP, 2/3" detector) attached to a microscope (BXFM, Olympus Corporation, Center Valley, PA, USA) mounted over the test system allowed images of the region around the razor sharpened notch tip to be taken. The images were taken at a frequency of 4 Hz.

4.2.1.3 Results for preliminary study 1

Fig 4.1 illustrates an example of significant crack deflection during transverse directed fracture in a bovine SENB specimen that had no side grooves. To minimize crack deflections and allow straighter growth, double sided grooves were used in this preliminary study. However, it was observed the crack extension was not clearly visible within the side groove under the optical microscope (see Fig. 4.2). There was evidence of crack growth indicated by the whitening ahead of the razor-sharpened crack tip however the exact location of the current crack tip was not discernible. Consequently, grooves created on only one side of the SENB specimens were considered. For this, a groove was cut on only one side

and the crack extensions were imaged under the optical microscope on the other side without a groove. This approach has been previously employed in fatigue and fracture testing of dentin [288,289].

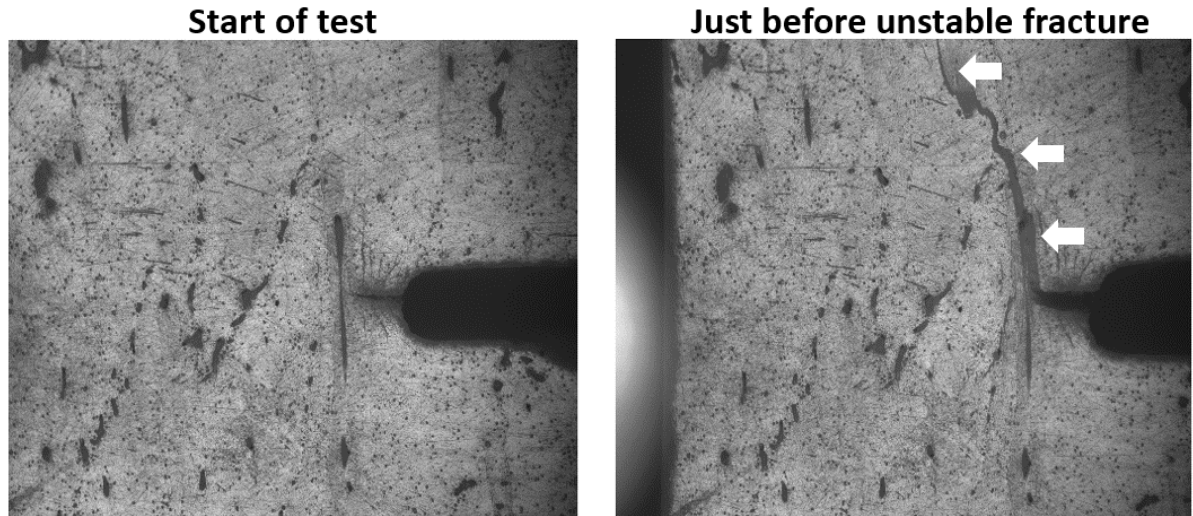


Figure 4.1: Illustration of significant crack deflection during human cortical bone fracture testing in the absence of side grooves. The image on the left shows the sharpened pre-crack at the start of the test. The image on the right shows how the crack deflects almost at an angle of 90° to actual path of crack propagation. The white arrows indicate this crack deflection.

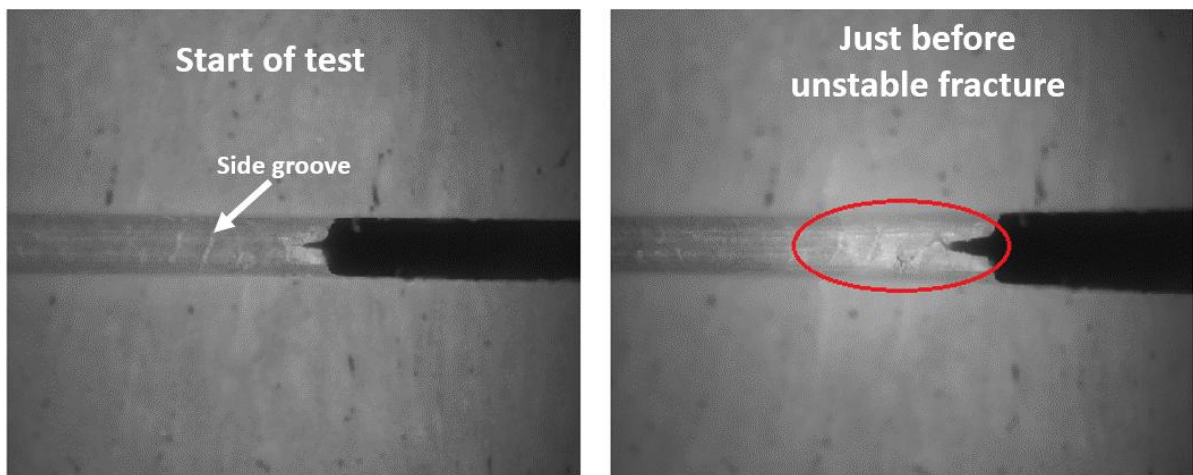


Figure 4.2: Illustration of crack extension inside a side groove for a double sided grooved human cortical bone fracture test sample. The image on left shows the region around crack tip at the start of the test whereas the image on the right shows the region around the crack tip just before unstable fracture. For the image on the right, there is evidence of crack extension indicated by

the whitening highlighted by the red circle within the side groove. However, it is difficult to pinpoint exactly where the crack tip is to determine the crack extension at this point.

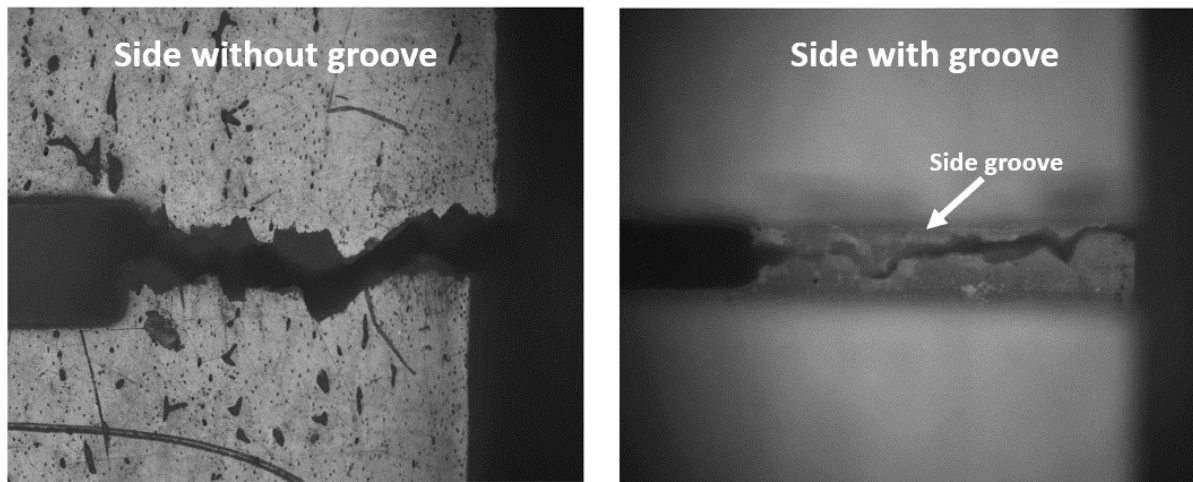


Figure 4.3: Illustration of crack growth path in a one-sided grooved human cortical bone fracture test sample. These images were taken post-fracture test. The image on the left shows the side without a groove and demonstrates a relatively straight crack growth path. The image on the right shows the side with a groove and demonstrates how the side groove controls crack growth by forcing it to stay within the groove. See Figs 4.10 and 4.17 for images of crack growth taken prior to unstable crack growth.

The one-sided groove was found to keep crack growth relatively straight while allowing for crack extension measurement using an optical microscope (see Fig. 4.3). Based on these results, it was decided one-sided grooves would be used in the preparation of the SENB specimens for the main part of this study.

4.2.2 Preliminary study 2: Matching displacement rate between the two loading regimes.

The second preliminary study investigated how to match displacement rates between the two loading regimes employed in this study to generate comparable load versus load line deflection curves. The first loading regime involved loading specimens continuously at a constant rate until unstable fracture. This was referred to as the monotonic loading regime or group. The second loading regime involved a series of regularly spaced load-partial unload cycles during the fracture test, necessary when using the UC method, until unstable fracture. This was referred to as the load-unload loading regime or group. To match displacement rates, two case studies were explored. In the first case study, the same displacement rates were used for both loading regimes on paired specimens. This meant the load-unload

loading regime fracture test took considerably longer (by a factor of 5) to reach unstable fracture. The second case study defined the displacement rates such that the time taken to the deflections at the unloading points for the load-unload loading regime matched the time taken to the same deflections for the monotonic loading regime. In other words, the average deflection rate for both loading regimes were matched.

4.2.2.1 SENB specimen preparation and fracture testing

Paired SENB specimens were prepared from the posterior diaphysis of the tibiae of two young steers (1.5-2 years) which was sourced fresh frozen from a local farm and stored dry in a -20°C freezer. The preparation of the SENB specimen was the same as described in section 4.2.1.1 with a groove on only side of the uncracked ligament region. For fracture testing, the SENB specimens underwent displacement controlled three-point fracture tests with the same mechanical test system and set-up described in section 4.2.1.2. As previously mentioned, two case studies were explored in this preliminary study.

For the first case study, one of the paired specimens underwent a monotonic loading regime at a displacement rate of 0.3 mm/min. The other pair underwent a load-unload loading regime at a displacement rate of 0.3 mm/min. The load-unload loading regime consisted of repeated cycles of loading by 75µm and partial unloading by 50µm with a one second dwell period in between the loading and unloading cycles. For the second case study, the average deflection rate was matched between the monotonic and load-unload loading regimes for the paired SENB samples. The average deflection rate was defined as the net deflection of the specimen divided by the time taken to obtain that net deflection. A displacement rate of 0.3 mm/min was maintained for the monotonic loading regime. For the load-unload loading regime, a displacement rate of 1.5 mm/min was utilised. The load-unload loading regime employed the same repeated cycles of loading by 75 µm and partial unloading by 50 µm. This meant there was a net deflection of 25µm after each load-reload cycle. Since a total displacement of 125µm (75+50) was travelled to achieve the net deflection of 25µm, the average deflection rate will be given as:

$$\text{average deflection rate} = \frac{\text{net deflection}}{\text{time taken to net deflection}}$$

$$\text{where, time taken to net deflection} = \frac{\text{total displacement travelled to achieve net deflection}}{\text{displacement rate}}$$

therefore, average deflection rate = $\frac{25}{\frac{125}{1.5}} = 0.3 \text{ mm/min}$

Consequently, the average deflection rate of the load-unload loading regime matched that of the monotonic loading regime where the displacement rate of 0.3 mm/min was equal to its average deflection rate. The loads and load-line deflections during the fracture tests were acquired at the frequency of 4 Hz for both loading regimes in the first case study. For the second case study, the loads and load line deflections were acquired at a frequency of 4 Hz and 20 Hz for the monotonic and load-unload loading regimes respectively. To account for slight geometric differences between the paired SENB specimens, the recorded load was normalized by multiplying by the dimensionless compliance and dividing by the effective thickness of the specimen. This process is briefly discussed in Appendix A.1. Using this normalized load, the normalized load versus load line deflection curves were compared between the paired SENB specimens for the two case studies explored.

4.2.2.2 Results for preliminary study 2

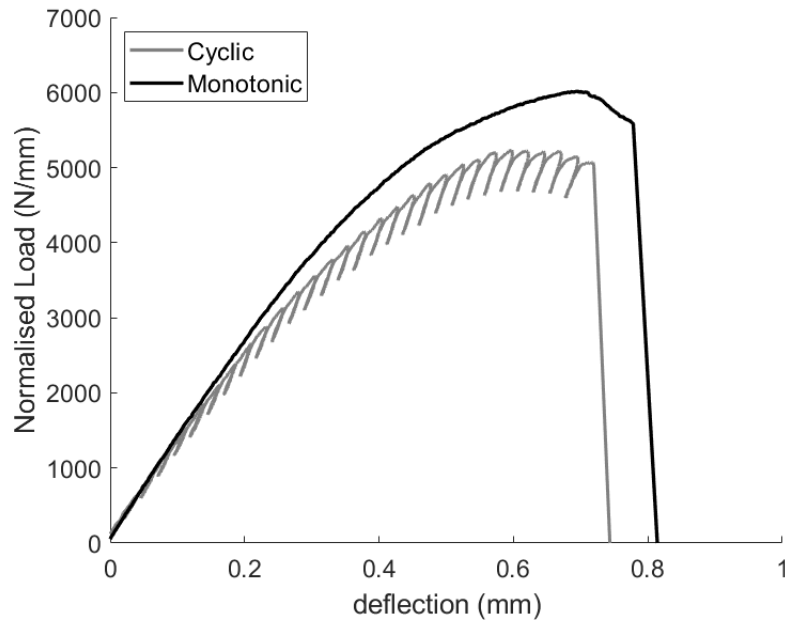


Figure 4.4: Representative normalised load vs load-line deflection curve for paired samples when the same constant displacement rate is used for the two loading regimes, that is, cyclic and monotonic. It is noticed there is a considerable difference between the two curves.

For the first case study where the same displacement rates were used in both loading regimes, it was found there was a considerable difference between the area under the normalized load versus load-line

deflection curves for the two loading regimes even when accounting for the slight geometric differences between the paired specimens (see Fig. 4.4). In the second case study where the average deflection rates were matched between the two loading regimes, the area under the normalized load versus load-line deflection curves were found to be comparable after accounting for geometric differences (see Fig. 4.5). Based on these results, it was decided that the average deflection rates would be matched for the two loading regimes in order to obtain comparable normalized load versus load line deflection curves to ascertain the impact of the UC method and load-unload loading on cortical bone fracture toughness assessment.

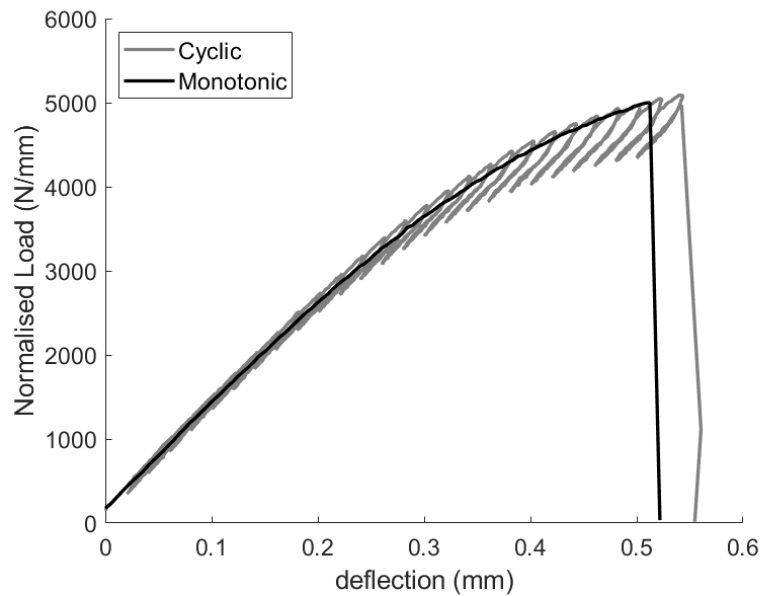


Figure 4.5: Representative normalised load vs load-line deflection curve for paired samples when the time taken to deflections at unloading points are matched for the two loading regimes, that is, cyclic and monotonic. In this case, the two load versus deflection curves match closely.

4.3 Materials and methods

4.3.1 Specimen preparation

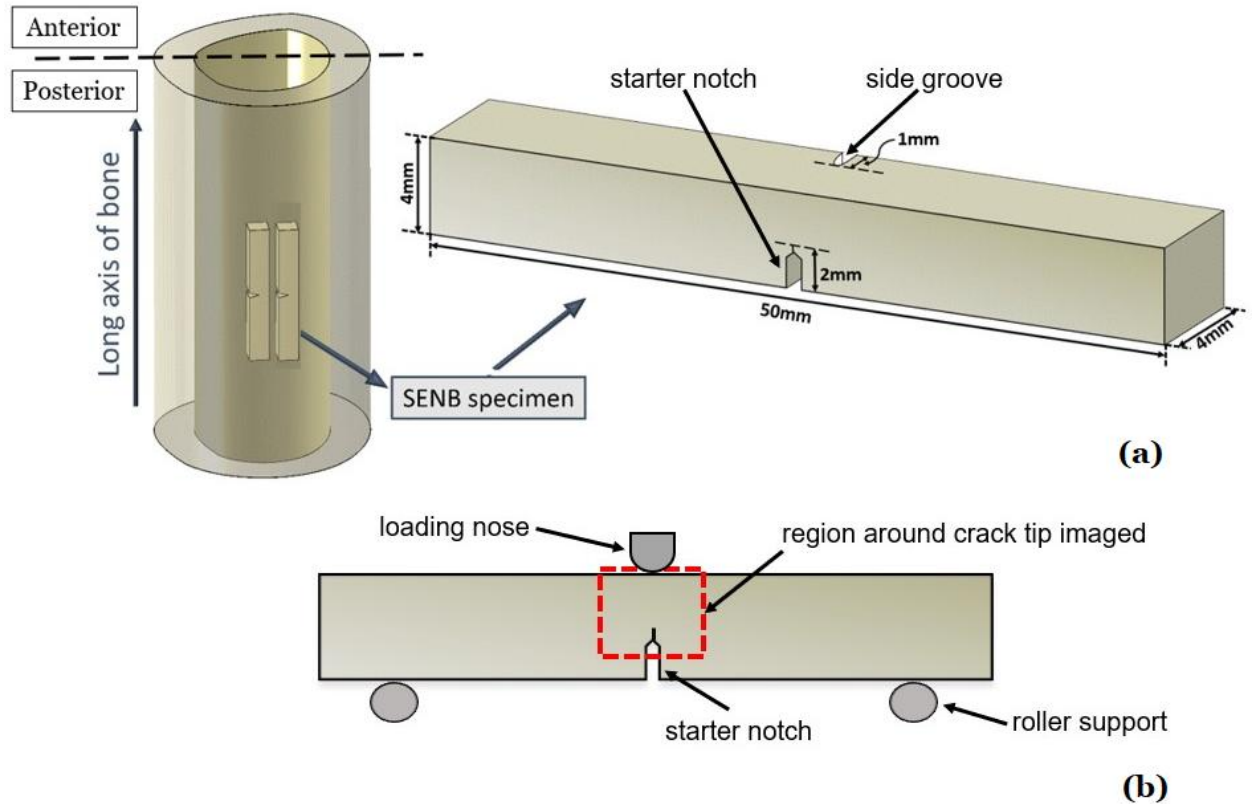


Figure 4.6: (a) Illustration of bovine tibia diaphysis as well as a single edged notched bend (SENB) specimen with groove on only one side. The paired SENB specimens were obtained from the posterior diaphysis of the tibia, and their length was directed along the long axis of the bone with the starter notch directed in the circumferential direction of the long bone. (b) Illustration of the 3-point single edged notched bend (SENB) fracture test as well as the region around the crack tip that was imaged by the microscope with a digital camera.

Tibiae from four young steers (1.5-2 years) were sourced fresh frozen from the same local farm and stored dry in a -20°C freezer. From these, 10 matched pairs of single edged notched bend (SENB) specimens of cortical bone were prepared from the posterior diaphysis (see Fig. 4.6a). The matched pairs were prepared such that they were taken adjacent to each other from the posterior diaphysis. The SENB specimens were prepared as described in section 4.2.1.1 with dimensions of width ~ 4 mm, thickness ~ 4 mm and length ~ 50 mm and with grooves of depth 1 mm on only one side of the uncracked

ligament region along the width (see Fig. 4.6a). This was based on the results of the first preliminary study discussed in section 4.2.1. Placing the grooves on only one side allowed optical crack extension measurement on the other side without a groove while still driving relatively straight crack growth. The grooves were prepared following guidelines from the ASTM E1820 standard [143]. From this, the depth of the side groove should not exceed a quarter of the initial thickness, and the root of the side groove should be at an angle less than 90°. As such, a depth of 1 mm and an elliptic shaped root were used in preparing the side grooves. Fig. 4.6a shows an illustration of the final SENB specimen geometry with dimensions. The prepared specimens were kept in saline-soaked gauze at -20 °C until testing.

4.3.2 Modulus screening

Prior to creating the starter notches and side grooves in the SENB specimens, the unnotched beams were screened to calculate their elastic moduli. Using the unnotched beams allowed simple beam theory to apply. This was conducted by non destructively and monotonically loading them in three-point bending to a deflection of 0.15 mm. This approximates to a stress value 50 % below the elastic limit point of bovine cortical bone in three-point bending [22]. This was carried out in displacement control at a rate of 0.3mm/min. The span for the tests was 40 mm. Load and load-line deflection were acquired at a frequency of 5 Hz. From this data, the loading slope was calculated by fitting a line between the 5th and 95th percentile of the data points. Using the loading slope and dimensions of the specimens, the elastic moduli were computed as [141]:

$$E = \frac{MS^3}{4BW^3} \quad (4.1)$$

where M is the loading slope

S is the span of the test

B is the thickness of the specimen

W is the width of the specimen

After screening the specimens for their elastic moduli, they were notched, and side grooved as described in the preceding section and prepped for fracture testing. The elastic moduli values were used when calculating for the tearing moduli, one of the fracture toughness measures, of the specimens given in Eq. 4.5.

4.3.3 Fracture testing

Before fracture testing, the specimens were kept in 1X phosphate buffered saline (PBS; Fisher Scientific, Hampton, NH, USA) overnight at 4 °C and then for 4 hours at room temperature to re-hydrate in case of any water loss. All specimens underwent a displacement controlled three-point SENB fracture test (see Fig. 4.6b). This was conducted using a micromechanical test system (μ TS, Psylotech, IL, USA). The span for the fracture tests was 40 mm. The paired SENB specimens were split between the two different loading groups described in Section 4.2, that is, the load-unload and monotonic loading groups. For the monotonic loading group, testing was conducted at a constant displacement rate of 0.3 mm/min. For the load-unload loading group, testing was conducted by repetitive loading for 75 μ m and partial unloading for 50 μ m at a constant displacement rate of 1.5 mm/min. There was a dwell period of one second between the loading and unloading cycles to reduce hysteresis for easier measurement of the unloading compliances. From the second preliminary study, these testing protocols matched the average deflection rates between the loading regimes as discussed in section 4.2.2. During each fracture test, a digital camera (Point Grey, 5MP, 2/3" detector) attached to a microscope (BXFM, Olympus Corporation, Center Valley, PA, USA) with a 5x magnification objective lens (Olympus Corp., Center Valley, PA, USA) mounted over the test system allowed images of the region around the sharpened notch tip to be taken (see Fig. 4.6b). The images were captured under green light to ensure phase contrast. The images were taken at a frequency of 4 Hz for the monotonic loaded specimens and 20 Hz for the load-unload loaded specimens. The load and load-line deflection during the fracture tests were acquired at the same frequencies as the image acquisition frequencies for the respective loading regimes.

4.3.4 Crack extension measurements

For this study, there were two methods of crack extension measurement: (1) unloading compliance method (UC) and (2) optical method. The unloading compliance method was and could only be applied to the load-unload loading group whereas the optical method of crack extension measurement was applied to both the monotonic and load-unload loading groups.

4.3.4.1 Unloading compliance (UC) method

As previously mentioned, it has been found that the standard isotropic UC calibration equations widely available in ASTM standards and textbooks did not accurately predict crack extension for anisotropic biological materials [148,149]. To rectify this, a finite element analysis was performed to develop a

new UC calibration equation for bovine cortical bone. A 3D model of the one-sided grooved SENB specimen was modelled in the commercially available ABAQUSTM software (Dassault Systèmes, France) according to the given dimensions in Fig. 4.6a. Because a plane of symmetry could be applied along the transverse plane of the specimen, only half of the specimen was modelled reducing computation cost. A roller boundary condition was placed at the base of the specimen to replicate the 3-point experimental setup. In addition, a displacement boundary condition was placed on the top of the specimen in line with the crack front to mimic the experimental displacement-controlled loading (see Fig. 4.7). The model was meshed with quadratic tetrahedral elements of size 0.4 mm. Because, the unloading compliance method is based on linear elastic behaviour, only the elastic properties of bovine cortical bone were considered with transverse isotropy assumed [290] (see Table 4.1). In addition, because only elastic properties were considered, no crack tip mesh refinement was carried out to save computational time [291]. A mesh sensitivity study was carried out to ensure convergence using this approach (See Appendix A.2 and Fig. A.1). To obtain the new UC calibration equation, multiple simulations were run varying the initial crack length from 0.8 mm to 3.2 mm at a constant interval of 0.4 mm. That is, a crack length to width (a/W) ratio running from 0.2 to 0.8 at intervals of 0.1 were simulated. The specimens were loaded to 0.1 mm in 10 fixed steps and fully unloaded. The load and load-line deflection were tracked from which the unloading compliance for each crack length simulated was computed from the load versus deflection curves.

These unloading compliances (C) were converted to a normalised form, μ using the equation [291]:

$$\mu = \left(1 + \sqrt{\frac{4WE B_e C}{S}} \right)^{-1} \quad (4.2)$$

with $B_e = B - \frac{(B-B_N)^2}{B}$

where W is the width

E is the effective elastic modulus

B_e is the effective thickness of the specimen

B is the overall thickness of the specimen

B_N is the net thickness of the specimen accounting for the side groove

C is the unloading compliance

S is the span for the test.

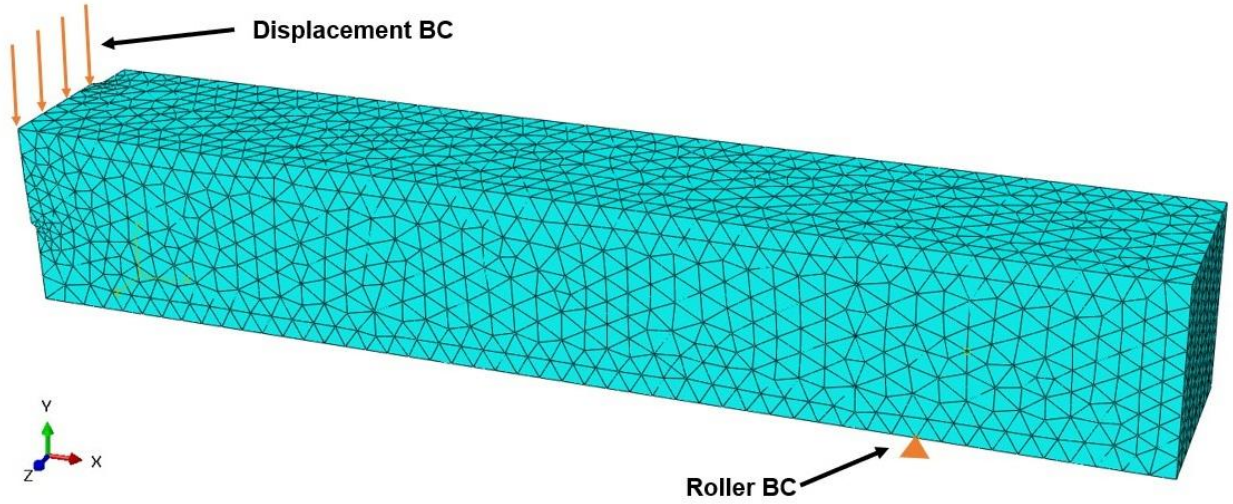


Figure 4.7: 3D finite element model of half of a SENB specimen showing the boundary conditions (BC) used.

Table 4.1: Elastic properties of bovine cortical bone used in finite element simulations. E refers to elastic modulus, G refers to the shear modulus and ν , the Poisson ratio. The subscripts 1 represents longitudinal direction with subscripts 2 and 3 representing the transverse circumferential and transverse radial directions respectively

Property	E_1 (MPa)	E_2 (MPa)	ν_{12}	G_{12} (MPa)	G_{13} (MPa)	G_{23} (MPa)
Value	19100	10400	0.3	4000	4000	3500

The UC calibration equation was obtained by plotting the dimensionless compliance (μ) versus the normalised crack length, which is crack length divided by width (a/W) and fitted with a polynomial function between the two (See Fig. 4.8). In this case, a second order polynomial function was found to be the best fit ($\text{Adj-R}^2 = 0.99$) and was given by:

$$\frac{a_i}{W} = 0.9712 - 3.569\mu - 66.07\mu^2 \quad (4.3)$$

where a_i is the current crack extension plus length of the starter notch.

Using this equation, the crack extensions at the unloading points of the specimens in the load-unload loaded group were estimated.

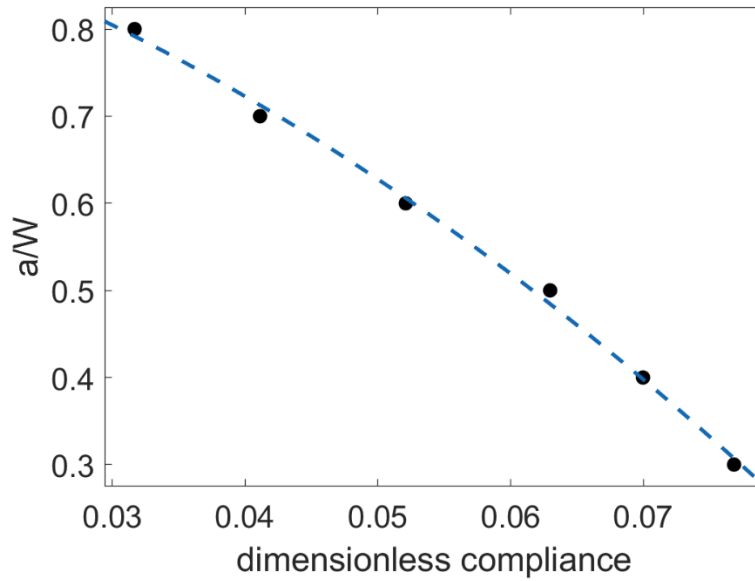


Figure 4.8: Plot of dimensionless compliance versus normalized crack length (a/W) derived from ABAQUS simulations and used to compute the standard UC calibration equation for this study.

4.3.4.1.1 Correction on crack extension obtained from UC method

An interesting realization from the load-unload loaded specimens was that the unloading compliances from their load versus load-line deflection curves initially decreased, contrary to expectation. An increase in the crack length should lead to an increase in the unloading compliance. Consequently, when Eq. 4.3 was utilised in the case of this decreasing unloading compliance, an apparent negative crack growth was obtained for the initial part of the fracture test. This apparent negative crack growth is unrealistic therefore this was corrected using an offset approach. The offset approach was based on work done by Seok [292] and Tertuliano and co-workers [293]. In this approach, the apparent negative crack growth obtained is proposed to coincide with the period of crack blunting. As such, the point at which the unloading compliance reaches its lowest value and subsequently increases, closely matches the start of actual crack extension. Hence the crack extension values are shifted such that the crack extension at this point is zero with the preceding crack extension values equated to zero. Since the

subsequent unloading compliances are greater, no negative crack extension values are obtained. Fig. 4.9 presents an illustration of the offset technique.

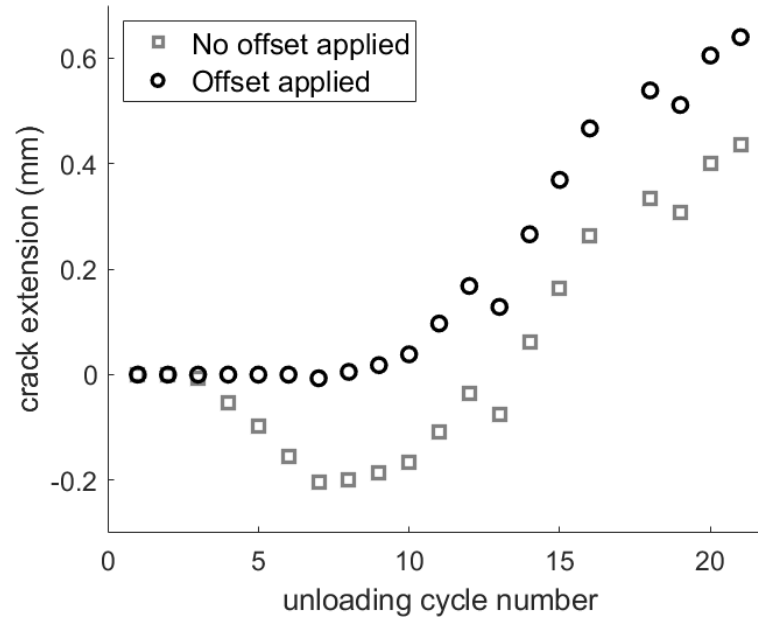


Figure 4.9: Representative plot of unloading cycle number versus crack extension illustrating the application of the offset approach to correct the initial apparent negative crack extension obtained from using the unloading compliance method to estimate crack extension.

4.3.4.2 Optical method

The optical method utilised the images taken of the crack tip region during the fracture test. To confirm the images taken were representative of physical reality, a number of pre-testing evaluations were undertaken. First, using a 2-axis linear translation stage (Newport, Irvine, CA, USA) the errors associated with taking the images was evaluated. This was done by moving a piece of computer-generated speckle pattern paper at a known distance and evaluate the image using digital image correlation to ascertain if this known distance will be matched. It was found that the errors from these were less than a pixel. In addition to the above, spatial resolution and distortion correction was performed to ensure the images were well calibrated. The spatial resolution was measured by using a microscope calibration ruler slide of length 1mm with divisions of 0.01 mm. The distortion correction was conducted following the VIC-2D software manual provided by Correlated Solutions (Irmo, SC,

US), suppliers for the microscope-camera system [294] . This was to correct for geometric distortions at the edges of the images that arise because of the shape of the objective lens [295].

Fig. 4.10 illustrates how the crack extension measurement was conducted in this method. The initial unbroken ligament length prior to fracture testing was measured (l_o). For any subsequent images for which the crack extension was to be measured, the current unbroken ligament length was measured (l_i) and subtracted from l_o to give the crack extension ($\Delta a_i = l_o - l_i$). This was performed using ImageJ (NIH, USA). The resolution of the microscope setup (1.38 $\mu\text{m}/\text{pixel}$) was used as the measurement scale in ImageJ. The crack extension measurement was conducted on selected images. For the load-unload loading group, the selected images corresponded to the start of unloading in the fracture test. For the monotonic loading group, the selected images were such that the corresponding deflection at that point matched the deflections at the start of unloading in the fracture test of the load-unload loading group. This allowed for direct comparison between the two methods of crack extension measurement specifically in the case of the load-unload loading group.

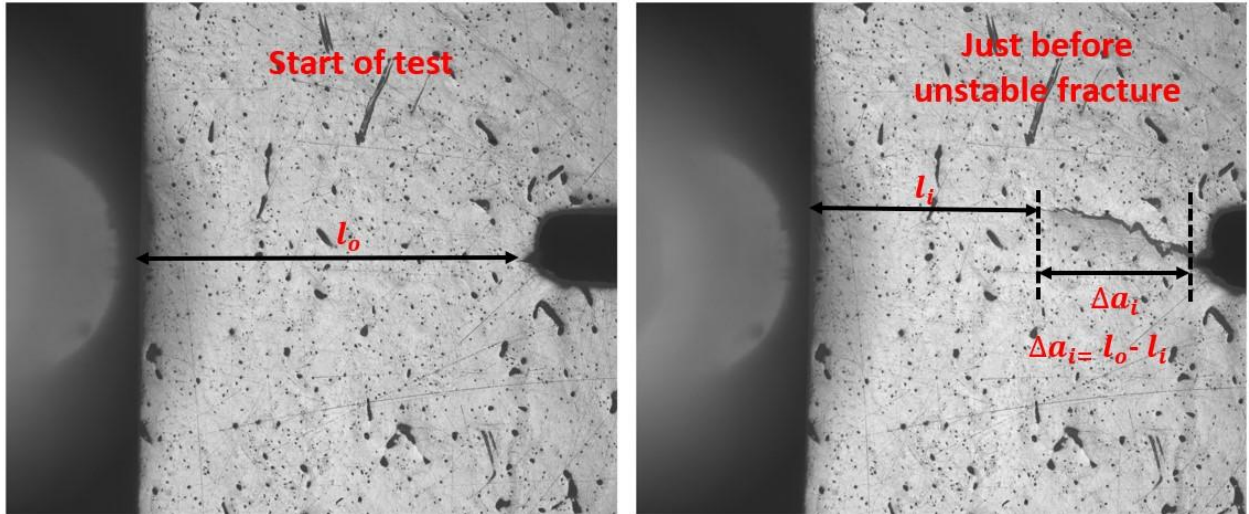


Figure 4.10: Illustration of how crack extension is measured by the optical method. The initial unbroken ligament length known as l_o is measured from an image taken at the start of the fracture test. In subsequent images, the unbroken ligament length, l_i , is measured and the crack extension at that point in the test can be computed as $\Delta a_i = l_o - l_i$

4.3.5 J-Resistance (J-R) curve analysis

Using the load versus load-line deflection curves from the fracture tests as well as the crack extensions, three different approaches of J-R curves were computed for each SENB specimen pair. Fig. 4.11 gives

an overview of how the loading regimes and crack extension measurement methods combine to form the various approaches stated below. These were:

1. Optical microscope (OM) approach: This involved the monotonic loaded group specimens and crack extensions were measured by the optical method. This approach serves as the “ground truth” and hence is the reference or control for comparison with the other two approaches.
2. Unloading compliance (UC) approach: This involved the load-unload loaded group specimens and crack extensions were determined using the UC method.
3. Optically measured compliance (OM-C) approach: This also involved the same load-unload loaded group specimens in the UC approach, but crack extensions were measured by the optical method.

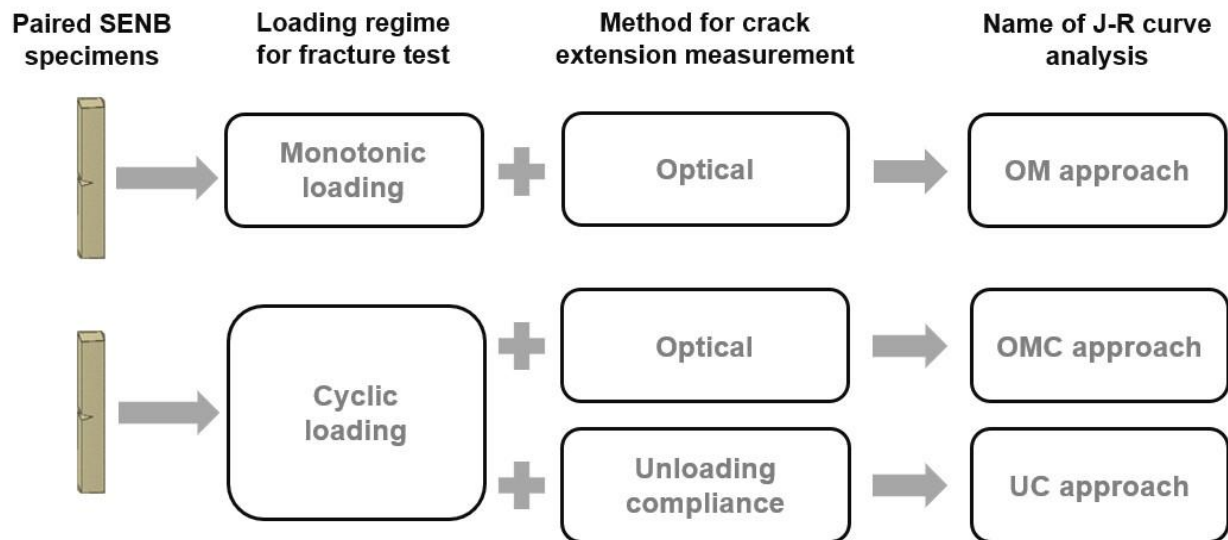


Figure 4.11: An overview of the methods scheme after specimen preparation culminating into three different J-R curve analysis groups given in section 4.2.6.

This design allowed the individual impact of crack extension estimation by the UC method on the accuracy of fracture toughness measures to be examined by comparing the OM-C and UC approaches. Comparison between the OM-C and OM approaches highlighted the individual effect of the load-unload loading on fracture toughness measures whereas comparing the OM and UC approaches revealed the combined effect of the load-unload loading and crack extension estimated by the UC method.

For each crack extension measurement, the corresponding J-integral was computed by using an incremental approach given by [285]:

$$J_i = \left(J_{i-1} \frac{\eta_{i-1}}{B_N b_{i-1}} A_{i-1,i} \right) \left(1 - \frac{\gamma_{i-1}}{b_{i-1}} (a_i - a_{i-1}) \right) \quad (4.4)$$

where $\eta_{i-1} = 1.9$, a dimensionless geometry factor

$\gamma_{i-1} = 0.9$, a geometry factor related to η

B_N is the net thickness of the specimen (accounting for side groove)

b_{i-1} is the uncracked ligament length from the previous step

$A_{i-1,i}$ is the net change of the area under the load-deflection curve at corresponding point

a_i is the current crack extension plus starter notch length

a_{i-1} is the crack extension plus starter notch length from previous step.

The J-integral equation above is equivalent to the J-integral equation given in ASTM E1820 [285] discussed in section 2.3.4. The one difference is that total J is used in Eq. 4.4 whereas in ASTM E1820, total J is separated into its elastic (J_{el}) and plastic (J_{pl}) components. In the ASTM E1820 equation, the unloading compliance must be known or calculated to compute both J_{el} and J_{pl} because the area under the load versus deflection curve needs to be separated into elastic and plastic regions. While the unloading compliance can be determined in the load-unload loading group, this was not the case for the monotonic loading group. As noted earlier, the incremental approach to computing J-integral mitigates errors associated with the load-unload cycles as well as the growing crack [285]. An empirical equation for calculating compliance, given in ASTM E1820 was found to produce inaccurate results when compared to the initial compliance of the specimens before crack growth or microdamage formation. Consequently, Eq. 4.4 was used in both groups to eliminate the need for determining the unloading compliance in the monotonic loading group.

The calculated J-integral values were plotted against the measured or computed crack extensions (Δa) to generate the J-R curves. A power law fit of the form $J = c_1 (\Delta a)^{c_2}$ was used to fit the data points to create a fitted J- resistance curve. This was conducted using a custom-made MATLABTM script (The MathWorks Inc., Natick, MA, USA). From each J-R curve, three single value fracture toughness measures were computed: (1) the crack initiation fracture toughness (J_{IC}), (2) the crack propagation

fracture toughness or the tearing modulus (T_R) and (3) the instability fracture toughness (J_{int}) (see Fig. 4.12). J_{IC} was computed as the intersection between the fitted J-R curve and a 0.05 mm offset construction line. The construction line had a slope twice the flow stress of bovine cortical bone. The flow stress is simply the average of a material's yield and ultimate strengths. The 0.05 mm offset used is different from the 0.2 mm offset indicated in ASTM E1820. The 0.05 mm offset was used because the optical measurements of crack extension indicated that this was the crack extension on average beyond which there were a significant change in the rate of crack extension between unloading points (more on this in the section 4.5). Further, ASTM E1820 uses the 0.2 mm offset to account for crack blunting prior to true crack growth which is erroneously detected as crack extension by the UC method. When, the crack extension is optically measured, crack blunting is not measured as crack extension, hence a 0.05 mm offset was a reasonable point to calculate J_{IC} . The tearing modulus was calculated by using the slope of a line fitted to the latter part of the J-resistance curve. Specifically, this was the region corresponding to crack extensions between 0.15 mm and a quarter of the initial unbroken ligament ($0.25l_0$; see Fig. 4.10) [143,296]. The crack extensions between 0.15 mm and a quarter of the initial unbroken ligament were chosen based on exclusion lines defined in ASTM E1820 standard. A line of best fit was plotted for data points in this region and the slope of the line gave the modulus of toughness (dJ/da). The tearing modulus (T_R) was subsequently calculated as [97]:

$$T_R = \frac{E}{\sigma^2} \times \frac{dJ}{da} \quad (4.5)$$

where E is the elastic modulus obtained from the modulus screening step described in section 4.3.2

σ is the flow stress which is the average of the yield and ultimate strength

J_{int} was calculated as the last valid J-integral value as indicated in the ASTM E1820 standard. That is, the last computed J-integral value with a crack extension equal to or less than a quarter of the initial unbroken ligament. This normally signifies the J-integral at the point of unstable crack growth.

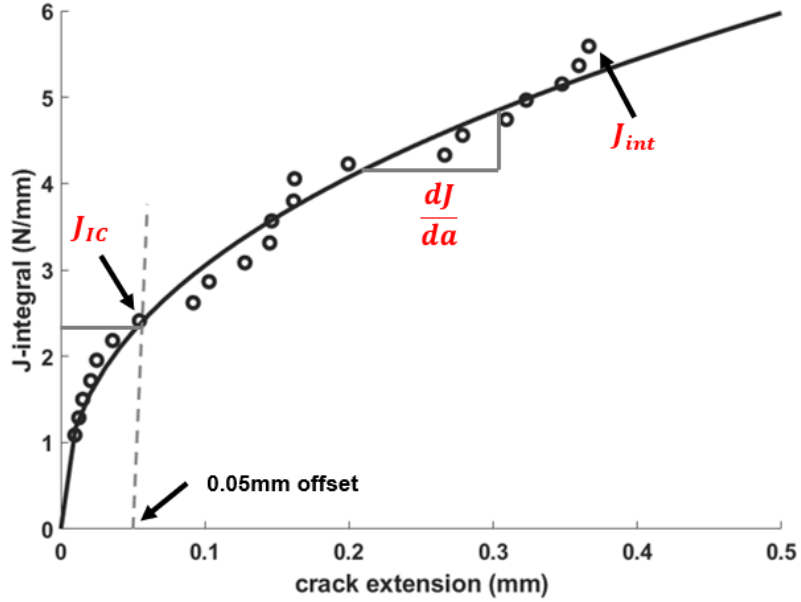


Figure 4.12: Illustration of fracture toughness measurements computed from J-R curves of the various specimens.

4.3.6 Crack extension comparison

For the load-unload loading group specimens, crack extension was determined by both the UC and optical methods. Hence these two measures were compared to test for the accuracy of the UC method in estimating crack extension. For each specimen, this was done by comparing crack extensions above 0.05 mm that were optically measured to their corresponding crack extension determined by the UC method and computing the differences as a mean absolute percentage error (*MAPE*) given as:

$$MAPE = \frac{100 * \sum \left| \frac{\Delta a_{UC} - \Delta a_{OMC}}{\Delta a_{OMC}} \right|}{N} \% \quad (4.6)$$

where Δa_{UC} represents the crack extension computed using the UC method

Δa_{OMC} represents the crack extension measured using the optical method

N is the number of unloads performed in the test hence the number of crack extensions measured optically or computed by the UC method.

4.3.7 Statistical analysis

To evaluate for statistical differences, a series of two-tailed paired t-tests were used to compare the three fracture toughness measures among the J-R curve approaches. Similarly, a series of F-test for equality of variances were conducted to compare the three fracture toughness measures taken from the J-R curves among the J-R curve approaches. A paired t-test compares the means of two measurements taken from the same sample to assess whether there is a difference whereas an F-test compares the variances of two measurements to assess whether there is a difference. As such, the paired t-test evaluated the accuracy of the OM-C and UC approaches as compared to the OM approach whereas the F-test evaluated the precision of the OM-C and UC approaches in comparison to the OM approach. For both tests, a p-value below 0.05 indicated statistical significance. These were conducted using the Data Analysis tool in Microsoft Excel® (Microsoft, Redmond, WA, USA). In addition, Pearson correlations were performed between the three J-R curve approaches for the various fracture toughness measures to assess if the errors were systematic. Again, significance was assessed as a p-value below 0.05. This was conducted in MATLAB Statistics Toolbox (The MathWorks Inc., Natick, MA).

4.4 Results

Fig. 4.13a shows representative normalised load versus load-line deflection curves for the two loading groups. The normalised load was calculated by multiplying the load by the dimensionless compliance of the specimen and dividing by its effective thickness as described in Appendix A.1. This accounted for slight geometric differences between the paired specimens. Fig. 4.13b shows representative J-R curves for the three different approaches employed for J-R curve analyses. Bar charts of the three fracture toughness measures computed are presented in Fig. 4.14 for each of the three different J-R curve analyses groups. For all three fracture toughness measures, the OM approach was found to be statistically different from the UC approach. The UC approach overestimated J_{IC} by 27%, T_R by 95% and J_{int} by 31% on average as compared to the OM approach. The OM-C approach was only significantly different from the OM approach for the J_{int} fracture toughness measure, overestimating it by 20% on average. The OM-C approach was however found not to be statistically different from the UC approach for all three fracture toughness parameters. Additionally, the crack extension at which J_{int} was taken was also computed and a bar chart is shown in Fig. 4.14d. There was no statistical difference found between the three approaches for this measure.

Table 4.2 presents the p-values of the series of F-test statistical analysis conducted. There were statistical differences between the OM and UC approaches as well as OM-C and UC approaches for both the crack initiation fracture toughness (J_{IC}) and the tearing modulus (T_R), both considered material properties.

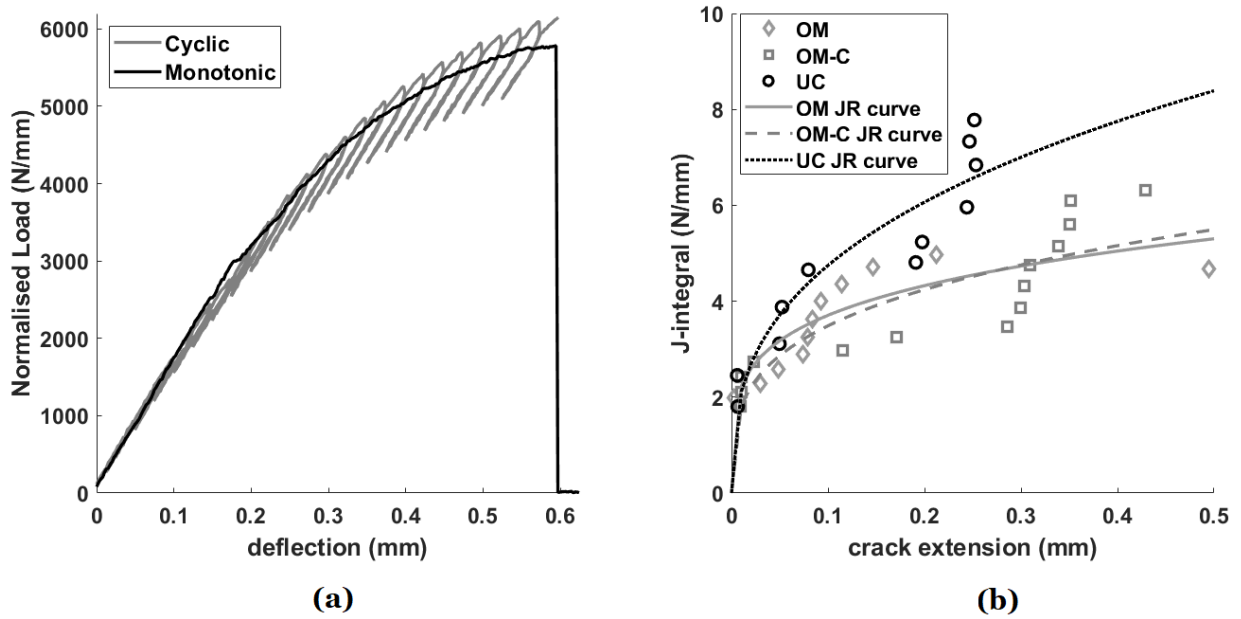


Figure 4.13: (a) Representative normalised load versus load-line deflection curves for the two loading groups. (b) Representative J-resistance curves for the three different approaches of analyses.

Table 4.2: P-values for F-test of variance between the various J-R curve analyses groups for the various fracture toughness parameters computed

J-R curve approaches compared	J_{IC}	T_R	J_{int}	Δa at J_{int}
OM vs OM-C	0.46	0.37	0.39	0.44
OM vs UC	<0.01	<0.01	0.17	0.42
OM-C vs UC	<0.01	<0.01	0.25	0.48

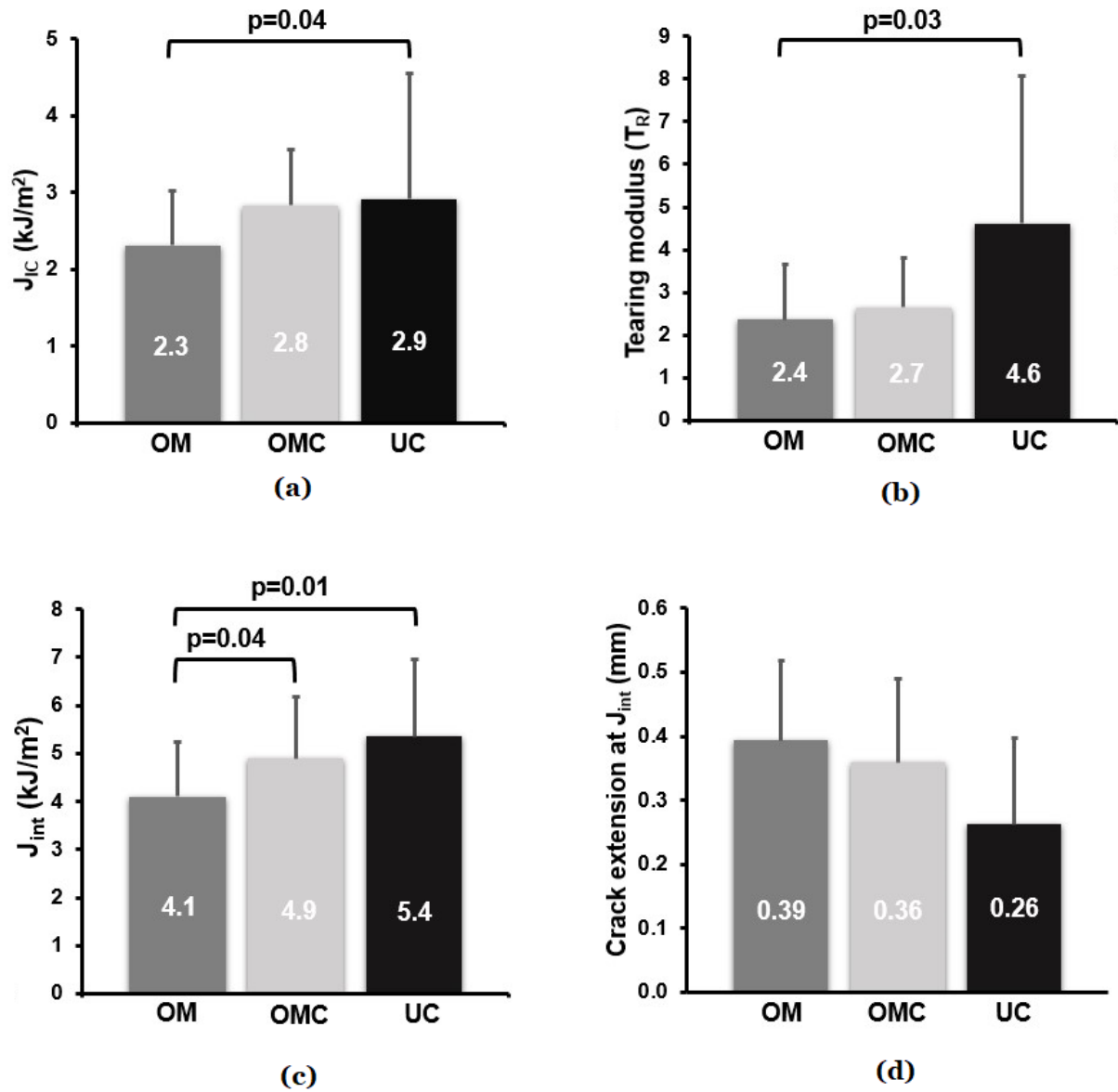


Figure 4.14: Bar charts of the three fracture toughness measurements computed from the three J-R analyses approaches: (a) crack initiation toughness; J_{IC} (b) tearing modulus; T_R (c) J-instability; J_{int} (d) crack extension at J-instability. The error bars represent one standard deviation and groups statistically different from each other are shown.

Table 4.3 presents the correlation coefficients from the Pearson correlation between the different J-R curve approaches for the various fracture toughness measures. The UC approach correlated positively

with the OM approach for both the J_{IC} and T_R . The OMC and UC approaches were positively correlated only for J_{IC} whereas OM and OMC approaches were positively correlated for only J_{int} .

Table 4.3: Pearson correlation coefficient table between the fracture toughness measures of the three different J-R curve approaches. The superscripts indicate: * p value < 0.05, **p-value < 0.01, * p -value < 0.001, , ns represents not significant (p-value > 0.05)**

For J_{IC}	Correlation coefficient	OM	OMC	UC
	OM	-	ns	0.64*
	OMC	ns	-	0.75**
	UC	0.64*	0.75**	-

For T_R	Correlation coefficient	OM	OMC	UC
	OM	-	ns	0.65*
	OMC	ns	-	Ns
	UC	0.65*	ns	-

For J_{int}	Correlation coefficient	OM	OMC	UC
	OM	-	0.63*	Ns
	OMC	0.63*	-	0.76*
	UC	ns	0.76*	-

In addition to the above, a MAPE of 72.8 ± 39.2 % (mean $\pm$ standard deviation) was obtained comparing crack extension measured in the OM-C and UC approaches using the optical and UC methods, respectively. The crack extension generated from the UC method was predominantly lower than the corresponding crack extension determined using the optical method for the load-unload loaded SENB specimens (see Fig. 4.15). This was opposite to what was hypothesized.

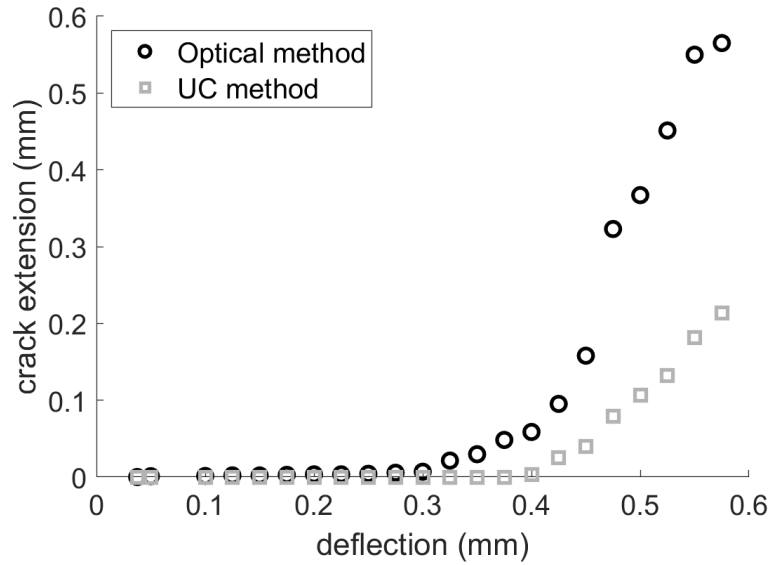


Figure 4.15: Representative plot comparing crack extension measured by the UC method and optical method for the same load-unload loaded SENB specimen.

4.5 Discussion

In this study, the impact on the accuracy and precision when using the UC method to quantify J-integral fracture toughness of bovine cortical bone was examined. Results showed that the UC method inaccurately predicted crack extension for bovine cortical bone with an average underestimation error of 73 % when compared to optical measurements. This clearly violates the 15 % error allowance given in ASTM E1820 for crack extension estimations when utilising the UC method [143]. Surprisingly though, for all three fracture toughness measures, there was no statistical difference between the OM-C and UC approaches despite the considerable error in crack extension measurement between the two approaches. Conversely, there was a statistical difference between UC and the OM approach for all three fracture toughness measures. These seem to suggest that statistical difference between the UC and OM approaches is due to the combined effects of crack extension estimated by the UC method and the energy dissipated due to the load-unload loading. OM-C was statistically different from the OM approach for only the J_{int} fracture toughness measure. For the OM-C specimens, the load-unload loading leads to energy dissipation due to cortical bone's viscoelastic nature reducing the energy available for crack growth. This, in turn, increases the apparent fracture toughness value. Therefore, it is possible, at

J_{int} , the energy lost to load-unload loading may be sufficient to detect a higher fracture toughness measure for the OM-C specimens compared to the OM specimens explaining the statistical difference.

From the F-test statistical analysis, it was found that there was a statistical difference between the OM and UC approaches as well as the OM-C and UC approaches for both the crack initiation (J_{IC}) and crack propagation (T_R) fracture toughness. Because of the higher variance observed in the UC approach, these results indicate that the UC approach produced less precise measures of these fracture toughness parameters as both are considered material properties of cortical bone hence these measures should be fairly repeatable.

Results from the Pearson correlation showed a positive relationship between UC and OM approaches for both crack initiation (J_{IC}) and crack propagation (T_R) fracture toughness despite their difference in the F-test analysis. This suggest there might be a systematic error at play in the UC approach compared to the OM approach. Surprisingly, the OM and OM-C approaches only correlated for J_{int} . One reason for this might be the impact of the load-partial load cycle on the growth of the crack which is discussed later and highlighted in Fig. 4.18. Another reason might be that the analysis is not adequately powered due to the number of data points (10).

In the load-unload group specimens, an unexpected finding was that the unloading compliance decreased during the initial stages of the fracture test. Consequently, when using the UC method for crack extension estimation, an apparent negative crack growth was obtained which is unrealistic. Interestingly, in canvassing the literature, this initial decrease in compliance was not uncommon, reported in studies involving other materials especially ductile metals [292,297–299]. The apparent negative crack growth has been attributed to a few factors including friction at contacts, indentation or bending of loading pins and misalignment of loading train [292]. Verstraete et al. [298] using experimental and FE analyses argued the apparent negative crack extension was due to rotation effects from induced bending moments around the crack tip. They employed single edged notched tensile (SENT) specimens in their study. Interestingly, the apparent negative crack growth was not realised in their FE model when the material property was assigned as linear elastic. Conversely, when an elastic plastic material property was used, it resulted in this apparent negative crack growth. In addition, Andrade et al. [297] noted an initial decrease in the compliance for 3D simulated SENB specimens of structural steel when plasticity was included. They also simulated a case of no plasticity for which the initial decrease in compliance was not observed. In work done by Seok [292], precautions were taken

to minimise inaccuracies resulting from specimen and testing setup however an initial apparent negative crack growth was still obtained using the UC method. It was argued and shown analytically that the plastic zone initially formed around the crack tip prior to true crack extension contributed to this negative crack growth. The plastic zone is thought to exert compressive residual stresses around the crack tip which leads to a decrease in unloading compliance when there is no crack extension.

For cortical bone, the equivalent of this plastic zone is the micro-damage process zone (MDPZ) which forms before and after crack growth initiation. To show this, digital image correlation (DIC) was used to compute the strain field distribution for one of the load-unload group specimens from which the MDPZ formed was estimated. This was performed using the DIC software, Vic2D (v6, Correlated solutions, Irmo, SC, US). The MDPZ was estimated from the Lagrange strain field as regions above a threshold of 0.5% [112,300]. Fig. 4.16 shows this estimated MDPZ (represented by the red regions) formed at crack growth initiation, at peak load and just before unstable fracture. It is apparent that the MDPZ continues to form until unstable fracture. The MDPZ is comprised of diffuse sub micron-sized cracks that result in volume expansion in the material [110,115]. However, due to the surrounding material which restricts this expansion, compressive stresses are induced in this region around the crack tip [115]. Hence, based on work done by Seok [292], the initial decrease in unloading compliance observed with the load-unload group specimens might be due to the formation of the MDPZ prior to any actual crack extension. This was contrary to our hypothesis that micro-damage formation would result in an overestimation of crack extension by the UC method. As noted, an offset technique was adopted to rectify this initial negative crack growth hence, the impact of micro-damage formation on the initial crack extensions computed by the UC method is significantly reduced. This might explain why there is no statistical difference for J_{IC} between the UC and OM-C approaches. Further, the absence of statistical difference validates the use of the offset technique in dealing with the apparent negative crack growth at the start of the fracture test.

It is worth noting that, in cortical bone, the MDPZ continues to form even after crack growth onset (see Fig. 4.16). Consequently, more compressive residual stresses may be exerted around the crack tip decreasing the actual material unloading compliance leading to a lower-than-expected unloading compliance. This will result in an underestimation of the crack extension computed by the UC method which might partly explain this observation in this study. Significant underestimation of crack extension using the UC method has also been found for ductile metals such as steel that undergo extensive stable crack extension [298,299]. Other factors have also been attributed to the underestimation of crack

extension in these ductile metals and include stress triaxiality, side grooves, specimen rotation, closure, crack tip plasticity and crack tip tunneling [297]. It is possible these also contribute to the underestimation of the crack extension using the UC method in this study.

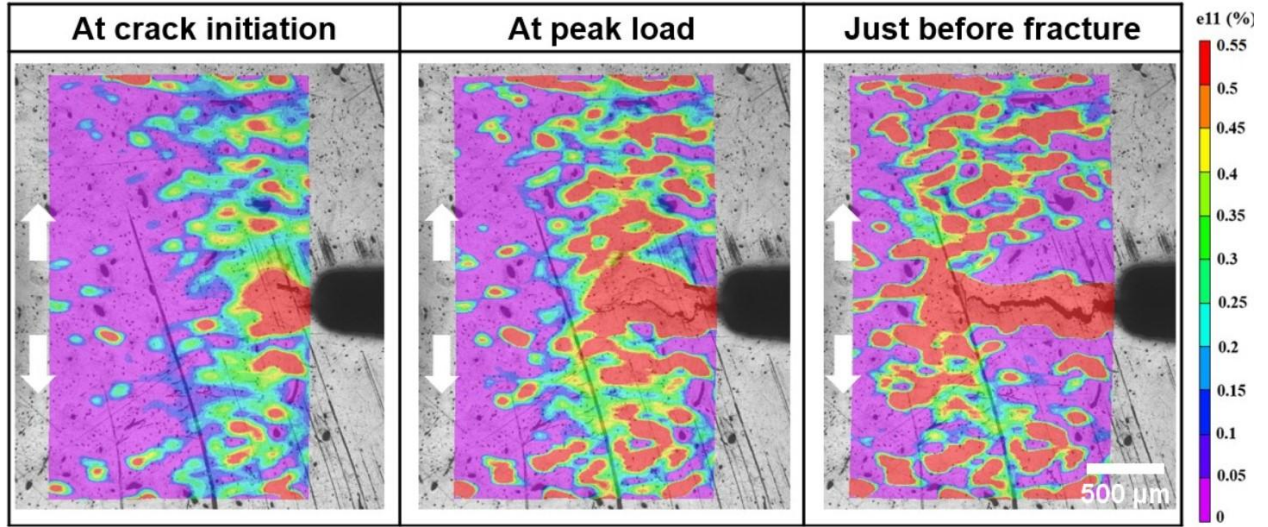


Figure 4.16: Lagrange strain field distributions ahead of the crack tip region for one of the monotonically loaded specimens at crack growth initiation, peak load and just before unstable fracture. The red regions represent the MDPZ which is denoted as regions with a strain greater than 0.5% (see legend). The white arrows point in the direction of the e_{11} strain, that is, in the longitudinal direction.

Another interesting realization from this study was that the use of load-unload loading drove relatively straighter crack growth as compared to the monotonic loaded specimens (see Fig. 4.17). Fig. 4.18a presents a plot of crack extensions measured using the optical method for both loading regimes (load-unload and monotonic) plotted against their corresponding deflections at the time of measure. From this figure, it was noticed at larger deflections, crack extension was larger in the monotonic loaded specimen compared to the corresponding crack extension measured for the load-unload group specimen. As such, the average crack extension rate was slower in the load-unload group specimens than their corresponding monotonic loaded specimens (see Fig. 4.18b). A reasonable explanation for the slower average crack extension rate is that the load-unload loading reduces the energy available for crack growth because of hysteresis, as noted earlier. Consequently, the impact of crack deflection as an extrinsic toughening mechanism may be diminished allowing for straighter crack growth in the load-unload group specimens. In addition, the relatively straighter crack growth is reflected in the J-R curves for the OM-C approach. For the OM-C approach, in which the load-unload group specimens were used,

an average root-mean square error (RMSE) of 0.32 was obtained between the data points and the power law fitted to these points. For the OM approach (monotonically loaded), an average RMSE of 0.4 was obtained indicating more deviation from the power law shape.

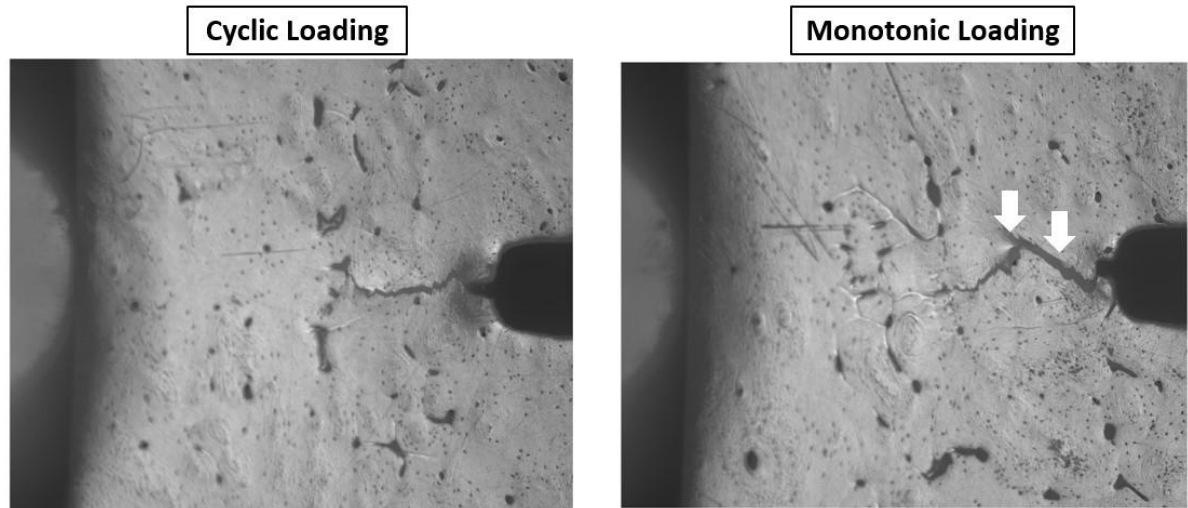


Figure 4.17: Representative comparison of crack growth path between equivalent cyclically loaded (left) and monotonically loaded (right) specimens. The cyclically loaded specimen exhibits relatively straight crack growth whereas the monotonically loaded specimen undergoes noticeable crack deflection indicated by the white arrows. However, the side grooves still drive a relatively straight crack path in the monotonically loaded specimen compared to specimens without side grooves (see Fig. 4.1).

In addition, Fig. 4.18a shows the 0.05 mm offset line that was used to compute J_{IC} . It was observed that there is little increase in crack extension below this line. Above this crack extension limit, there is an initial significant increase in crack extension between the deflections at the unloading points. This informed the use of the 0.05 mm offset line for calculating J_{IC} . In the monotonically loaded specimens predominately, it was noticed there was an instantaneous increase in crack extension just beyond the 0.5 mm offset line as shown in Fig. 4.18a. This resulted in a crack extension increase of about 150-200 μm for a net deflection of 25 μm . This is particularly interesting as the increase in crack extension is on the same length scale as the osteonal diameter suggesting they may play a role in stopping this instantaneous crack growth from leading to catastrophic fracture [301,302].

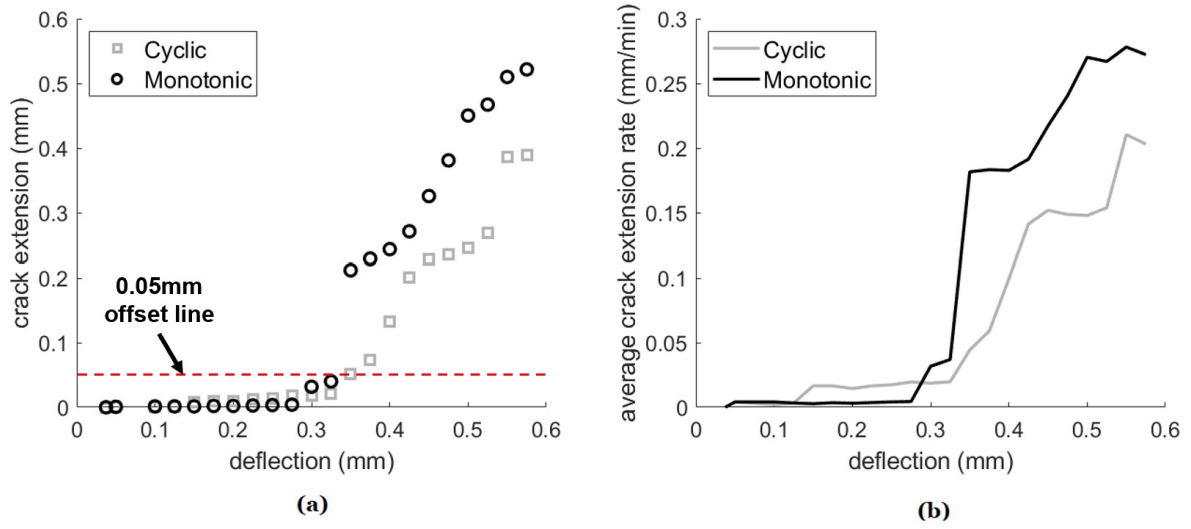


Figure 4.18: (a) Representative crack extension data for respective loading groups measured using the optical method plotted against their corresponding deflection at time of measure. (b) Corresponding average crack extension rate plotted against deflection for the two loading groups.

The importance of cortical bone fracture mechanics to finding and understanding key determinants of bone fragility and fracture makes it essential that the correct approach is applied to obtain accurate and precise fracture toughness measures. This work clearly shows that using the UC method produces uncertainties in the measurement of cortical bone fracture toughness. This highlights a need for standardized procedures for fracture testing of cortical bone. Such standardization will allow reproducibility of studies and results between laboratories. This will allow various laboratories to verify and build upon each others' findings. To assist with developing a standardized procedure, studies specific to cortical bone need to be carried out to ascertain how testing specifications "borrowed" from structural materials translate to cortical bone fracture testing. These include testing configurations (3-point vs 4-point, compact tension, or single edged notched bend, etc.) and valid test specimen dimensions (thickness, span to width ratios, etc.). From this study, it is recommended an optical method is adopted for crack extension measurement until a suitable and validated unloading compliance method for cortical bone is developed. In addition, it is proposed a 0.05 mm offset be used for calculating for J_{IC} as opposed to the 0.2 mm offset defined in ASTM E1820 when an optical method is used to measure crack extension. This value was determined from analysis of the average crack extension rate which typically accelerated around an extension of 0.05 mm. However, it is important to note that, the crack extension rate is a function of displacement rate hence the proposed offset only applies to quasi-static

fracture tests in the range of 10^{-2} mm/s. In all, standardized testing methods will be impactful in studies seeking to better understand the cortical bone fracture process and provide knowledge that contribute to the development of better predictive and preventive tools.

A limitation of this study was the fracture testing was conducted in ambient air. This was necessary to measure crack extension by the optical method. However, setting up and testing each specimen took less than 10 minutes. From a post hoc experiment on the fractured SENB specimens, it was found less than a 1% water loss by weight occurs after 10 minutes in ambient air (see Table A.1 in Appendix A.3). This suggest there is minimal effect on the fracture test results associated with the hydration state of the specimens. Also, a recent study showed comparable fracture toughness values for sheep cortical bone between testing in ambient air and a bio-bath for the first 500 μm of crack extension [303], which is the range of crack extension used to generate the J-R curves in this study.

4.6 Conclusion

The aim of the present study was to evaluate the effect of using the unloading compliance (UC) method for quantifying fracture toughness in bovine cortical bone. It was found that the UC method underestimated crack extension by 73% on average when compared to an optical method of crack extension measurement. Furthermore, the added effect of the load-unload loading needed when using this method led to significantly different fracture toughness measures using the J-based R-curve approach. Consequently, an optical approach to measuring crack extension in cortical bone fracture toughness testing is recommended to be used instead of the unloading compliance method. Ultimately, this highlights a need towards developing standardized procedures for cortical bone fracture testing methods. Based on results from this work, the following concluding comments are made:

- The OM approach of monotonic loading and using an optical microscope to track crack extension provides the most accurate results over the entire range of the J-R curve.
- Optical based methods (OM and OM-C approaches) are also more precise.
- If J_{IC} is the fracture toughness measure of interest, the OM-C approach may be used to allow for straighter crack growth because of the load-unload loading employed. Straight crack growth is especially important for Mode I fracture testing of cortical bone to avoid mixed mode effects.
- If the UC method, which is less expensive in terms of equipment, must be applied to cortical bone, work must be done to establish appropriate standard unloading compliance calibration equations for different types of cortical bone similar to work done by Gibeling's group [148,149] and correction

factors incorporated to account for the initial reduced unloading compliance due to MDPZ formation as well as the energy lost during the unloading cycles due to the viscoelastic nature of cortical bone.

Chapter 5: Study 2

Oxidative damage as a means of collagen network connectivity degradation associated with reduced human cortical bone fracture resistance

5.1 Introduction

Cortical bone is a hierarchical biological nanocomposite comprising of bone crystals known as hydroxyapatite, an organic phase and water [48]. The organic phase consists of type I collagen, which makes up 90% by volume of the organic phase, and non collagenous proteins (NCPs) such as osteopontin, osteonectin and decorin [52,304]. Altogether, the organic phase is estimated to make up 45% by volume of the cortical bone tissue [49]. The organic phase is thought to be instrumental to cortical bone's inelastic (post yield) behaviour, that is, its ultimate strength and strain as well as toughness [83,84,254]. This is attributed to the collagen molecules' ability to withstand large strains reaching up to 50 % for tensile strains [104,108]. The inelastic behaviour of a material is important to its ability to resist fracture as it provides mechanisms of greater energy dissipation. As such, it has been shown that the organic phase is important to many toughening mechanisms in cortical bone tissue including but not limited to fibril bridging, micro-damage formation and collagen unraveling. These mechanisms stabilize cracks against unstable crack growth under normal physiological loading [17]. Unsurprisingly, modern bone quality studies have demonstrated the importance of the organic phase to the fracture resistance of cortical bone [17,50,93]. Among these, is a study in the late 1990s [19] and another in the early 2000s [23] that established the degradation of the collagen network was associated with reduced fracture toughness associated with aging. More recently, a study in which a general and heterogenous population donor group was used, also showed a strong positive correlation between collagen network connectivity and the fracture toughness of bone, helping to explain 49% of the variance observed in their fracture toughness measure [24]. Collagen network connectivity is measured using a technique known as hydrothermal isometric tension (HIT) testing [20] and provides a measure of how well the collagen molecules are linked together, increasing with crosslinking but decreasing with fragmentation of the polypeptide alpha chains [21,22]. Although, it is well established that the degradation of the collagen network connectivity and organisation is associated with reduced fracture resistance, the mechanisms by which these occur remain elusive.

One possible explanation for the degradation of the collagen network connectivity and organisation is oxidative damage but this has received little attention. Oxidative damage entails the deleterious changes that occur in cells, tissues and macromolecules (proteins, lipids, nucleic acids) because of oxidative stress [37]. Oxidative stress occurs when there is an abundance of reactive oxygen species (ROS) compared to antioxidants that scavenge these ROS [37]. Interestingly, oxidative stress is regarded as a prominent feature of normal aging as well as many acute and chronic diseases such as osteoporosis, osteoarthritis, cancers, diabetes, hypertension, cardiovascular diseases, chronic kidney diseases and others [38,40]. These conditions are all associated with poor bone quality. Oxidative stress is known to lead to protein oxidation in long lived proteins, of which collagen is a prime example [200]. This happens because the ROS attack and react with the backbone of the protein polypeptide chains which can eventually lead to fragmentation of the polypeptide chains or disrupt the side chain groups of the protein chains which can in turn lead to conformational changes in the protein structure [41,200]. These oxidation reactions between ROS and the protein structure predominantly leads to the formation of carbonyl containing compounds [200]. Therefore, carbonylation is a stable biomarker widely used to measure the extent of protein oxidation and oxidative damage in proteins [200,201].

Interestingly, another consequence of oxidative stress is the formation of advanced glycation end products (AGEs). These form from highly reactive carbonyl containing compounds such as glyoxal, glycolaldehyde and methylglyoxal reacting with the amine groups on a protein. These highly reactive compounds result from the ROS oxidizing sugars such as glucose and fructose [36,305]. The accumulation of AGEs is widely accepted as an indicator of poor bone quality [239,274]. This is because they have been found to accumulate in the serum, urine and other tissues such as the skin of osteoporosis, type 2 diabetes (T2D) and chronic kidney disease (CKD) patients as well as with aging [31,32,254–257,259]. Both T2D and CKD are associated with increased fracture risk when compared to controls despite T2D patients reporting normal to high BMD levels [13,14,221,306]. AGEs may also form through non-enzymatic glycation (NEG) reactions where a reducing sugar such as glucose reacts directly with the amine group on a protein [39]. Consequently, hyperglycemia associated with T2D is thought to lead to excessive formation of AGEs [274]

To explain how AGEs, which accumulate in the organic matrix of cortical bone with age and disease, affect bone fragility, in-vitro models were developed [26,28,33,253]. Typically, pentosidine, an AGE crosslink and one of the first AGEs to be isolated and quantified, was measured and used as a biomarker for all other AGEs that formed in vitro. Alternatively, other studies utilised a measure termed total

fluorescence AGE (tfAGE) which uses a fluorometric microplate-based assay to quantify AGEs that fluoresce within roughly a 370 nm excitation and 440 nm emission spectrum. These *in vitro* studies showed that the accumulation of pentosidine or higher tfAGE was associated with reduced post yield properties of bone and bone collagen as well as the toughness and fracture resistance of cortical bone [23,26,28,33,254]. The reduction in post yield strain, toughness and fracture resistance were consistent with changes observed in age-related bone fragility studies [146] therefore situating pentosidine and AGE crosslinks, in general as possible determinants of bone fragility. In addition, the same *in vitro* models showed that the accumulation of the afore-mentioned AGE biomarkers resulted in a stronger, stiffer, more connected but less ductile collagen network due to increased crosslinking [26,33,251,253]. Therefore, it is widely thought that AGEs lead to over-crosslinking of the collagen network making them stiff and less ductile which ultimately results in the bone's reduced ability to resist fracture. However, some other studies contradict this idea.

First, from the *ex vivo* studies discussed earlier [19,23,24], a less organised, less strong and less connected collagen network was associated with a reduction in post yield strain, toughness and fracture resistance, consistent with changes observed in other age-related bone fragility studies [146,254,307]. This is contrary to the findings from the *in-vitro* models where the collagen network is stronger, stiffer and more connected. Secondly, there are two broad classes of AGEs that have been identified; (1) crosslinks that form covalent bonds between two neighbouring collagen molecules and (2) adducts that form as modifications to the side groups of the collagen molecules [308]. Recent studies have highlighted that AGE adducts which are mostly non-fluorescent such as carboxyl-methyl-lysine (CML) are much more abundant (20 to 200 times) than pentosidine in bone including cancellous bone [34,35]. In addition, the relative abundance of these AGE adducts are on the same order of magnitude as the lysyl oxidase (LOX) enzymatic crosslinks whereas pentosidine is two orders of magnitude lower [34,172]. Hence, at the very least, the idea of “over-crosslinking” of the collagen network is not the only mechanism by which AGEs negatively impact bone collagen quality and might be of minor consequence compared to the impact of AGE adducts in bone fragility. Overall, pentosidine may not be a causative factor for bone fragility but may be indicative of elevated oxidative stress which results in degradation of the collagen network connectivity through various mechanisms such as collagen chain fragmentation.

Although, oxidative damage has been shown to affect bone cell metabolism specifically bone remodeling [309] as well as cause damage to collagen in other tissues [43,310], its possible impact

specifically on bone collagen network connectivity has not been investigated. In addition, ex vivo measures of biomarkers of oxidative damage, including various AGEs beyond pentosidine and especially AGE adducts, in human cortical bone tissue from a large heterogeneous human donor cohort is lacking. Furthermore, the mechanisms by which oxidative damage may impact collagen network properties and bone quality as well as how these explain the variations observed in cortical bone fracture resistance remain inconclusive. Consequently, in this study carbonyl content as well as three AGE adducts [carboxy-methyl-lysine (CML), carboxy-ethyl-lysine (CEL) and 5-hydroxymethyl-imidazolone (MG-H1)] and pentosidine, all biomarkers for oxidative damage, were measured ex vivo in cadaveric human femoral cortical bone specimens taken from a large, wide age ranging heterogeneous donor group that included those with a history of T2D and/or CKD. In addition, fracture toughness tests along with various characterization tests to obtain measures of key determinants of bone fragility as well as measures of collagen network quality were carried out. Statistical models were developed to investigate the relationships between the measures of oxidative damage, cortical bone fracture toughness, collagen network properties and other key determinants of bone fragility.

It was hypothesized that the measures of oxidative damage would be significantly higher across the board in the T2D and/or CKD donor group compared to the controls (non-T2D and non-CKD group). Furthermore, it was hypothesized that the carbonyl content would correlate strongly and positively with measures of collagen fragmentation whereas AGE content would correlate strongly and negatively with LOX catalysed crosslinks. Consequently, carbonyl content and AGE content would negatively correlate with collagen network connectivity, which in turn would strongly explain, through statistical models, variations observed in cortical bone fracture toughness measures. Therefore, it was also hypothesized that cortical bone fracture toughness measures will be significantly lower in the T2D and/or CKD donor group compared to the controls.

5.2 Materials and methods

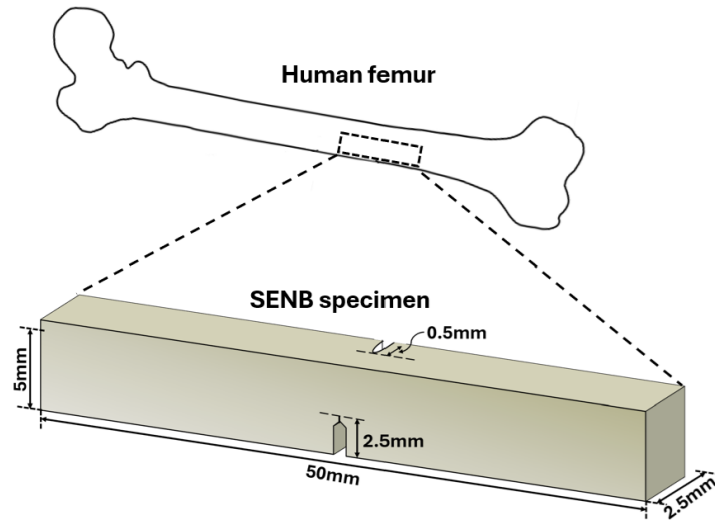
5.2.1 Specimen acquisition and fracture test specimen preparation

After ethics approval from the University of Waterloo's Office of Research Ethics Committee (ORE #40888), eighty-one (81) cadaveric human femora (43 male and 38 female) were acquired from various tissue banks across North America [Mount Sinai Allograft Technologies (Toronto, ON, CA), Lake Superior Centre for Regenerative Medicine (Thunder Bay, ON, CA), National Disease Research Interchange (Philadelphia, PA, USA), United Tissue Network (Phoenix, AZ, USA), Innoved Institute

(Elk Grove Village, IL, USA), and Science Care (Phoenix, AZ, USA)]. The donors spanned a wide age range between 19 to 103 years (Mean $\pm$ SD = 68.7 ± 17.7 years). Of the donors, 35 (19 males and 16 females) had a history of type 2 diabetes (T2D) and/or chronic kidney disease (CKD) (Mean $\pm$ SD = 69.9 ± 13.3 years). Of this T2D/CKD donor group, 22 donors were reported to have a history of only T2D, 5 donors reported to have only CKD and 8 reported to have both T2D and CKD. The remaining femurs (24 males and 22 females) were from donors without a history of T2D and/or CKD (Mean $\pm$ SD = 68.0 ± 20.0 years) and this will be referred to as the control group. Across both the T2D/CKD and control groups, some of the donors had a history of other morbidities besides T2D and CKD such as osteoporosis, osteoarthritis, heart diseases and hypertension. However, donors with a history of heavy smoking and/or drinking, cancer, liver disease, hysterectomy/ovariectomy, radiation/radiotherapy or chemotherapy treatment, surgeries such as arthroplasty of hip or knee, taking selective serotonin reuptake inhibitors, bisphosphonates or other antiresorptive medications and glucocorticoids as well as sex hormone imbalances were excluded. The femurs were stored in a -80°C freezer until specimen preparation.

From each femur, a single edged notched bend (SENB) specimen was prepared for 3-point bending fracture tests. The preparation of the SENB specimens was consistent with work presented in chapter 4 where cortical bone fracture testing approaches were investigated with minor modifications made to specimen geometry. The process is briefly described as follows. The femurs were rid of any remaining soft tissues using a knife and scalpels. A pathology bone band saw with an irrigated diamond blade (IMEB, California, USA) was then used to isolate the lateral diaphysis of each femur and the bone marrow was removed. Next, a rectangular block from the distal lateral diaphysis was cut using the same bone band saw. Afterwards, using a mini-CNC vertical mill (Sherline, California, USA), this rectangular block was reduced to a cortical bone beam of dimensions $\sim 52 \text{ mm} \times 8 \text{ mm} \times 3.5 \text{ mm}$. The length of the block aligned with the long axis of the femur. Using a combination of a low-speed metallurgical saw with a wafering diamond blade (Isomet, Buehler, IL, USA) together with polishing cloths and slurry, a final beam of dimensions $\sim 50 \text{ mm} \times 5 \text{ mm} \times 2.5 \text{ mm}$ was obtained. Polishing involved using a series of diamond discs and polycrystalline diamond suspension with a final finishing grade of $6 \mu\text{m}$ (MetaDi Supreme, Buehler, IL, USA). A starter notch with a razor sharpened tip of length $\sim 2.5 \text{ mm}$ along the width of the beam was created at the mid-point of the length of the specimen using the low-speed metallurgical saw and an ultra-sharp razor blade (American Line, Extra Keen single edge blades). The width of the specimen was directed along the circumferential direction of the

(A)



(B)

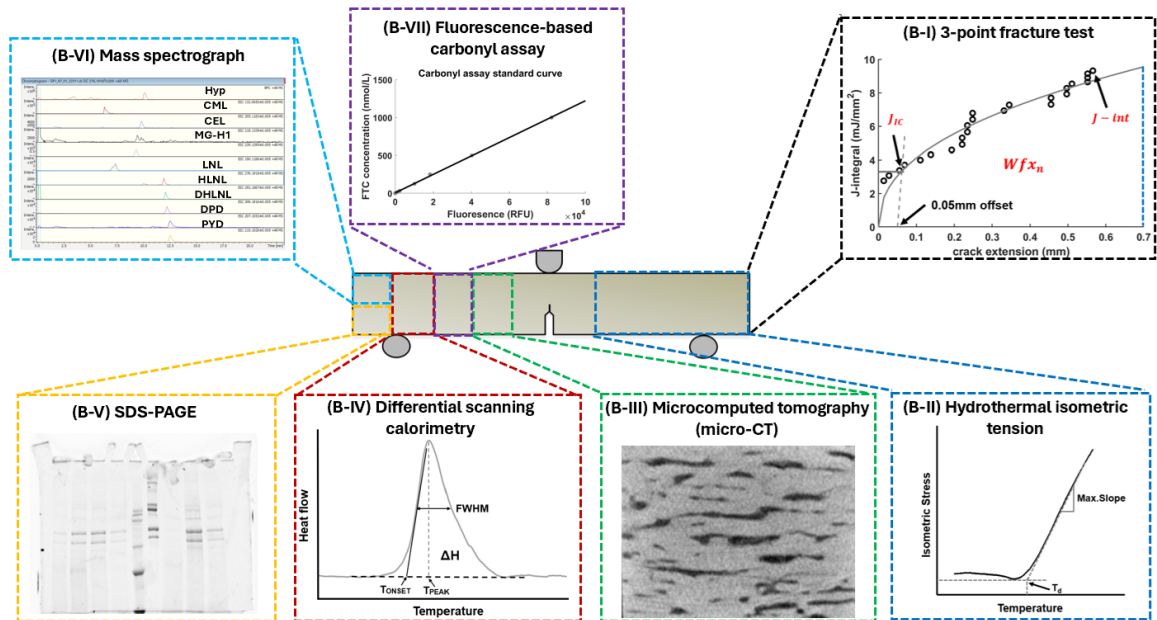


Figure 5.1: (A) Illustration of a single edged notched bend (SENB) specimen having a side groove on one side with dimensions as shown and prepared from the lateral distal diaphysis of a human femur. (B) Schematic illustrating the various methods carried out in this study which were (B-1) 3-point fracture testing, (B-II) hydrothermal isometric tension (HIT) testing, (B-III) microcomputed tomography (micro-CT), (B-IV) differential scanning calorimetry (B-V) sodium

dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), (B-VI) liquid chromatography-mass spectrometry and (B-VII) fluorescence-based protein carbonyl content assay.

femur. Lastly, utilizing the low-speed metallurgical saw again, a side groove of depth 0.5 mm was cut on one side of the unbroken ligament segment ahead of the starter notch. Side grooves promote straighter crack growth during fracture testing which is important for Mode I (tension) fracture. The side groove was created on only one side to allow for optical measurement of the advancing crack tip on the other side during the fracture test. Fig. 5.1A gives an illustration of the final SENB specimen and its dimension. The prepared beams were stored in saline soaked gauzes and kept in a -80 °C freezer until testing. The exact dimensions of each specimen were measured using digital calipers (MasterCraft, Vonore, TN, USA) prior to testing.

5.2.2 Fracture testing of SENB specimens

Prior to the fracture tests, specimens were re-hydrated in case of any water loss during specimen preparation by immersing each in 1X phosphate buffered saline (PBS) overnight at 4°C and then at room temperature for 4 hours. The specimens were tested using a mechanical test system (μ TS, Psylotech, IL, USA) under displacement controlled three-point bending. The fracture testing regimen as well as the computation of the fracture toughness measures were informed by the study described and discussed in chapter 4 of this thesis. A quasi-static progressive multiple load-unload testing regime was adopted where the specimens were loaded for 75 μ m and unloaded for 50 μ m at a constant loading rate of 0.03 mm/s [311]. This regimen was chosen to allow for straighter and greater stable crack growth [311] thus engaging the organic matrix of the specimens, which is the primary component of bone being analyzed in this study. Above the mechanical test system was a digital camera (Point Grey, 5MP, 2/3" detector) attached to an optical microscope (BXFM, Olympus Corporation, Center Valley, PA, USA) that allowed images of the region around the growing crack to be acquired during the fracture test. These were taken at a frequency of 10 Hz for the loading cycle and 15 Hz for the unloading cycle. The load and load-line deflection for the test were also recorded at the same frequencies.

Crack extensions at the start of each unloading cycle were computed by analyzing images taken during the fracture test using digital image correlation (DIC). Digital image correlation (DIC) was carried out using the commercially available Vic-2D software (v6, Correlated solutions, SC, USA). This approach was based on work done by Lai and co-workers albeit for graphite specimens [312]. The main idea behind using DIC is that crack growth results in displacement discontinuities in the vicinity of the crack

and this can be captured in DIC. The point at which the displacement discontinuity ends coincides with the tip of the growing crack (see Appendix B.1 for more details). Using this approach, the growing crack tip was found more efficiently and objectively compared to manual measurements. The crack extensions for each SENB specimen were calculated using a custom MATLAB (The MathWorks Inc., Natick, MA) code that incorporated the DIC measured displacements based on the above approach. Access to the MATLAB code can be found in Appendix B.2 with the steps to use it documented as well.

5.2.3 Computing J-integral resistance curves and fracture toughness measures

Using the load versus load-line deflection curves (see Fig. 5.2A) recorded during the fracture tests as well as the crack extension measurements computed using the DIC-based approach, J-integral resistance (J-R) curves were developed for each specimen. Since crack extensions were calculated at the start of the unloading cycle during the fracture test, each corresponding J-integral fracture toughness was computed at the same point using the incremental approach given as [285]:

$$J_i = \left(J_{i-1} \frac{\eta_{i-1}}{B_N b_{i-1}} A_{i-1,i} \right) \left(1 - \frac{\gamma_{i-1}}{b_{i-1}} (a_i - a_{i-1}) \right) \quad (5.1)$$

where $\eta_{i-1} = 1.9$, a dimensionless geometry factor

$\gamma_{i-1} = 0.9$, a geometry factor related to η

B_N is the effective thickness of the specimen (accounting for side groove)

b_{i-1} is the uncracked ligament length from the previous step

$A_{i-1,i}$ is the net change of the area under the load-deflection curve at corresponding point

a_i is the current crack extension plus starter notch length

a_{i-1} is the crack extension plus starter notch length from previous step.

It is important to note this equation is equivalent to the incremental J-integral equation given in ASTM E1820 standard [143]. The only difference between the two is the J-integral is separated into elastic and plastic components in the ASTM E1820 standard. The above equation was used to eliminate the need of finding the unloading compliance during each unloading step.

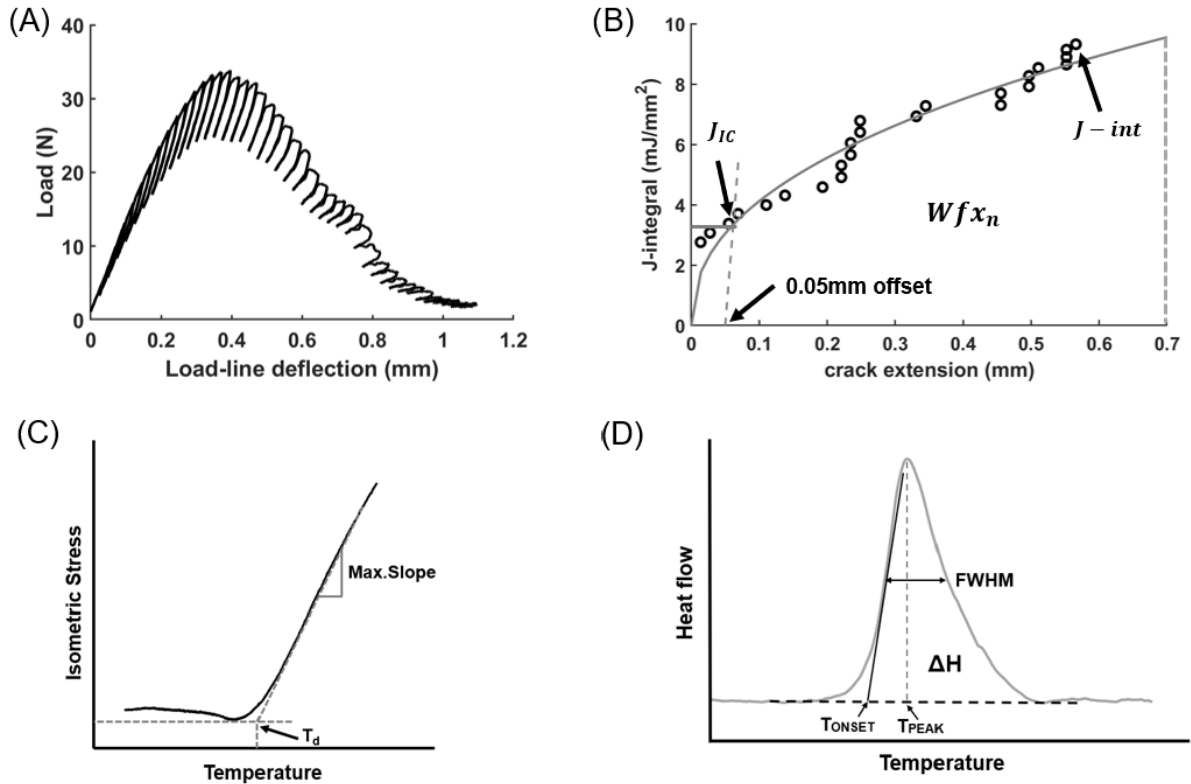


Figure 5.2: Representative curves for (A) load versus load-line deflection (B) J-integral resistance curve (C) hydrothermal isometric tension test (D) differential scanning calorimetry analysis.

J-integral resistance curves were computed by plotting the calculated J-integral values against their corresponding crack extensions (see Fig. 5.2B). The data points were then fitted to a power law of the form $J=c_1(\Delta a)^{c_2}$ to create a fitted J-R (resistance) curve. From the J-R curves, three parameters were derived which will be referenced as fracture toughness measures hereafter. These were: (1) the crack initiation fracture toughness (J_{IC}), (2) crack instability fracture toughness ($J-int$) and (3) the normalized work to fracture (Wfx_n) (see Fig. 5.2B). J_{IC} corresponds to the onset of crack growth at the razor sharpened notch. J_{IC} was computed as the intersection between a 0.05 mm offset line with slope twice the flow stress of human femoral cortical bone and the fitted J-R curve power law line. The flow stress is the average of the “yield” and ultimate tensile stresses of a specific material [97]. A value of 141.5 MPa was used corresponding to the average of the yield and ultimate tensile stresses for human femoral bone [313]. $J-int$ denotes the fracture toughness at which crack growth transitions to unstable fracture. $J-int$ was found as the last valid J-integral fracture toughness value of the J-R curve. From ASTM

E1820, only crack extensions less than or equal to a quarter of the initial unbroken ligament (width minus length of razor sharpened notch) are considered valid to use when plotting J-R curves. Based on this definition, J-int is found as the last J-integral value before or at the maximum allowable crack extension for that J-R curve. Wf_{x_n} gives a measure of the total energy dissipated during the stable fracture process per unit crack extension. It is computed as the area under the power law fitted J-R curve up to the last valid crack extension value according to the ASTM E1820 standard. Because the J-integral normalizes the energy dissipated to grow the crack to the unbroken ligament area, Wf_{x_n} is also normalized to the unbroken ligament area.

5.2.4 Specimen division and de-mineralization

After fracture testing, each SENB specimen was divided into various portions schematically shown in Fig. 5.1B. The portions were used in various characterization tests including: (1) micro-computed tomography (micro-CT), (2) hydrothermal isometric tension (HIT) testing, (3) differential scanning calorimetry (DSC), (4) liquid chromatography-mass spectrometry (LC-MS), (5) biochemical assays and (6) sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The location taken for each characterization test was kept consistent between SENB specimens in an attempt to minimize inter-specimen variability. In addition, the portions used in the HIT, DSC, LC-MS, biochemical assays and SDS-PAGE were all demineralized beforehand. This was done by soaking the relevant portions in 50 ml of 0.5 M ethylenediaminetetraacetic acid (EDTA) at room temperature for six weeks changing the EDTA solution every three or four days. Full demineralization of the specimens were confirmed using thermogravimetric analysis (TGA).

5.2.5 Micro-computed tomography

Micro-computed tomography was carried out on a region of the SENB specimen about 5 mm distal to the razor-sharpened starter notch. This section was chosen to exclude the micro-damage process zone that forms around the growing crack during the fracture test but still be close enough to capture the local microstructure [99,111]. The dimensions of the samples used were approximately 5 mm × 3 mm × 3 mm. The micro-CT samples were mounted in microcentrifuge tubes and fixed in place using double-sided tape. The samples were submerged in 1X PBS containing 1 mM of calcium ions to prevent calcium leaching and to keep the samples hydrated.

Each specimen was scanned using an inCiTe 3D X-ray microscopy system (KA Imaging, ON, Canada) with an isotropic voxel size of 7 μm with the following settings: tube voltage of 80 kVp, tube current of 166 μA , integration time of 1500 ms, 1000 projections and an averaging frame of 5. A 1-mm aluminium filter was used to aid beam hardening and a micro-CT hydroxyapatite phantom (QRM-D32, PTW, Freiburg, Germany) was included to allow for density calibration. Each scan involved three samples and took approximately 7 hours to complete. The samples were re-constructed using VGStudio software (Volume Graphics, NC, USA) with settings of high ring artifact and low beam hardening corrections.

After reconstruction, a volume of interest (VOI) was selected to eliminate edge effects and this VOI was analyzed to obtain cortical porosity, apparent volumetric bone mineral density (avBMD), tissue mineral density (TMD), extent of mineralization (M_{peak}) and mineralization heterogeneity [153]. These were obtained by analyzing the reconstructed samples in Dragonfly software (Comet, Montreal, QC, Canada). Cortical porosity was calculated as the fraction of the voxels in percentage that had a bone mineral density of less than 700 mgHA/cm^3 to the total number of voxels in the VOI. avBMD was calculated as the average of the volumetric bone mineral density for all the voxels in the VOI. TMD was calculated as the average of all the voxels in the VOI with a volumetric bone mineral density greater than 700 mgHA/cm^3 which is the actual bone tissue matrix. The extent of mineralization (M_{peak}) was computed as the mode (gray scale value occurring the most) of the histogram of all voxels within the VOI with a volumetric bone mineral density greater than 700 mgHA/cm^3 whereas the mineralisation heterogeneity was computed as the full width at half maximum for the same histogram .

5.2.6 Thermal and thermo-mechanical testing of de-mineralized human cortical bone samples: hydrothermal isometric tension and differential scanning calorimetry testing

Demineralized portions of the SENB specimens were used to characterize the quality of the collagen network by testing with hydrothermal isometric tension (HIT) and differential scanning calorimetry (DSC). These were conducted using methods consistent with previous studies [21,22].

The HIT test was carried out using a custom-built HIT system which allows for the simultaneous testing of six samples [314]. Two HIT samples of dimensions $\sim 2.5 \text{ mm} \times 1.5 \text{ mm} \times 20 \text{ mm}$ (width $\times$ thickness $\times$ length) were prepared from each SENB specimen's demineralized portion using a custom-made parallel ultra-sharp razor blade system (American Line, Extra Keen single edge blades). The samples were held in the HIT system at a fixed length using custom-made tissue grips at a pre-load of 2N. These

were subsequently placed in a 4 L water bath and heated from room temperature ($\sim 23^{\circ}\text{C}$) to 90°C at a rate of $1.4^{\circ}\text{C}/\text{min}$. The temperature of the water bath was recorded by means of a resistive temperature device (RTD) whereas load cells recorded the isometric forces generated by the HIT samples during the test. As the water is heated, the initial force is characterized by stress relaxation due to the bone collagen's viscoelastic nature. At a transition temperature, known as the denaturation temperature (T_d), the cortical bone collagen network being semi-crystalline begins to melt (denature), uncoiling from its triple helix structure. The denaturation temperature gives a measure of the nativity and stability of the collagen network. The uncoiling of the triple helices of collagen molecules drive the samples to shrink, however due to the isometric constraint, they generate increasing isometric tensile forces. The isometric forces recorded were converted to stresses using the initial cross-sectional areas of the samples which were measured prior to the test using digital calipers (MasterCraft, Vonore, TN, USA). From the isometric stress versus temperature curve, the maximum rate of change in the stress, referred to as maximum slope (Max.Slope), and denaturation temperature (T_d) were computed (see Fig. 5.2C). Max.Slope gives a measure of the connectivity of the collagen network which is thought to be a function of the crosslinking and the alpha chain lengths [21,22,315]. The denaturation temperature was computed as the temperature at which the minimum stress and the maximum slope line intersects. Because two HIT samples per specimen were used, the mean of their T_d and Max.Slope values were assigned to that specimen.

Differential scanning calorimetry works by measuring the heat flow difference between a sealed pan containing a sample and an empty sealed reference pan during a controlled heating cycle. As indicated earlier, when the collagen network is heated, it melts or denatures. This will cause more heat to be absorbed in the sealed pan with the demineralized sample compared to the empty reference pan. This is represented as an endotherm in the heat flow versus temperature curve from a DSC test and characterizes the nativity and thermal stability of the collagen network. For each SENB specimen, two cylindrical disks were prepared using a 4-mm diameter cylindrical biopsy punch (Integra, York, PA, USA) from the assigned demineralized portion for DSC. The wet mass of each sample was measured before testing using an analytical balance. The DSC test was performed using a TA Instruments DSC Q-2000 system (TA instruments, New Castle, DE, USA). The prepared cylindrical disk samples were hermetically sealed in aluminium pans and heated from room temperature to 95°C at a rate of $1.4^{\circ}\text{C}/\text{min}$ to mimic the heating rate for HIT. Hermetically sealed pans were used to ensure the mass of the samples were conserved by trapping water vapor inside as well as generate an even heat flux during

heating. After the DSC test, the samples were lyophilized by puncturing a hole in the pans and placing in a Laconco freeze dryer (FreeZone, Kansas City, MO, USA) for 30 hours. The samples were reweighed afterwards to obtain their dry masses. The heat flow versus temperature curves were analyzed using Universal Analysis Software (TA instruments, New Castle, DE, USA) to obtain the temperature at onset of denaturation (T_{onset}), temperature at which the maximum heat flow was recorded (T_{peak}), the full width of the endotherm at half of the maximum heat flow (FWHM), which gives a measure of heterogeneity or disorder with the collagen network and the enthalpy of denaturation (ΔH) from the denaturation endotherm (see Fig 5.2D). The enthalpy of denaturation was normalized to both the wet mass and dry mass of the samples. These were done by dividing the total energy absorbed by each sample, calculated as the total area under the endotherm, by the wet mass and dry mass of that sample.

5.2.7 Liquid chromatography - mass spectrometry

Liquid chromatography - mass spectrometry (LC-MS) combines the functionality of liquid chromatography and mass spectrometry allowing both qualitative and quantitative assessment of known and unknown components of a solution or mixture.

In this study, LC-MS was used to measure the lysyl oxidase (LOX) catalysed crosslinks, both mature and immature, as well as four different advanced glycation end products (AGEs). The LOX crosslinks analyzed were lysinonorleucine (LNL), hydroxylysinonorleucine (HLNL) and dihydroxylysinonorleucine (DHLNL), which are immature LOX crosslinks, as well as pyridinoline (PYD) and deoxypyridinoline (DPD) crosslinks, which are mature LOX crosslinks. For the AGEs, pentosidine, which is an AGE crosslink, alongside three AGE adducts; carboxy-methyl-lysine (CML), carboxy-ethyl-lysine (CEL), 5-hydro-5-methyl-4-imidazolone-2-yl-ornithine 1 (MG-H1) were measured. CML and CEL form from reactions between lysine on the collagen molecules and oxidized carbonyl compounds such as glyoxal, glycolaldehyde and methylglyoxal whereas MG-H1 forms by the reaction between arginine and methylglyoxal [265,316]. As such, CML and CEL were referred to as lysine-based AGEs whereas MG-H1 was referred to as an arginine-based AGE.

5.2.7.1 Chemical reagents

CML, CEL, MG-H1, [$^2\text{H}_2$]-CML, [$^2\text{H}_4$]-CEL, and [$^2\text{H}_3$]-MG-H1 were purchased from PolyPeptide Laboratories (Strasbourg, France).

Pentosidine was purchased from Cayman Chemical Company (MI, USA). [$^2\text{H}_4$]-Pentosidine was purchased from Alsachim (Illkirch Graffenstaden, France).

Lysinonorleucine hydrochloride (LNL), (2*S*, 2'*S*, 5*S*)-5-hydroxy lysinonorleucine hydrochloride (HLNL) and (5*S*, 5'*S*)-Dihydroxy lysinonorleucine (DHLNL) were purchased from Toronto Research Chemicals (Toronto, Canada). [$^2\text{H}_4$]-LNL and [$^2\text{H}_4$]-HLNL were purchased from Shinsei Chemical Company Ltd., (Osaka, Japan). [$^2\text{H}_4$]-DHLNL was purchased from Toronto Research Chemicals (Toronto, Canada).

Pyridinoline (PYD) and deoxypyridinoline (DPD) were purchased from TLC Pharmaceutical Standards (Ontario, Canada). [$^2\text{H}_4$]-DPD was purchased from Iris Biotech GmbH (Marktredwitz, Germany). [$^2\text{H}_6$]-PYD was purchased from Toronto Research Chemicals (Toronto, Canada).

L-hydroxyproline was purchased from Nacalai Tesque (Kyoto, Japan). [$^2\text{H}_3$]-L-hydroxyproline was purchased from C D N Isotopes Inc., (Quebec, Canada).

ESI-L Low Concentration Tuning Mix was purchased from Agilent Technologies (CA, USA).

High-performance liquid chromatography (HPLC)-grade distilled water (H_2O), acetonitrile (MeCN) and formic acid were purchased from Nacalai Tesque (Kyoto, Japan).

All other chemicals were purchased from Wako Pure Chemical Inc (Osaka, Japan).

5.2.7.2 Sample pretreatment

The demineralized cortical bone sample portions were pulverized using a Multi-beads Shocker (Yasui Kikai, Osaka, Japan). Samples were then washed with phosphate-buffered saline (0.15 mol/L NaCl in sodium phosphate buffer, pH 7.4), delipidated with chloroform/methanol (2:1, v/v) mixture during 24 hours at 4°C and demineralized with 0.5 mol/L EDTA in 50 mmol/L Tris buffer (pH 7.4) for 96 hours at 4°C. Delipidated and demineralized bone powders were reduced by sodium borohydride in sodium borate buffer (100 mmol/L of boric acid and 1 mmol/L diethylenetriamine pentaacetic acid; pH 9.1) for 4 hours at ambient temperature. Approximately 0.1 mg of bone powders were spiked with isotope-labelled internal standards (ISTDs) in the following quantities: 5 nmol of [$^2\text{H}_3$]-L-OH-pro, 10 pmol of [$^2\text{H}_2$]-CML, [$^2\text{H}_4$]-CEL, [$^2\text{H}_3$]-MG-H1, [$^2\text{H}_4$]-LNL, [$^2\text{H}_4$]-HLNL, [$^2\text{H}_4$]-DHLNL, [$^2\text{H}_4$]-DPD, and [$^2\text{H}_6$]-PYD. The same quantities of ISTDs were also spiked to six different concentrations of each authentic standard to facilitate the construction of external calibration curves. Reduced bone powders were

hydrolyzed in 6 N hydrochloric acid (HCl) at 110°C for 18 h and dried using centrifugal evaporation under reduced pressure.

Separate bone powders (5 mg) were reduced and hydrolyzed to quantitate pentosidine. Bone hydrolysates were resuspended in MeCN/acetic acid/H₂O (8:1:1, v/v/v) mixture and spiked with 20 pmol of [²H₄]-pentosidine, then cleaned up by a Bond Elut-Cellulose SPE column from Agilent Technologies (CA, USA). The column was washed with MeCN/acetic acid/H₂O (8:1:1, v/v/v) mixture and eluted with H₂O. The eluate was dried, re-suspended in 200 µL of 0.1% (v/v) formic acid, and 10 µL aliquots were injected into the LC-MS.

5.2.7.3 LC-MS analysis

The measurement was performed according to the method of Arakawa et al. [34]. For analysis of OH-pro, CML, CEL, MG-H1 and enzymatic crosslinks, 0.1 mg of hydrolysate was loaded on a SeQuant ZIC-HILIC column (5 µm, 150 × 2.1 mm) by Merck Millipore (MA, USA), installed on a LaChrom Elite L-2000U from Hitachi (Tokyo, Japan). The separations were performed at a flow rate of 200 µL/min, at 40°C in a linear gradient mode, with eluents A and B being water and MeCN, both containing 0.1% (v/v) formic acid. After an isocratic step at 80% eluent B for 2 mins, analytes were separated in the gradient to 10% eluent B for 14 mins. After a second isocratic segment (3 mins at 10% eluent B) for wash, a third isocratic segment (3 mins at 80% eluent B) was run for re-equilibration. For analysis of pentosidine, 5 mg of hydrolysate was loaded on a SunShell RP-AQUA column (2.6 µm, 150 × 2.1 mm) by ChromaNik Technologies (Osaka, Japan), installed on a same LC system and with the same eluents. The separation was performed at a flow rate of 200 µL/min, at 40°C in an isocratic mode. Pentosidine was separated in an isocratic step at 0% eluent B for 4 mins. After a second isocratic segment (6 min at 60% eluent B) for wash, a third isocratic segment (3 mins at 0% eluent B) was run for re-equilibration.

Detection was performed using a maXis 3G Ultra-High Resolution Quadrupole Time-of-Flight mass spectrometer (QqTOF-MS) by Bruker Daltonics (Bremen, Germany), equipped with an electrospray ionization source. The instrument was operated in positive ion mode, using a mass to charge ratio (*m/z*) range of 50–1000. Capillary voltage of ion source was set to 4500 V, nebulizer gas flow was 0.4 bar, and dry gas flow was 4.0 L/min. Dry temperature was set at 200°C.

Post-run calibration for each sample was performed by injecting 20 µL of ESI-L Low Concentration Tuning Mix at the beginning of each run via the 6-port diverter valve. At each calibration, the measured

masses of sodium formate cluster ions were compared with theoretical ones (m/z 90.976645–974.813156) to achieve 5 ppm mass accuracy. Target analytes and their ISTDs are summarized in Table 5.1. The extracted ion chromatograms (theoretical $m/z \pm 0.005$) were constructed for target analytes and ISTDs, and peak area ratios (target/ISTD) were calculated. The amounts of target analytes in samples were determined by comparing peak area ratios with the 6-point calibration curves of the external standards spiked with ISTDs, respectively. The contents of AGEs and enzymatic crosslinks were standardized to OH-pro amounts to surrogate collagen amounts and expressed as $\mu\text{mol/mol}$ of OH-pro.

HyStar 3.2 was used for HPLC operation, otofControl 4.0 for QqTOF-MS operation, Data Analysis 4.3 for data processing, and QuantAnalysis 4.3 for quantitation were by Bruker Daltonics (Bremen, Germany).

Table 5.1: Compositional formulas and theoretical mass-charge ratios (m/z) of the analytes and their isotope-labelled internal standards.

Analyte	Compositional formula	Theoretical m/z	Isotope-labelled internal standard	Theoretical m/z
Amino acid				
OH-pro	$\text{C}_5\text{H}_9\text{NO}_3$	132.0655 ($\text{M} + \text{H}^+$)	$[\text{}^2\text{H}_3]$ OH-pro	135.0844 ($\text{M} + \text{H}^+$)
AGEs				
CML	$\text{C}_8\text{H}_{16}\text{N}_2\text{O}_4$	205.1183 ($\text{M} + \text{H}^+$)	$[\text{}^2\text{H}_2]$ CML	207.1308 ($\text{M} + \text{H}^+$)
CEL	$\text{C}_9\text{H}_{18}\text{N}_2\text{O}_4$	219.1339 ($\text{M} + \text{H}^+$)	$[\text{}^2\text{H}_4]$ CEL	223.1590 ($\text{M} + \text{H}^+$)
MG-H1	$\text{C}_9\text{H}_{16}\text{N}_4\text{O}_3$	229.1295 ($\text{M} + \text{H}^+$)	$[\text{}^2\text{H}_3]$ MG-H1	232.1483 ($\text{M} + \text{H}^+$)
Pentosidine	$\text{C}_{17}\text{H}_{26}\text{N}_6\text{O}_4$	190.1080 ($\text{M} + 2\text{H}^+$)	$[\text{}^2\text{H}_4]$ Pentosidine	192.1206 ($\text{M} + 2\text{H}^+$)
Enzymatic crosslinks				
DHLNL	$\text{C}_{12}\text{H}_{26}\text{N}_3\text{O}_6$	308.1816 ($\text{M} + \text{H}^+$)	–	–
HLNL	$\text{C}_{12}\text{H}_{26}\text{N}_3\text{O}_5$	292.1867 ($\text{M} + \text{H}^+$)	–	–
LNL	$\text{C}_{12}\text{H}_{26}\text{N}_3\text{O}_4$	276.1918 ($\text{M} + \text{H}^+$)	–	–
PYD	$\text{C}_{18}\text{H}_{30}\text{N}_4\text{O}_8$	215.1026 ($\text{M} + 2\text{H}^+$)	–	–
DPD	$\text{C}_{18}\text{H}_{30}\text{N}_4\text{O}_7$	207.1052 ($\text{M} + 2\text{H}^+$)	–	–

5.2.8 Biochemical analysis of the de-mineralized cortical bone specimen

To obtain the extent of oxidative damage to the collagen network of the cortical bone tissue, carbonyl content, which is widely regarded as a stable biomarker for oxidative damage, was quantified in a demineralized portion of each SENB sample. This was carried out using a fluorescence-based microplate assay developed by Mohanty and co-workers [215]. This approach uses fluorescein-5-thiosemicarbazide (FTC) which binds specifically to ketones and aldehydes, carbonyl-containing compounds. The associated fluorescence from the bound FTC is measured to quantify the amount of protein carbonyls present. Since FTC binds only to ketones and aldehydes, the carbonyl content measure will only quantify oxidative damage to the collagen network from the direct pathway of protein oxidation that give rise to these compounds. FTC does not bind to the AGEs mentioned in section 5.2.7. To expose the carbonyl containing compounds within the collagen network structure, trypsin digestion was initially performed. Trypsin is a proteolytic enzyme which breaks down the collagen peptide chains by cleaving the peptide bond between the carboxyl group of arginine or lysine and the amino group on a neighbouring amino acid [317].

Roughly 15 to 20 mg (wet mass) of demineralized bone sample was isolated, cut into very small pieces using an ultra sharp razor blade and placed in a microcentrifuge tube for each specimen. 100 µl of Hank's buffer was added and incubated at 85 °C for an hour. Trypsin enzyme powder from porcine pancreas (Millipore Sigma, St. Louis, MO, USA,) was dissolved in Hank's buffer such that a 1 mg/ml solution was formed. After incubation with the Hank's buffer, 100 µl of the 1 mg/ml trypsin digest solution was added to each sample and incubated for 16 hours (carried out overnight) at 37 °C. After this, the incubated samples were reheated at 85 °C for an hour before a further 50 µl of the 1 mg/ml trypsin solution was added and incubated at 37 °C for 3 hours. An additional 50 µl of the same trypsin solution was added and incubated for a further 16 hours (carried out overnight) at 37 °C. The last steps of reheating at 85 °C and the addition of two 50 µl trypsin solution with their associated incubation times at 37 °C were repeated one more time. After this, the samples were centrifuged at 5000 revolutions per minutes (RPM) for 5 minutes and the supernatants collected. These supernatants were used in a fluorescence-based microplate carbonyl assay to determine the number of carbonyls present as well as a colorimetric-based hydroxyproline assay to determine the amount of collagen present.

5.2.8.1 Fluorescence-based microplate carbonyl assay

After trypsin digestion, 50 µl aliquots of the trypsin digested supernatants were mixed with 50 µl of 0.2 mM FTC dissolved in dimethyl sulfoxide (DMSO; Millipore Sigma, St. Louis, MO, USA) and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (Cell Biolabs Inc., San Diego, CA, USA) in micro-centrifuge tubes. The resulting solutions were vortexed and incubated at room temperature overnight in the dark to give sufficient time for the FTC to bind to the carbonyls in the trypsin digested supernatants. After overnight incubation, 400 µl of cold 30 % trichloroacetic acid (Fisher Scientific, Hampton, NH, USA) was added to the solutions to precipitate the FTC bound collagen into pellets. The microcentrifuge tubes containing the solutions were then put on ice for 15 minutes and centrifuged at 15,000 RPM for 10 minutes afterwards. The supernatants were then decanted to leave the precipitated collagen pellets. Three acetone washes were performed to rid the precipitated collagen pellets of any unbound FTC. The acetone washes were carried out as follows: 1 ml of cold 99.9 % acetone was added to each microcentrifuge tube, vortexed extensively, put on ice for 15 minutes and centrifuged at 15,000 RPM for 10 minutes. For the third and final acetone wash, the centrifugation was carried out for 15 minutes at 15,000 RPM. After each centrifugation, the supernatants were decanted, eliminating the unbound FTC. After completing the acetone washes, the samples were left to air dry in a fume hood for an hour and 30 minutes to allow any remaining acetone to evaporate. The air-dried collagen pellets were then resolubilized using 6 M guanidine hydrochloride (Cell Biolabs Inc., San Diego, CA, USA) and dissolved in a sodium phosphate monobasic buffer (Cell Biolabs Inc., San Diego, CA, USA). Triplicates of 100 µl aliquots were taken from each dissolved solution and placed in allocated wells in a black opaque 96 well microplate (Greiner Bio-One, Monroe, NC, USA). Fluorescence was measured using a Bio Tek Synergy H4 Hybrid multi-mode microplate reader (Agilent, Santa Clara, CA, USA) at an excitation of 480 nm and emission of 530 nm. Fluorescence was then compared against a standard curve prepared from an FTC standard stock solution to compute the number of carbonyls present in each trypsin digested supernatant. The carbonyl content concentration found for each sample was normalized to the collagen content concentration. The amount of collagen content in each trypsin digested supernatant was measured using a colorimetric-based hydroxyproline assay.

5.2.8.2 Colorimetric-based hydroxyproline assay

Hydroxyproline has been found to be unique to as well as of a consistent amount in mammalian collagen, making it a good biomarker for measuring collagen content. Hydroxyproline makes up about 14% of all the amino acids that form type I bone collagen in mammals [162]. Colorimetric assays have been developed that allows the amount of hydroxyproline in a sample to be measured. The method adopted in this study closely follows work done by Hofman and co-workers [163] as well as previous work from Willett's group [21] and is discussed below.

First, to assess the hydroxyproline content, the demineralized sample must be acid hydrolysed to liberate the amino acids. For each sample, a 25 μ l aliquot from the trypsin digested supernatant was mixed with the same volume of 12 N hydrochloric acid (HCl) in an acid hydrolysis tube and incubated for 4 hours at 110 °C to break down the collagen into its amino acids. The product of this incubation termed the hydrolysate was then neutralized using sodium hydroxide (NaOH). The neutralized hydrolysate was then diluted 1:30 using de-ionised water due to its high concentration of amino acids. Hydroxyproline standards of known concentrations were prepared using a standard stock of trans-4-hydroxy-L-proline (Millipore Sigma, St. Louis, MO, USA). 80 μ l of the diluted neutralized hydrolysate and prepared hydroxyproline standards of known concentrations were aliquoted into assigned wells in a clear transparent 96-well microplate (Corning, Glendale, AZ, USA). To each well, 40 μ l of 0.05 N chloramine-T was added and incubated for 20 minutes at room temperature after which 40 μ l of Ehrlich's Reagent (15% (w/v) p-dimethylaminobenzaldehyde dissolved in 2:1 (v/v) iso-propanol: perchloric acid) was also added and incubated for 20 minutes at 60 °C. The microplate was allowed to cool down and absorbance at 560 nm was measured using a SpectraMax Plus 384 microplate reader (Molecular Devices, San Jose, CA, USA). Hydroxyproline content of each sample was then computed by comparing with a standard curve generated from absorbance readings of the hydroxyproline standards of known concentrations. Carbonyl content for each sample was finally calculated as carbonyl content in μ mol per hydroxyproline content in mol (μ mol/mol of OH-pro).

5.2.9 Sodium dodecyl sulfate polyacrylamide gel electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out to evaluate the amount of fragmentation in the collagen polypeptide chains of each specimen. This was done by measuring the relative intensity of the intact alpha chain bands of each specimen. SDS-PAGE works by using an electric field to separate proteins such as collagen into its polypeptides according to their

molecular weight or mass to charge ratio [183]. To prepare the samples for SDS-PAGE, a portion of the demineralized samples were first pepsin digested. Pepsin acts by cleaving peptide bonds in the telopeptide region liberating the individual procollagen chains and fragments that can then be separated with SDS-PAGE [318]. The method closely followed a previous established protocol from work done by Burton et al. [21] with a few modifications.

Approximately 20 to 25 mg of the demineralized portion of each sample was isolated, cut into very small pieces and then lyophilized overnight. The dry mass of each lyophilized sample was between 10 to 15 mg. Pepsin from porcine gastric mucosa (lyophilized powder, Millipore Sigma, St. Louis, MO, USA) was dissolved in 0.5 M acetic acid (HOAc) to form a 4 mg/ml solution and used to digest the lyophilized demineralized bone pieces. The amount of pepsin solution added to each sample was based on a 1:10 (w/w) pepsin to dry mass of bone sample ratio.

The pepsin digest was performed at room temperature for 144 hours (6 days). After the initial 72 hours of the pepsin digest, the supernatant was aliquoted from the undigested bone pieces for each sample after centrifuging at 10,000 RPM for 5 mins. The collected supernatant was stored in a -20 °C freezer. Fresh pepsin digest solution was added to the undigested samples and the pepsin digest process continued for a further 72 hours. Next, the supernatant was collected after centrifugation and added to the prior collected supernatant. The combined supernatants were neutralized using NaOH. Concentrates of the collected supernatants were prepared by placing the neutralized supernatants into separate 15 ml centrifugal filter units with a 10 kDa molecular weight cut-off (AMICON Ultra, Millipore Sigma, St. Louis, MO, USA, Massachusetts, US), and centrifuging for 40 mins at 4000 RPM.

Using a 25 µl aliquot of each sample's concentrate, acid hydrolysis was carried out as described for the trypsin digested supernatants in section 5.2.8.2. The hydrolysate for each sample was then used in a colorimetric based hydroxyproline assay. This followed the same protocol described in section 5.2.8.2, but a dilution ratio of 1:3 was used because the pepsin digest concentrates contained a smaller amount of collagen compared to the trypsin digest supernatants. From the hydroxyproline assay, collagen content was determined by dividing hydroxyproline content by a factor of 0.14 based on hydroxyproline forming about 14% of the collagen molecule.

In preparation for use in SDS-PAGE, another 25 µl aliquot of each sample's concentrate was mixed with a buffer solution in a 1:1 concentrate to buffer ratio. The buffer solution consisted of 4x Laemmli buffer (Bio-Rad, Hercules, CA, US) and 2-mercapto-ethanol (Millipore Sigma, St. Louis, MO, USA,)

in a 95:5 (v/v) ratio. The 4x Laemmli buffer (pH = 6.8) contained 227.8 mM Tris-hydrochloride, 44.4% (v/v) glycerol and 4.4% lithium dodecyl sulfate (LDS), a substitute for sodium dodecyl sulfate (SDS). Similar to SDS, LDS gives the components of the collagen the same charge therefore separation is only based on molecular weight. The sample-buffer mixture is then heated at 95 °C for 5 minutes and then allowed to cool. Based on the collagen concentration measured using the hydroxyproline assay, a volume containing 30 µg of collagen for each sample-buffer mixture was aliquoted and used in the SDS PAGE analysis. These were loaded into pre-cast 7.5% polyacrylamide gels with 10 wells (Mini-protean TGX, Bio-Rad, Hercules, CA, US). For each gel, an unstained protein standard (Precision Plus, Bio-Rad, Hercules, CA, US), purified rat tail collagen (RTC) (Millipore Sigma, St. Louis, MO, USA,) and the pepsin-HOAc solution used for digestion were included as a molecular weight standard, a positive control and a negative control, respectively. These were put in wells number 5, 6 and 10 respectively for all the gels run with the remaining wells loaded with the sample concentrates (see Fig. 5.3). The gels were run in 700 ml of running buffer (pH = 8.3) containing 2.5 mM tris(hydroxymethyl)aminomethane, 19.2 mM glycine, 0.01% SDS (Bio-Rad, Hercules, CA, US) at a voltage of 200 V for 50 minutes. Once completed, the gels were stained with Coomassie blue (Bio-Rad, Hercules, CA, US) for two hours and de-stained using a laboratory prepared 10% (v/v) methanol plus 10% (v/v) HOAc aqueous de-staining solution for 36 hours. Coomassie blue binds specifically to proteins whereas the de-staining solution removes any unbound Coomassie blue from the gel [319]. The resulting Coomassie blue bands from the de-stained gel (see Fig 5.3) were then analyzed using a gel imaging system (ChemiDoc MP, Bio-Rad, Hercules, CA, US). The intensity of the intact alpha-I and alpha-II chains which are between 100 and 150 kDa were then analyzed using ImageJ [186]. The gel function under the analyze option on the menu bar in ImageJ was used to analyze the gels and this gave histograms of the intensity of the alpha-I and alpha-II bands due to the Coomassie blue staining. The area under the histogram for the two bands were computed to give a measure of the intensity of the intact alpha chains. The alpha chain intensity is indicative of the number of intact alpha chains present. These were then normalized by dividing by the intensity of the RTC alpha chains from their corresponding gel. The same RTC solution was used in every gel run for this study.

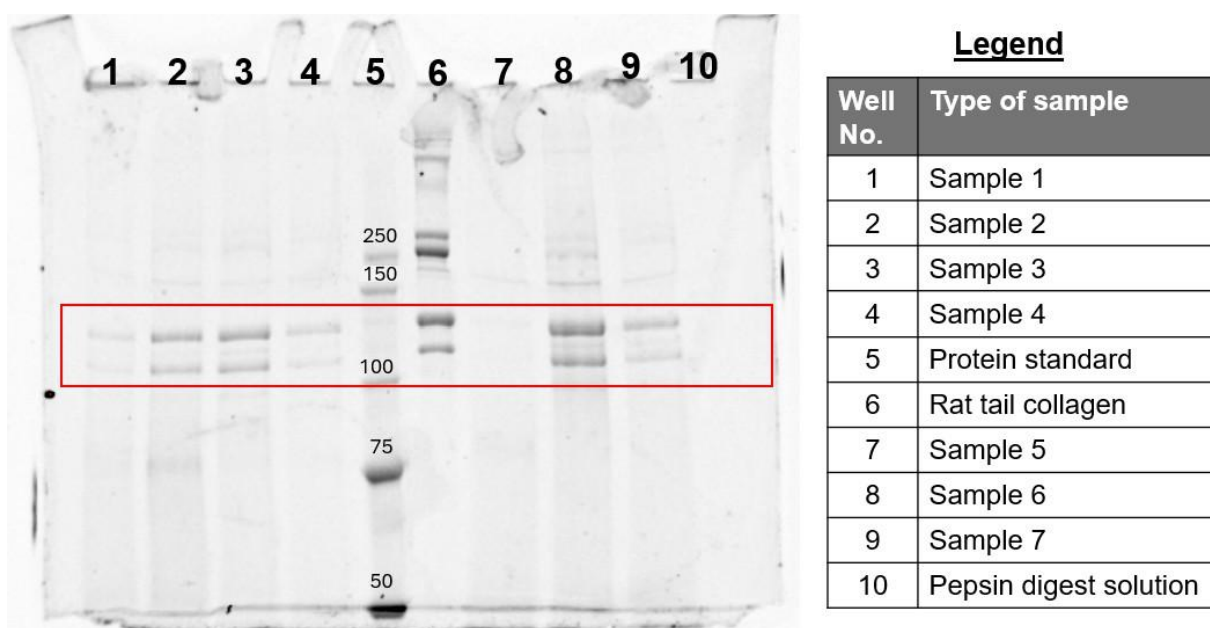


Figure 5.3: Representative SDS-PAGE gel showing polypeptide bands from Coomassie Blue staining. The numbers on top of the gel denote the well number, with the legend on the right side of the figure indicating what type of sample was put in each well. Well 5 contained the protein standard and the number directly on top of each band in well 5 represents the molecular weight of the band in kDa. The red box highlights the two α chain bands for each sample.

In addition to the method described above, a preliminary study was carried out where aliquots of the concentrates to be used in the SDS-PAGE analyses were first incubated with FTC overnight. The idea here was to assess which polypeptides of the type I collagen was associated with the measured carbonyls [213]. The FTC incubation was done by mixing an aliquot of the concentrate with 0.2 mM FTC solution dissolved in dimethyl sulfoxide (DMSO; Millipore Sigma, St. Louis, MO, USA,) and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (Cell Biolabs Inc., San Diego, CA, USA) in a micro-centrifuge tube in a 9:1 (v/v) ratio. The resulting solution was vortexed and incubated at room temperature overnight in the dark to give sufficient time for the FTC to bind to the carbonyls in the concentrate. After this, the same process of mixing with a buffer solution, heating at 95°C for 5 minutes and allowing to cool as described above was followed. Similar to above, a volume containing 30 μ g of collagen for each sample-buffer mixture was aliquoted and used in the SDS PAGE analysis. However, for this, pre-cast 4-20% gradient polyacrylamide gels each with 10 wells (Mini-protean TGX, Bio-Rad, Hercules, CA, US) were used. These gels allow separation of polypeptides within a range of roughly 2 to 400 kDa. An unstained protein standard (Precision Plus, Bio-Rad, Hercules, CA, US),

purified rat tail collagen (RTC) (Millipore Sigma, St. Louis, MO, USA,) and the pepsin-HOAc solution used for digestion were still included as a molecular weight standard, a positive control and a negative control, respectively for these gels. Whereas the RTC and pepsin solution were incubated with FTC, the unstained protein standard was not and therefore did not show any fluorescence. The gels were run in 700 ml of running buffer at a voltage of 200V for 45 minutes. Once completed, the gels were imaged using a gel imaging system (ChemiDoc MP, Bio-Rad, Hercules, CA, US) but under a fluorescence-based setting to detect the bound FTC to the various polypeptides. After this, Coomassie blue staining and de-staining was carried out following the same proceeding above. The gels were then re-scanned to image the Coomassie blue stained polypeptides. This procedure was however unsuccessful in consistently detecting the carbonyls (see Appendix B.3 for images and more details). The unbound FTC tended to bleed through the wells and/or overshadow the alpha chain bound FTC bands settling towards the base of the gels. Consequently, the gel was changed to a 7.5% pre-cast polyacrylamide gel to ensure the unbound FTC migrated out of the gel. This change improved the noise from the unbound FTC however the intensities of the alpha chain bands associated with the bound FTC were weak especially when compared to the Coomassie Blue staining. That is, there was a high noise to signal ratio for the fluorescence-based imaging of the gels. As such, only analyses and results from the Coomassie Blue staining is presented in this thesis work.

5.2.10 Statistical analysis

To test the hypotheses that measures of oxidative damage were higher and fracture toughness measures were lower in the T2D/CKD group compared to the control group, one tail student t-tests were conducted. In fact, this was conducted for all parameters measured from the various characterization tests (fracture test, micro-CT, HIT, DSC, carbonyl assay, SDS-PAGE and mass spectrometry) to test for differences that might exist between the T2D/CKD and the control groups. In addition, since the donors from which the specimens were taken were not age matched between the T2D/CKD and control groups, analysis of covariance (ANCOVA) were performed for all the parameters measured, with age acting as a covariate and T2D/CKD or control group as the categorical variables to ascertain whether differences found between groups could be attributed to age.

To test the hypothesis that the measures of oxidative damage would correlate strongly and negatively with collagen network connectivity, which in turn would strongly explain variations observed in cortical bone fracture toughness measures, Spearman correlations were conducted, and multiple linear

regression (MLR) models were developed. The Spearman correlations were performed to assess the association among all parameters measured from the various characterization tests (fracture test, micro-CT, HIT, DSC, carbonyl assay, SDS-PAGE and mass spectrometry). Spearman correlations were used because not all variables followed a normal distribution, and these were tested using the Kolmogorov-Smirnov test. Various MLR models were developed to investigate how the characterization measures of collagen network quality and AGEs explain the variations in fracture toughness measures between specimens. The fracture toughness measures were used as the dependent/response variables whereas all other characterization measures or parameters were considered as independent/explanatory variables. T2D/CKD and control groups were also included as a possible categorical explanatory variable. Age was a significant covariate with many of the explanatory variables and hence was removed from the analysis. Sex was also not included in the analysis as it is not a material property. Many different combinations of the explanatory variables were considered, and a bi-directional stepwise regression approach was adopted to generate the final MLR models. In addition, interaction terms between relevant explanatory variables were also considered in generating the MLR models. If two explanatory variables were considered to be co-variables ($r > 0.55$ from Spearman's correlation), they were not used in the same model to prevent multi-collinearity. If an explanatory variable or interaction term was not significant ($p > 0.05$) in the model, the model was re-analyzed without it. If two or more explanatory variables or interaction terms were not significant ($p > 0.05$) in the model, then the one with the highest p-value was eliminated and the model re-analyzed. Linearity and normality were checked for the models to ensure they met these assumptions for MLR models (see Appendix B.4 for more details).

T-tests, ANCOVA and Spearman's correlation were conducted with MATLAB Statistics Toolbox (The MathWorks Inc., Natick, MA) whereas the MLR models were developed using the open source RStudio software (Posit, Boston, MA). In all cases, a p-value of less than 0.05 was considered statistically significant.

5.3 Results

Data from three of the specimens were excluded from the various analyses presented in this study. Two of these specimens were excluded because of significantly higher porosity values ($> 30\%$). These were more than three standard deviations away from the mean of the porosity calculated for all the other specimens. Hence, they were determined to be outliers. One of these specimens was from a donor with

a history of Huntington's chorea (a neurodegenerative disease) as well as hypertension and was observed during specimen preparation to have suffered from cortical bone thinning. The other sample was from a donor that was paralyzed for 11 years. The third specimen was excluded due to an error that occurred during the conduction of its LC-MS analysis. Consequently, the various results and statistical analyses involved 78 samples. Excluding these three specimens, Table 5.2 gives a breakdown of the new number of specimens by sex and T2D/CKD or control group as well as their associated mean and standard deviation age for the various groups.

Table 5.2: Breakdown of the number of donors divided by sex as well as disease group (T2D/CKD group or control group) and their associated mean and standard deviation age.

	Control	T2D/CKD	Total
Number of males	24	19	43
Age (Mean $\pm$ SD)	(62.9 $\pm$ 23.4 years)	(73.6 $\pm$ 11.2 years)	(67.7 $\pm$ 19.6 years)
Number of females	20	15	35
Age (Mean $\pm$ SD)	(73.8 $\pm$ 15.4 years)	(65.5 $\pm$ 15.4 years)	(70.3 $\pm$ 15.7 years)
Total	44	34	78
Age (Mean $\pm$ SD)	(67.9 $\pm$ 20.4 years)	(70.1 $\pm$ 13.4 years)	(68.8 $\pm$ 17.6 years)

Fig. 5.4 presents images showing the region around the razor-sharpened tip of the starter notch illustrating the progression of crack growth during the fracture test, that is, before the commencement of the fracture test (Fig. 5.4A), at the start of crack growth initiation (Fig. 5.4B), at the point of crack growth instability where crack growth accelerates (Fig. 5.4C), and beyond the crack growth instability point (Fig. 5.4D). It can be observed that although they were some deflections in the path of the crack growth, the side groove was able to maintain a relatively straight path which is important for quantifying Mode I fracture toughness.

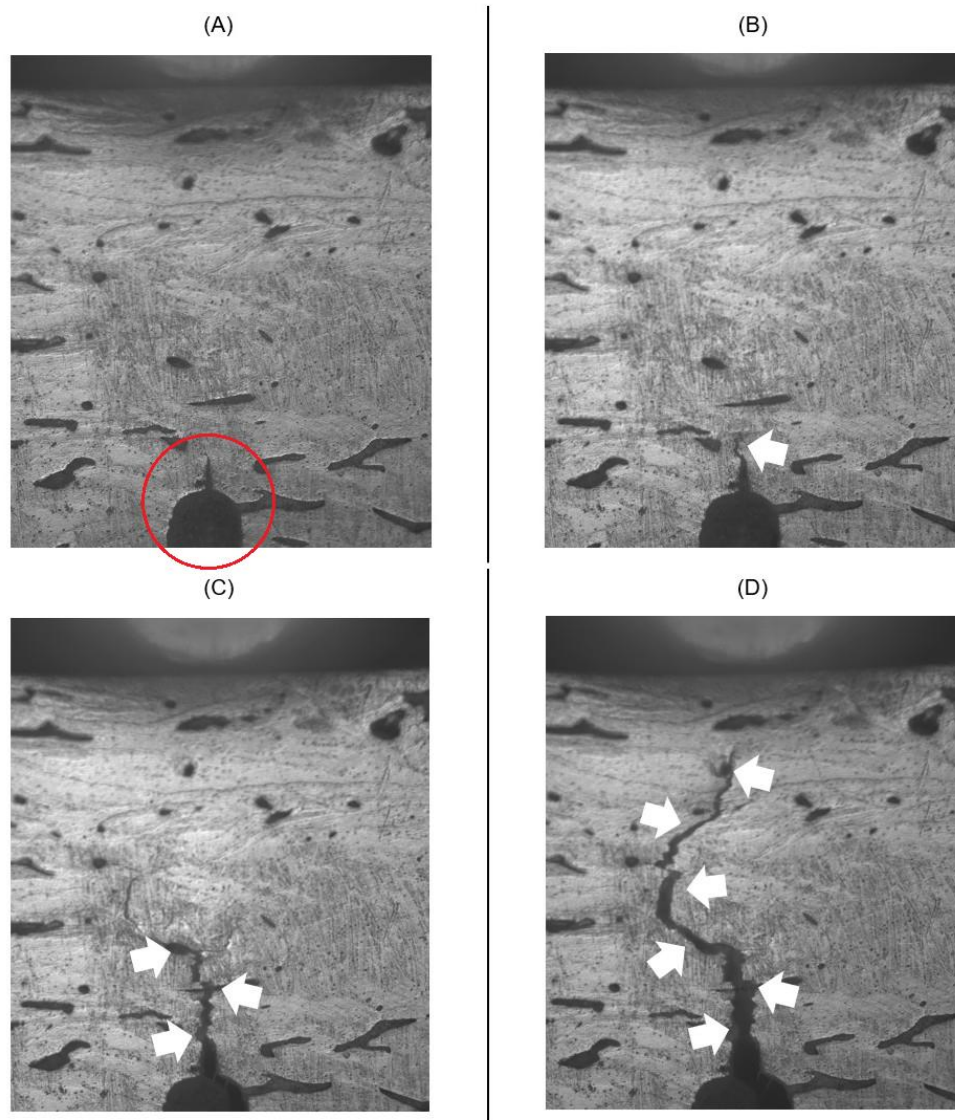


Figure 5.4: Illustration showing the progression of crack growth from the razor-sharpened notch (highlighted by the red circle in (A)) at (A) start of fracture test, (B) start of crack growth initiation, (C) crack growth instability point (D) beyond crack growth instability point. The white arrows highlight the path of the crack growth.

5.3.1 AGE adducts and Pentosidine

Fig. 5.5 presents column plots of the three AGE adducts, CML, CEL and MG-H1 as well as pentosidine. MG-H1 was the most abundant of all the AGEs. More interestingly, MG-H1 (Mean $\pm$ SD = 4294 ± 1884 $\mu\text{mol/mol}$ of OH-pro) was roughly 830 times more abundant than pentosidine (Mean $\pm$ SD =

5.183 ± 2.595 $\mu\text{mol/mol}$ of OH-pro). Similarly, CML (Mean $\pm$ SD = 730.4 ± 320.5 $\mu\text{mol/mol}$ of hydroxyproline) and CEL (Mean $\pm$ SD = 356.0 ± 186.6 $\mu\text{mol/mol}$ of OH-pro) were about 140 and 70 times more abundant than pentosidine respectively. This is consistent with findings from a recent study of human cancellous bone [34].

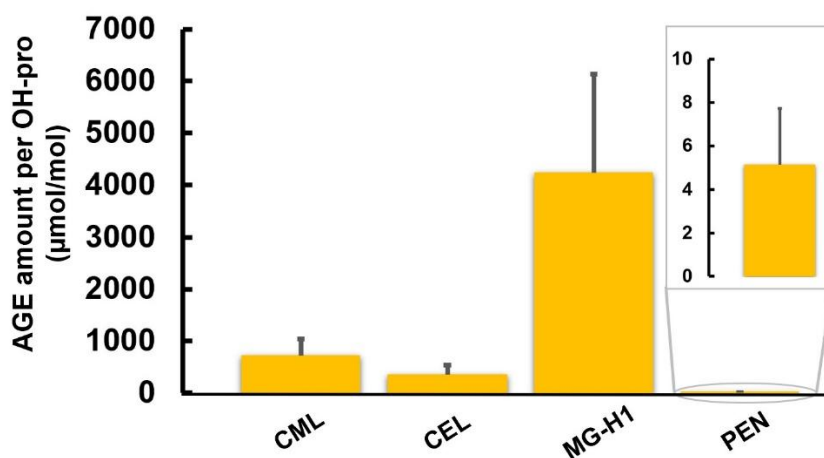


Figure 5.5: Column plots showing the average amount of the various AGE adducts and pentosidine measured ex vivo in human femoral cortical bone specimens. OH-pro represents hydroxyproline.

5.3.2 T-test between T2D/CKD group and control group

The one-sided t-tests revealed that the various AGE adducts as well as pentosidine were significantly higher in the T2D/CKD group compared to the control group. Fig. 5.6A provides column plots of the AGE adducts as well as pentosidine comparing the T2D/CKD and control groups. The AGE adducts were between 57% to 63% higher ($p < 0.001$) on average in the T2D/CKD group compared to the control group. In addition, pentosidine was 22 % higher ($p = 0.039$) in the T2D/CKD group compared to the control group. Surprisingly, carbonyl content was not significantly different between the two groups ($p = 0.64$). However, along with the AGEs, HLNL content, an immature LOX crosslink ($p = 0.035$) and enthalpy of denaturation (ΔH) per dry mass of sample ($p = 0.039$) were significantly different between the two groups. HLNL was 8% higher and ΔH per dry mass was 11% higher in the control group compared to the T2D/CKD group (see Fig 5.6B and 5.6C). From the ANCOVA analysis, none of the differences for these variables between the groups were attributed to age. All three fracture toughness measures were not statistically different between the two groups ($p > 0.32$). Table 5.3

provides a summary of the descriptive statistics of the control and T2D/CKD groups for the fracture toughness measures as well as the measures from all the various characterization tests.

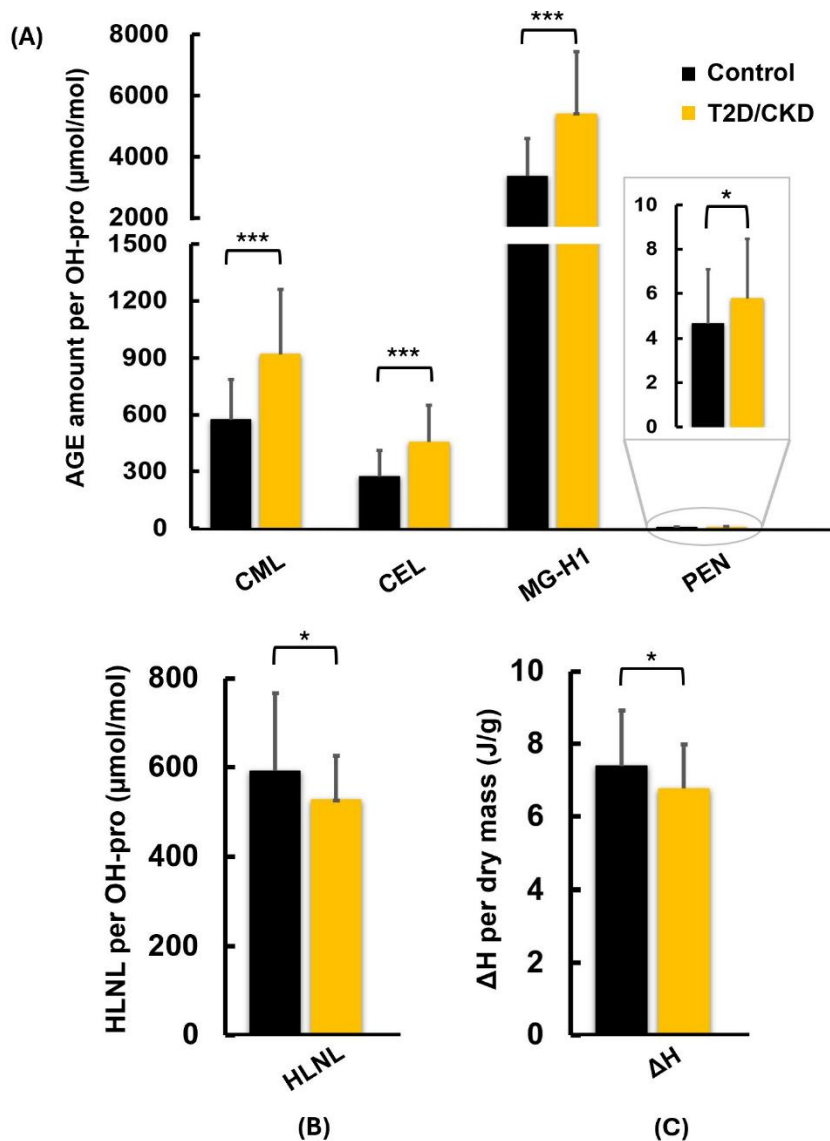


Figure 5.6: Column plots of (A) the various AGEs (B) HLNL (C) Enthalpy of denaturation per dry mass of collagen, comparing the T2D/CKD and control groups. Note * signifies p value < 0.05, ** signifies p-value < 0.01, *** signifies p -value < 0.001 and OH-pro represents hydroxyproline

Table 5.3: Summary of descriptive statistics (mean $\pm$ standard deviation) between the T2D/CKD group and control group for the fracture toughness measures and various characterisation tests measures along with their one tail t test p-value comparing the two groups

Characterization measures (units)		Control group	T2D/CKD group	p-value
Fracture toughness measures	J _{IC} (kJ/m ²)	4.06 $\pm$ 1.40	4.23 $\pm$ 1.80	0.680
	J-int (kJ/m ²)	6.26 $\pm$ 2.41	6.02 $\pm$ 2.03	0.325
	Wf _{x_n} (J/m)	3.26 $\pm$ 1.11	3.22 $\pm$ 1.14	0.441
Collagen quality-based properties	Max.Slope (kPa/°C)	55.0 $\pm$ 10.1	55.1 $\pm$ 9.51	0.455
	T _d (°C)	64.1 $\pm$ 0.92	64.0 $\pm$ 0.99	0.617
	T _{onset} (°C)	54.2 $\pm$ 1.82	54.3 $\pm$ 1.50	0.663
	T _{peak} (°C)	59.2 $\pm$ 1.33	59.5 $\pm$ 1.06	0.786
	FWHM (°C)	8.31 $\pm$ 1.78	8.49 $\pm$ 1.51	0.316
	Δ H/wet mass (J/g)	3.69 $\pm$ 0.70	3.53 $\pm$ 0.55	0.151
	Δ H/dry mass (J/g)	7.34 $\pm$ 1.54	6.77 $\pm$ 1.21	0.039
	Intact alpha chains	2.43 $\pm$ 3.13	2.04 $\pm$ 4.60	0.036
LOX immature crosslinks	LNL (μmol/mol of OH-pro)	68.5 $\pm$ 19.1	67.4 $\pm$ 17.4	0.394
	HLNL (μmol/mol of OH-pro)	582.6 $\pm$ 169.4	526.1 $\pm$ 99.64	0.035
	DHLNL (μmol/mol of OH-pro)	819.7 $\pm$ 200.4	838.7 $\pm$ 168.3	0.670
Mature LOX crosslinks	DPD (μmol/mol of OH-pro)	149.7 $\pm$ 44.0	150.8 $\pm$ 37.9	0.548
	PYD (μmol/mol of OH-pro)	648.6 $\pm$ 98.1	638.9 $\pm$ 82.8	0.322
AGEs	CEL (μmol/mol of OH-pro)	270.1 $\pm$ 137.9	455.4 $\pm$ 196.1	< 0.001
	CML (μmol/mol of OH-pro)	584.3 $\pm$ 212.6	919.4 $\pm$ 339.8	< 0.001
	MG-H1 (μmol/mol of OH-pro)	3440 $\pm$ 1201	5400 $\pm$ 2041	< 0.001
	PEN (μmol/mol of OH-pro)	4.73 $\pm$ 2.45	5.77 $\pm$ 2.69	0.039
Oxidative damage	Carbonyls (μmol/mol of OH-pro)	97.2 $\pm$ 44.1	93.7 $\pm$ 39.3	0.639
Bone mineral-based properties	Porosity (%)	6.37 $\pm$ 4.75	6.10 $\pm$ 5.66	0.590
	avBMD (mg HA/cm ³)	1065 $\pm$ 60.17	1063.0 $\pm$ 58.07	0.448
	TMD (mg HA/cm ³)	1111 $\pm$ 42.54	1108 $\pm$ 47.48	0.374
	Mineralization peak (mg HA/cm ³)	1115 $\pm$ 46.45	1110 $\pm$ 54.38	0.324
	Mineral Heterogeneity(mg HA/cm ³)	250.6 $\pm$ 22.3	253.2 $\pm$ 20.6	0.297

5.3.3 Results from Spearman's correlation

Table 5.4 details the Spearman correlation coefficients for relationships between the three fracture toughness measures and measures from the other characterization tests as well as age. Porosity, Max.Slope, T_{onset} , ΔH per wet mass of sample, CEL, HLNL and DHLNL all had significant correlations with all three fracture toughness parameters. Max.Slope had a moderately strong positive correlation ($0.43 < r < 0.54$, $p < 0.001$) with all three fracture toughness measures. As expected, porosity had a negative correlation albeit weak to moderate with all three toughness measures. CEL, an AGE adduct, had a weak negative correlation with the fracture toughness measures. Conversely T_{onset} , ΔH per wet mass of sample, HLNL and DHLNL, both immature LOX crosslinks, had weak positive correlations with the fracture toughness measures. Both Age and FWHM correlated negatively with only J-int. The other AGEs besides CEL as well as carbonyl content did not have significant correlations with the fracture toughness measures.

Table 5.5 reports the Spearman correlation coefficients for relationships between the AGEs, carbonyl content and the other characterization test measures excluding the fracture toughness measures. The three AGE adducts correlated positively and strongly among themselves as well as with pentosidine ($r = 0.60 - 0.71$, $p < 0.001$). All the AGEs including pentosidine correlated positively with age. However, the AGEs correlated most strongly and negatively with HLNL, one of the immature LOX crosslinks ($r = -0.55$ to -0.71 , $p < 0.01$) as well as the intact alpha chains ($r = -0.46$ to -0.59 , $p < 0.01$). Another immature LOX crosslink, LNL, and denaturation temperature (T_d) also had negative correlations with all the AGEs but did not meet significance for CML ($p = 0.080$, $p = 0.077$ respectively) and MG-H1 ($p = 0.079$, $p = 0.73$ respectively). All AGEs had significant negative correlations with ΔH normalized to both wet and dry mass of the samples except CML which was just outside significance for its relationship with ΔH per wet mass ($p = 0.058$). All AGEs had significant positive correlations with FWHM except MG-H1 which also was just outside significance ($p = 0.077$). CEL but not the other AGEs had significant negative correlations with DHLNL and T_{onset} . Interestingly, pentosidine had a negative correlation with Max.Slope however the AGEs adducts did not correlate with Max.Slope. Carbonyl content had moderate positive correlations with the AGE adducts whereas its correlation with pentosidine did not meet significance ($p = 0.068$). In addition, carbonyl content had a positive correlation with T_{peak} but more interestingly, a moderate negative correlation with the intact alpha chains.

Table 5.4: Spearman's correlation coefficient table between the fracture toughness measures and the other characterization test measures and age. The superscripts indicate: * p value < 0.05, **p-value < 0.01, * p -value < 0.001, + p-value < 0.1, ns represents not significant (p-value > 0.1)**

Characterization measures		J _{IC}	J-int	Wf _{x_n}
Age		- 0.20+	- 0.24*	- 0.18+
AGEs	CEL	- 0.23 *	- 0.27*	- 0.26 *
	CML	ns	ns	ns
	MG-H1	ns	ns	ns
	Pentosidine	ns	ns	ns
Oxidative damage measure	Carbonyl content	ns	ns	ns
Bone mineral-based properties	Porosity	- 0.28*	-0.26*	-0.28*
	avBMD	ns	ns	ns
	TMD	ns	ns	ns
	Mineral Heterogeneity	ns	ns	ns
Collagen quality-based properties	Max.Slope	0.43***	0.54***	0.52***
	T _d	ns	ns	ns
	T _{onset}	0.31**	0.31**	0.27*
	T _{peak}	ns	ns	ns
	FWHM	- 0.19+	-0.24***	ns
	ΔH (wet mass)	0.28*	0.36**	0.36**
	ΔH (dry mass)	ns	0.19+	ns
	Intact alpha chains	0.21+	ns	ns
Immature LOX crosslinks	LNL	ns	0.22+	ns
	HLNL	0.27*	0.29*	0.26*
	DHLNL	0.32*	0.25*	0.29*
Mature LOX crosslinks	DPD	ns	ns	ns
	PYD	ns	ns	ns

Table 5.5: Spearman's correlation coefficient table between AGEs, carbonyl content and the other characterization test measures and age. The superscripts indicate: * p value < 0.05, **p-value < 0.01, * p -value < 0.001, + p-value < 0.1, ns represents not significant**

Characterization measures		CML	CEL	MG-H1	PEN	Carbonyls
Age		0.37***	0.42***	0.28***	0.33**	ns
AGEs	CML	-	0.96***	0.88***	0.71***	0.32**
	CEL	0.96***	-	0.87***	0.67***	0.29**
	MG-H1	0.88***	0.87***	-	0.60***	0.31**
	Pentosidine	0.71***	0.67***	0.60***	-	0.21+
Oxidative damage measure	Carbonyl content	0.32**	0.29**	0.31**	0.21+	-
Collagen quality-based properties	Max.Slope	ns	ns	ns	- 0.24*	ns
	T _d	-0.20+	- 0.26*	- 0.20+	- 0.25*	ns
	T _{onset}	ns	- 0.29**	ns	ns	ns
	T _{peak}	ns	ns	ns	ns	0.39***
	FWHM	0.30**	0.37***	0.20+	0.29**	ns
	ΔH (wet mass)	- 0.22+	- 0.26*	- 0.30**	- 0.31**	ns
	ΔH (dry mass)	- 0.26*	- 0.27*	- 0.29**	- 0.35**	ns
	Intact alpha chains	- 0.56***	- 0.56***	- 0.59***	- 0.46***	- 0.37***
Immature LOX crosslinks	LNL	- 0.20+	- 0.25*	- 0.20+	- 0.23*	ns
	HLNL	- 0.62***	- 0.71***	- 0.55***	- 0.56***	ns
	DHLNL	ns	- 0.28*	ns	ns	ns
Mature LOX crosslinks	DPD	ns	ns	ns	0.20+	ns
	PYD	ns	ns	ns	0.21+	ns

5.3.4 Multiple linear regression model results

For the multiple linear regression (MLR) models, it was found that a base model combining Max.Slope and porosity explained all three fracture toughness measures, explaining 29.2 % of variation in J_{IC}, 41.8 % in J-int and 37.9 % in Wf_{x_n} (see Tables 5.4, 5.5 and 5.6). In all cases, Max.Slope had a positive

standardized beta coefficient (β) highlighting a positive contribution to the fracture toughness measures. On the other hand, porosity had a negative standardized beta co-efficient highlighting a negative contribution to the fracture toughness measures. Other MLR models were built using these two explanatory variables as the basis and adopting a bi-directional stepwise approach to adding and/or eliminating other explanatory variables.

Table 5.6 provides a summary of the various MLR models that explain the variation in J_{IC} including the base model of Max.Slope and Porosity. A combination of Max.Slope, avBMD, M_{peak} and ΔH per wet mass best explained the variation in J_{IC} ($adj-R^2 = 31.5\%$). When Max.Slope, porosity and ΔH per wet mass were combined, they closely matched the adjusted R^2 of the previous model ($adj-R^2 = 31.3\%$). However ΔH per wet mass was just outside significance in the model. Alternatively, when an interaction term between Max.Slope and ΔH per wet mass was introduced as part of the latter MLR model, the same adjusted R^2 as the best explaining model was obtained ($adj-R^2 = 31.5\%$). Across the multiple models for J_{IC} , Max.Slope was a significant contributor to J_{IC} signified by its strong β values reaching as large as 0.506.

Table 5.7 provides a summary of the MLR models that explain the variation in J-int. J-int was best explained by a combination of Max.Slope, porosity, HLNL and sex with interaction terms between Max.Slope and porosity as well as HLNL with an $adj-R^2 = 49.8\%$ (highlighted in bold in Table 5.6). Max.Slope had the largest absolute standardized beta co-efficient value ($\beta = 0.436$) in explaining the variation in J-int in this model. A general interesting trend for the J-int based MLR models was the multiple interactions terms between Max.Slope and collagen-based properties as well as porosity that were relevant to explaining the variations in J-int. These collagen-based properties were HLNL, ΔH per wet mass of sample, intact alpha chains and the lysine-based AGE adducts (CML plus CEL). The inclusion of these interaction terms with Max.Slope produced models with an $adj-R^2$ that roughly matched the $adj-R^2$ of models in which they were used as independent terms. For these, HLNL, ΔH per wet mass, intact alpha chains and their interactions with Max.Slope contributed positively to J-int highlighted by their positive β . In contrast, the lysine-based AGE adducts and their interaction with Max.Slope had a negative impact on J-int highlighted by their negative β . When MG-H1 was used in place of the lysine-based AGE adducts, MG_H1 did not reach significance as an explanatory variable ($p = 0.185$). Once again it was observed that, Max.Slope had relatively strong β values ($\beta = 0.436 - 0.683$) across the various models highlighting its relevance to J-int. Pentosidine and carbonyl content were not detected as explanatory variables for J-int.

Table 5.8 presents a summary of the MLR models for Wf_{x_n} . Wf_{x_n} was best explained by a combination of Max.Slope, porosity and the intact alpha chains with interactions between Max.Slope and porosity as well as the intact alpha chains with an $\text{adj-R}^2 = 41.3\%$ (highlighted in bold in Table 5.7). Like J-int, there were multiple interaction terms between Max.Slope and collagen-based properties that were relevant to explaining the variation in Wf_{x_n} . The collagen-based properties included HLNL, ΔH per wet mass and the intact alpha chains. When AGE adducts or their interaction with Max.Slope were used in the model, the model reached significance however, the AGE adducts or their interaction with Max.Slope as an explanatory variable was not significant. Similarly to J-int, Max.Slope had relatively strong β values ($\beta = 0.349 - 0.588$) across the various MLR models for Wf_{x_n} . Again, pentosidine and carbonyl content were not detected as explanatory variables for Wf_{x_n} . In addition, for all three fracture toughness measures, T2D/CKD and control groups were also not detected as a categorical explanatory variable.

Table 5.6: Multiple linear regression (MLR) models table showing combinations of various explanatory variables and their associated standardized beta co-efficient (β) and p-value that explained the variation observed in the crack initiation fracture toughness J_{Ic} . P-value < 0.05 is considered significant.

Fracture toughness measures	Explanatory variables				Adj-R ²
J_{Ic}	Max.Slope ($\beta = 0.452$, $p < 0.001$)	Porosity ($\beta = -0.273$, $p = 0.006$)			29.2
	Max.Slope ($\beta = 0.506$, $p < 0.001$)	Max.Slope: Porosity ($\beta = -0.270$, $p = 0.006$)			29.1
	Max.Slope ($\beta = 0.304$, $p = 0.014$)	avBMD ($\beta = 0.347$, $p = 0.003$)	Mpeak ($\beta = -0.334$, $p = 0.006$)	$\Delta H(\text{wet mass})$ ($\beta = 0.241$, $p = 0.048$)	31.5
	Max.Slope ($\beta = 0.325$, $p = 0.008$)	Porosity ($\beta = -0.306$, $p = 0.002$)	$\Delta H(\text{wet mass})$ ($\beta = 0.213$, $p = 0.074$)		31.3
	Max.Slope: $\Delta H(\text{wet mass})$ ($\beta = 0.473$, $p < 0.001$)	Porosity ($\beta = -0.310$, $p = 0.002$)			31.5

Table 5.7: Multiple linear regression (MLR) models table showing combinations of various explanatory variables and their associated standardized beta co-efficient (β) and p-value that explained the variation observed in the crack instability fracture toughness J-int. P-value < 0.05 is considered significant.

Fracture toughness measures	Explanatory variables			Adj-R ²
J-int	Max.Slope ($\beta = 0.574$, $p < 0.001$)	Porosity ($\beta = -0.256$, $p = 0.005$)		41.8
	Max.Slope ($\beta = 0.627$, $p < 0.001$)	Max.Slope: Porosity ($\beta = -0.284$, $p = 0.001$)		43.4
	Max.Slope: $\Delta H(\text{wet mass})$ ($\beta = 0.569$, $p < 0.001$)	Porosity ($\beta = -0.304$, $p = 0.001$)		41.6
	Max.Slope ($\beta = 0.573$, $p < 0.001$)	Max.Slope: Porosity ($\beta = -0.248$, $p = 0.004$)	HLNL ($\beta = 0.217$, $p = 0.014$)	47.2
	Max.Slope ($\beta = 0.417$, $p = 0.001$)	Max.Slope: Porosity ($\beta = -0.243$, $p = 0.005$)	Max.Slope: HLNL ($\beta = 0.304$, $p = 0.010$)	47.6
	Max.Slope ($\beta = 0.617$, $p < 0.001$)	Maxslope:Porosity ($\beta = -0.330$, $p < 0.001$)	Alpha chain ($\beta = 0.193$, $p = 0.013$)	46.3
	Max.Slope ($\beta = 0.596$, $p < 0.001$)	Maxslope:Porosity ($\beta = -0.328$, $p < 0.001$)	Max.Slope: Alpha chain ($\beta = 0.201$, $p = 0.023$)	46.6
	Max.Slope ($\beta = 0.557$, $p < 0.001$)	Porosity ($\beta = -0.267$, $p = 0.003$)	Lysine based AGEs ($\beta = -0.187$, $p = 0.031$)	44.6
	Max.Slope ($\beta = 0.620$, $p < 0.001$)	Porosity ($\beta = -0.262$, $p = 0.003$)	Max.Slope: Lysine based AGEs ($\beta = -0.189$, $p = 0.034$)	44.5
	Max.Slope ($\beta = 0.564$, $p < 0.001$)	Porosity ($\beta = -0.273$, $p = 0.003$)	<i>MG-HI</i> ($\beta = -0.117$, $p = 0.185$)	42.4
	Max.Slope ($\beta = 0.436$, $p < 0.001$)	Max.Slope: Porosity ($\beta = -0.185$, $p = 0.037$)	Max.Slope: HLNL ($\beta = 0.326$, $p = 0.005$)	Sex ($\beta = 0.178$, $p = 0.046$)

Table 5.8: Multiple linear regression (MLR) models table showing combinations of various explanatory variables and their associated standardized beta co-efficient (β) and p-value that explained the variation observed in the normalized work to fracture Wf_{x_n} . P-value < 0.05 is considered significant.

Fracture toughness measures	Explanatory variables			Adj-R ²
Wf_{x_n}	Max.Slope ($\beta = 0.533$, $p < 0.001$)	Porosity ($\beta = -0.272$, $p = 0.004$)		37.9
	Max.Slope ($\beta = 0.588$, $p < 0.001$)	Max.Slope: Porosity ($\beta = -0.285$, $p = 0.002$)		38.7
	Max.Slope: $\Delta H(\text{wet mass})$ ($\beta = 0.536$, $p < 0.001$)	Porosity ($\beta = -0.316$, $p = 0.001$)		38.7
	Max.Slope ($\beta = 0.495$, $p < 0.001$)	Porosity: HLNL ($\beta = -0.271$, $p = 0.003$)	HLNL ($\beta = 0.211$, $p = 0.022$)	40.6
	Max.Slope ($\beta = 0.349$, $p < 0.001$)	Porosity: HLNL ($\beta = -0.267$, $p = 0.003$)	Max.Slope: HLNL ($\beta = 0.288$, $p = 0.019$)	40.9
	Max.Slope ($\beta = 0.579$, $p < 0.001$)	Maxslope: Porosity ($\beta = -0.327$, $p = 0.001$)	Alpha chain ($\beta = 0.177$, $p = 0.054$)	41.0
	Max.Slope ($\beta = 0.559$, $p < 0.001$)	Maxslope: Porosity ($\beta = -0.326$, $p = 0.001$)	Max.Slope: Alpha chain ($\beta = 0.188$, $p = 0.042$)	41.3
	Max.Slope ($\beta = 0.520$, $p < 0.001$)	Porosity ($\beta = -0.281$, $p = 0.003$)	<i>Lysine based AGEs</i> ($\beta = -0.141$, $p = 0.119$)	39.1
	Max.Slope ($\beta = 0.567$, $p < 0.001$)	Porosity ($\beta = -0.277$, $p = 0.003$)	<i>Max.Slope: Lysine based AGEs</i> ($\beta = -0.140$, $p = 0.133$)	39.0

5.4 Discussion

In this study, various measures of oxidative damage (carbonyl content, three AGE adducts and pentosidine, an AGE crosslink) were measured *ex vivo* in human femoral cortical bone specimens from 81 donors with and without a history of T2D and/or CKD. In addition, cortical bone fracture toughness measures, cortical porosity and mineralization profiles as well as collagen network properties were also measured in the same specimens. The overarching goal was to investigate the impact of oxidative damage of the bone collagen network on its connectivity and organisation and its associated impact on cortical bone fracture resistance. In addition, this was carried out considering the context of two bone affecting diseases: T2D and CKD, which both have higher incidence of fractures in their patients and have been associated with the accumulation of AGEs in the body, by-products of oxidative damage.

5.4.1 AGE adducts versus pentosidine

First, the results showed that the AGE adducts are much more abundant (70 to 800 times) than pentosidine in human cortical bone. This reinforces findings from Arakawa and co-workers [34] where a similar trend (20 to 200 times more abundant) was found for human cancellous bone. In addition, Thomas and others [35] also found CML to be 40 to 100 times more abundant compared to pentosidine in 6 human tibiae. It is important to note though that pentosidine correlated strongly and positively ($r > 0.6$, $p < 0.001$) with all the AGE adducts implying it could be a suitable biomarker or indicator for the accumulation of AGE adducts. The relative abundance of the AGE adducts over pentosidine suggest they may play a much larger role in the mechanisms that lead to bone fragility as compared to pentosidine and probably AGE crosslinks in general. It is noteworthy to mention that there are other AGE crosslinks such as glucosepane and methylglyoxal-lysine dimer (MOLD) that have not yet been detected in bone. Glucosepane has been measured in skin and been found to be about 70 times more abundant than pentosidine [269] but there is no indication that this same ratio exists in bone.

5.4.2 T2D/CKD group versus control group

From the one tailed t-tests, it was found that the various AGE adducts, CML, CEL and MG-H1 were all significantly higher in the T2D/CKD group compared to the controls as was hypothesized. This is consistent with a very recent study where the same AGE adducts (CML, CEL and MG-H1) were measured in femoral mid-diaphysis samples taken from a cohort of 11 men with T2D and 18 men without T2D [320]. This result is further supported by studies where AGE adducts have been shown to accumulate in various other tissues and fluids such as the skin, urine and serum for T2D and CKD

patients [31,32,34,256,259,268]. Pentosidine was also found to be higher in the T2D/CKD group which is consistent with previous studies [31,239]. These results reinforce the idea that hyperglycemia in T2D [274] and uremia in CKD [321] promote elevated levels of AGE formation possibly because of the role they play in glycation and glycoxidative reactions. Surprisingly, carbonyl content was not significantly different between the two groups contrary to what was hypothesized. It is important to note though that donors belonging to the control groups had other morbidities aside from T2D and CKD such as osteoporosis, hypertension and osteoarthritis which are also associated with elevated levels of oxidative stress. This can therefore give rise to ketone and aldehyde containing compounds in their collagen network and this will be quantified by the carbonyl content assay. As such, the elevated oxidative stress from other morbidities may still lead to the formation of ketone and aldehyde containing compounds in the collagen network of the control group at the same quantity as the T2D/CKD group possibly explaining why there is no difference in carbonyl content between the two groups.

Two other characterization measures, HLNL, an immature LOX crosslink and ΔH normalized to the dry mass of the collagen sample were also found to be significantly different between the T2D/CKD and control groups. Interestingly, both measures correlated negatively with the AGE adducts. HLNL had strong correlations with the AGE adducts whereas ΔH per dry mass had weak to moderate correlations with the AGE adducts (see Table 5.5). This suggests the difference for these two characterization measures between the groups could be tied to the greater amount of AGE adducts in the T2D/CKD group.

All three fracture toughness measures were not significantly different between the two groups in contrast to hypothesis. However, this is in agreement with a very recent study in 2023 where various microstructural parameters and mechanical properties were measured and compared in a cohort of age-matched men with and without T2D [322]. For fracture testing, samples were taken from both the mid-diaphysis of the femur whereas samples were taken from both the mid-diaphysis of the femur and the 12th thoracic vertebrae for the microstructural analysis. They found no statistical difference for J-based fracture toughness measures between the T2D group and non-T2D group. In fact, there was also no difference between cortical porosity, cortical avBMD, TMD and Raman spectra measures between the two groups [322]. Furthermore, this is also consistent with work done by Nyman's group in collaboration with the Willett group [323] (unpublished). This involved a larger cohort of 120 donors of both sexes, 60 with T2D and 60 without T2D and samples taken from the femoral mid-diaphysis. No statistical difference was found for measures of J-based fracture toughness between the two groups

as well. These results contrast with many diabetic small animal (rat or mouse) models where the progression of T2D in these animals is associated with lower fracture toughness of their bone [324–327]. This discrepancy suggest that the controlled nature of disease progression in these animal models may not be representative of the nature and possible differences in T2D disease progression in humans and its effect on human bones.

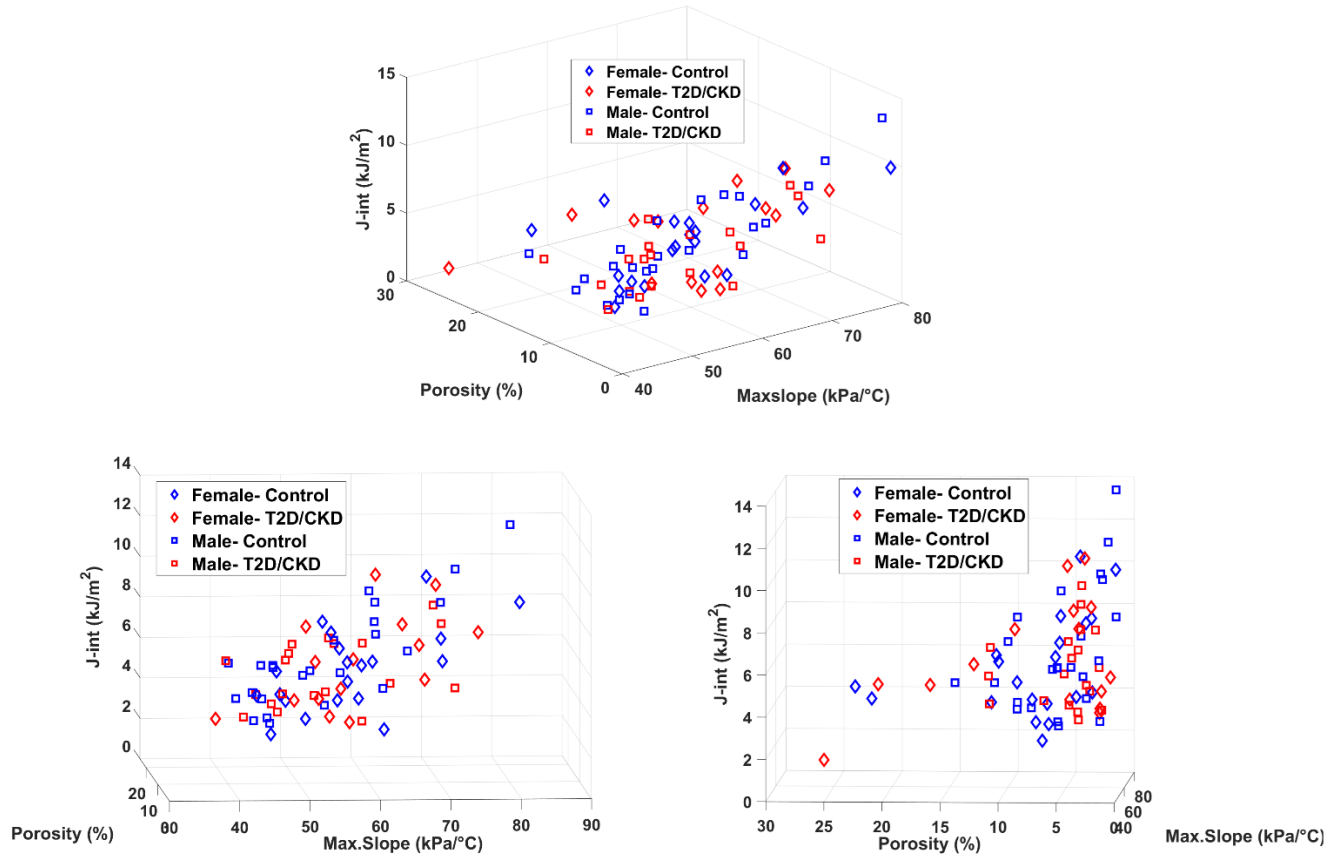


Figure 5.7: 3D scatter plots of J-int versus porosity and Max.Slope highlighting the control and T2D/CKD groups as well as males and females.

Further, the lack of a statistical difference for the fracture toughness measures between the two groups was highlighted by the T2D/CKD and control groups not being an explanatory variable in the MLR models although they were included as possible categorical dependent variables. Fig. 5.7 presents a 3D scatter plot of the fracture toughness measure J-int against Max.Slope and Porosity, two prominent explanatory variables for J-int. It can be observed from the figure that the data points for the T2D/CKD

group are widely spread as well as significantly overlap with data points for the control group. All these suggest that the consequence of bone affecting diseases such as T2D and CKD in relation to cortical bone fracture resistance may happen on a continuum level rather than placing them in categorical bins of disease and control groups. In other words, it is the bone tissue quality changes caused by these bone affecting diseases that are better predictors of reduced fracture resistance, and these changes may not be the same across a disease group but based on disease progression, its management and other factors. For instance, in T2D, it is possible factors such as the duration of T2D, and glycemic control may play a role in the extent of oxidative damage of collagen and therefore its impact on cortical bone fracture resistance may vary from one patient to the next.

5.4.3 Relationship between measures of oxidative damage, collagen network properties and cortical bone fracture resistance

From the Spearman's correlation analysis, one of the interesting results was the relatively strong correlation between AGE adducts and HLNL as was hypothesized (see Table 5.5). This result is consistent with work done by Arakawa and co-workers [34] where they also found a relatively strong negative correlation between HLNL and the AGEs: CML, CEL as well as pentosidine ($r = 0.66 - 0.68$, $p < 0.001$) albeit for human tibial cancellous bone. In addition, it was found in a diabetic rat model study that as pentosidine increased in the diabetic rats with disease progression, the number of immature crosslinks decreased [328]. They reasoned that the reduction in immature crosslink formation was as a result of vitamin B6 deficiency caused by hyperglycemia which suppressed the action of the lysyl oxidase (LOX) enzyme vital to crosslink formation. In addition, a few recent studies have highlighted specific "hotspot" sites at which AGE adducts form specifically depending on environmental conditions [25,329]. Among these sites are the helical lysine and hydroxy-lysine residue sites in the telopeptide region specific to the formation of LOX mediated crosslinks [329,330]. Note, CEL and CML form on lysine and hydroxylysine residues on the collagen molecule. All this lends to the idea that AGE adducts may disrupt the formation of immature LOX crosslinks by (1) competing for the lysine and hydroxylysine residue sites in the telopeptide regions meant for the formation of the immature LOX crosslinks and/or (2) forming at these sites as a result of the action of the LOX enzyme being suppressed thereby disrupting the formation of the immature LOX crosslinks. In these cases, it will be the lysine-based AGE adducts (CEL and CML) that will compete with the formation of immature LOX crosslinks between the collagen molecules. Fig. 5.8 presents a scatter plot of HLNL versus the lysine-based AGE adducts to highlight the relationship between the two variables.

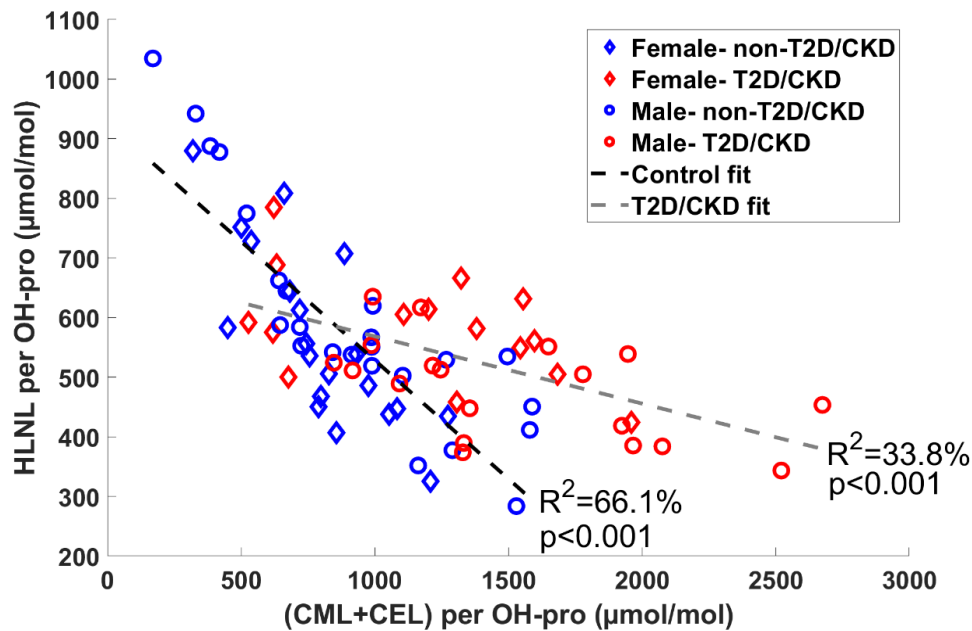


Figure 5.8: Scatter plot of HLNL versus the sum of CML and CEL also referred to as the lysine- based AGE adducts (both normalized to hydroxyproline content).

It is important to note though, for the AGE adducts to disrupt LOX crosslink formation, this must happen early when new bone is deposited prior to mineralization. This unmineralized new bone is called osteoid. It is thought that immature LOX crosslinks convert to mature LOX crosslinks by the time mineralization is complete in newly formed bone in adults [331]. Therefore, it is hypothesized that this inhibition of LOX crosslinking by AGE adducts will occur during bone remodeling as the osteoid is laid. Such a mechanism however would require many years for it to eventually lead to bone fragility which might partly explain the gradual time course of bone fragility in T2D and other diseases. Well controlled animal or in-vitro models will be needed to confirm this hypothesis and to better understand this potential mechanism or ascertain whether this correlation does not necessarily mean causation.

Another thing that stands out from Fig. 5.8 is the difference in the regression lines for the T2D/CKD group and the control group, with the control group having a steeper regression line ($R^2 = 66.1\%$) compared to the T2D/CKD group ($R^2 = 33.8\%$). Using ANCOVA, it was confirmed these regression lines were statistically different from each other ($p < 0.001$). This seems to suggest two different mechanisms may be at play, one within each group. However, it has been widely reported that bone turnover is slower in T2D than non-diabetics [332,333]. Bone turnover is the overall rate at which bone

remodeling occurs [334]. Consequently, this might explain the less steep regression line for the T2D/CKD group. That is, the disruption of LOX crosslink formation by the lysine-based AGE adducts will be less pronounced because of the slower bone turnover even though there is a higher amount of AGE adducts in the T2D/CKD group compared to the control group. Another observation, based on the slope of the fitted line for the control group, is that it seems on average there is a trade of one HLNL for two AGE adducts. This is consistent with stoichiometry as two lysine/hydroxylysine residues are required to form the HLNL crosslink, and these two residues can each accommodate an AGE adduct.

Also of note is that DHLNL, another immature LOX crosslink did not have significant correlations with the AGE adducts except CEL. DHLNL is formed by the reaction of a helical hydroxylysine residue with a N-terminal telopeptide hydroxylysine aldehyde whereas HLNL is formed by the reaction of a helical hydroxylysine residue with a C-terminal telopeptide lysine aldehyde [79]. A possible explanation for the non-significant DHLNL correlations with the other AGE adducts could be because the disruption of LOX crosslink might favour or be more specific to the C-terminal telopeptide region. This cannot be verified from the current study's data and more studies will be needed to investigate this possibility.

The LOX crosslinks provide stability to the collagen network by forming intermolecular bonds between the individual collagen molecules hence have been shown to be important to bone strength [78,79]. Therefore, a disruption to the formation of the immature LOX crosslinks might lead to reduced collagen network stability. This reduced collagen network stability is highlighted by the positive and negative correlations of T_d with HLNL ($r = 0.46$, $p < 0.001$; see Table 5.9) as well as the AGE adducts (see Table 5.5) respectively. T_d gives a measure of the thermal stability of the collagen network. A consequence of this reduced collagen network stability could be changes to the structure of the collagen molecules. In addition, the formation of AGE adducts such as MG-H1 on the side chain groups of the collagen molecules may also directly lead to changes in the collagen molecular structure [274].

This idea of collagen structural changes due to AGEs is further supported by a study in which ribose incubation was used to generate AGEs and Raman spectroscopy was used to assess the bone tissue composition [197]. They found that AGE accumulation from the ribose incubation resulted in a decrease in the amide I sub peak ratios (1668/1638 and 1668/1610). The amide I denotes the carbon-oxygen double bond which is involved in hydrogen bonding between neighbouring side chain groups. Hence, they concluded that this decrease reflected modifications in the organic matrix likely being

changes to the collagen structure. These structural changes to the collagen molecules may explain the correlation between FWHM and the AGE adducts as well as the even stronger correlation with HLNL ($r = -0.60$, $p < 0.001$; (see Table 5.9). FWHM gives a measure of the collagen network disorder or heterogeneity which will increase with greater collagen molecular structural changes.

Table 5.9: Spearman's correlation coefficient table among and between the measures of collagen network quality and the LOX crosslinks. The superscript indicates: * p value < 0.05, **p-value < 0.01, * p-value < 0.001, + p-value < 0.1, ns represents not significant**

Characterization measures		Max.Slope	T _d	FWHM	ΔH (wet mass)	ΔH (dry mass)	Intact alpha chains	HLNL
Collagen quality-based properties	Max.Slope	-	ns	-0.26*	0.56***	0.31**	0.20+	0.21+
	T _d	ns	-	-0.51***	ns	ns	0.19+	0.46***
	T _{onset}	0.33**	0.56***	-0.76***	ns	ns	ns	0.52***
	T _{peak}	ns	0.44***	-0.38***	ns	ns	ns	0.26*
	FWHM	-0.26*	-0.51***	-	ns	ns	-0.23*	-0.60***
	ΔH (wet mass)	0.56***	ns	ns	-	0.73***	0.36**	ns
	ΔH (dry mass)	0.31**	ns	ns	0.73***	-	0.37***	ns
	Intact alpha chains	0.20+	0.19+	-0.23*	0.36**	0.37***	-	0.54***
Immature LOX crosslinks	LNL	ns	0.38***	-0.46***	ns	ns	0.45***	0.58***
	HLNL	0.21+	0.46***	-0.60***	ns	ns	0.54***	-
	DHLNL	ns	0.23*	-0.41***	ns	-0.27*	ns	0.51***
Mature LOX crosslinks	DPD	-0.25*	ns	ns	-0.29**	-0.20+	ns	0.23*
	PYD	ns	ns	ns	-0.27*	-0.29**	ns	0.25*

Another possible consequence of reduced stability may be that the collagen molecules become more susceptible to fragmentation/scission. In a study where ribose pre-treatment was used to counter the deleterious effect of gamma irradiation necessary for the sterilization of allografts, it was found that the increased crosslinking in the collagen network due to the ribose incubation protected the collagen network from the polypeptide chain scission effect of the gamma irradiation [22]. This highlights that increased crosslinking provides greater stability to the collagen network which in turns keeps the polypeptide chains intact. Therefore, the reduced stability of the collagen network due to the disruption

of the immature LOX crosslinks may make the collagen molecules more susceptible to fragmentation/scission by ROS and probably proteolytic enzymes from bone resorption processes. One of the main ways ROS damage proteins is by attacking the backbone of the protein polypeptide chains which can ultimately lead to fragmentation. Consequently, this will lead to reduced stable and intact polypeptide chains making up the collagen molecule. This is reflected in the strong positive correlation between HLNL and the intact alpha chains in this study ($r = 0.54$, $p < 0.001$; see Table 5.9) as well as the negative correlation between the AGEs adducts and the intact alpha chains (Table 5.5). The by-products of fragmentation of the collagen molecules by the ROS are mainly carbonyl containing compounds which were measured by the carbonyl content measure in this study. Therefore, the negative correlation between carbonyl content and intact alpha chains as was hypothesized may be a result of the fragmentation of the alpha chains by the ROS.

The reduction in intact alpha chains from fragmentation will result in less energy dissipation when the collagen denatures. Denaturation of collagen involves the breaking of the hydrogen bonds that bind the single procollagen chains into a triple helix structure and the subsequent unwinding of the chains [335]. Less intact alpha chains mean there are fewer hydrogen bonds to break hence less energy dissipation during denaturation. The thermal energy dissipated during collagen denaturation was measured in this study through DSC as ΔH normalized to wet mass or dry mass of the collagen sample. Consequently, the intact alpha chains correlated positively with the two enthalpies of denaturation ($r = 0.37 - 0.38$, $p < 0.01$; see Table 5.9) and maps how AGEs may indirectly impact ΔH explaining the moderate negative correlation between the two variables (Table 5.5). To add, a study by Xiaodu Wang and colleagues [271] showed that an increase in pentosidine concentration was associated with increased existing collagen network denaturation in ex vivo human femoral cortical bone samples. This suggest one of the structural changes to collagen may be denaturation. That is, the native state of the triple helix of the collagen molecule is negatively impacted by the AGE adducts. This is a known consequence of oxidative damage in proteins [39]. This may further explain the negative correlation between the AGE adducts and ΔH . Although, the LOX crosslinks help stabilize the collagen network they do not contribute towards ΔH as measured here as they are not known to be heat labile bonds [20]. This explains why a correlation between HLNL and both ΔH was not detected (see Table 5.9).

In summary, the AGE adducts are proposed to disrupt the formation of the immature LOX crosslinks during bone remodeling, ultimately resulting in reduced collagen network stability and collagen structural changes which are highlighted by the correlation between the AGE adducts and various

measures of collagen network quality: the intact alpha chains, the enthalpies of denaturation (ΔH), denaturation temperature (T_d) and collagen network disorder or heterogeneity measured as FWHM.

While among the AGEs, only CEL correlated with the measures of fracture toughness, the fracture toughness measures correlated positively with HLNL as well as ΔH per wet mass of collagen and negatively with FWHM (except for Wf_{x_n}). Interestingly, all these variables had significant correlations with the AGE adducts in this study. The relationship between these collagen-based properties and the fracture toughness measures suggest that the disruption of the formation of the immature LOX crosslinks which may lead to collagen structural changes impacts the fracture resistance of cortical bone. In fact, this is analogous to osteo-lathyrism models. In osteo-lathyrism models, β -aminopropionitrile (BAPH), a toxin found in common sweet pea, is used to suppress the action of the LOX enzyme in animals such as rats. This in turn has been shown to disrupt the formation of the LOX crosslinks [86,277,279]. The disruption in LOX crosslinks formation has been associated with a decrease in bone strength as well as the fracture resistance of bone in these models [280,281,336]. This highlights an indirect way AGE adducts may impact cortical bone fracture resistance through disruption of the formation of the immature LOX crosslinks. In addition to the above, porosity correlated negatively with all three measures of fracture toughness, consistent with literature [153,337,338]. Further, Max.Slope had a moderately strong positive relationship with all three fracture toughness measures, with J-int being the strongest (see Table 5.4). Fig. 5.9 presents a scatter plot between J-int and Max.Slope showing the distribution of the T2D/CKD and control groups' specimens. The relationship between J-int and Max.Slope is consistent with a previous study where for a similarly large heterogenous donor group, a positive relationship ($R^2 = 35.1\%$, $p < 0.001$) with Max.Slope and J-int was found [24].

Surprisingly, Max.Slope did not correlate with the AGE adducts or carbonyl content contrary to what was hypothesized. However, the MLR models revealed an interesting trend where relevant explanatory variables towards explaining the variation in J-int and Wf_{x_n} , explained the variance in J-int and Wf_{x_n} as independent terms roughly equal to when they formed an interaction term with Max.Slope. Specifically, for J-int, it was observed that HLNL (adj- R^2 of 47.2% vs 47.6%), the intact alpha chains (adj- R^2 of 46.3% vs 46.6%) and the lysine-based AGEs (adj- R^2 of 44.6% vs 44.5%) all follow this trend. This indicates these afore-mentioned explanatory variables affect the contribution of collagen network connectivity (Max.Slope) towards cortical bone fracture resistance. Consequently, the AGE

adducts, specifically the lysine-based AGEs, although they do not correlate with Max.Slope might still affect the collagen network connectivity due to their disruption of the collagen network structure.

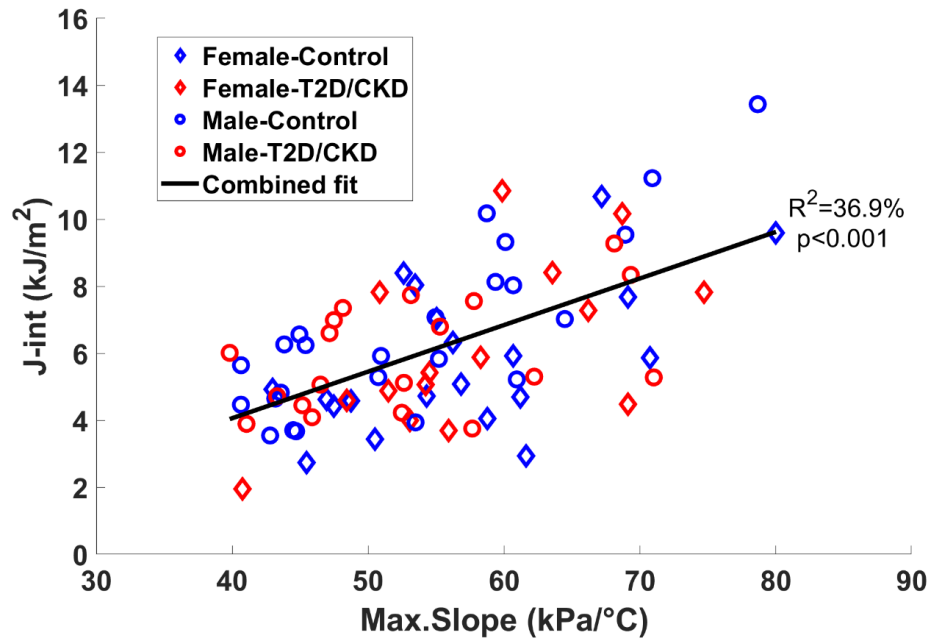


Figure 5.9: Scatter plot of Max.Slope versus J-int also showing distribution between the two groups: T2D/CKD (red markers) and control (blue markers). The circles represent males, the diamonds females.

Consistent with work from Nyman's group [24], the importance of Max.Slope to cortical fracture resistance was highlighted in the MLR models. The relatively large beta-coefficients for Max.Slope for J-int and W_{fx_n} suggest it is integral to explaining these fracture toughness measures. Max.Slope is a measure of the collagen network connectivity. Its strong relationship with J-int and W_{fx_n} in this study highlights the importance of the collagen network connectivity to sustained stable crack growth in the cortical bone tissue. From various studies using gamma radiation and ribose incubation, it has been found that Max.Slope is a function of crosslinking and polypeptide chain lengths of the collagen molecules; increasing with greater crosslinking and decreasing with fragmentation of the polypeptide chains [21,22,33,141]. A previous ex vivo cortical bone study showed that Max.Slope did not correlate with ex vivo measures of matured LOX crosslinks or pentosidine [24]. Interestingly in this study, Max.Slope did not have significant correlations with the immature LOX crosslinks and the intact alpha chains however its correlation with HLNL ($r = 0.21$ $p = 0.065$) and intact alpha chains ($r = 0.20$ $p = 0.078$) fell just outside significance. This hints at a potential relationship between HLNL as well as the

intact alpha chains and the collagen network connectivity. This was observed in the MLR models where HLNL and intact alpha chains formed interaction terms with Max.Slope, the measure of collagen network connectivity towards explaining J-int and Wf_{x_n} .

It has been shown that once denaturation has been exhausted in collagen molecules, the denatured collagen behaves as a rubber polymer [339]. Consequently, in a recent paper, which sought to give a theoretical framework to Max.Slope, the generation of isometric stress after denaturation was complete that constitutes Max.Slope, was modelled using a rubber thermo-elasticity formulation [315]. Therefore, Max.Slope essentially measures the amount of tensile stress the network of denatured gelatinous-like procollagens can sustain without failure. This means for a higher Max.Slope, the collagen network can better stabilize the cortical bone tissue during crack growth increasing cortical bone fracture resistance. Therefore, Max.Slope might quantify collagen network quality beyond its connectivity (crosslinking and chain lengths). This may explain why there is no significant correlations between Max.Slope and HLNL as well as the intact alpha chains and as an extension with the AGE adducts. However, interestingly, crosslinking in and the molecular weight of the material are important factors affecting the rubber elasticity of rubber polymers [340]. This may explain the effect of HLNL, one of LOX crosslinks, and the intact alpha chains on the collagen network's connectivity contribution to the fracture toughness measures as evidenced in the MLR models.

ΔH normalized to wet mass was another parameter to have an interaction term with Max.Slope. A recent paper, using fluorescently labeled collagen hybridizing peptides showed that collagen molecules mechanically denature during cortical bone fracture [106]. In this paper, the authors highlighted the importance of water to this mechanism, and this may explain why the ΔH normalized to wet mass of the sample and not the dry mass was the relevant explanatory variable to the fracture toughness measures. From a thermo-mechanical framework [20,315], the measurement of ΔH and Max.Slope are uncoupled. That is, ΔH is the energy dissipated during the entire collagen network denaturation process whereas Max.Slope is measured once collagen denaturation is exhausted. Therefore, it was surprising that an interaction term between these two variables was an important contributor to explaining the fracture toughness measures. The interaction might be because ΔH gives a measure of the strength of the intramolecular bonds within the collagen molecule and this might be indicative of the structural quality of the individual procollagen chains which are important to quantifying Max.Slope. In other words, there may be a relationship between collagen network nativity and connectivity. Another interesting finding was that there was an interaction term between Max.Slope and porosity that was

relevant to explaining the variation in the fracture toughness measures. Increasing porosity for a fixed cortical bone volume means less cortical bone tissue resulting in higher stresses within the material. As such, the collagen network during fracture will bear greater stresses explaining the effect of porosity on the collagen network connectivity in its relationship with the fracture toughness measures.

Another interesting observation from the MLR models was that the impact of the bone mineral-based properties (avBMD, M_{peak} , porosity) on J_{IC} was greater as compared to the J-int and W_{fx_n} . This was indicated by the higher β for avBMD and M_{peak} predominantly compared to porosity in the various MLR model for J-int and W_{fx_n} . The most prominent toughening mechanism during cortical bone fracture is considered to be the formation of the micro-damage process zone (MDPZ) ahead of and around the growing crack [112,341,342]. This MDPZ is theorized to originate from sliding processes at the collagen-bone mineral interfaces highlighting the contribution of the bone mineral to the formation of the MDPZ [107,112]. The onset of microdamage formation which precedes crack growth initiation highlights how the bone mineral contributes to explaining J_{IC} . After the onset of crack growth, the MDPZ continues to form and is thought that a rate dependent mechanism termed “strain rate hardening” enables the continual formation [113]. Due to the rate dependency of this mechanism, it must be driven by the organic phase of bone (collagen, NCP) and water bound to the matrix of the bone tissue [343,344]. Collagen has superior inelastic (post yield) behaviour and is thought to stabilize the bone tissue to allow for the formation of MDPZ thereby ensure stable crack growth [17]. Hence, there is a greater contribution from the collagen network for J-int and W_{fx_n} compared to J_{IC} . This is in agreement with work done by the Nyman group [24,135,153]. In one of their studies, they found porosity to correlate with K-init, which is the K based equivalent of J_{IC} , with an R^2 of 41.7% as well as J-int but with a lower R^2 of 14.7% [153]. Conversely, Max.Slope, a measure of collagen network connectivity was found to correlate with K-init with an R^2 of 24.4% whereas with J-int, it had an R^2 of 35.1% [24]. In addition, they developed general linear models using different key determinants of bone fragility to explain K-init and J-int measured from specimens taken from the femoral diaphysis of a large heterogenous group of 60 donors. avBMD was more prominent in explaining K-int with a $\beta = 0.43$ in their best model compared to J-int where pore water, a surrogate for porosity had a $\beta = -0.2$ [135]. Max.Slope was less prominent in explaining K-int with a $\beta = 0.37$ in their best model compared to J-int where Max.Slope had a $\beta = 0.48$ [24].

Altogether, although the above illustrates the importance of the collagen network to cortical bone fracture resistance, it also highlights the complexity of understanding the mechanism by which the

collagen network contributes to cortical bone fracture resistance. This may explain the limited significant correlations between measures of collagen network quality and its connectivity (Max.Slope) as well as the fracture toughness measures. Although, there were a few direct correlations between the collagen network quality measures and the fracture toughness measures, there are complex interactions between these measures and the collagen network connectivity that in turn impacts cortical bone fracture resistance. For instance, the negative correlation between pentosidine and Max.Slope might be a result of AGE adducts inhibiting the formation of the LOX crosslinks hence ultimately leading to a lower Max.Slope. However, since pentosidine correlates positively with the AGE adducts this can drive the negative correlation observed in the study. In fact, a correlation between pentosidine and Max.Slope will be expected to be positive because increased crosslinking should lead to an increase in Max.Slope. However, the amount of pentosidine as measured in this study is too low to effect such a change. In addition, pentosidine was not detected as an explanatory variable in any of the fracture toughness measures highlighting it might be an indicator but not a cause of reduced cortical bone fracture toughness.

Carbonyl content, a measure of oxidative damage was also not detected as a significant explanatory variable in the various MLR models developed. It however correlated negatively with the intact alpha chains, which was a significant explanatory variable for MLR models explaining the variation in J-int and Wfx_n. An explanation for its absence in the MLR models may be the measure's lack of specificity. Unlike the AGE adducts and PEN measures, carbonyl content measures a wide variety of products from protein oxidation. Most likely, the impact of these products on the collagen network will be different and some may be more deleterious to the collagen molecules and its structure. Here using methods that allow for better specificity when measuring carbonyl content will be of benefit. One such approach is the use of high-performance liquid chromatography (HPLC) to measure carbonyl content [200].

To conclude, it is thought that oxidative damage plays an indirect role in negatively affecting cortical bone fracture resistance. It is proposed that AGE adducts disrupt the formation of immature LOX crosslinks during bone remodeling impacting the stability of the collagen network as well as leading to collagen structural changes which in turns affects the contribution of the collagen network connectivity towards stabilize the cortical tissue during fracture resulting in reduced cortical bone fracture resistance. The disruption of the immature LOX crosslinks is thought to be analogous to osteo-lathyrism models where reduced LOX crosslinking have been showed to result in reduced fracture resistance. In addition,

it is also proposed that the collagen network may be susceptible to fragmentation by ROS also impacting the contribution of the collagen network connectivity to cortical bone fracture resistance.

5.4.4 Limitations

As with all studies, this study has limitations. First, not all AGEs existing in cortical bone were measured. This is because there are about ~750 different AGEs which are difficult to isolate and measure. For instance, glucosepane which might be more abundant than pentosidine in bone, does not survive acid hydrolysis which is necessary to analyse the bone material with mass spectrometry. Another limitation is the donors between the T2D/CKD and control groups were not age-matched or body mass index (BMI) matched. The groups were not age matched in order to collect as many cadaveric human femurs as possible. This approach was taken as the main aim of the study was to investigate the potential fundamental mechanisms by which oxidative damage impacts cortical bone fracture resistance and not just the differences between the two groups. BMI was not matched as this information was not made available from the various tissue banks for all the donors. In addition, T2D duration and HbA1c, a measure of blood glucose level over the past two to three months, was not accounted for in this study also because this information was not available for all donors from the tissue banks nor was the necessary biological tissue available to carry out this measurement. Despite the above limitations, the study provides insights into the potential mechanisms by which oxidative damage may affect cortical bone fracture resistance irrespective of disease state.

5.5 Conclusion

In conclusion, measures of oxidative damage, carbonyl content and AGEs, were measured in cortical bone specimens from a heterogeneous group of 80 donors. The AGE adducts, CML, CEL and MG-H1 and the AGE crosslink, pentosidine were all found to be significantly higher in the T2D and/or CKD donor group compared to controls whereas carbonyl content was not. For all donors, the AGE adducts were about 70 to 800 times more abundant than pentosidine. The AGE adducts correlated strongly and negatively with the immature LOX crosslink, HLNL. This suggests AGE adducts may competitively inhibit formation of immature LOX-mediated crosslinks. It was proposed that this might occur during bone remodeling. Animal or in-vitro models are needed to establish and investigate this proposed mechanism. These AGE adducts were also thought to alter the structure of the collagen molecules, partly as a result of the inhibition of LOX crosslink formation. These changes in collagen network interact negatively with the collagen network connectivity which has been shown previously

and again in this study to be vital to cortical bone fracture resistance. Ultimately, all these lead to reduced cortical bone fracture toughness highlighting an indirect pathway by which AGE adducts may affect cortical bone fracture resistance. On the other hand, carbonyl content, a biomarker for oxidative damage although correlated with the AGE adducts and pentosidine was not a significant explanatory variable for fracture toughness measures and may have suffered from a lack of specificity in the method used to measure. However, it correlated with the intact alpha chains which was an explanatory variable for two of the measures of cortical bone resistance. Better methods for measuring protein carbonylation should be explored in the future to investigate this potentially important relationship with fracture toughness. Overall, this study proposes that AGE adducts disrupt the formation of immature LOX crosslinks, reducing the collagen network stability and connectivity. In addition, ROS are proposed to attack the collagen molecules polypeptide directly also leading to fragmentation of the alpha chains. These affect the mechanical energy dissipation mechanisms of collagen network which negatively impacts the contribution of collagen network connectivity to cortical bone fracture resistance

Chapter 6: Overall Conclusion and Future Work

6.1 Summary of findings and conclusions

The over-arching goal of this thesis work was to investigate oxidative damage as a cause of collagen network connectivity degradation leading to reduced cortical bone fracture resistance. However, first fracture toughness testing methods for cortical bone were critically examined to establish the best method to use. This was because fracture toughness testing of cortical bone was an integral part of achieving the over-arching goal of this work.

From this study, it was found that the unloading compliance method widely used to estimate crack extension in cortical bone fracture toughness testing underestimated crack extension measurements by 73 % on average. It was proposed that the MDPZ that forms around the crack tip during fracture exerts compressive residual stresses on the bone tissue producing lower than expected unloading compliance values thereby partly explaining the observed underestimation. This finding was contrary to the hypothesis that the UC method would overestimate crack extension. In addition to the above, this underestimation combined with the energy dissipated during the unload-reload cycles employed in the fracture test led to inaccurate and less precise fracture toughness measures when compared to the standard fracture test of using monotonic loading and measuring crack extension optically. When the load-unload type of loading was combined with crack extension measured using the optical method in computing the J-R curve (OM-C approach), there was no difference between the measured J_{IC} and that measured from the standard test. However, there was a difference between the two approaches for J-int presumably due to the energy dissipation associated with the load-unload loading. Finally, it was proposed that when the crack extension is optically measured, a 0.05 mm offset more accurately quantified the crack initiation fracture toughness (J_{IC}) opposed to the 0.2 mm offset stated in ASTM E1820. Overall, it was concluded that the use of the unloading compliance method for cortical bone fracture testing is not recommended, and the best method is to optically measure crack extension as well as use a monotonic loading scheme for fracture testing of cortical bone.

The second study focused on investigating oxidative damage as a means of collagen network degradation associated with reduced fracture resistance. It was found that the AGE adducts; CML, CEL and MG-H1 were 70 to 800 times more abundant than pentosidine, an AGE crosslink in human cortical bone irrespective of a history with T2D and/or CKD or not. However, both the AGE adducts and pentosidine were 20 % to 65 % greater in quantity in the T2D/CKD group compared to the control

group in agreement with what was hypothesized. The greater quantity of AGEs were attributed to hyperglycemia in T2D and uremia in CKD which seems to favor glycation and glycoxidation reactions. Carbonyl content was found not be different between the two groups probably because of elevated oxidative stress implications from other morbidities besides T2D and CKD in the control group. More interestingly, fracture toughness measures were not detectably different between the two groups contrary to what was hypothesized. However, this is consistent with other very recent studies [320,323]. The AGE adducts correlated strongly with HLNL, an immature LOX crosslink and moderately with other collagen network quality-based properties such as the intact alpha chains, enthalpies of denaturation and collagen network disorder or heterogeneity but not with collagen network connectivity. Carbonyl content was also found to correlate with the intact alpha chains but not collagen network connectivity. Interestingly, the MLR models showed that interaction terms between collagen network connectivity and the collagen network quality-based properties listed above as well as the immature LOX crosslink, HLNL were important to explaining the variation observed in the fracture toughness measures particularly J-int and W_{fx_n} . These results agreed with the hypotheses on the relationships between the oxidative damage biomarkers, immature crosslinking and collagen chain fragmentation. However, there is some nuance regarding the hypothesis on the relationship between the oxidative damage biomarkers and collagen network connectivity. Although they did not directly correlate with each other, the interaction terms between them were relevant in explaining the fracture toughness measures. Based on all these results, it was proposed that oxidative damage indirectly affects cortical bone fracture resistance by disrupting the formation of immature LOX crosslinks analogous to osteo-lathyrism models. The disruption of the formation of the immature LOX crosslinks was thought to affect the collagen network stability thereby leading to altered collagen structure. This in turn interacted negatively with the collagen network connectivity which was found to be central to explaining the fracture toughness measures alongside cortical porosity. In addition, it was proposed that the influence of T2D and CKD on cortical bone fracture resistance be treated on a continuum as it was not an explanatory variable for the fracture toughness measures nor was there a significant difference between the T2D/CKD group and the control group for these measures. Overall, it was concluded that changes to the collagen network structure caused by oxidative damage negatively impacts the contribution of collagen network connectivity to cortical bone's fracture resistance.

6.2 Contributions to the field

One of the key elements for a doctoral level research work is the advancement of knowledge and understanding it provides. As such, the original and meaningful contributions from this thesis work are presented, separated into the themes of the two main studies that form this thesis work.

For study 1 on critically examining cortical bone fracture toughness methods, the following original and/or meaningful contributions to the field were made:

1. To the best of my knowledge, this is the first study that compared using the optical and the unloading compliance (UC) methods of measuring crack extension towards the determination of the fracture toughness of cortical bone.
2. This study was the first to show that the widely used UC method leads to inaccurate crack extension estimation for cortical bone beyond correcting for the empirically derived calibration equations developed for metals. The isotropy-based calibration equations used for metals have been previously found to produce errors when used for cortical bone [148,149].
3. This study proposed the inaccuracies in the fracture toughness measures for cortical bone using the UC approach were partly due to the formation of the MDPZ in addition to energy dissipation during the load-unload loading cycles employed for this approach. This knowledge is important towards developing more accurate calibration equations specific to cortical bone for the UC method.
4. This work showed that an optical method of measuring crack extension along with the use of a monotonic loading scheme provides the most accurate and precise measurement of cortical bone fracture toughness.
5. A 0.05 mm offset was proposed to be used when calculating for human cortical bone crack initiation fracture toughness from J-R curves where the crack extension was measured by an optical method.
6. It was also shown that SENB bovine cortical bone specimens with a groove on only one side allowed for relatively straight crack growth during transverse directed fracture which is important for accurate measurement of Mode I fracture toughness.

For Study 2 on investigating oxidative damage as a cause of collagen network connectivity degradation associated with reduced cortical bone fracture resistance, the following original and/or meaningful contributions to the field were made:

1. The AGE adducts, CML, CEL and MG-H1 were shown to be much more abundant than pentosidine in human cortical bone in a large heterogenous donor cohort over a wide age range. To the best of my knowledge, this is the first study to show that, CEL and MG-H1 are more abundant in ex vivo human cortical bone than pentosidine. This has previously been shown in cancellous bone.
2. The AGE adducts, CML, CEL and MG-H1 were shown to more abundant in the cortical bone of T2D and/or CKD donors compared to controls without the diseases for a large heterogenous donor cohort over a wide age range (19 to 103 years). These had only been previously shown for a smaller sized male donor cohort.
3. This study reinforces very recent reports of no statistical difference in fracture toughness measures of cortical bone between T2D and non-T2D contrary to findings in diabetic animal models
4. This study establishes a strong negative relationship between AGE adducts and immature LOX crosslinks in human cortical bone previously established in human cancellous bone and a diabetic rat model.
5. This study provides greater understanding of how oxidative damage indirectly impacts fracture toughness through changes to the collagen structure that affect the collagen network connectivity contribution to fracture toughness
6. It was also proposed that the influence of oxidative damage due to oxidative stress impacts cortical bone fracture resistance on a continuum, and this is irrespective of a specific disease group.

6.3 Significance and Impact

The first sub-objective of this thesis work was to determine the best method and approach to assess the fracture toughness of cortical bone material accurately and precisely. This is important because fracture toughness measures provide the most comprehensive measure of the cortical bone tissue's ability to resist fracture. Using these measures of cortical bone fracture resistance, possible key determinants contributing to age and disease-related bone fragility and fracture can be investigated. This can be done by investigating how changes to these key determinants correspond to changes in the measured fracture toughness of the bone tissue. It is therefore of utmost importance that the appropriate methods and techniques are applied in quantifying cortical bone fracture resistance. From this study, the widely used UC method was shown to produce significant errors in estimating cortical bone crack extensions during fracture testing. This demonstrates the need for a shift from using the UC method in cortical bone

fracture toughness testing to optically tracking crack growth. However, this raises the question of needing additional equipment hence increased cost for undertaking cortical bone fracture testing. Considering this, an alternate approach may be to develop calibration equations specific to cortical bone that account for the sources of the inaccuracies, proposed in this work to be MDPZ formation and energy dissipation during the load-unload loading. The development of such calibration equations will be hugely impactful, allowing for accurate measurement of cortical bone fracture toughness without the need for additional equipment to track the growing crack. Overall, this study highlights the need for the development of a rigorous and standardized protocol for cortical bone fracture testing and fracture toughness measurement that can be applied across different laboratories.

The second sub-objective and over-arching goal of this thesis was to investigate oxidative damage as a cause of collagen network connectivity and quality degradation associated with reduced cortical bone fracture resistance. It was proposed that oxidative damage indirectly impacts cortical bone fracture resistance by disrupting the immature LOX crosslinks and altering the collagen structure thereby affecting the connectivity of the collagen network. This highlights oxidative damage of collagen as a key contributor to bone fragility and fracture. This provides greater mechanistic understanding towards bone fragility and fracture. Based on this understanding, developing robust and measurable biomarkers of oxidative damage in bone could be vital to development of better tools for detection, prevention and treatment of bone fragility. In addition, it also supports the idea of looking into AGE inhibitor or breaker interventions as a means for preventing and treating bone fragility and fracture. The pre-eminence of Max.Slope as an explanatory variable for cortical bone fracture resistance remains intriguing. While more studies are needed to better understand what exactly this variable quantifies, it begs the question whether a biomarker for this variable, if one could be found, might be relevant to fracture risk prediction. The findings from these studies could ultimately lead to better detection, prevention and therapies against bone fragility and fracture.

6.4 Future Work

This thesis highlights the role oxidative damage may play in the age and disease related reduction in cortical bone fracture resistance in addition to the shortcomings of the UC method towards cortical bone J-R curve determination. Some of the findings in this study do highlight the need for further work and some of these are listed below:

1. The development of standardized calibration equations for various sites of human cortical bone that accurately estimate crack extension will be very impactful for J-R curve fracture testing of human cortical bone. These calibration equations will have to account for the errors associated with the formation of the MPDZ as well as energy dissipation during the load-unload loading cycles.
2. Although J-integral is widely used to quantify the fracture toughness of cortical bone, it might not be the most appropriate as the J-integral is based of the theory of non-linear elasticity while cortical bone is considered to be quasi brittle. It will be worthwhile to apply quasi brittle fracture mechanics approaches such as discrete fracture mechanics and compare to J-integral based fracture toughness measures.
3. The digital image correlation-based assessment of crack extension measurement can be made more effective and efficient by exploring machine learning models such as the convolutional neural network to detect the location of the crack tip thereby computing the crack extension.
4. The development of a better approach for analyzing carbonyl content specific to human cortical bone will enhance studies into the effects of oxidative damage on cortical bone fracture resistance.
5. The development of in vitro or animal models to verify that the association between AGEs and immature LOX crosslink is causative and not just correlative.
6. The development of such an in vitro or animal model will be beneficial towards further studies into establishing the pathway by which oxidative damage affects cortical bone fracture resistance as well as testing of drugs to combat the negative effects of oxidative stress and damage
7. Further investigations into what exactly Max.Slope, a measure from hydrothermal isometric tension testing, quantifies will be beneficial towards a better understanding of how this measure is a strong explanatory variable for human cortical bone fracture toughness.

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Appendix A: Study 1 Supplementary material

A.1 Normalization of load for load versus load-line deflection curve

To account for the geometric differences (slightly different widths, thicknesses, and initial crack lengths) between paired specimens, the dimensionless compliance (Z_{ll}) together with the effective thickness was used to normalize the load to allow for a direct comparison. This is based on the fact that elastic modulus is a material property.

To work this out, first, the relationship between load and deflection in a 3-point single edged notched bend (SENB) test is defined which is given as [97] :

$$P = \frac{EB_e}{Z_{ll}} \Delta \quad (\text{A.1})$$

where P is the load

Δ is the deflection

Z_{ll} is the dimensionless compliance

E is the elastic modulus

B_e is the effective thickness of the sample

Re-arranging Equation 1:

$$P \frac{Z_{ll}}{B_e} = E \Delta \quad (\text{A.2})$$

From equation 2, the load is normalized by multiplying by the dimensionless compliance (Z_{ll}) and dividing by the effective thickness (B_e). Consequently, the normalized load is related to the deflection by the elastic modulus which should be of similar value between the paired samples allowing direct comparison.

The dimensionless compliance (Z_{ll}) for a 3-point SENB sample is calculated as [97]:

$$Z_{II} = \frac{S^3(1-\nu^2)}{W^3} \left[0.25 + 0.6 \left(\frac{W}{S} \right)^2 (1 + \nu) \right] + 1.5 \left(\frac{S}{W} \right)^2 \left(\frac{\frac{a}{W}}{1 - \frac{a}{W}} \right)^2 \left[5.58 - 19.57 \left(\frac{a}{W} \right) + 36.82 \left(\frac{a}{W} \right)^2 - 34.94 \left(\frac{a}{W} \right)^3 + 12.77 \left(\frac{a}{W} \right)^4 \right] \quad (A.3)$$

where S is the span equal to 40 mm

ν is the Poisson's ratio equal to 0.3

W is the width of the sample

a is the crack length

The effective thickness (B_e) is also given by [143]:

$$B_e = B - \frac{(B - B_N)^2}{B} \quad (A.4)$$

where B is the full thickness of the sample

B_N is the net thickness of the sample accounting for the side groove

A.2 Mesh sensitivity for FE model

To ensure the FE model used to compute the new unloading compliance calibration equation was adequately meshed, a sensitivity study was conducted. This was carried out using an equivalent single edge notched bend (SENB) model with a crack length to width (a/W) ratio of 0.5 as described in the section 4.3.4.1 . The size of the quadratic tetrahedral elements was varied between 0.1 mm, 0.2 mm, 0.4 mm as well as 0.8 mm and simulated for each case. In all cases, the sample was loaded to a 0.1 mm deflection using the displacement control boundary condition. The load and deflection were tracked and plotted to test for convergence. Figure A.1 presents the results of the mesh sensitivity study. As noticed, mesh sizes of 0.1mm, 0.2mm and 0.4mm produced identical load versus deflection curves, therefore a mesh size of 0.4 mm was used for the subsequent simulations.

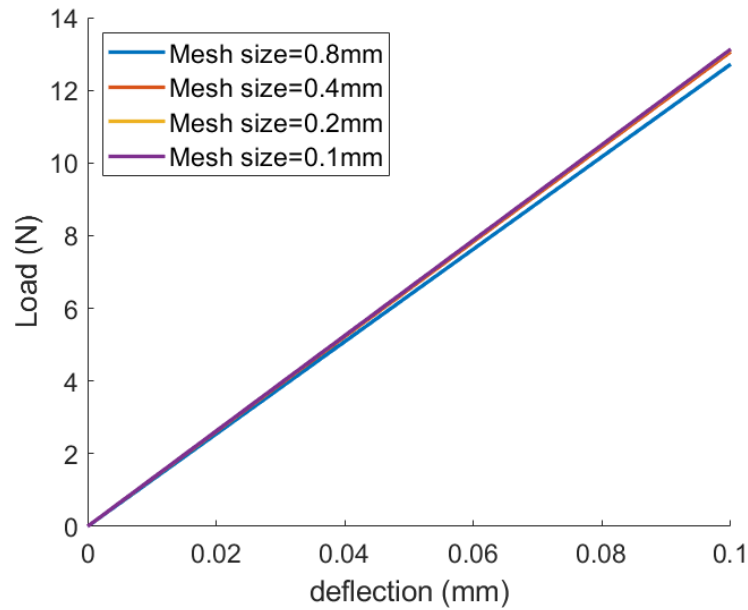


Figure A.1: Load versus deflection curves obtained from mesh sensitivity study for FE model used to generate a new unloading compliance calibration equation

A.3 Approximate water loss during fracture test

To provide a rough estimate of how much water is lost from the SENB specimens during fracture testing, a post hoc experiment was carried out on the fractured SENB specimens. After re-hydrating the specimens, their initial weight in grams (g) were measured and left in ambient air for 10 minutes and then re-weighed. 10 mins was chosen as this represents the maximum time it took to set up and test the specimens in ambient air. The table below (Table A.1) provides the results for five samples showing a percent weight change of less than 1% in all cases. This indicates there is minimal water loss during the fracture tests.

Table A.1: Post hoc experimental dataset on evaluating percent weight loss of SENB specimens after 10 minutes in ambient air.

Specimen Number	Initial weight of SENB specimen (g)	Weight of SENB specimen after 10 mins in ambient air (g)	Percent change in weight (%)
Specimen 1	1.63	1.62	0.4
Specimen 2	1.54	1.53	0.7
Specimen 3	1.59	1.58	0.4
Specimen 4	1.59	1.58	0.6
Specimen 5	1.60	1.595	0.5

Appendix B: Study 2 Supplementary material

B.1 Crack extension measurement using digital image correlation (DIC)

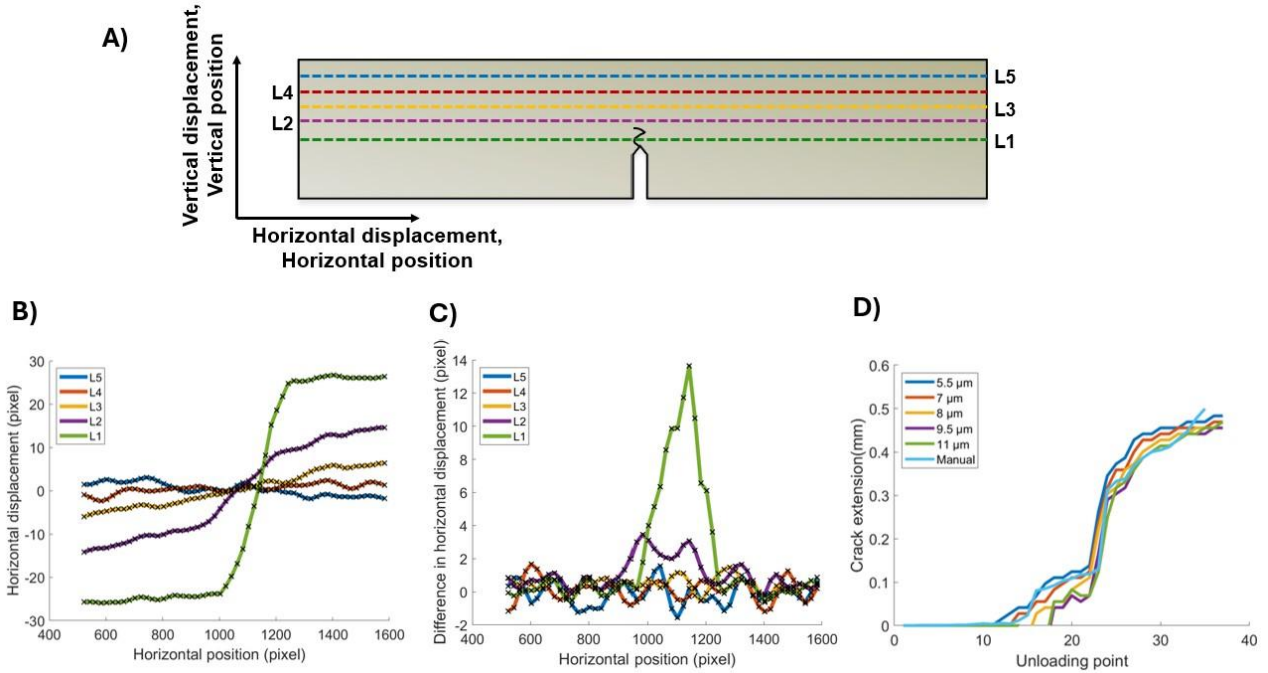


Figure B.1: (A) Two-dimensional representation of a single edged notched bend (SENB) sample showing 5 horizontal paths along the length. (B) Plot of the horizontal displacement (left to right) along the various horizontal path against the positions on the horizontal path. (C) Plot of horizontal displacement difference between successive horizontal positions against the corresponding horizontal position. (D) Results of convergence analysis of various threshold displacement difference compared to a manual method of determination.

Fig. B.1 gives a schematic illustration of how DIC is used to locate the position of the crack tip and hence aid in the computation of crack extensions for the single edged notched bend (SENB) specimens used in the 3-point fracture tests. Fig. B.1(A) shows five different horizontal paths labelled L1 to L5 ahead of the razor sharpened starter notch. Fig. B.1(B) gives the horizontal displacement plotted along these horizontal paths. The specific data points are depicted by the black cross markers. It can be observed for L1 which passes through the growing crack, there is a step change in displacement about the mid-point of this horizontal path highlighted by the greater displacements between the data points along the mid-point of L1. This represents the displacement continuity created as a result of the growing crack. For paths L2, L3, L4 and L5, this step change in displacement is not noticed and that is because

they are above the growing crack. Paths L2 to L5 are not parallel to each other because of the deformation gradient created across the sample as a result of the beam undergoing bending. To figure out a path with a displacement continuity, the difference between the horizontal displacement at a certain horizontal position and the horizontal displacement four positions away was taken. This is presented in Fig. B.1(C) as a plot of the differences between the horizontal displacements and the corresponding initial horizontal position. From this plot, data points related to path L1 have the highest difference highlighting the displacement continuity due to crack growth. It can be noticed that path L2 also has a few data points with differences higher than that associated with L3, L4 and L5. To determine a threshold difference value above which crack growth can be said to have occurred, a convergence analysis was performed. That is, various difference values were chosen, and their results were used to ascertain the minimum value they will converge. In addition, the results from this was compared to a manual determination of the crack extension using the optically captured images. Fig. B.1(D) presents a representative plot of the convergence analysis showing the crack extension at various points of the fracture test for the various threshold difference values chosen. In addition, the crack extension obtained by using a manual method is also shown. From these convergence analysis on 10 samples, it was obtained that a threshold displacement difference of 6 pixels equivalent to about 8 μm between two points separated horizontally by roughly 50 μm signified crack growth. Using this threshold, the crack extensions for all other fracture tests were determined.

B.2 Using MATLAB based crack tip tracking method to compute J-R curve data point

The MATLAB code used to compute crack extension from DIC derived displacement fields from fracture tests and generate J-R curve data points for specimens in this study can be found here: [JRdatapts_PhD.m](#).

To use the code, the following steps are needed.

1. Obtain the load deflection data csv file from the fracture test.
2. Perform digital image correlation (DIC) on the regions of crack growth from images produced from the fracture test to calculate the displacement fields.
3. Export the DIC displacement fields from the software as a csv file. This can be done on vic-2D system by going to the “Export” menu option and choosing “Data”.

4. The name of the DIC displacement field csv file should match the name of the load deflection data csv file.
5. Using the excel template found here: [Sample dimensions for thesis.xlsx](#) , input the widths, lengths, thicknesses, net thicknesses and initial crack lengths of the specimens using the names of the load deflection data csv file as their sample ID.
6. Place these files in the same file directory and change line 15 in the code to match this directory.
7. Run the code to generate images of the displacement field with crack extension representation as well as an excel file containing the crack extension values. In addition, the code will generate a plot of the J-R data point as well as an excel file containing the J-R curve data points.

B.3 Preliminary study with SDS PAGE on fluorescence-based imaging

As described in section 5.2.9, a preliminary study was carried out to evaluate whether fluorescence-based imaging of the gels using fluorescein-5-thiosemicarbazide (FTC) could detect the location of the carbonyl groups within the bone collagen network. This approach was however unsuccessful in consistently detecting the carbonyls. The unbound FTC tended to bleed through the wells and/or overshadow the alpha chain bound FTC bands settling towards the base of the gel (see Fig. B.2). Consequently, the gel was changed to a 7.5% pre-cast polyacrylamide gel to ensure the unbound FTC migrated out of the gel. This change improved the noise from the unbound FTC however the intensities of the bands associated with the bound FTC were weak especially when compared to the Coomassie Blue staining (see Fig. B.3). Therefore, this approach was not used in this thesis work.

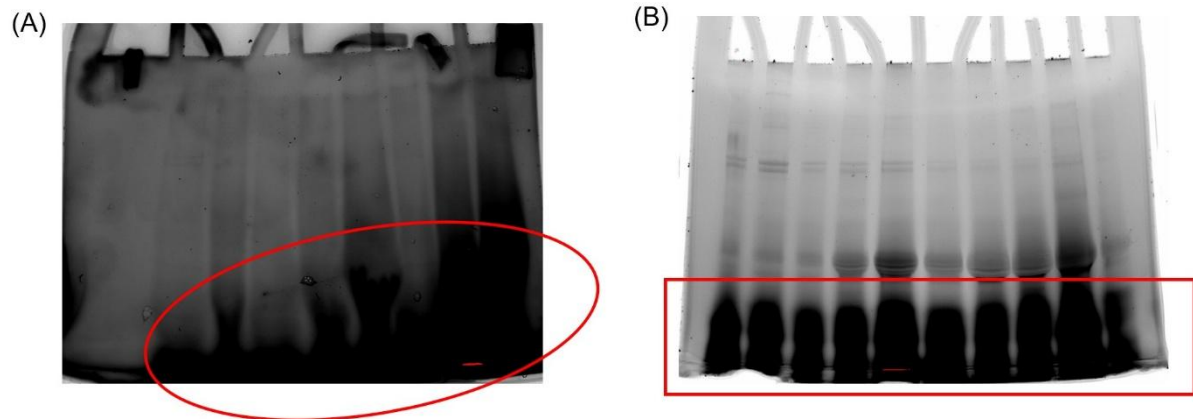


Figure B.2: SDS PAGE gels showing (A) how the unbound FTC seeped through the different wells creating the blob of fluorescence signal highlighted by the red circle and (B) the unbound FTC at the base of the wells of the gels highlighted by the red rectangle overshadowing the other bands because of their high intensity.

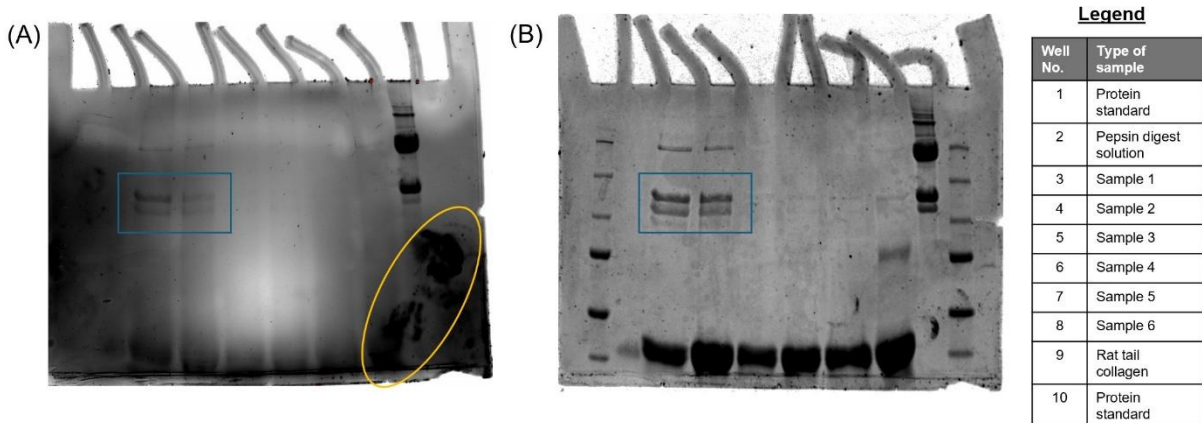


Figure B.3: Comparison between (A) an FTC bound fluorescence scanned SDS PAGE gel and (B) a Coomassie blue stained and scanned SDS PAGE gel. Note the same samples are placed in each well of the gels according to the legend on the right. It is observed the fluorescence scanned gel produces a weaker signal with significant background noise and artifacts (yellow circle) compared to the Coomassie blue stained gel. For example, the alpha chains for samples in well 3 and 4 are much brighter in the Coomassie blue stained gel compared to the fluorescence scanned gel. These are highlighted in the blue boxes.

B.4 Checking normality and linearity assumptions for the multiple linear regression models

Two important assumptions of the multiple linear regression (MLR) analysis is linearity and normality. That is, the dataset must follow a normal distribution and the relationship between dependent variable and combination of independent variables must be linear. Normality can be assessed using a residual plot where the residual (actual value minus predicted value) of the data points is on the y-axis with the value of the predicted variable on the x-axis. For a dataset that is normally distributed, the data points will be roughly symmetric about the line the residual is equal to zero ($y = 0$) and have no clear or distinctive shape to their distribution. The RStudio software provides a red line that indicates these two measures for the residual plots. The residual plots for J_{IC} , J-int and Wfx_n are showed in Fig. B.4. In all cases, the red line is roughly about the line where the y-axis is zero. Although, there were some deviations in the line, it was not considerable enough hence the dataset was considered to be normally distributed

Linearity can be assessed using a Q-Q plot. This plot has standardized residual on the y-axis and the theoretical quantiles on the x -axis. Standardized residuals are calculated as the observed value minus the predicted value divided by the standard error of the predicted values whereas the theoretical quantiles are the percentiles of a standard normal distribution curve (mean = 0 , standard deviation =1). For linearity, the data points should form roughly a straight diagonal line where the value of the y-axis is equal to the value of the x-axis (that is, $y = x$). The Q-Q plots for J_{IC} , J-int and Wfx_n are presented in Fig B.5. J-int and Wfx_n have the data points roughly on the line where $y = x$. J_{IC} has some deviation from the straight line at both tails especially the lower tail. This signifies there is some non- linearity in the relationship between the independent variables and J_{IC} however this was considered not too significant to affect the final model.

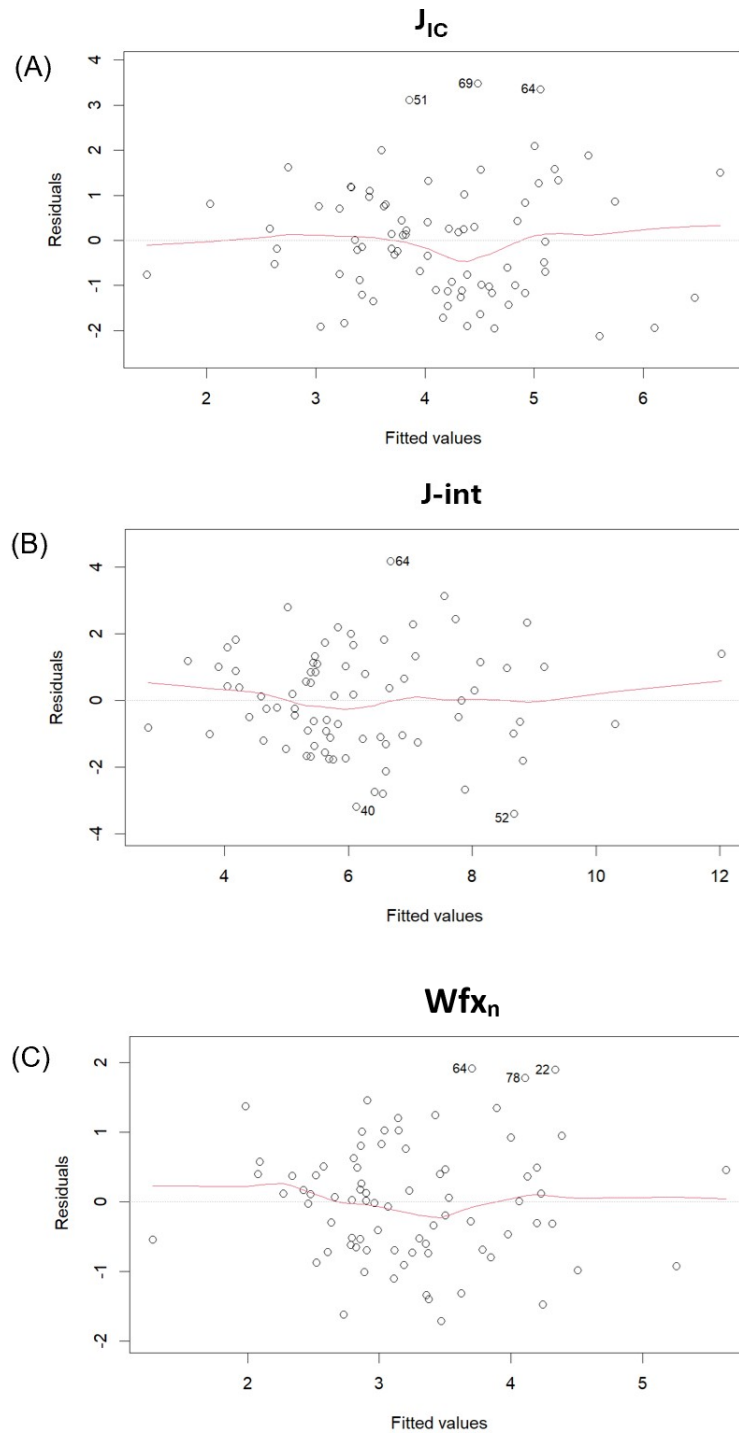


Figure B.4: Residual plots for MLR models explaining (A) J_{IC} (B) J_{int} (C) Wfx_n with the red lines showing the MLR models meet the assumption of linearity.

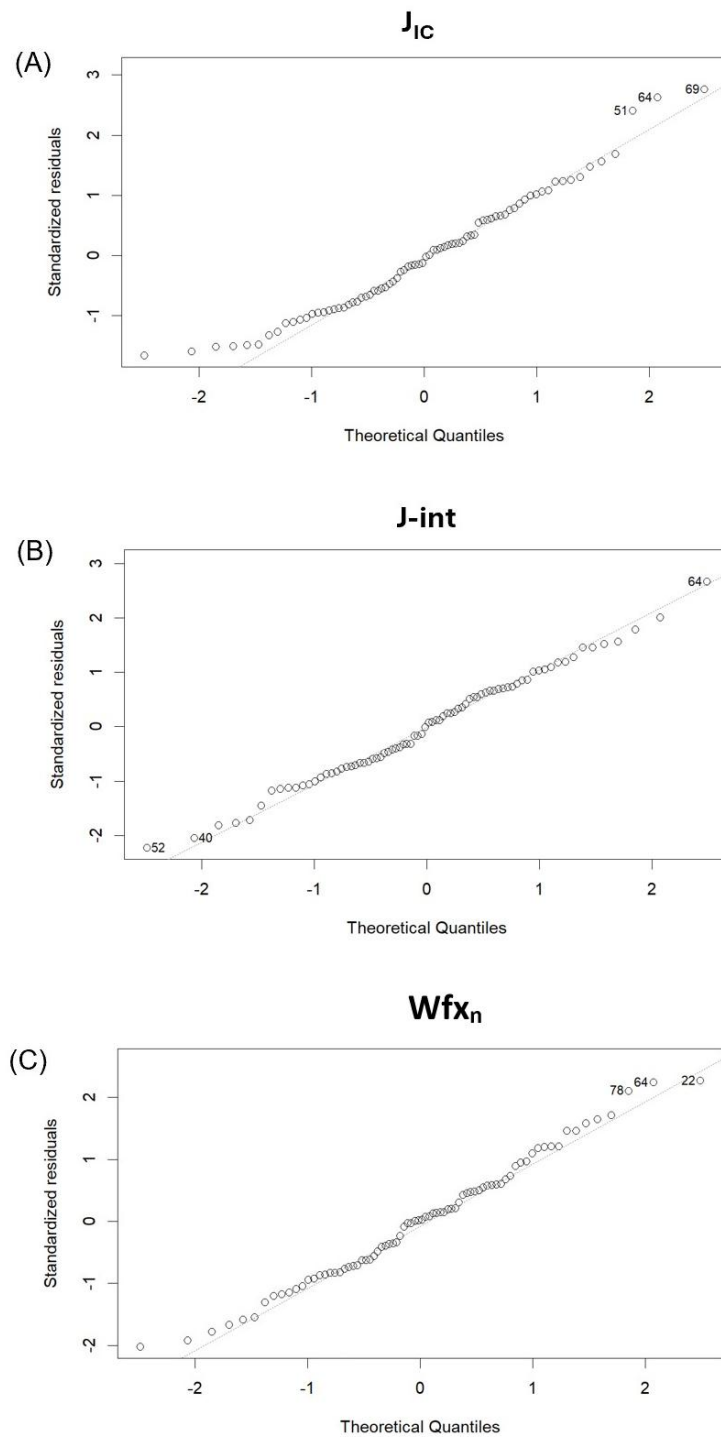


Figure B.5: Q-Q plots of the MLR models explaining (A) J_{IC} (B) J_{int} (C) Wfx_n showing the MLR models roughly meet the linearity assumption.