Skeletal Fragility in Type 2 Diabetes Mellitus: Bone Mechanical Properties, Microarchitecture, and Advanced Glycation End-Products in the Proximal Femur

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Abstract

Osteoporosis is a metabolic bone disorder characterized by depleted bone mass, remodeling of microarchitecture, and an increased incidence of fractures. Predominantly diagnosed through a measurement of low bone mineral density (BMD), osteoporosis is widely recognized as a highly prevalent condition affecting roughly one in two women and one in five men over the age of fifty.

Diagnostic criteria for osteoporosis, however, fail to accommodate the growing population of patients who suffer from type 2 diabetes mellitus (T2DM), a metabolic disorder characterized by peripheral insulin resistance, high circulating glucose, and insufficient insulin production. Although data on fracture risk is inconsistent, several studies have indicated an increased incidence of fractures in patients with T2DM; paradoxically, BMD is found to be normal or elevated in these patients, even when values were adjusted for BMI.

A possible explanation lies in the perturbed whole body metabolism of T2DM. Many chronic complications from diabetes arise from poor glycemic control and the accumulation of advanced glycation end-products (AGEs). In vitro studies have shown that the non-enzymatic glycation of amine residues on collagen and other proteins leads to increased microdamage in bone. Currently unknown are the mechanical and biological implications of increased AGE content on bone health in diabetic and healthy patients. To address this, the Bouxsein lab at Beth Israel Deaconess Medical Center (BIDMC) have recruited a group of diabetic and non-diabetic patients...
undergoing elective total hip replacement (THR) as donors of bone and plasma samples. From the samples collected, I hypothesized that AGE accumulation and bone fragility, as assessed through bone biomechanics, will be greater in bone from diabetic patients as compared to non-diabetic controls.

In 20 diabetic and 34 control patients, biomechanical properties and microarchitecture were characterized through cyclic reference point indentation (cRPI) and microcomputed tomography (microCT), respectively. Following completion of AGE quantification in bone and serum samples, correlations between AGE content and structural properties were assessed, with adjustments for confounding factors. Diabetic patients were found to have increased AGE content in cortical bone and increased indentation distances, as assessed by cRPI, although no relationships were found between bone AGE content and cRPI values. A weakly positive correlation between serum AGEs and total bone AGEs was also noted. Although diabetic patient recruitment was limited by surgical criteria to only those with excellent glycemic control, this study is novel for its investigation of AGE accumulation and structural assessment of bone from diabetic patients, and it is the first to note a correlation between serum total AGEs and bone AGEs in a cohort including T2DM patients.
Introduction

Bone Structure and Strength

1.5 billion years ago, tectonic plate activity left large deposits of calcium carbonate in the oceans; in response, unicellular organisms developed hardened, mineralized body parts which are considered the earliest precursors to bone (1,2). The human skeleton in its current composition is vastly more complicated than the Cambrian fossils of ancient sea life. Instead of simple shells and hardened plates, the human skeleton is composed of 213 bones, adapted to support and protect the body (3).

At a macroscopic level, bones can be broadly categorized by their shape. Flat bones, such as the skull and scapula, shield internal organs (3). Short bones are cuboidal in shape and found in the wrists and ankles, and irregular bones are those that do not fit in any of the above categories (3). Long bones, consisting of a diaphysis and epiphysis, generally support the body’s weight and facilitate movement (3).

The diaphysis of long bones is a hollow tube surrounded by hard cortical bone, while the epiphysis is a spongy head of trabecular bone with a thin, dense covering of cortical bone (3). Compressive and tensile forces that long bones are subjected to help determine the orientation of spongy trabeculae and distribution of cortical bone (see Image 1) (3).
Image 1: Representation of forces and histology of femoral head. Long bones, such as the femur, consist of a shell of cortical bone surrounding spongy trabecular bone (see inset of microCT on femoral neck). Trabecular bone remodeling is influenced by the tensile and compressive forces to which the femoral head is subjected.

At a microscopic level, both cortical and trabecular bone are comprised of structural units called osteons (3). In dense cortical bone, osteons are cylindrical and lamellar, but within this arrangement there is also cortical
porosity, which is increased with active bone remodeling (3). Trabecular osteons are similarly lamellar, however their semilunar shape and arrangement in a rod and plate configuration differentiates them from cortical osteons (3,4). In both cortical and trabecular bone, osteon arrangement and bone microarchitecture are important determinants of fracture risk. (5-7).

As a tissue, bone consists mainly of a collagenous extracellular matrix mineralized from calcium hydroxyapatite (3). The extracellular collagen gives the bone elasticity while the mineralization of the matrix provides the mechanical rigidity that allows for load bearing, and defects in bone collagen or mineralization can impair structural integrity and lead to increased fractures (8).

Mechanical properties of bone can be assessed indirectly through analysis of bone microarchitecture and density by high-resolution computed tomography (CT) and magnetic resonance (MR) (9). Through these imaging techniques, microscopic assessment of characteristics such as cortical porosity, trabecular thickness, trabecular separations, trabecular number, bone volume, and tissue mineral density can be quantified in both ex vivo bone specimens and in living organisms, making it particularly well suited for longitudinal studies (9).

Alternatively, mechanical properties can be assessed directly through ex vivo mechanical testing at the whole bone, bulk tissue, or microbeam level to characterize structural strength, stiffness and stress, or through indentation testing to assess microscale material properties (9-15). Because of its scale, microindentation allows for mechanical characterization of individual
osteons and trabeculae; consequently, specimens undergoing micro- and nano-indentation are largely preserved, allowing for further structural, mechanical, and compositional testing to occur (11).

**Osteoporosis**

Osteoporosis is a metabolic bone disordered characterized by decreased bone strength, depleted bone mass, deteriorated remodeling of bone microarchitecture, and an increased incidence of fractures. A widely prevalent condition, affecting roughly one in two women and one in five men over the age of fifty, osteoporosis can have a devastating affect upon quality and length of life (16). Osteoporotic fractures, typically occurring from a low trauma incident such as a fall from standing height, are associated with increased mortality of up to 20 percent and the development of chronic pain and limited mobility (17).

Properties that influence bone strength include bone mineral density (BMD), bone size, bone shape, mineralization, and microarchitecture; of these properties, the most readily measured is BMD through dual energy x-ray absorptiometry (DXA) (18). To be diagnosed as osteoporotic, patients have their BMD value compared to the average value found in a gender matched population at 30 years of age; if the patient value is found to be greater than 2.5 standard deviations below this average (a “T-Score” of -2.5), the patient is identified as having low BMD and diagnosed with osteoporosis (19). Patients with BMD insufficiently low for an osteoporosis diagnosis may also be at risk for the fragility fractures that plague osteoporotic patients; indeed, even though reduced BMD correlates strongly with increased
fracture incidence, the majority of fragility fractures occur in the larger patient population diagnosed with osteopenia, which is defined as low BMD as measured by a T-score of -1.0 to -2.5 (20,21).

Clinically, the diagnosis of osteoporosis may be applied in the setting of a fragility fracture, even when the T-score for BMD is insufficiently low, since the treatment for osteoporosis seeks to attenuate bone loss, with the overall goal of decreasing the incidence and severity of osteoporotic fractures. For those patients who do not meet the diagnostic criteria of osteoporosis on the basis of their DXA-BMD result alone, improved diagnostic and screening criteria are needed. One approach has been the development of assays to quantify serum levels of bone turnover, including c-terminal peptide (CTX), a marker of bone resorption, and procollagen type 1 peptide (P1NP), a marker of bone formation (22).

Recognizing that other clinical factors correlate with bone loss, the Fracture Risk Assessment Tool (FRAX) was developed by the World Health Organization to compute the 10 year fracture risk for a patient, given their age, glucocorticoid usage, smoking history, personal and family history of a previous low impact fracture, and BMD (23). FRAX improves the predictive power of physicians treating to prevent fragility fractures because it uses factors that have been validated to correlate with the development of osteoporosis, as glucocorticoids have been shown to inhibit bone turnover, personal and family history indicate a propensity for fracture, and bone turnover is known to decrease with age (24).
Type 2 Diabetes Mellitus

FRAX is an imperfect tool since it does not account for every factor and condition known to affect bone strength. Type 2 diabetes mellitus (T2DM) is one such condition. Although data on fracture risk is inconsistent, several studies have indicated an increased incidence of foot, ankle, and humerus fractures in patients with T2DM; paradoxically, BMD is found to be normal or elevated in these patients (25-27). Commonly associated with central weight gain and elevated body mass index (BMI), T2DM is a metabolic disorder characterized by peripheral insulin resistance, high circulating glucose, and insufficient insulin production. Because the percentage of overweight and obese individuals has increased in nearly every region of the world since the 1970s, T2DM has developed into a pandemic, with 9.3% of Americans thought to be diabetic in the most recent report from the Centers for Disease Control (CDC) (28-30).

T2DM is a metabolic condition, but the associated comorbidities and complications affect nearly every organ system. Hypertension, cardiovascular disease (CVD), renal disease, neuropathy, non-alcoholic fatty liver disease (NAFLD), and dyslipidemia have all been found to occur alongside and because of T2DM (31,32). Some of these conditions, such as renal disease and neuropathy, could explain the increased incidence of fractures seen in diabetics. Renal disease can effect calcium homeostasis through the malabsorption of dietary calcium from a lack of activated vitamin D, produced in the proximal convoluted tubules in the kidney, and from secondary hyperparathyroidism (33). Neuropathy in the distal limbs has been shown to destabilize posture, which could increase the risk of falling
for diabetic patients, and could potentially explain the increased fracture rates seen (34). Analyses of diabetic populations, however, indicate that renal disease and neuropathy are strongly associated with increased fracture, but their presence alone are insufficient in explaining the increased incidence of fractures (35-37).

The trend of increased BMD in the same population at increased risk for fracture suggests that perhaps T2DM is affecting bone quality. Since T2DM is strongly correlated with increased body mass index (BMI), the elevated BMD values seen in T2DM patients may be attributable to increased weight bearing (27). Intriguingly, even when BMD values are adjusted for BMI, diabetic patients are still found to have a higher than expected BMD, suggesting increased density is a real phenomenon in the setting of T2DM (38,39).

With increased BMD, other markers of bone quality have been investigated as the causative factors for fractures. Bone microarchitecture and trabecular density have been examined in the bones of diabetic and control patients, yet no significant differences were observed (40). Porosity in cortical bone, however, was observed to be greater in diabetic bone, yet differences between T2DM and non-T2DM are small and these results, when assessed by finite element analysis, were found to have negligible effects on bone strength (41,42). To date, none of the examined factors contributing to bone quality have revealed an apparent biomechanical mechanism to explain diabetic skeletal fragility.
**Advanced Glycation End Products**

Many of the chronic complications from diabetes result from poor glycemic control and the accumulation of a heterogeneous group of compounds called advanced glycation end-products (AGEs). In both the healthy and diabetic states, excessive levels of circulating glucose can lead to non-enzymatic glycation on amino acid residues on plasma proteins, collagen fibers, and extracellular sugars (43,44). The reliability of this process is such that glycated hemoglobin (HbA1c), an Amadori product (see Image 2), is routinely used as a biomarker for glycemic control in diabetic patients (45-47).

AGEs are formed following the addition of glucose and other reducing sugars to amine residues in a series of steps known as the Maillard Reaction (44). In the first stage, the reducing sugar react with a free amino group to form an unstable Schiff base, which then rearranges to form a more stable Amadori product (44,48). Degradation of the Amadori product to a stable, fluorescent AGE is the final step in this process (see Image 2) (44,48).
AGEs have been shown to accumulate with higher average blood glucose levels and age, and excessive AGE accumulation can cause vascular and neuronal damage, resulting in diabetic retinopathy, cardiovascular disease, and neuropathy (44,47,48,50). Effects of AGE accumulation can be via direct alteration of protein function through structural interference of the non-enzymatic glucose adduct or through receptor mediated signaling (51,52).

Identification of a receptor for AGEs (RAGE) revealed that accumulation of AGES and increased expression of RAGE result in activation of inflammatory pathways, which possibly contribute to the pathogenesis of diabetic complications (52). RAGE is a member of the immunoglobulin receptor superfamily and is expressed in many cell types, including mesenchymal stem cells (MSCs), osteoblasts, and osteoclasts (53). Multiple ligands bind and activate RAGE, including AGES, pro-inflammatory cytokine S100, Alzheimer's Disease related amyloid β peptide, and macrophage derived pro-

Image 2: Formation of AGES via the Maillard Reaction. Non-enzymatic glycation of free amino groups forms the unstable Schiff base, rearranges to an Amadori product, then degrades to an AGE. Shown above are several species of AGES, including carboxymethyllysine (CML), glycoxal-lysine dimer (GOLD), and pentosidine. As illustrated, both GOLD and pentosidine form protein crosslinks, whereas CML does not. This image is adapted from Oliveira et al (49).
inflammatory amphoterin (HMGB1) (54-58). RAGE is best characterized for its pro-inflammatory effects, as RAGE activation causes downstream activation of NF-κB, resulting in transcription of pro-inflammatory cytokines and RAGE itself (59,60).

Truncated forms of RAGE have been identified, including soluble RAGE (sRAGE), the proteolytically cleaved extracellular domain of RAGE, and endogenous secretory RAGE (esRAGE), a RAGE splice variant (61-63). Both sRAGE and esRAGE act as decoy receptors, competing with RAGE in binding AGEs (64,65). AGE accumulation is known to be a risk factor in cardiovascular disease, and exogenous sRAGE administration has been shown to reduce the progress of rapid onset atherosclerosis in diabetic apolipoprotein E mice (64). Notably, esRAGE levels have been found to be inversely associated with vertebral fracture risk in T2DM patients over 50 years of age (66).
The relationship between serum AGEs, sRAGE, and esRAGE is dependent on many factors, including disease state. The literature reports conflicting relationships of sRAGE and T2DM status, with both increased and decreased sRAGE noted in diabetic patients as compared to controls (68,69). Given that sRAGE is derived from the proteolytic cleavage of RAGE, increased tissue expression of RAGE in response to increased serum AGES could explain the positive correlation of sRAGE with T2DM seen in some reports (69).

In contrast, esRAGE levels has consistently been noted to be lower in T2DM as compared to controls and lower levels are associated with increased atherosclerosis, metabolic syndrome, and inflammatory markers (70,71).
Species of AGEs include those that form crosslinks between collagen fibers (e.g. pentosidine, vesperlysines, crossline) and those that don’t (e.g. carboxymethyllysine, carboxyethyllysine, pyrraline); both are different from the enzymatic glycation of collagen, which is known to strengthen bone (72).

In the only study to examine AGE content in bone from diabetic patients, pentosidine content in bone was found to be higher in a small group of diabetic patients (73).

Serum and skin AGE measurements have been investigated as biomarkers for diabetic complications, allowing for treatment before onset of hyperglycemia induced illness (45,74,75). Correlation of serum and tissue resident AGES, however, is variable, suggesting that the best measure of AGE accumulation in a tissue is through direct assessment of the tissue in question (74).

Quantification of AGES in bone has previously been accomplished through exploitation of the compounds’ natural fluorescence (72). Pentosidine has been isolated from lyophilized, acid-hydrolyzed bone and measured with high performance liquid chromatography (HPLC), then normalized to collagen content (76). Because this method selects for pentosidine, a specific AGE species that crosslinks collagen yet constitutes less than 1% of AGE content in bone, it does not quantify the total bone AGES (72). Measurement of bulk fluorescence in lyophilized, acid-hydrolyzed bone samples has previously been used, although this method does not distinguish between fluorescent enzymatic crosslinks between collagen (72). In vitro studies have shown that AGE accumulation following incubation of bone samples with a reducing sugar leads to increased microdamage in bone, perhaps triggering
apoptosis in nearby osteocytes and affecting bone porosity (77). Currently unknown, however, are the mechanical and biological implications of increased AGE content on bone health in diabetic and healthy patients.

Aims

Despite increased BMD and no significant differences in bone microarchitecture, diabetic patients have an increased incidence of fracture as compared to non-diabetic controls. Past investigations into diabetic skeletal fragility have considered AGE accumulation as a potential mediator of weakened diabetic bone, yet these studies were limited to either small patient numbers (n = 20) or focused on bone biomechanics following in vitro glycation (72,73,77,78). Currently, the Bouxsein lab at Beth Israel Deaconess Medical Center (BIDMC) have recruited a group of diabetic and non-diabetic patients undergoing elective total hip replacement (THR) as donors of bone and plasma samples.

I hypothesize that AGE accumulation and bone fragility, as assessed through bone biomechanics, will be greater in bone from diabetic patients as compared to age-, gender-, and BMI- matched controls. The aims of this project are:

1. To characterize biomechanical properties and microarchitecture in bone specimens from diabetic and non-diabetic patients undergoing elective THR.
2. To quantify AGE content in bone specimens collected from diabetic and non-diabetic patients, and compare these measurements to the biomechanical and microarchitecture properties measured in Aim 1.
3. To quantify serum levels of AGEs, esRAGE, and markers of bone turnover, and assess the relationship with the bone AGEs values collected in Aim 2. Additionally, to analyze these serum markers as predictors for the biomechanical and microarchitecture properties quantified in Aim 1.
Materials and Methods

Patient recruitment

Patients undergoing total hip replacement (THR) at Beth Israel Deaconess Medical Center (BIDMC) were evaluated for inclusion as subjects in this IRB approved study. Those patients with an age over 40 years and no history of metabolic bone disease or recent use of medications known to impact bone, such as anti-retroviral therapy for HIV or use of osteoporosis medications within the past year. Additionally, thiazolidendiones (TZDs) and glucocorticoid use within the past three months were considered exclusionary, as was diagnosis of pre-diabetes.

Pending availability of research staff, patients were contacted and written informed consent attained. Access to the patient’s online medical record, blood samples for HbA1c and pentosidine measurements, and discarded femoral head and neck surgical specimens were provided by each patient after informed written consent.

Study participants were classified as T2DM subjects if they met one of five criteria: 1) Had a recorded HbA1c greater than or equal to 6.5 within the past two years, 2) Had a recorded HbA1c greater than or equal to 6.5 more than 2 years previously and are currently using a medication for T2DM (e.g. Metformin, sulfonylureas, insulin), 3) Have a fasting blood glucose measurement of greater than or equal to 125 mg/dL in the past two years, 4) Have a fasting blood glucose measurement greater than or equal to 125 mg/dL more than two years previously and are currently using a medication
for T2DM, including metformin, 5) Are currently using a medication for T2DM other than metformin.
Subjects were classified as non-diabetic controls if they had no recorded past history or current indications of T2DM.

**Specimen Collection**

All blood samples collected were stored at -80°C until use.
Femoral head and neck surgical specimens were collected within 24-48 hours after the surgery. They were wrapped in saline soaked surgical gauze and frozen at -20°C. Upon use, specimens were thawed and a 3 mm cross section from the posterior half of the femoral neck was cut and used in reference point indentation, microcomputed tomography, and quantification of AGEs. Cross sections were stored in saline soaked gauze at -20°C until use.
Image 4: Specimen sectioning of femoral neck. The posterior half was sectioned; cRPI and microCT were performed on the medial half, AGEs were measured on the lateral half.

**Cyclic Reference Point Indentation**

Cyclic reference point indentation (cRPI) was used to assess nano-scale mechanical properties of the bone specimens. Cross sections were thawed to room temperature and wrapped in saline soaked gauze to ensure adequate hydration prior to indentation. Using a Biodent Hfc (Active Life Scientific, Santa Barbara, CA), indentation tests along the cortical bone were conducted from the medial to lateral aspects of the proximal side of the longitudinal axis of the femoral neck. Approximately 8 to 10 indentations were made 1 mm apart with a beveled reference probe with a blunted end (~5 mm
cannula length) and test probe with a spherical tip (2.5 μm radius point) that tapers from a 90° cone shape to a cylindrical shaft (BP2 probes, Active Life Scientific, Santa Barbara, CA). A force of 6 N at 2 Hz for 20 cycles comprised each indentation, with outcomes averaged. The following variables were measured: indentation distance (ID, μm [indentation distance into the bone during the first cycle]), creep indentation distance (CID, μm [total indentation distance during the hold step of the first cycle]), total indentation distance (TID, μm [total indentation distance into the bone across all cycles]), indentation distance increase (IDI, μm [difference in indentation distance into the bone between the first and last cycles]), average energy dissipated (avg ED, μJ [area enclosed by the test’s hysteresis loop from the third to last cycle]), average unloading slope (avg US, N/μm [average unloading slope from 3rd to last cycle]), and average loading slope (avg LS, N/μm [average loading slope from 3rd to last cycle]).

Image 5: Force-Displacement results from cRPI. Image from Granke et al (79).
Microcomputed Tomography

Femoral neck cross sections were imaged by microcomputed tomography (µCT, Scanco Medical, Brütisellen, Switzerland) to assess cortical tissue mineral density (Ct. TMD), and cortical porosity (Ct. Po. %). Images were obtained at 15 µm voxel nominal resolution (X-ray tube current 114 mA, effective energy 70 kV, 300 ms integration time) and segmented using a threshold of 601.8 mgHA/cm³.

Fluorimetric Assay for Advanced Glycation End Products

Lateral femoral neck cross sections were separated into cortical and trabecular bone specimens then defatted in isopropyl ether (three 15-minute washes at room temperature). Samples were lyophilized overnight and hydrolyzed in 6N hydrochloric acid for 20 hours at 110°C. The light and temperature sensitive hydrolysates were stored at -80°C in complete darkness until use.

Hydrolysate fluorescence was measured using a microplate reader (Synergy MX, BioTek, Winooski, VT) at 360/460 nm excitation/emission, and normalized to a quinine sulfate standard. Sample dilutions were determined from pilot assays of randomly selected samples from each batch of hydrolyzed samples. Collagen content was determined by adding chloramine-T solution to the remaining hydrolysates and hydroxyproline standards and incubating for 20 minutes at room temperature to oxidize hydroxyproline. A 3.15M perchloric acid solution was added and incubated
for 5 minutes at room temperature to eliminate residual chloramine-T. A p-dimethylaminobenzaldehyde solution was added and incubated for 20 minutes at 60°C. Samples and standards were then cooled in complete darkness for 5 minutes and absorbance was measured at 570 nm using a microplate reader. Collagen content was calculated from hydroxyproline content, and total AGEs were weighted by this value.

Initially, the experimental protocol called for AGE quantification of the posterior-medial femoral neck, the same portion that had undergone microCT and cRPI analysis. Clinical mishandling of samples prior to research acquisition and inter-user variability of the AGE fluorometric assay, however, necessitated AGE quantification of the posterior-lateral femoral neck for all samples.

**Serum Measurements**

HbA1c measurements were performed by a commercial laboratory (LabCorp). Serum levels of pentosidine (Lifeome BioLabs, Oceanside, CA; ELISA kit # CEA264Ge) and total AGEs (Cell Biolabs, Inc., San Diego, CA; ELISA kit # STA-817) were both measured using commercially available enzyme linked immuno-sorbent assay (ELISA) kits according to manufacturers’ protocols by collaborators at Maine Medical Center. Serum C-terminal peptide (CTX) and procollagen type 1 peptide (P1NP) were measured via ELISA by collaborators at Maine Medical Center. Serum esRAGE was measured via ELISA (B-Bridge International, Cupertino, CA; ELISA kit #K1009-1).
Statistical Analyses

Distributions for all variables were plotted to identify potential outliers. No outliers were identified and distribution were verified as approximately normal or log normal for each continuous variable. Basic demographics and clinical characteristics were calculated for control and diabetic groups and compared via t-test of normal or log transformed data. Differences between control and T2DM for microCT, cRPI, AGE, and serum results assessed using ANCOVA. Covariates included age, gender, race, and BMI, and were verified to have a parallel linear relationship. In the single exception to this validation of ANCOVA assumptions, age was found to have a non-parallel linear relationship with disease status and with BMI. Analysis was repeated in subdivided age cohorts, and found to return no interacting terms under these conditions. Values are reported as unadjusted means, significance was assigned following an unpaired Student’s t-Test of least square means.

Scatter plots were developed using Adaptive Lasso with AICc validation to prevent model overfit. Covariate significance was assessed and regression models were adjusted accordingly. For bone AGE and cRPI values, covariates considered were age, gender, race, BMI, CTX, P1NP, and esRAGE. For microCT values, covariates considered were age, gender, race, BMI, esRAGE, pentosidine and serum AGEs. Of the regression models generated, only 2 returned coefficients, which were discarded as negligible as both were <0.006.

Scatterplots shown are of unadjusted values, correlation was assessed with Pearson correlation coefficient.
Results

Basic Demographics
Subjects undergoing elective total hip replacement (THR) were recruited from BIDMC. 54 subjects in total were recruited (34 control, 20 T2DM). Of these, several bone specimens (10 control, 5 T2DM) were mishandled and unavailable for analysis. Additional exclusions were necessary due to specimen quality and unavailability of the posterior medial femoral neck for imaging and indentation (4 control, 3 T2DM).
In both groups, 55% of subject were male (Table 1). No statistically significant differences in age, height, weight, or BMI were found. Self identified race showed a predominance of Caucasian subjects, although both groups had similar African-American enrollment. Only one Asian subject was enrolled as a control. HbA1c was elevated in T2DM (6.99%) as compared to control (5.70%).
<table>
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<th>Controls (n = 34)</th>
<th>T2DM (n = 20)</th>
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<tr>
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<td>62.26 ± 10.94</td>
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<td>***6.99 ± 1.33</td>
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Table 1: Demographics of the study cohort. Clinical characteristics are reported as averages ± standard deviation. HbA1c was measured at time of enrollment (pre-admission testing, PAT). Diabetes medications reported here were those listed in the patients’ online medical records at BIDMC. *** p < 0.001
## Bone Microarchitecture, Biomechanical Properties, AGEs, and Serum Markers

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<tr>
<td>Cortical tissue mineral density (mgHA/ccm)</td>
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<td>916 ± 47</td>
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<td>Cortical porosity (%)</td>
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### Reference Point Indentation

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<td>Indentation distance (µm)</td>
<td>71.36 ± 18.12</td>
<td>73.41 ± 16.69</td>
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<tr>
<td>Creep indentation distance (µm)</td>
<td>5.88 ± 1.31</td>
<td>6.91 ± 1.50*</td>
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<tr>
<td>Total indentation distance (µm)</td>
<td>80.03 ± 20.10</td>
<td>83.91 ± 19.47</td>
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<tr>
<td>Indentation distance increase (µm)</td>
<td>13.58 ± 4.29</td>
<td>16.45 ± 4.72*</td>
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<tr>
<td>Average energy dissipation (µJ)</td>
<td>19.33 ± 5.35</td>
<td>19.97 ± 5.70</td>
</tr>
<tr>
<td>Average loading slope (N/µm)</td>
<td>0.40 ± 0.06</td>
<td>0.39 ± 0.06</td>
</tr>
<tr>
<td>Average unloading slope (N/µm)</td>
<td>0.53 ± 0.07</td>
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### Advanced Glycation End-Products (AGES)

<table>
<thead>
<tr>
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<th>T2DM</th>
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<tr>
<td>n = 23</td>
<td>n = 18</td>
<td></td>
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<tr>
<td>Cortical bone AGES (ng quinine/mg collagen)</td>
<td>178 ± 53</td>
<td>216 ± 62*</td>
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<tr>
<td>Trabecular bone AGES (ng quinine/mg collagen)</td>
<td>211 ± 60</td>
<td>222 ± 49</td>
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### Serum Markers

<table>
<thead>
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<tbody>
<tr>
<td>n = 23</td>
<td>n = 15</td>
<td></td>
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<tr>
<td>Serum pentosidine (ng/mL)</td>
<td>46.63 ± 11.48</td>
<td>53.53 ± 11.71</td>
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<tr>
<td>Serum AGES (µg/mL)</td>
<td>32.28 ± 9.15</td>
<td>29.17 ± 7.09</td>
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<tr>
<td>Serum esRAGE (ng/mL)</td>
<td>0.232 ± 0.155</td>
<td>0.285 ± 0.188</td>
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<tr>
<td>Serum CTX (ng/mL)</td>
<td>0.361 ± 0.186</td>
<td>0.202 ± 0.092*</td>
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<tr>
<td>Serum P1NP (ng/mL)</td>
<td>67.83 ± 26.10</td>
<td>54.64 ± 29.60</td>
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</table>

Table 2: Structural, mechanical, and biochemical results. Values are reported as averages ± standard deviation, significance calculated via ANCOVA w/ effects from age, gender, race, and BMI accounted for. *p< 0.05 #p ≤ 0.0509

Microarchitecture, biomechanical, and biochemical analyses were performed and analyzed for differences between the control and T2DM groups (Table 2). microCT of the posterior medial femoral neck was performed to assess for cortical porosity and tissue mineral density. No statistically significant
difference between the bone microarchitecture properties of control and T2DM subjects was found.

Biomechanical material properties were assessed via cRPI. Of the values collected, creep indentation distance (CID) and indentation distance increase (IDI) were found to be increased in the cortical bone of T2DM subjects as compared to controls.

Lateral femoral neck specimens were collected and analyzed for AGE content in the cortical and trabecular bone. Cortical AGEs trended higher in T2DM subjects, although this result was not statistically significant (p ≤ 0.0509). No difference was noted between control and T2DM subjects in trabecular AGE content.

Serum AGEs (pentosidine and total AGEs), receptors (esRAGE), and markers of bone turnover (CTX, P1NP) were evaluated via ELISA. Serum levels of AGEs and esRAGE did not differ between control and T2DM subjects. CTX, a marker of bone resorption, was found to be decreased in T2DM subjects as compared to controls.

**Bone and Serum AGEs**

To determine if serum AGEs correlate with bone AGEs, Pearson correlation coefficients were obtained on unadjusted scatter plots following verification of null covariate coefficients for age, gender, race, BMI, CTX, P1NP, and esRAGE. ANCOVA showed no difference between T2DM and control populations in the relation of bone AGEs with their serum counterparts. Cortical AGEs showed a weakly positively correlation with serum pentosidine (r = 0.39, p < 0.05) and weakly positive association with serum
AGEs \((r = 0.28, p \leq 0.087)\). Trabecular AGEs showed a weakly positive correlation with serum pentosidine \((r = 0.34, p < 0.05)\) and serum AGEs \((r = 0.398, p < 0.05)\). No relation was noted between HbA1c and bone AGEs, serum AGEs, or serum pentosidine.
Figure 1: Cortical and trabecular bone AGEs in relation to serum pentosidine and total AGEs. Bone AGEs as measured through a fluorometric assay were plotted against serum values of pentosidine and total AGEs from the same subject. Regression models generated showed no statistically significant covariance from age, gender, race, BMI, esRAGE, CTX, and P1NP. ANCOVA showed no difference in linear regression of control vs T2DM subjects. Scatterplots shown here are of unadjusted values. Pearson correlation shows a positive relationship between bone AGEs and their serum counterparts (pentosidine, serum AGEs).
Bone Biomechanical Properties and AGEs

To determine if AGE measurements correlated with bone biomechanical properties, Pearson correlation coefficients were obtained on unadjusted scatter plots following verification of null covariate coefficients for age, gender, race, BMI, CTX, P1NP, and esRAGE. ANCOVA showed no difference between T2DM and control populations in the relation of cRPI values with AGEs. Cortical and trabecular AGEs showed no correlation with bone biomechanical properties. Serum AGE values were positively correlated with US ($r = 0.415$, $p < 0.05$) and positively associated with average US ($r = 0.320$, $p \leq 0.057$) and average LS ($r = 0.323$, $p \leq 0.055$). Serum AGES were negatively correlated with ID ($r = -0.413$, $p < 0.05$), CID ($r = -0.544$, $p < 0.001$), TID ($r = -0.481$, $p < 0.01$), IDI ($r = -0.577$, $p < 0.001$), average CID ($r = -0.577$, $p < 0.001$), and average ED ($r = -0.481$, $p < 0.01$). Serum pentosidine and bone biomechanical property plots are not shown, as no significant correlation was found.
Figure 2: Bone biomechanical properties in relation to AGEs. Biomechanical properties of the medial femoral neck were measured with cRPI. Cortical and trabecular AGEs were quantified via a fluorometric assay. Serum AGEs were quantified via ELISA. Regression models generated showed no statistically significant covariance from age, gender, race, BMI, esRAGE, CTX, and P1NP. ANCOVA showed no difference in linear regression of control vs T2DM subjects.
Scatterplots shown here are of unadjusted values. Pearson correlation showed no correlation between bone AGEs and cRPI results; serum AGEs showed a positive correlation with US, Average US, and Average LS and a negative correlation with ID, CID, TID, IDI, Average CID, and Average ED. * p<0.05 **p<0.01 ***p<0.001

**Bone Microarchitecture and AGEs**

To determine if AGE measurements correlated with bone microarchitecture properties as measured through microCT, an Adaptive Lasso regression on age, gender, race, BMI, esRAGE, pentosidine, serum AGEs, CTX and P1NP was performed. All variables except CTX returned with a null covariate coefficient.

ANCOVA showed no difference between T2DM and control populations in the relation of microarchitecture values with AGEs.

CTX and P1NP are markers of bone resorption and formation, respectively. To determine their correlation with cortical porosity and TMD, Pearson correlation coefficients were obtained on unadjusted scatter plots following verification of null covariate coefficients for age, gender, BMI, esRAGE, pentosidine, serum AGEs, and bone AGEs. Race returned with a coefficient when the subject was Asian; given only one participant identified as Asian, this was discarded. ANCOVA showed no difference between T2DM and control populations in the relation of microarchitecture values with AGEs.
Figure 3: Bone microarchitecture in relation to bone AGEs and CTX. Bone microarchitecture was assessed via microCT. Cortical and trabecular AGEs were quantified via fluorometric assay. CTX, a marker of bone turnover, was quantified via ELISA. Regression models generated showed no statistically significant covariance from age, gender, race, BMI, esRAGE, pentosidine, and serum AGEs. Cortical porosity and TMD as a function of P1NP are not shown as no significant correlation was noted. ANCOVA showed no difference in linear regression of control vs T2DM subjects. Scatterplots shown here are of unadjusted values. Pearson correlation showed no correlation between bone AGEs and microarchitecture variables. CTX was positively correlated with cortical porosity and negatively correlated with TMD. *p<0.05
Discussion

Comparison of Control and Diabetic Subjects

Cyclic reference point indentation (cRPI) data showed similar material properties between control and diabetic specimens, with the exception of increased creep indentation distance (CID) and indentation distance increase (IDI) in diabetic bone samples. CID, measured as the total indentation distance during the hold on the first indentation cycle, and IDI, measured as the difference in indentation distance between the first and last cycles, have previously been found to positively correlate with each other (80). CID is a measure of the bone’s resistance to change under constant pressure, and it is inversely correlated with crack growth toughness and damage susceptibility (81). IDI is a measure of the bone’s post-yield mechanical properties and has been negatively correlated with crack growth toughness (81,82). Elevated CID and IDI, therefore, correlate with decreased toughness, or increased brittleness, in diabetic bone, and is similar to results reported in a diabetic rat model and consistent with reports of increased fragility in patients with T2DM (11,83).

However, interpretation of cRPI results is still an area of active research, and the argument can be made for the importance of bending and compression tests to provide information beyond the material properties described by cRPI (84). Preservation of the bone sample following biomechanical testing via cRPI allows for further analysis, including the second aim of this project, AGE quantification.
AGE content was increased in cortical bone samples from T2DM subjects, but trabecular bone AGE content was not significantly different between the two groups. Increased cortical AGEs is consistent with a prior report noting increased pentosidine content in the tibias of T2DM men, although this study is limited by its focus on one species of AGE in a mixture of both cortical and trabecular bone (73).

Trabecular AGE findings were similar to those reported by Pritchard et al (85). In their examination of femoral trabecular bone, no differences in pentosidine content were noted between control and T2DM patients (85). Given that trabecular bone is more actively remodeled than cortical bone, differences in AGE content between these anatomic sites may potentially be reflective of short and long term AGE accumulation, respectively (86).

Pritchard et al described femoral samples acquired from elective THR; unfortunately, HbA1c levels for this cohort were not acquired, which would have provided further insight into the recent state of glycemic control for these patients and may provide context regarding differences in bone AGE levels (85).

HbA1c is routinely used as a marker of glycemic control and diagnostic test for diabetic patients, and although it is formed by the non-enzymatic glycation of hemoglobin, it is not an AGE but rather an Amadori product (87). Because the synthesis of AGEs via the Maillard reaction requires Amadori product formation following the non-enzymatic glycation of protein residues by glucose (see Image 2), the state of AGE exposure is sometimes extrapolated from HbA1c levels (88). Although several studies have shown a positive correlation with tissue AGEs and HbA1c, others have
shown no correlation (88,89). HbA1c and bone AGEs were not found to have a correlation in the results reported here, which is likely influenced by the relatively small cohort size and narrow range of HbA1c values.

Aim 3 of this project sought to investigate the relationship of serum markers with bone AGEs. Serum total AGEs and pentosidine were weakly positively correlated with cortical and trabecular AGE levels. Previously, a weak positive correlation was reported between serum pentosidine and cortical pentosidine in femoral bone specimens; this study differs notably from the project described here by the exclusion of diabetic subjects and the focus on pentosidine (90). Pentosidine is but one of several dozen AGEs, however it is among the most commonly quantified individual AGEs in bone, despite its weak correlation with total AGEs (76,91-93). In this project, the AGEs quantified in cortical and trabecular bone included all fluorescent AGE species, including pentosidine, and it is the first report on total serum and bone AGE correlation in a cohort including T2DM patients.

**Correlations of Bone AGEs, Serum AGEs, and Bone Biomechanical Properties**

Our results may suggest that bone AGE content could be inferred by serum total AGEs and pentosidine content, however further validation of this weakly positive correlation in a small group of patients is necessary. Additionally, the relationship between AGEs and bone biomechanical and microarchitecture properties requires further investigation. In this study, bone AGEs were not related to cRPI and microCT results. Previously, in vitro incubation of trabecular bone cores with a ribosylating sugar found a
correlation between increased AGE content and increased propensity to fracture (94). Although cRPI data on cortical bone cannot be directly compared to trabecular compression testing, no correlation between bone AGE content and cRPI values was unexpected. Of note, average AGE levels for the in vitro results reported previously were significantly elevated than the endogenous values reported here (94). Interpreting the physiological relevance of the results reported here, however, must be done in the context of the study cohort size and tight glycemic control. Further characterization in a larger group with a broader range of diabetic disease severity may reveal a correlation between bone AGEs and biomechanical properties that was not observed here.

Serum AGEs were negatively correlated with indentation measures (IDI, CID, total indentation distance (TID), and indentation distance increase (IDI)). These results were unexpected. This study noted an increase in CID and IDI in diabetic patients, and although there was no increase seen in serum AGEs in the diabetic cohort, previous reports have noted this (95). Furthermore, prior reports in the literature have shown decreased bone mineral strength index (BMSi) from in vivo RPI to be positively correlated with skin AGEs (83). Extrapolating from these results and the prior reports, serum AGEs were thought to likely correlate positively with indentation measures.

Additionally, serum AGEs were shown here to have a positive correlation with loading slope (LS) and unloading slope (US), properties that describe material stiffness. Increased serum AGEs, therefore, correlate with increased
bone stiffness. This correlation also ran counter to expectations, as decreased US was described in the femurs of a diabetic rat model (11).

The correlations between serum AGEs and cRPI values would seem to suggest that increased serum AGEs correlates with improved material properties and thus a greater resistance to fracture. The correlation of serum AGEs with bone AGEs would seem to indicate a direct effect of circulating AGEs on the composition of bone, however no correlations between bone AGEs and cRPI were noted.

AGEs elicit their affects through more than one manner, however. Direct AGE accumulation and material properties were studied in this project, but AGEs also activate cell signaling pathways through its receptor, RAGE. In bone, RAGE signaling is necessary for osteoclast differentiation, and upregulation of RAGE has been reported in osteoblast differentiation (53).

Currently evolving is our understanding of how RAGE signaling impacts bone remodeling through its actions on osteoclasts and osteoblasts.

Modulating the effect of AGEs on RAGE signaling are circulating sRAGE and esRAGE, which act as decoy receptors for some species of AGEs, and previous reports in the literature note the esRAGE/pentosidine ratio is predictive for fracture risk in T2DM patients (96). In the results reported here, serum esRAGE was quantified and evaluated as a covariate for the relationship between cRPI values and serum AGEs, but no significant interaction was noted on regression analysis. Unaccounted variables, however, include RAGE and sRAGE expression. Variations in the expression and proteolysis of RAGE could confound the relationship between serum
AGES and bone function, particularly in this subset of patients with a relatively narrow distribution of serum pentosidineline and AGES. Future investigation of serum AGES, bone AGES, bone biomechanical properties, and bone microarchitecture can be more rigorous through cohort expansion and inclusion of patients with less controlled T2DM. Surgical subject recruitment following the same model as described here could accommodate the former, however inclusion of a significant number of patients with poorly controlled T2DM would be difficult given the elective nature of the procedure. Fortunately, characterization of serum AGES, serum pentosidine, skin AGES, and bone mechanical and microarchitecture properties can be performed in a minimally invasive manner (71,95,97-99). Although removal of the surgical component in study design would eliminate the chance to quantify bone AGES, the advantages to transitioning future studies to a minimally invasive design would allow greater inclusion of poorly controlled T2DM and longitudinal characterization of in vivo bone health with serum AGES. Alternatively, future investigations in patients recruited from elective orthopedic surgeries would continue to allow for direct quantification of bone AGES. To investigate the role of RAGE expression and signaling, processing of harvested samples for marrow sera, isolation of bone marrow mesenchymal stem cells (BM-MSCs) via fluorescence activated cell sorting (FACS), and preservation of bone mRNA should be considered. Analysis of marrow sera for soluble factors would allow for a local characterization of potential upstream effectors of RAGE, such as sRAGE and esRAGE, and potential downstream effects, such as IGF-1, sclerostin, and RANKL.
Isolation of BM-MSCs has previously been described and would provide the opportunity to measure cellular expression of RAGE in osteoblast precursors (102) In addition to BM-MSCs, expression of RAGE in bone can be quantified via qRT-PCR following isolation of RNA from fresh bone samples (103).

**Strengths and Limitations**

The results of this project should be considered in the context of several limitations. Previously reported differences in diabetic subjects include increased cortical porosity at the tibia and radius, decreased serum esRAGE, increased serum pentosidine, and increased serum AGEs\((71,95,98,99)\). The absence of any significant difference for these values between diabetic and control subjects suggests the diabetic cohort collected may not be representative of the wider diabetic population. Recruitment of diabetic and control subjects was performed from patients undergoing elective total hip replacement (THR), and as such, diabetic patients showed well controlled blood glucose with relatively low HbA1c values.

Additionally, all subjects recruited were known to have osteoarthritis (OA), necessitating their THR. OA may cause sclerosis of the subchondral bone of the femoral head, which would impact bone density measurements \((104)\). This study sought to minimize effects from OA by analyzing bone form the femoral neck, which is minimally impacted by the disorder \((104,105)\).
Conclusions

In conclusion, this project demonstrated increased indentation distances in cortical bone from diabetic subjects as compared to non-diabetic controls. Notably, this is the first time serum total AGEs and pentosidine have been shown to correlated with total bone AGEs in a cohort including T2DM subject; additionally, cortical bone from T2DM subjects was also found to have increased AGE accumulation. Although bone AGE values were not correlated with cRPI and microCT findings, further work is needed to elucidate the underlying relationships between AGEs and skeletal fragility.
Acknowledgments

Funding for this work was provided by NIH R21AR070366. This project was only possible with the gracious guidance and help from Mary Bouxsein, Lamya Karim, and Julia Moulton. Patient recruitment and AGE quantification was performed by Julia Moulton and the author. MicroCT and cRPI was performed by Julia Moulton with contributions from the author. ELISAs at BIDMC and all statistical analyses were performed by the author. Drs. Ayres and Abdeen from BIDMC were essential collaborators in facilitating patient contacts and specimen acquisition. Dr. Breggia from Maine Medical Center graciously measured serum AGEs and pentosidine. Special thanks to everyone at the Center for Advanced Orthopedic Studies for a warm, collaborative, and fun environment, and to John Dyer for acting as a sounding board of all things statistical.
<table>
<thead>
<tr>
<th>Term</th>
<th>Definition</th>
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<td>AGE</td>
<td>Advanced Glycation End-product</td>
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<tr>
<td>ANCOVA</td>
<td>Analysis of Covariance</td>
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<td>HPLC</td>
<td>High Performance Liquid Chromatography</td>
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ID: Indentation Distance
IDI: Indentation Distance Increase
IGF1: Insulin-like Growth Factor 1
MR: Magnetic Resonance
mRNA: Messenger RNA
MSC: Mesenchymal Stem Cell
NAFLD: Non-alcoholic Fatty Liver Disease
OA: Osteoarthritis
PiNP: Procollagen Type 1 Peptide
qRT-PCR: Quantitative Reverse Transcription Polymerase Chain Reaction
RAGE: Receptor for Advanced Glycation End-products
RANKL: Receptor Activator of Nuclear Factor Kappa B
sRAGE: Soluble Receptor for Advanced Glycation End-products
T2DM: Type 2 Diabetes Mellitus
THR: Total Hip Replacement
TID: Total Indentation Distance
TMD: Tissue Mineral Density
TZD: Thiazolidendiones
μCT: MicroCT or MicroComputed Tomography
Citations


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