HISTOMORPHOMETRIC ANALYSIS OF BONE IN HEALTHY, YOUNG PATIENTS WITH TYPE 1 DIABETES MELLITUS

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Patients with Type 1 Diabetes Mellitus (DM) have markedly increased risk of fracture, but little is known about abnormalities in bone micro-architecture or remodeling properties that might give insight into the pathogenesis of skeletal fragility in these patients. This is a case-control study comparing bone histomorphometric results from iliac biopsies in 18 otherwise healthy subjects with Type 1 Diabetes Mellitus with those from healthy age- and sex- matched non-diabetic control subjects. Five of the diabetics had histories of low-trauma fracture. Transilial bone biopsies were obtained after tetracycline labeling. The biopsy specimens were fixed, embedded, sectioned and quantitative histomorphometry was performed as previously described (Recker et al., 1988). Two sections, >250 μm apart, were read from the central part of each biopsy. Overall there were no significant differences between diabetics and controls in histomorphometric measurements. However, fracturing diabetics had structural and dynamic trends different from nonfracturing diabetics. In conclusion, Type 1 Diabetes Mellitus does not result in abnormalities in bone histomorphometric variables in the absence of manifest complications from the diabetes. However, diabetics suffering fractures may have defects in their skeletal microarchitecture that may underlie the presence of excess skeletal fragility.
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LIST OF ABBREVIATIONS

Ac.F = activation frequency
BCE = bone collagen equivalent
BFR/BS = bone formation rate (bone surface referent)
BFR/BV = bone formation rate (bone volume referent)
BMD = bone mineral density
BSAP = bone specific alkaline phosphatase
BV/TV = bone volume
CTX = C-telopeptide
DXA = dual-energy x-ray absorptiometry
ES/BS = eroded surface (bone surface referent)
FP = formation period
FSH = follicle stimulating hormone
GAD = glutamic acid decarboxylase antibodies
GFR = glomerular filtration rate
HgA1c = glycated hemoglobin
IGF-1 = insulin like growth factor
MAR = mineral apposition rate
μCT = micro-computed tomography
Mlt = mineralization lag time
MS/BS = mineralizing surface (bone surface referent)
MS/OS = mineralizing surface (osteoid surface referent)
NOD = non obese diabetic
NTX = crosslinked N-telopeptide, type 1 collagen

Ob.S/BS = osteoblast surface (bone surface referent)

OS/BS = osteoid surface (bone surface referent)

O.Th = osteoid thickness

PTH = parathyroid hormone

pQCT = peripheral quantitative computed tomography

Rm.P = remodeling period

RP = resorption period

Scr = serum creatinine

Tb.N = trabecular number

Tb.Sp = trabecular separation

Tb.Th = trabecular thickness

TIBM = Type 1 Diabetes Mellitus

W.Th = wall thickness

1,25 (OH)2D = 1,25- dihydroxyvitamin D

25(OH)D = 25-hydroxyvitamin D
INTRODUCTION

Diabetes Mellitus is a chronic disease with many complications. One of the most recently recognized complications is the propensity of these patients to fracture. Little is known about the mechanisms that lead to decreased bone strength. Few well-controlled human studies have been done; hence, the goal of this thesis is to evaluate critically the existing literature and present new data in this field.

REVIEW OF THE LITERATURE

Type 1 Diabetes Mellitus

Diabetes Mellitus is a group of diseases characterized by high blood glucose levels due to insulin deficiency (Type 1) or insulin resistance (Type 2) (Unger, 1998). Type 1 Diabetes Mellitus (T1DM), previously known as Juvenile Diabetes, is an autoimmune disorder which destroys the pancreatic β cells that produce insulin. It is most commonly diagnosed in children or young adults, but can occur later in life. T1DM accounts for 5-10% of all cases of diabetes (US Department of Health and Human Services 2011). Because of the young age of disease onset, these patients live for many years with both the disease and the many disease complications that may arise. T1DM complications are classically confined to the vascular system. Microvascular disease leads to nephropathy, retinopathy and neuropathy (US Department of Health and Human Services 2011). Macrovascular disease leads to ischemic heart disease, cerebrovascular and peripheral vascular disease (US Department of Health and Human Services 2011). The risk of the disease to the skeleton in the form of increased fracture risk has only recently been explored.
Fracture Risk

Diabetes as a risk factor for fracture has been documented in the literature (Ahmed et al., 2006; Forsen et al., 1999). Although both T1DM as well as T2DM have been associated with an increased risk of fracture (Forsen et al., 1999; Vestergaard, 2007), this review will be focused on reports of T1DM risk. Two recent meta-analyses found an increased risk of hip fracture in patients with Type 1 DM (Janghorbani et al., 2007; Vestergaard, 2007). Janghorbani and colleagues reviewed one case-control and five cohort studies of T1DM all of which had found increased risk of hip fracture and calculated a summary RR of 6.3 (95% CI 2.6-15.1) for hip fracture in T1DM (Janghorbani et al., 2007). Vestergaard reviewed 5 of the same studies and found similar results with a RR of 6.94 (95% CI 3.25-14.78) for hip fracture in patients with T1DM (Vestergaard, 2007). One of the cohort studies, the Iowa Women’s Health Study found the highest risk for fracture; 12.25 times higher in postmenopausal women with T1 DM (Nicodemus & Folsom, 2001). Among the other cohort studies, one found an increased risk of any fracture; not just hip fracture (Ahmed et al., 2006). Some studies found that the presence of other diabetic complications (including retinopathy, neuropathy, nephropathy and cardiovascular disease) increased the risk for fracture (Ahmed et al., 2006; Miao et al., 2005). The largest cohort study was from the Swedish Inpatient Register in which researchers followed 24,605 relatively young T1DM patients (mean age 21 years) who were hospitalized for their diabetes. After an average of 10 years of follow-up, 121 had sustained their first hip fracture. RR for hip fracture was 7.6 (95% CI 5.9-9.6) in men and 9.8 (95% CI 7.3-12.9) in women compared to the age and sex matched Swedish population. Those hospitalized at any time with diabetic complications were at
particularly high risk for hip fracture (17-42 times the expected rate) (Miao et al., 2005).
A population survey of the Norway population (Tromsø study) identified 81 validated cases of Type 1 Diabetes in their survey. Of these, 8 fractures were reported. The age adjusted RR for all non vertebral fractures was approximately 3.0 for both men and women with Type 1 DM. The age adjusted RR for hip fracture was 17.8 (95% CI 5.6-56.8) in men with T1DM and 8.6 (95% CI 1.2-61.5) in women with T1DM. Adjustment for confounding variables had no effect on the results (Ahmed et al., 2006). The Nord-Trøndelag Health Survey, another survey of the older (50-74 years of age) population in Norway, identified 51 subjects with T1DM. Four fractures were identified. In this study, the age adjusted RR for hip fracture was 5.7 (95% CI 1.8-17.9) in women while it was not statistically significantly higher in men. The epidemiological studies contain no information about the circumstances surrounding these fractures or their morphology. The causative factors for these increased fractures is not known, but bone mineral density (BMD) is one measurable risk factor that can be ascertained.

**Bone Mineral Density in Type 1 Diabetes Mellitus**

Bone mineral density (BMD) as measured by dual-energy x-ray absorptiometry (DXA) is the gold standard for assessing the skeleton in typical postmenopausal osteoporosis. The diagnosis of osteoporosis in postmenopausal women and elderly men is based on a comparison of BMD (measured by DXA) to the average BMD of young adults at the time of peak bone mass. This is defined as the T-score. A T-score >2.5 SD below the mean peak bone mass defines osteoporosis while a T-score >1.0 SD and <2.5 SD below the mean peak bone mass defines osteopenia (World Health Organization Technical Report Series, 1994). The bone field is moving away from using the term “osteopenia” to
describe lower bone mass, but the literature related to bone mass in diabetes uses this term freely. This is because the relationship between T-scores and fracture risk is not a threshold relationship, but rather a non-linear one with the risk rising markedly as a function of both age and BMD. I will use the T-score to describe bone mass measurements, whenever possible, and avoid using terms such as “osteoporosis” and “osteopenia” unless I am specifically referring to postmenopausal osteoporosis in which fracture risk related to bone mass is well described. Longitudinal studies have shown that in postmenopausal women and elderly men relative risk of fracture doubles for every 1.0 reduction in T-score (Marshall et al., 1996). There are other factors such as increasing age and co-morbidities that play a strong role in predicting fracture risk, as T-score alone does not nearly account for all risk of fracture. However, DXA is a noninvasive, clinically available tool that has been used widely to measure BMD in a variety of patients of both sexes, of all ages and ethnicities and with multiple co-morbidities including Type 1 DM. It is the best tool we have to estimate risk of fracture.

Many small studies examining BMD in Type 1 DM have been done in both children and adults with the diagnosis (Chobot et al., 2010; Mastrandrea et al., September 2008; Saha et al., 2009; Strotmeyer et al., 2006). While many of them found overall somewhat lower BMD in T1DM compared to controls (Mastrandrea et al., September 2008; Strotmeyer et al., 2006), some did not see a difference, perhaps because of a small sample size (Bridges et al., 2005; Hampson et al., 1998). Vestergaard conducted a meta-analysis of 34 studies that included BMD measurements of Type 1 Diabetes, and concluded that BMD of both the hip and spine were lower in Type 1 DM (Vestergaard, 2007). In some of these studies, lower BMD was linked to microvascular complications (Clausen et al., 1997;
Rozadilla et al., 2000). Vestergaard used the observed BMD to calculate an expected relative fracture risk. The expected risk was 1.42 which is quite different from the relative risk he observed of 6.94 (Vestergaard, 2007). Because the diabetics are fracturing at a younger age than the general population (Miao et al., 2005), this leads us to believe there are significant factors that lead to fracture in Type 1 DM other than BMD.

**Glucose Control and Disease Duration and Relationship to Bone Mineral Density**

The epidemiologic studies do not record glucose control and cannot easily assess disease duration. It is more difficult to understand the role of glucose control or disease duration in increasing fracture risk. Only one epidemiologic study found an effect of disease duration on fracture risk but it was a retrospective cohort study and could not differentiate between Type 1 DM and Type 2 DM (Leslie et al., 2007). Given the higher incidence of Type 2 DM in the population this was likely mostly patients with Type 2 DM and it may not be applicable to Type 1 DM. However, smaller studies assessing bone mineral density have been able to obtain current glucose control with HgA1c measurement and accurately document disease duration. The results are not consistent, possibly due to the small numbers of subjects, and the wide range of ages of the subjects.

A study in 27 children with Type 1 DM (mean age 13 years) observed a negative correlation between lumbar BMD and HgA1c and disease duration. In contrast, another study of 55 children with Type 1 DM (mean age males-10 years, females-11 years) showed no correlation between BMD and HgA1c values or disease duration (Pascual et al., 1998). Another study of 39 girls (mean age 16 years) with Type 1 DM also showed no correlation between BMD and HgA1c or disease duration (Liu et al., 2003).
In adults, a case controlled longitudinal study of premenopausal women (mean age 28 years) with Type 1 DM found a correlation between low BMD at heel and forearm and poor glycemic control (using current HgA1c measures as well as a 10 year average of control) (Danielson et al., 2009). This has not been observed in cross sectional studies. For example, in 35 otherwise healthy Type 1 DM patients in the Netherlands, BMD was lower than matched controls but no correlation was seen with HgA1c levels (Kemink et al., 2000). The same findings were seen in 35 men with Type 1 DM (mean age 49.3 years) (Bridges et al., 2005) and in 31 females with Type 1 Diabetes (mean age 42 years) (Hampson et al., 1998).

Another cross sectional study of 32 young adults at the time of diabetes diagnosis found lower BMD in the diabetics but no correlation with HgA1c values (Lopez Ibarra et al., 2001). This is somewhat unexpected if we suppose that bone density decreases with disease duration.

Many of these studies had negative findings, but were small and under powered for finding an effect of glucose control or disease duration. The difficulty with finding an effect of glucose control is that if it has any effect it is likely long term with bone mass decreasing slowly over time. HgA1c represents average glucose control over the past 2-3 months, but does not give any indication of long term glucose control. The one longitudinal study that measured HgA1c over 10 years and was able to get a 10 year average did find an effect on BMD (Danielson et al., 2009). So, theoretically, while hyperglycemia should be detrimental to bone, it is difficult to show in vivo, and may require long term studies to prove.

**Bone Remodeling**
As bone mineral density does not explain much of the fracture risk in Type 1 Diabetes or even in typical postmenopausal osteoporosis patients, we must examine other factors that play a major role in fracture risk such as rates of bone remodeling or turnover.

Remodeling of bone is a constant activity throughout life needed for the purposes of replacing old, fatigued, damaged bone with new bone and for providing access to the skeletal stores of calcium and phosphorous. Modeling is a similar but separate activity occurring mainly during growth that shapes a growing bone as it elongates (Frost, 1983). Bone remodeling is accomplished in four distinct phases: activation, resorption, reversal, and formation. Activation is the first phase in which osteoclast precursors are recruited to a specific site on bone, and seal themselves to the bone matrix. During the resorption phase, osteoclasts dissolve and digest the bone matrix, leaving cavities called Howship’s lacunae (Parfitt, 2002). These temporary cavities can cause focal weakness in the bone. During the reversal phase signals are sent to recruit osteoblasts to the bone cavity. During the formation phase, osteoblasts lay down an organic matrix and trigger mineralization of bone (Parfitt, 2002). This process of resorption and formation is coupled in remodeling to ensure that formation will follow resorption and maintain a constant amount of bone mineral.

Various hormones and cytokines can influence the rate of remodeling. The most well known hormone to influence this process is estrogen (Recker et al., 2004). Menopause and the concomitant loss of estrogen results in increased bone remodeling. The remodeling rates double resulting in an increase in fracture risk, independent of BMD (Garnero et al., 1996; Garnero, 2000; Recker et al., 2004). The association of increased rates of bone remodeling and increased fracture risk recently has emerged, in great part
because antiresorptive medications have reduced fracture by a much greater degree than can be accounted for by a gain in bone mass alone (Heaney, 2003). One example comes from fitting the MORE (Multiple Outcomes of Raloxifene Evaluation) trial data to a model which showed that raloxifene (a selective estrogen receptor modulator) decreased fracture risk by 30-40% before any increase in bone mass (Sarkar et al., 2002). So theoretically, an increase in remodeling could potentially play a role in increasing fracture risk in Type 1 Diabetics as well, but this is not the current prevailing theory.

While it is evident that an increase in bone remodeling rate is detrimental to the skeleton, there is also evidence that a decrease in remodeling is harmful. The most extreme example is radiation necrosis in which the bone cells are dead due to radiation and there is no remodeling activity (Abbott et al., 1996). Another more recent, but less extreme, example is that of atypical fractures of the femur associated with bisphosphonate treatment (Lenart et al., 2008). These fractures are unique in that they are subtrochanteric and are potentially caused by suppressing remodeling to such a degree that damage cannot be repaired (Lenart et al., 2008). Low remodeling rates have been postulated to be the cause for fractures in diabetes (Krakauer et al., 1995).

**Treatment options**

The importance of understanding the remodeling rate is never so apparent as when considering treatment options. The two classes of treatment for postmenopausal osteoporosis include antiresorptive or anabolic drug categories; based on their action on bone remodeling (Delmas, 2002). The antiresorptives, which include bisphosphonates, selective estrogen receptor modulators, and denosumab, act by decreasing the osteoclasts action; albeit by different mechanisms (Recker & Armas, 2011). Their overall effect is to
decrease bone remodeling. Teriparatide is the only currently commercially available anabolic agent which increases bone remodeling as well as modeling (Lindsay et al., 2007; Quattrocchi & Kourlas, 2004). A choice of treatment for diabetic patients with fractures requires knowledge of the underlying bone remodeling rate. A high rate of remodeling would be better treated by an antiresorptive while a low rate of remodeling would be best treated by an anabolic agent. If remodeling rates are not the underlying problem with diabetic bone disease, a new explanation for etiology and treatment is warranted.

**Biomarkers of Bone Turnover or Remodeling in Type 1 Diabetes**

Low remodeling rates are a tenable cause for the increase in fracture in diabetic humans. The difficulty arises in assessing remodeling *in vivo*. One method that has been widely used is to measure products of the processes of bone resorption and formation which liberate bone mineral, collagen and collagen byproducts into the circulation (Vasikaran et al., 2006). Many studies of diabetic bone disease have used serum or urine biomarkers of bone remodeling as a noninvasive way of assessing remodeling (Fassbender et al., 2009; Gerdhem et al., 2005). These studies have had varied results. When compared to healthy controls, some studies have reported increased bone resorption markers (Fassbender et al., 2009) and others have shown decreased bone resorption markers (Gerdhem et al., 2005). There are also reports of both increased and decreased bone formation markers (Lumachi et al., 2009; Miazgowski & Czekalski, 1998). Interestingly, other studies have observed no difference in bone markers between diabetics and controls (Liu et al., 2003; Valerio et al., 2002). This lack of consistent data from bone remodeling markers just adds confusion to the field. The reasons for these disparate findings between laboratories are
that bone markers are subject to marked intra- and inter-assay variability as well as individual variability because of diurnal variation in excretion of the markers. The variability in urine-based markers can be between 20-30% and the variability in serum-based markers between 10-15% (Camacho & Kleerekoper, 2006) making interpretation of marker data fraught with difficulty.

**Fluorochrome Labeling of Transiliac Bone Biopsies**

A more direct measure of bone remodeling in humans can be made from transiliac bone biopsies after labeling with an oral fluorochrome labeling agent given in a split dose dosing regimen. The fluorochrome label deposits at the sites of active bone mineralization and allows rates of change to be determined from measurement of the fluorescent labels (Frost, 1963). This transiliac biopsy and labeling method allows direct measurements of static structure and calculation of dynamic variables (Parfitt et al., 1987). These variables will be defined in detail in the methods section. The transiliac site is used because the bone specimen is relatively easily obtained, and the procedure is less invasive and less dangerous than obtaining a rib biopsy, well tolerated by patient volunteers, and can be done in outpatient surgical centers under moderate sedation (Rao, 1983). The transiliac bone is mainly comprised of trabecular bone, and the remodeling is representative of remodeling at similar skeletal sites such as the vertebral body (Mellish et al., 1991).

**Histomorphometry in Humans with Diabetes Mellitus**

Despite the advantages of using histomorphometric analysis in humans, there are limited data on human bone histomorphometry in diabetic patients. There has only been one study which reported transiliac bone biopsies on diabetic humans (Krakauer et al., 1995).
Of the eight diabetic patients biopsied, only two had Type 1 DM. From these 8 bone biopsies, decreased bone formation rate and decreased osteoblasts (bone forming cells) were observed compared to an existent set of histomorphometric data from premenopausal females. The authors concluded from these limited data that diabetics have low bone remodeling (Krakauer et al., 1995). Low remodeling rates could lead to fracture by not repairing small damaged areas in the bone. This damage could accumulate until the bone becomes fragile enough to fracture. While a tenable theory, this small study cannot definitively represent the remodeling status of bone in Type 1 DM.

**Animal Models of Type 1 Diabetes Mellitus**

The best support that the underlying bone remodeling status is low in diabetes comes from rat and mice studies. The three most common models are the diabetic BB (BioBreeding) rat; the streptozotocin induced diabetic rat and the non-obese diabetic (NOD) mouse model. The diabetic BB rat is a model of spontaneous diabetes (Crisá et al., 1992) while the streptozotocin chemically destroys the pancreatic B cells, inducing diabetes (Lampeter et al., 1989). The NOD mouse is another model of spontaneous diabetes (Kolb, 1987). Many histomorphometric studies in these models have shown decreased bone formation (Verhaeghe et al., 1989; Verhaeghe et al., 1990) and decreased numbers of osteoblasts and osteoclasts (Goodman & Hori, 1984). These studies have also shown decreased osteoid surface (Hamada et al., 2007). Overall, a decrease in bone remodeling rate has been found, possibly as a result of the effects of diabetes and/or hyperglycemia on the bone forming and resorbing cells or their precursors. Alternatively, microvascular complications could directly affect bone forming and resorbing cells.
While these animal studies have not examined microvascular complications, other researchers have reported that the degree of hyperglycemia strongly correlates with poor healing of an induced bone defect in mice (Follak et al., 2004a). Decreasing the hyperglycemia, in turn, improves fracture healing (Follak, et al., 2004b; Follak et al., 2005).

**Cellular Effects of Hyperglycemia**

There are many theories on the potential mechanisms underlying diabetes bone disease and this section is by no means an exhaustive review. The main metabolic effect of Type 1 DM is hyperglycemia due to an absolute insulin deficiency. Hyperglycemia can have direct and indirect effects on the cells which are responsible for bone formation and bone resorption (i.e. osteoblasts and osteoclasts). There is potential for an effect on osteocytes as well, but this area has not been well explored. The effect of hyperglycemia on osteoblasts and osteoclasts has been defined. Hyperglycemia results in hyperosmolality. In response to this, virtually all cells lose some water and shrink. (Kwon et al., 1995) The cells must adapt to this by modulating ion transportation and/or changing metabolic pathways. (Kwon et al., 1995)

**Osteoblasts**

Zayzafoon et.al. demonstrated that osteoblasts in cell culture (MC3T3 –E1 cells) decreased osteocalcin expression while increasing expression of collagen 1 and c-june under acute (24°) hyperglycemic hyperosmolality. (Zayzafoon et al., 2000; Zayzafoon et al., 2002) Botolin et al showed that chronic hyperglycemia of osteoblasts in vitro increased their alkaline phosphatase production while decreasing osteocalcin, VEGF, GAPDH, and MMP-13 expression. (Botolin et al., 2006) This was not due to
hyperosmolar conditions. This suggests that hyperglycemia may directly affect bone formation by osteoblasts.

**Osteoclasts**

Osteoclasts (OC) are important for bone resorption and lack of OC number or activity could lead to low levels of bone remodeling. Cell work using the murine monocytic cell line RAW 264.7, showed that high glucose levels inhibited their differentiation to mature osteoclasts and inhibited RANK-L signaling. (Wittrant et al., 2008) Hyperglycemia may decrease bone remodeling by inhibiting both osteoclast differentiation and function in diabetes. (Wittrant et al., 2008) It is probable that all bone cells are effected to some degree by the fluctuations in glucose level that are so common, especially in Type 1 DM. Further cellular research is important to find exactly what mechanism(s) are contributing to bone fragility.

So in conclusion, we know that Type 1 diabetics have an increased risk of fracture, we know that BMD does not explain all of this risk, and the rate of bone remodeling and bone microarchitecture may explain more of the risk. Treatment or prevention regimens must address the underlying bone problem in diabetic humans in order to decrease fracture. These regimens cannot rationally be developed or tested without a thorough understanding of the underlying problem.
STATEMENT OF THE PROBLEM AND HYPOTHESIS

We have little, if any, knowledge of the bone remodeling rate and bone microarchitecture in humans with Type 1 Diabetes Mellitus. The diabetic complications can cause bone disease in and of themselves irrespective of the duration or control of the disease. This study addresses these questions through histomorphometric analysis of transiliac bone biopsies in Type 1 DM patients who do not have microvascular or macrovascular complications. There are several methods that can quantify bone structure including 2D micro-computed tomography (μCT) measurements of iliac biopsies (which correlates well with histomorphometric measurements) (Akhter et al., 2007) and peripheral quantitative computed tomography (pQCT) (Saha et al., 2009). However, histomorphometric analysis of fluorochrome labeled specimens is the only way to make accurate measurements of dynamic bone tissue remodeling. Serum measurements of bone turnover markers have several problems which we have addressed previously, and while they are helpful in some instances (for example, in a clinical trial to show suppression of resorption markers with an agent such as a bisphosphonate) they are less helpful in quantifying bone remodeling in a population such as this one. Bone histomorphometry has been chosen to describe bone remodeling and bone structure in this study.

The primary hypothesis is that activation frequency (a measure of bone remodeling activity) will be lower in young, otherwise healthy subjects with Type 1 Diabetes Mellitus compared to age- and sex- matched nondiabetic control subjects.

Specific Aims

1. To recruit and enroll 15 Type 1 diabetic subjects and 15 healthy age- and sex- matched controls.
2. To perform a transilial biopsy on each subject after tetracycline fluorochrome labeling.

3. To quantify bone remodeling and microstructure by standard bone static and dynamic histomorphometry on biopsy specimens. To quantify bone structure further by performing DXA of spine, hip and total body bone mineral.

4. To gather general health information from the subjects on diabetes duration, treatment, lifestyle and dietary factors, and family history that may influence bone health, and to perform clinical laboratory measures including serum 25(OH)D, creatinine, glycated hemoglobin, and IGF-1 that will be used in interpreting the data.

**Long term Goal**

My long-term goal is to decrease fractures in patients with Diabetes Mellitus. In order to achieve this, bone remodeling rates and bone micro-structure must be measured in humans with Type 1 DM.
MATERIALS AND METHODS

**Design:** This was a cross-sectional, case-control study of bone histomorphometric variables from iliac biopsies in otherwise healthy subjects with Type 1 DM compared with the same data from healthy age- and sex- matched non-diabetic control subjects.

**Subjects:** A convenient sample of 23 Type 1 DM Caucasian subjects (8 M, 15 F) and an equal number of Caucasian age-matched (within 5 years) and sex-matched control subjects were recruited from the community through newspaper, TV, radio and website advertising. In compliance with the World Medical Association Declaration of Helsinki – Ethical Principles for Medical Research Involving Human Subjects, all subjects provided a signed written consent approved by the Creighton University Institutional Review Board. (Appendix)

The diabetic subjects had a diagnosis of Type 1 DM clinically defined as onset before age 50, with an acute presentation or diabetic ketoacidosis, and with normal body mass index (BMI). If history was equivocal, glutamic acid decarboxylase (GAD) antibodies > 1.45 U/mL were used to confirm the diagnosis. All diabetic subjects were on insulin treatment. Glycated hemoglobin (HgA1c) was used to determine glucose control before the biopsy. Good health of all subjects was determined by medical history, clinical examination and blood and chemical analysis including: complete metabolic panel, thyroid stimulating hormone, intact parathyroid hormone, phosphorus, 25-hydroxyvitamin D, and transglutaminase antibody titers. Each subject’s weight and height were measured. Height was measured in triplicate and the average used using a Harpenden stadiometer (Seritex, Inc., Carlstadt, NJ). Fracture history was obtained by self-report. Non-traumatic fracture or fractures were defined as less than or equal to a fall from a standing height (digit and
Facial fractures were not included. Activity levels were assessed using a validated Baecke questionnaire, a recording tool which measures qualitative and quantitative indices, addressing dimensions such as occupational, leisure and sport related physical activities (Baecke et al. 1982). All the female patients were premenopausal defined as having regular monthly periods with a follicle stimulating hormone (FSH) level within the normal laboratory range (< 22.5 mIU/mL). None of the subjects were on medications that are known to interfere with bone metabolism including steroids, anticonvulsants, diuretics, bisphosphonates, metformin, and glitazones. The subjects were excluded if they had impaired kidney function defined as a calculated glomerular filtration rate (GFR) less than 60 mL/min/1.73m², a history of cancer other than skin cancer, unstable angina, myocardial infarction, uncontrolled hypertension, untreated hypothyroidism, hyperthyroidism, malabsorption, metabolic bone disease, active rheumatoid arthritis or collagen disease. Of the 23 subjects that were recruited, 1 subject’s biopsy was not obtained because of obese body habitus, 2 subjects were found to have an excluding diagnosis (celiac disease and renal failure respectively) and 2 subjects did not have comparator control data available at the time of analysis, leaving 18 pairs to be reported.

**Analytical Methods**

**Bone Mineral Density**

Bone mineral density and bone mineral content measurements were made by dual energy X-ray absorptiometry (DXA) with a Hologic 4500 instrument (QDR4500A model, Hologic Inc. Waltham, MA). The anterior/posterior spine, whole body and left hip were measured. The densitometer was operated by certified radiological technicians and the coefficient of variation for repeated measures of BMD was 1.0%
Laboratory Analytical Methods

Glycated hemoglobin (HgA1c) was measured in our clinical laboratory using Roche Integra 700 (Roche Applied Sciences, Indianapolis, IN) and Beckman Coulter UniCel 600i DxC analyzers (Beckman Coulter, Brea, CA). FSH was measured in our clinical laboratory using the Beckman Coulter UniCel 600i DxC (Beckman Coulter, Brea, CA). Serum creatinine, calcium and phosphorus were measured in our clinical laboratory using Roche Integra 700 analyzers (Roche Applied Sciences, Indianapolis, IN) and Beckman Coulter UniCel 600i DxC (Beckman Coulter, Brea, CA). Glomerular filtration rate was calculated using the Modification of Diet in Renal Disease (MDRD) Study equation:

\[
GFR (\text{mL/min/1.73 m}^2) = 175 \times (S_{cr})^{-1.154} \times (\text{Age})^{-0.203} \times (0.742 \text{ if female}) \text{ (Levey et al., 2006)}.
\]

Bone specific alkaline phosphatase was measured in our clinical laboratory using Beckman Access 2 analyzers (Beckman Coulter, Brea, CA). Insulin like growth factor 1 (IGF-1) was measured by ARUP Laboratories (Salt Lake City, UT) using quantitative chemiluminescence immunoassay. Urine Cross-Linked N-Telopeptide, was measured by ARUP Laboratories (Salt Lake City, UT) using quantitative chemiluminescence immunoassay. Serum collagen type 1 cross-linked C-telopeptide (CTX) was measured by ELISA at Esoterix labs (Austin, TX). 1,25(OH)2 D was measured by ARUP Laboratories (Salt Lake City, UT) using DiaSorin radioimmunoassay. Serum 25(OH)D levels were analyzed in the Creighton University Osteoporosis Research laboratory by liquid phase radioimmunoassay, IDS kit (ImmuNoDiagnostic Systems, Fountain Hills, AZ). Intact parathyroid hormone (hPTH 1-84) was analyzed in the Creighton University Osteoporosis Research laboratory by immunoradiometric assay (IRMA) using the DiaSorin N-tact PTH SP IRMA kit (DiaSorin Inc., MN, USA). Osteocalcin levels were
analyzed in the Creighton University Osteoporosis Research laboratory by radioimmunoassay method (CisBio US, Inc. Bedford, MA).

**Transilial Bone Biopsy**

Each subject was given *in vivo* double tetracycline labeling as follows: oral tetracycline hydrochloride 250 mg four times daily for three days (label 1), followed by 14 days drug free interval, and then tetracycline hydrochloride 250 mg four times daily for three days (label 2). The biopsy was performed 5 to 14 days after the last label was administered. The biopsy was performed in the outpatient surgery department at Creighton University Medical Center. It was in the morning hours after an overnight fast. After placement on the operating table, the hip was elevated about 1-2 inches on a folded towel. Blood pressure, electrocardiogram and pulse oximetry were monitored throughout the procedure. After indwelling intravenous access was obtained, the subject was sedated using ≈ five mg midazolam given through the indwelling intravenous catheter (2 subjects requested no sedation and were awake during the procedure and tolerated it well). The skin and subcutaneous tissue in the area approximately five cm inferior and posterior to the anterior-superior spine was anesthetized with 1% lidocaine (Rao, 1983). The inner and outer wall of the ilium was anesthetized as well. The bone biopsy was obtained through a five cm incision using a trephine of 7.5 mm inner diameter (Medical Innovations International, Inc., Rochester, Minnesota). The bony defect was packed with absorbable surgical cellulose, the wound closed with 5-0 monofilament suture and a dressing applied. The subject was cautioned regarding graduated physical activity, and the sutures removed seven days later, after which full activity was allowed (Rao, 1983).
The specimen was placed immediately in 70% ethanol as fixative and transported to the histomorphometry laboratory for standard histomorphometry.

Bone Specimen Preparation and Evaluation

The technicians who performed the histomorphometry were blinded to the subjects’ diabetes status. The bone specimens were dehydrated by sequentially increasing ethanol concentrations. They were placed in 70% ethanol for 48 hours, 95% ethanol for 48 hours and 100% ethanol for 48 hours. The specimens were placed in fresh ethanol twice daily and kept under a vacuum with intermittent releasing. The specimens continued to be dehydrated with acetone and then gradually changed to the embedding agent. They were placed in 100% acetone for 48 hours, then 50% acetone/50% methacrylate for 24 hours, then 1% benzoyl peroxide/methyl methacrylate for 24 hours and kept under a vacuum with intermittent releasing. Finally they were placed in partially polymerized methyl methacrylate which was prepared by placing 276 ml methyl methacrylate monomer (Fisher Scientific, Fair Lawn, New Jersey) in an Erlenmeyer flask, adding 3 g of benzoyl peroxide (Fisher Scientific, Fair Lawn, New Jersey), 24 ml of Dibutyl Phthalate (Fisher Scientific, Fair Lawn, New Jersey), and stirring at low speed under a hood. One hundred fifty g of Poly methyl methacrylate (MW 25,000) 200 micron beads (Polysciences Inc.,
Warrington, PA) were added in three 50 gram batches. Stirring speed was slowly increased (over 2-3 hours) until mixture thickened and all beads dissolved (1-2 days). The preparation was refrigerated at 4°C until use. The bone specimens were put in a glass vial, the partially polymerized methyl methacrylate poured over them and the vial capped tightly and placed in a 45°C water bath for 5-7 days. After the methyl methacrylate had hardened, the vial was broken, freeing the embedded specimen. The methacrylate block was trimmed to expose the cutting face of the bone sample and to remove excess methacrylate from the sides of the specimen. Near-serial longitudinal sections were obtained with a motorized Leica RM2255 microtome (Leica Microsystems, Nussloch, Germany) with tungsten carbide-tipped knife blades (Leica Microsystems, Nussloch, Germany) sharpened at 55º angulation. Longitudinal sections from two areas of the specimen, separated by ~ 250-300 μm were prepared and mounted on slides. After drying overnight, the slides were placed in two changes of 100% acetone over a 30 minute period and allowed to dry overnight before staining.

The following sections were stained:

- five 5 μm sections with Goldner’s modification of Masson’s trichrome stain;
- two 5 μm sections with Toluidine blue;
- two 8 μm sections unstained (Baron et al, 1983).

An automatic stainer (Fisher Scientific, Pittsburgh, PA ) was used to stain slides with Goldner’s modification of Masson’s trichrome stain. (Appendix for reagent preparation) The automatic stainer takes the slides through the following steps. First, the tray of slides is placed in 30% EtOH for 2 minutes. Then the slides are transferred to
Weighert's working solution C for 20 minutes. After rinsing in tap water for approximately 2 minutes they are transferred to aqueous 1% HCl for 2 minutes and rinsed in tap water 2 minutes. They are then transferred to aqueous lithium carbonate for 2 minutes. After rinsing in running tap water for 5 minutes, they are transferred to Masson's Ponceau-Fuchsin working solution for 1hr then rinse with 1% acetic acid for 2 minutes. They are then transferred to phosphotungstic-phosphomolybdic acid solution for 5 minutes; rinsed twice in 1% acetic acid for 2 minutes and 5 minutes respectively. They are then transferred to 1% naphthol green B for 15 minutes and then to water rinse for 1 minute. They are rinsed twice in 95% EtOH for 2 minutes each and then transferred to 100% ethanol for 2 minutes. They are then transferred to alcoholic Safran solution for 10 minutes and then to 100% ethanol for 2 minutes and covered tightly. They are then rinsed in three changes of SafeClear for 2, 3, and 3 minutes, respectively before covering the slides with a cover slip.

Toluidine blue stain is made by adding 72 drops of 1% T-Blue stain to 400 ml of working buffer. The tray of slides sits in the stain for 24 hours before being rinsed in two changes of 100% EtOH, 10 sec and one minute respectively. They are then rinsed immediately in two changes of SafeClear for 2 min each.

All the microscope work was performed on an Olympus Microscope (BX 60, Hitschfels Instruments St. Louis, MO) using an operator interactive, semi-automatic image analysis system (Bioquant, Nashville, TN) linked to a camera lucida Optronics DEI -750CE (Meyer Instruments, Inc. Houston, Texas). The camera was calibrated for each magnification with a stage micrometer. The following surfaces were identified and traced with a mouse and cursor on the Bioquant screen:
• on osteoid without osteoblasts
• on osteoid with osteoblasts
• on resorptive surface without osteoclasts
• on resorptive surface with osteoclasts

Figure 2. A picture of a slide with Goldner’s stain (green=bone, pink=marrow space).

The Goldner Trichrome stain distinguishes mineralized bone matrix (green), cell type by nuclei structure (dark purple to black), and osteoid seams (bright red). Osteoblasts are cells lying along an osteoid covered surface. They are generally plump and light pink in color. The osteoclasts are recognizable as multinucleated cells with foamy cytoplasm lying close to an eroding surface (Kimmel & Jee, 1983). The Toluidine Blue stain distinguishes bone matrix (purple) from osteoid (light blue) and cement lines (deep violet).
Bone area was measured on the Goldner stained sections at 40X magnification. Osteoid length and width, eroded length, osteoblasts, and osteoclasts were identified and measured at 200X magnification on the Goldner sections. Osteoid width was measured at 2-3 places along the length of a single osteoid seam. Tetracycline labeling was examined under epifluorescence ultraviolet light at 200X magnification on the unstained sections, for bone surface bearing single and double label. All specimens had tetracycline label. Bone area was also measured on the unstained sections. Interlabel thickness measurement was made at 400X magnification under epifluorescence ultraviolet light on the Bioquant screen. Wall thickness was measured on Toluidine blue-stained sections at 100X magnification. The distance between cement lines and trabecular surface on completed, inactive trabecular osteons was measured on the Bioquant screen. The technicians making these measurements were trained and a standard slide measured monthly to assess repeatability of the measurements. The coefficient of variation for fourteen
measured variables ranged between 0.7 and 8.2%. All raw data were entered from the Bioquant directly into an Excel file (Microsoft, Redmond, WA) where calculations were performed, and results stored on a hard drive. Calculation of static and dynamic variables was done in a standard manner (Parfitt et al., 1987; Parfitt, 1983) (Appendix).

**Histomorphometry Variables**

Many variables can be measured or calculated from prepared bone specimens. They fall under static structural measurements or calculated variables of bone structure, identification of cells and dynamic measurements and calculated derived variables from the fluorochrome labeling. Below I give a more intuitive explanation of the variables. See the Appendix for the actual formulae used to calculate the histomorphometric variables from the measurements. Variables are named according to the nomenclature scheme approved by the American Society for Bone and Mineral Research and the formulae used are agreed upon by the American Society for Bone and Mineral Research (Parfitt et al., 1987). While all measurements such as perimeter, area and width are obtained from a 2 dimensional slide, the corresponding 3 dimensional results such as surface, volume and thickness are reported. The 3 dimensional thickness results are derived by multiplying the 2 dimensional width measurements by $\pi/4$ (Parfitt, 1983). Only 3 dimensional results are reported here. The commonly used referents for these results are tissue volume (TV), bone volume (BV), bone surface (BS) and osteoid surface (OS) (Parfitt et al., 1987).

**Static Structural Variables**

Bone volume (BV/TV) is the percent of total volume (TV) occupied by bone. Trabecular thickness (TbTh) is a calculated variable of the average distance across individual trabeculae and trabecular separation (TbSp) is a calculated variable of the average
distance between trabeculae. Trabecular number (TbN) is calculated by dividing BV/TV by TbTh. Eroded surface (ES/BS) is the percent of bone surface (BS) with Howship’s lacunae with and without osteoclasts. Wall thickness (WTh) is the average distance between trabecular surface and the cement line- representing a filled in Howship’s lacunae (multiplied by a factor of π/4 to correct for obliquity, the various “slants” at which one views this distance) (Parfitt 1983).

Osteoblasts surface (Obs/BS) and osteoclast surface (OcS/BS) are the percentages of bone surface occupied by these cells. Osteoid surface (OS/BS) is the percent of surface with unmineralized osteoid with and without osteoblasts representing the fraction of bone matrix laid down that was not yet mineralized at the time of the biopsy. Osteoid thickness is the average width of the unmineralized osteoid (multiplied by a factor of π/4 to correct for obliquity). Osteoid less than 3mm in width is not measured. This narrow layer of unmineralized osteoid represents what microscopists have called the “lamina limitans”, a thin layer of osteoid on a trabecular surface that is intermittently mineralized, and contains calcium in what is called the miscible calcium pool. It is not engaged in bone formation.

**Dynamic variables**

Mineralizing surface (MS/BS) is percent of trabecular surface that has fluorochrome labeling (average extent of double label plus one-half of the extent of single label) present (Schwartz & Recker, 1982). Mineralizing Osteoid Surface (MS/OS) is the ratio of MS/BS to OS/BS, expressed as a percent. Mineral appositional rate (MAR) is the average distance between the midpoints of the two fluorescent labels (adjusted for obliquity)
divided by the time between the midpoint of the label doses. This represents the thickness of new mineralized bone that was added during the labeling period.

**Derived dynamic variables**

There are several dynamic variables that are calculated from the measurements of double label. The Formation Period (FP) is the WTh divided by the MAR adjusted by the ratio of Mineralizing Surface to Osteoid Surface converting days to years. The Resorption Period (RP) is the FP multiplied by the ratio of the Eroded Surface to the Osteoid Surface. Remodeling Period (RmP) is the sum of RP and FP. Trabecular bone formation rates (BFR/BV and BFR/BS) are estimates of bone volume and surface that are replaced every year. Mineralization lag time (Mlt) is the average time interval between deposit of osteoid and its mineralization. It is calculated by dividing OTh by MAR multiplied by MS/OS and divided by 100. Activation frequency (AcF) is calculated by dividing the bone formation rate (BFR/BS) by average wall thickness (WTh) multiplied by 0.001. AcF is the best index of bone remodeling. It reflects the probability that a new remodeling unit will be initiated (Parfitt et al., 1987; Recker & Barger-Lux, 2006). It assumes that all sites of bone formation were preceded by bone resorption, an assumption that is nearly always correct, but may not be 100% correct. There is no way to confirm how much of the MS/BS in a given specimen may not have been preceded by resorption, but the evidence in specimens suggests that it is rare (Frost, 1983).

**Statistical Analysis**

Statistics were generated using the statistics package, PASW Statistics 18.0 (SPSS Inc., Chicago, Illinois). Nonparametric expressions and statistical tests were used as the data violated some of the assumptions needed for parametric tests. The following assumptions
were violated 1) many of the variables were not normally distributed (Kolmogorov-Smirnov test of normality) and 2) some of the variables violated homogeneity of variance (Levene’s Test for Equality of Variances). Comparisons between cases and controls were done using Wilcoxon Signed Ranks test. The diabetics who had sustained fractures were also compared to the non-fracturing diabetics using a Mann Whitney U test. Bivariate correlations were performed using Spearman’s rho. Statistical significance was set at P<0.05.

**Sample Size**

This project was a pilot project to assess the feasibility of the study and also to generate preliminary data for future grants. If this study was repeated with the goal of finding statistically significant differences in activation frequency between healthy diabetic subjects and controls, a sample size of 439 subjects will be needed, assuming a power of 80% and an α of 0.05.
RESULTS

Data from 18 subjects (7 male, 11 female) with a diagnosis of Type 1 Diabetes Mellitus and 18 control subjects who fulfilled the enrollment criteria were analyzed. A description of the subjects’ demographics is in Table 1. Their age range was 20-47 years and the diabetic subjects had been diagnosed with diabetes for 3-36 years. Of the diabetic subjects, 5 had previously sustained a non-traumatic fracture or fractures. Descriptions of the fracturing subjects are outlined in Table 2. Table 3 describes pertinent laboratory data. The diabetic subjects had a wide range of glucose control with HgA1c values ranging from 5.5-10.1%, and no differences in either vitamin D status or renal function when compared to the control subjects. Both groups’ 25(OH)D levels were comparable to values from other studies conducted in the same region (Armas et al., 2007). The diabetics had significantly lower levels of insulin like growth factor 1 (IGF-1). The calcium, phosphorus, PTH, and 1,25(OH)2D levels were within the reference laboratory’s normal range. Table 4 describes the serum and urine bone marker data. There was a significantly higher level of bone specific alkaline phosphatase (BSAP) in the diabetics than in controls (P=0.007, Wilcoxon signed ranks test) and a trend toward higher BSAP in the fracturing diabetics vs. the non-fracturing diabetics (P=0.095, Mann Whitney U). No differences were seen in the levels of osteocalcin between diabetics and controls. The bone resorption markers, urine crosslinked N-telopeptide, type 1 collagen (NTX) and C-telopeptide (CTX), were not different between diabetics and controls, but the fracturing diabetics had significantly higher CTX than the non fracturing diabetics (P=0.035, Mann Whitney U). Table 5 describes the DXA results of diabetic subjects and controls as well as the fracturing diabetics. There were no significant differences in DXA
Table 1 Subjects Demographics$^a$

<table>
<thead>
<tr>
<th></th>
<th>Diabetes</th>
<th>Control</th>
<th>P</th>
<th>Fracturing</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>18</td>
<td>18</td>
<td></td>
<td>5</td>
</tr>
<tr>
<td>Sex M,F</td>
<td>7,11</td>
<td>7,11</td>
<td></td>
<td>2,3</td>
</tr>
<tr>
<td>Age yrs</td>
<td>31.1 (25.4-38.0)</td>
<td>34.1(28.6-38.4)</td>
<td>NS$^b$</td>
<td>37.1(22.2-45.9)</td>
</tr>
<tr>
<td>Yrs Dx$^c$</td>
<td>15(8-25)</td>
<td>na$^d$</td>
<td></td>
<td>14(10-25)</td>
</tr>
<tr>
<td>Age at Dx</td>
<td>13(8-24)</td>
<td>na$^d$</td>
<td></td>
<td>13(10-28)</td>
</tr>
<tr>
<td>Height cm</td>
<td>171(166-174)</td>
<td>172(164-175)</td>
<td>NS$^b$</td>
<td>170(160-174)</td>
</tr>
<tr>
<td>Weight kg</td>
<td>75.6(70.4-85.4)</td>
<td>68.3(59.2-85.3)</td>
<td>0.043$^e$</td>
<td>74.9(69.9-89.0)</td>
</tr>
<tr>
<td>BMI</td>
<td>26.8(24.5-27.9)</td>
<td>23.9(21.5-24.9)</td>
<td>0.02$^e$</td>
<td>27.1(25.7-30.4)</td>
</tr>
</tbody>
</table>

Baecke Activity Scores

<table>
<thead>
<tr>
<th></th>
<th>Diabetes</th>
<th>Control</th>
<th>P</th>
<th>Fracturing</th>
</tr>
</thead>
<tbody>
<tr>
<td>Work</td>
<td>2.3(2.0-3.1)</td>
<td>2.2(2.0-2.6)</td>
<td>NS$^b$</td>
<td>2.4(1.9-3.9)</td>
</tr>
<tr>
<td>Sports</td>
<td>2.4(2.0-3.2)</td>
<td>2.3(1.9-2.6)</td>
<td>NS$^b$</td>
<td>2.3(2.1-3.3)</td>
</tr>
<tr>
<td>Leisure</td>
<td>2.5(2.0-3.1)</td>
<td>3.0(2.5-3.3)</td>
<td>NS$^b$</td>
<td>2.8(2.3-3.3)</td>
</tr>
</tbody>
</table>

$^a$Median (interquartile range)  
$^b$NS = not statistically significant by Wilcoxon Signed Ranks Test  
$^c$Yrs Dx = years since diagnosis of T1DM  
$^d$na = not applicable  
$^e$ Wilcoxon Signed Ranks Test
Table 2 Characteristics of Diabetic Subjects with Fracture

<table>
<thead>
<tr>
<th>ID</th>
<th>Current Age</th>
<th>Age at Diabetes</th>
<th>Diabetes Duration</th>
<th>Description of Fracture(s)</th>
<th>Age at last Fracture</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>21</td>
<td>7</td>
<td>14</td>
<td>R ankle, L ankle, elbow</td>
<td>18</td>
</tr>
<tr>
<td>2</td>
<td>22</td>
<td>12</td>
<td>10</td>
<td>R tibia</td>
<td>20</td>
</tr>
<tr>
<td>3</td>
<td>37</td>
<td>12</td>
<td>25</td>
<td>R tibia, fibula, ankle</td>
<td>36</td>
</tr>
<tr>
<td>4</td>
<td>45</td>
<td>35</td>
<td>10</td>
<td>L arm, knee, metatarsal</td>
<td>35</td>
</tr>
<tr>
<td>5</td>
<td>46</td>
<td>21</td>
<td>25</td>
<td>R proximal 5(^{th}) metatarsal</td>
<td>42</td>
</tr>
<tr>
<td></td>
<td>Diabetes</td>
<td>Control</td>
<td>P</td>
<td>Fracturing</td>
<td></td>
</tr>
<tr>
<td>------------------</td>
<td>----------</td>
<td>---------</td>
<td>-------</td>
<td>------------</td>
<td></td>
</tr>
<tr>
<td>N</td>
<td>18</td>
<td>18</td>
<td></td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>HgA1c %^b</td>
<td>6.8(6.3-8.3)</td>
<td>5.3(5.1-5.4)</td>
<td>&lt;0.001^c</td>
<td>8.1(6.9-9.7)</td>
<td></td>
</tr>
<tr>
<td>25(OH)D nmol/L^d</td>
<td>63(51-79)</td>
<td>68(56-86)</td>
<td>NS</td>
<td>61.6(52.0-72.5)</td>
<td></td>
</tr>
<tr>
<td>Creatinine mg/dl</td>
<td>0.8(0.7-0.8)</td>
<td>0.7(0.7-0.8)</td>
<td>NS</td>
<td>0.7(0.7-0.8)</td>
<td></td>
</tr>
<tr>
<td>IGF-1^e ng/ml</td>
<td>119(92-148)</td>
<td>158(127-240)</td>
<td>P=0.004^c</td>
<td>123(86-157)</td>
<td></td>
</tr>
<tr>
<td>Calcium mg/dl</td>
<td>9.5(9.2-9.5)</td>
<td>9.3(9.1-9.4)</td>
<td>NS</td>
<td>9.5(9.1-9.9)</td>
<td></td>
</tr>
<tr>
<td>Phosphorus mg/dl</td>
<td>3.5(3.1-3.9)</td>
<td>3.5(3.0-3.9)</td>
<td>NS</td>
<td>3.3(3.2-4.2)</td>
<td></td>
</tr>
<tr>
<td>1,25(OH)2D pg/ml</td>
<td>47(29-54)</td>
<td>43(33-51)</td>
<td>NS</td>
<td>26(18-66)</td>
<td></td>
</tr>
<tr>
<td>PTH pg/ml</td>
<td>18.5(15.1-23.4)</td>
<td>19.1(17.3-22.4)</td>
<td>NS</td>
<td>18.0(16.6-26.4)</td>
<td></td>
</tr>
</tbody>
</table>

^aMedian (interquartile range)  
^bHgA1c = Glycated hemoglobin  
^cWilcoxon Signed Ranks Test  
^d25(OH)D = 25-hydroxyvitamin D  
^eIGF-1 = Insulin-like growth factor 1  
^f1,25 (OH)2D = 1,25 dihydroxyvitamin D
Table 4  Laboratory Bone Marker Data

<table>
<thead>
<tr>
<th></th>
<th>Diabetes</th>
<th>Control</th>
<th>P</th>
<th>Fracturing</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>18</td>
<td>18</td>
<td></td>
<td>5</td>
</tr>
<tr>
<td>BSAP(^b) µg/L</td>
<td>14.2(12.3-17.0)</td>
<td>9.7(6.9-14.2)</td>
<td>P=0.007(^f)</td>
<td>16.7(13.8-17.8)(^g)</td>
</tr>
<tr>
<td>Osteocalcin ng/ml(^i)</td>
<td>23.0(15.2-23.0)</td>
<td>19.1(17.3-22.4)</td>
<td>NS</td>
<td>24.7(15.1-28.6)</td>
</tr>
<tr>
<td>CTX(^c) pg/ml</td>
<td>280(77-504)</td>
<td>293(130-535)</td>
<td>NS</td>
<td>658(283-671)(^h)</td>
</tr>
<tr>
<td>Urine NTX(^d) nM/BCE(^e)33(26-42)</td>
<td>24(20-41)</td>
<td>NS</td>
<td>41(36-50)(^g)</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\)Median (interquartile range)

\(^b\)BSAP = bone specific alkaline phosphatase

\(^c\)CTX= C-telopeptide

\(^d\)NTX= crosslinked N-telopeptide, type 1 collagen

\(^e\)BCE= bone collagen equivalent

\(^f\)Wilcoxon Signed Ranks Test

\(^g\)P=0.095 Mann Whitney U

\(^h\)P=0.035 Mann Whitney U

\(^i\)n= diabetes-17, controls-15
### Table 5 Dual Energy X-ray Absorptiometry (DXA) Results

<table>
<thead>
<tr>
<th></th>
<th>Diabetes</th>
<th>Control</th>
<th>P</th>
<th>Fracturing</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>18</td>
<td>18</td>
<td></td>
<td>5</td>
</tr>
<tr>
<td>L1-L4 Spine BMD</td>
<td>0.982(0.900–1.053)</td>
<td>1.079(0.955-1.137)</td>
<td>NS</td>
<td>0.973(0.863-1.125)</td>
</tr>
<tr>
<td>L1-L4 Spine T-score</td>
<td>-0.7(-1.5-0.0)</td>
<td>+0.3(-0.9-+0.6)</td>
<td>NS</td>
<td>-0.7(-1.9-+0.5)</td>
</tr>
<tr>
<td>Total Hip BMD</td>
<td>0.991(0.892–1.058)</td>
<td>0.970(0.885-1.093)</td>
<td>NS</td>
<td>0.862(0.841-1.045)</td>
</tr>
<tr>
<td>Total Hip T-score</td>
<td>0.0(-0.5–+0.6)</td>
<td>0.1(-0.7–+0.5)</td>
<td>NS</td>
<td>-0.8(-1.0-+0.4)</td>
</tr>
</tbody>
</table>

*aMedian (interquartile range)*

*bBMD = bone mineral density*

*cNS = not statistically significant by Wilcoxon Signed Ranks Test*
variables between cases and controls or between the fracturing diabetics and the nonfracturing diabetics.

**Histomorphometry Results**

Structural histomorphometry data are described in Table 6. There were no differences between diabetics and controls or in fracturing diabetics and nonfracturing diabetics in structural measurements including bone volume as a percent of total volume (BV/TV), wall thickness (W.Th), osteoid thickness (O.Th), and trabecular measures of thickness (Tb.Th), number (Tb.N), or separation (Tb.Sp). Surface data presented in Table 7 also showed no differences between diabetics and controls or in fracturing diabetics and nonfracturing diabetics in osteoid surface (OS/BS), osteoblast surface (Ob.S/BS), or eroding surface (ES/BS). Dynamic data are presented in Table 8 and Table 9. Diabetic subjects, when compared to controls, had no significant differences in mineral apposition rate (MAR), mineralizing surface (MS/BS), mineralizing osteoid (MS/OS), mineralization lag time (Mlt), bone formation rate (BFR/BS or BFR/BV), formation period (FP), remodeling period (Rm.P), or Activation frequency (Ac.F). There was a significant inverse correlation between Activation frequency and age (Spearman’s rho, 2-tailed significance, p=0.025). (Figure 4) There was no correlation between Activation frequency and HgA1c as a measure of glucose control or between Activation frequency and duration of disease.
Table 6 Histomorphometric Structural Data\(^a\)

<table>
<thead>
<tr>
<th>Variable</th>
<th>Diabetes</th>
<th>Controls</th>
<th>P</th>
<th>Fracturing</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>18</td>
<td>18</td>
<td>P</td>
<td>5</td>
</tr>
<tr>
<td>BV/TV %</td>
<td>20.08(15.81-27.26)</td>
<td>23.80(18.54-26.66)</td>
<td>NS(^b)</td>
<td>16.04(13.32-21.41)</td>
</tr>
<tr>
<td>W.Th μm</td>
<td>31.2(27.1-32.2)</td>
<td>29.9(28.6-30.6)</td>
<td>NS</td>
<td>32.3(26.7-34.9)</td>
</tr>
<tr>
<td>O.Th μm</td>
<td>6.75(6.08-7.78)</td>
<td>6.86(6.33-8.08)</td>
<td>NS</td>
<td>6.82(4.75-7.72)</td>
</tr>
<tr>
<td>Tb.Th μm</td>
<td>124.6(102.4-149.9)</td>
<td>128.9(106.2-144.5)</td>
<td>NS</td>
<td>102.0(100.7-134.5)</td>
</tr>
<tr>
<td>Tb.N/#/mm</td>
<td>1.61(1.40-1.79)</td>
<td>1.75(1.51-1.88)</td>
<td>NS</td>
<td>1.49(1.17-1.79)</td>
</tr>
<tr>
<td>Tb.Sp μm</td>
<td>605(548-703)</td>
<td>560(521-654)</td>
<td>NS</td>
<td>660(554-841)</td>
</tr>
</tbody>
</table>

\(^a\)Median (Interquartile Range)

\(^b\)NS = not statistically significant by Wilcoxon Signed Ranks Test
Table 7 Histomorphometric Surface Data

<table>
<thead>
<tr>
<th>Variable</th>
<th>Diabetes</th>
<th>Controls</th>
<th>P</th>
<th>Fracturing</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>18</td>
<td>18</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>OS/BS %</td>
<td>9.9(3.8-11.8)</td>
<td>11.2(3.0-13.1)</td>
<td>NS</td>
<td>4.1(1.9-10.8)</td>
</tr>
<tr>
<td>Ob.S/BS %</td>
<td>4.5(1.9-8.7)</td>
<td>4.2(1.6-9.3)</td>
<td>NS</td>
<td>1.2(0.9-7.9)</td>
</tr>
<tr>
<td>ES/BS %</td>
<td>1.0(0.8-1.4)</td>
<td>0.9(0.5-1.5)</td>
<td>NS</td>
<td>0.9(0.8-1.6)</td>
</tr>
</tbody>
</table>

\(^a\)Median (Interquartile Range)

\(^b\)NS = not statistically significant by Wilcoxon Signed Ranks Test
**Table 8 Histomorphometric Dynamic Data**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Diabetes</th>
<th>Controls</th>
<th>P</th>
<th>Fracturing</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>18</td>
<td>18</td>
<td></td>
<td>5</td>
</tr>
<tr>
<td>MAR μm/day</td>
<td>0.56(0.49-0.62)</td>
<td>0.54(0.49-0.58)</td>
<td>NSb</td>
<td>0.56(0.52-0.65)</td>
</tr>
<tr>
<td>MS/BS %</td>
<td>6.5(3.0-10.4)</td>
<td>6.6(3.3-11.4)</td>
<td>NS</td>
<td>2.7(1.7-11.4)</td>
</tr>
</tbody>
</table>

aMedian (Interquartile Range)

bdata calculated from double label plus half the single label

bNS = not statistically significant by Wilcoxon Signed Ranks Test
## Table 9 Histomorphometric Derived Dynamic Data

<table>
<thead>
<tr>
<th>Variable</th>
<th>Diabetes</th>
<th>Controls</th>
<th>P</th>
<th>Fracturing</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>18</td>
<td>18</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>MS/OS %</td>
<td>72.3(61.5-100)</td>
<td>73.3(53.8-100)</td>
<td>NS&lt;sup&gt;b&lt;/sup&gt;</td>
<td>96.1(63.9-100)</td>
</tr>
<tr>
<td>Mlt d</td>
<td>14.8(12.2-24.6)</td>
<td>18.1(11.4-26.0)</td>
<td>NS</td>
<td>13.6(9.2-14.8)</td>
</tr>
<tr>
<td>BFR/BS μm3/μm2/yr</td>
<td>12.18(4.94-22.63)</td>
<td>14.11(5.49-24.80)</td>
<td>NS</td>
<td>4.79(3.78-24.10)</td>
</tr>
<tr>
<td>BFR/BV %/yr</td>
<td>22.9(9.2-32.5)</td>
<td>21.1(11.4-34.1)</td>
<td>NS</td>
<td>9.4(7.5-35.9)</td>
</tr>
<tr>
<td>FP yrs</td>
<td>0.19(0.13-0.28)</td>
<td>0.19(0.13-0.36)</td>
<td>NS</td>
<td>0.15(0.12-0.24)</td>
</tr>
<tr>
<td>Rm.P yrs</td>
<td>0.22(0.16-0.31)</td>
<td>0.21(0.14-0.38)</td>
<td>NS</td>
<td>0.18(0.16-0.30)</td>
</tr>
<tr>
<td>Ac.F #/yr</td>
<td>0.42(0.18-0.69)</td>
<td>0.49(0.18-0.82)</td>
<td>NS</td>
<td>0.19(0.11-0.79)</td>
</tr>
</tbody>
</table>

<sup>a</sup>Median (Interquartile Range)

<sup>b</sup>NS = not statistically significant by Wilcoxon Signed Ranks Test
Figure 4. Scatterplot of all study subjects ♦ showing the relationship between age and activation frequency. (Spearman’s rho, 2-tailed significance, p=0.025).
Premenopausal women ■ (Recker et al., 2004)
Postmenopausal women ▲ 1 year after menopause (Recker et al., 2004)
Normal postmenopausal women ● mean 7 years after menopause (Recker et al., 1988; Recker et al., 2004)
DISCUSSION

There were no significant differences in histomorphometric analysis of transiliac biopsies from otherwise healthy subjects with Type 1 Diabetes Mellitus compared to age and sex matched controls. The primary hypothesis was that activation frequency will be lower in young, otherwise healthy subjects with Type 1 Diabetes Mellitus compared to age- and sex- matched nondiabetic control subjects. I have failed to reject this hypothesis and have accepted the null hypothesis. (Figure 5)

![Bar Graph showing the mean Activation Frequencies of Diabetics and Control Subjects. Error bars are ± SD.](image)

**Figure 5** Bar Graph showing the mean Activation Frequencies of Diabetics and Control Subjects. Error bars are ± SD.

This is the first case-controlled, fluorochrome-labeled histomorphometric study of Type 1 diabetic humans that I am aware of. The only other published human studies contained histomorphometric data on two male subjects (age 37 and 63) with Type 1 DM (Krakauer et al., 1995). They had diabetes for ~ 3 decades and had some degree of retinopathy and peripheral neuropathy. Glucose control was not reported and an existing set of
histomorphometric data from premenopausal women were used for comparison. This current study differs from the 2 subjects previously reported (Krakauer et al., 1995) in several respects: 1) these were healthy subjects with no reported diabetic complications and none identified on screening, 2) these had a shorter disease duration (an average of 15 vs. 30 years), and 3) these were compared to age and sex matched control subjects who were recruited and analyzed concurrently with the cases. Krakauer et al reported a low mean bone formation rate ($3.37 \pm 3.45 \mu m^3/\mu m^2/yr$ compared to a median bone formation rate of 12.18 $\mu m^3/\mu m^2/yr$ in this study) (Krakauer et al., 1995), indicating low remodeling in their group of subjects with Type 1 and Type 2 DM. The results are strikingly different from what I observed in the diabetic group, although more similar to those diabetics who had fractured (Table 9).

The activation frequency (a derived variable indicating remodeling) in this diabetic cohort of subjects, while no different than age matched controls, was much higher than what was observed in a previous study of premenopausal women (mean age 49.4 ± 1.9 yrs) (Recker et al., 2004). The median activation frequency for both diabetics and controls was 0.47/yr compared to 0.13/yr in the premenopausal women (Recker et al., 2004). This high activation frequency was even higher than in postmenopausal women who had a median activation frequency of 0.37/yr at the age of 60 (Recker et al., 2004). (Figure 4) Unfortunately, there are no published normative values for histomorphometry in males. The likely explanation for this higher activation frequency could be age related. There was an inverse correlation between age and AcF. (Figure 4) This younger cohort may still be in the process of building bone mass which is a separate process known as modeling. Activation frequency does not distinguish between modeling and remodeling.
Young women continue to gain bone well into their 20’s with total body bone mineral increasing by 12.5% during young women’s 3rd decade (Recker et al., 1992). Men continue to gain bone into their 20’s as well (Baxter-Jones et al., 2011). While this current study was not longitudinal it is probable that this cohort is still building bone mass accounting for the increased activation frequency.

This study examined diabetic subjects before diabetic complications could confound the subjects’ bone health. Epidemiological studies of fracture risk have shown an increase in fracture risk in Type 1 DM but have not always had documentation of diabetic complications (Ahmed et al., 2006; Forsen et al., 1999). Some of the studies that have documented complications have observed that diabetics with complications have a much higher risk of fracture, as high as 33 times higher in some studies (Miao et al., 2005; Vestergaard et al., 2009). While there were no differences between diabetic cases and controls, this could imply that deficits in bone remodeling rates and or microstructure could be directly or indirectly related to complications of the disease. To determine this will require further research on subjects with diabetic complications. An alternative explanation is that diabetes causes intrinsic material property defects at a level below what is identifiable by standard histomorphometry techniques. Some examples of this include intrinsic material property defects in the elasticity, toughness and strength of bone tissue. Chemical content of the bone tissue including size and shape of hydroxyapatite crystals, concentration of minerals in the bone matrix and organic makeup of the collagen molecules could also be affected by hyperglycemia either directly or indirectly which in turn can effect bone strength (Ruppel et al., 2008). Diabetes could also directly affect the cells responsible for bone formation and bone maintenance (Coe et
The osteocyte may play a role in the bone’s response to hyperglycemia and osmotic changes (Villarino et al., 2006). Some of these outcomes could potentially be explored by analyzing the remaining bone biopsy specimens from this current study.

The direct effect of hyperglycemia on the bone cannot be ignored. One of the reasons we may not have seen an effect of diabetes on bone structure or remodeling in this study could be the relatively good glucose control that these subjects had. The American Diabetes Association recommends that HgA1c be less than 7% (American Diabetes Association, 2011). While we had a wide range glucose control, half of our subjects met this goal.

There were no differences in any of the DXA results between diabetics or controls. (Figure 6) While the subjects were not matched for BMD at the time of recruitment, this lack of difference in BMD eliminated variation in histomorphometric variables that depend on bone mass.

Figure 6 Plot of Total Hip Bone Mineral Density (BMD) g/cm². (♦ controls, ■ diabetes, ▲ fracturing diabetics). No significant differences between the three groups.
BMD in Type 1 DM compared to controls. Strotmeyer et al. found a 3-8% lower BMD in 67 Type 1 diabetic premenopausal diabetic females compared to a group of non-diabetic women (Strotmeyer et al., 2006). Unlike our study, over half of their subjects reported microvascular complications which may have an influence on their bone mass (Strotmeyer et al., 2006). Although the authors were unable to find an effect of reported complications, disease duration or glucose control on BMD, they did find correlations between blindness and neuropathy on femoral neck BMD after adjusting for age and diabetes duration. (Strotmeyer et al., 2006). Mastrandrea et al. also found lower BMD in 26 Type 1 diabetic women (mean age 27 years) compared to a group of controls at the total hip, femoral neck and whole body, but not at the spine (Mastrandrea et al., 2008). They found no correlation between BMD and disease duration or glucose control and the degree of diabetic complications were not reported in this cohort. (Mastrandrea et al., 2008). As with risk of fracture, diabetic complications may play an important role in determining BMD. There are a few studies that found normal BMD in Type 1 DM, but these were small studies (one in 35 men [Bridges et al., 2005], one in 31 premenopausal women [Hampson et al., 1998]) and may not have had the power to see a difference. Vestergaard conducted a meta-analysis of BMD and fractures in Type 1 and Type 2 DM and while he showed overall a decreased BMD in the Type 1 diabetic subjects, he was able to calculate an expected relative fracture risk. The expected risk was 1.42, quite different from the observed relative risk of 6.94 (Vestergaard 2007). This perhaps points out that the real risk factor for fracture in Type 1 DM is bone quality rather than quantity. As with other similar studies of Type 1 DM (Bridges et al., 2005; Hampson et al., 1998), we found no correlation between BMD and glucose control (HgA1c) or disease duration.
There were significantly lower IGF-1 levels in the diabetic subjects. This has been observed in other diabetic cohorts and has been postulated to contribute to low bone mass in diabetics (Moyer-Mileur et al., 2008). However, there was no correlation between IGF-1 levels and any of the histomorphometric or DXA variables so it is difficult to attribute effects of low IGF-1 on bone in this study. Low IGF-1 may simply be a marker for nutrient intake (Isley et al., 1983) or result from the insulin deficiency of Type 1 DM which impairs hepatic IGF-1 synthesis (Edge et al., 1990).

**Strengths & Limitations**

The strengths of this study were that the control subjects were age and sex matched and analyzed concurrently with the diabetic cases, and the bone biopsies were processed and analyzed by standardized technology in a well established lab.

One of the limitations of this study was using a convenient sample of subjects. This will always be a limitation in any study using an invasive technique. Another limitation was that this group of diabetic subjects had reasonable glucose control (over half had HgA1c of <7%) (American Diabetes Association, 2011) and may not be representative of poorly controlled diabetic patients. If bone remodeling and structure are dependent on glucose homeostasis, variations in glucose control could have effects on bone which were not observed in this study. Also, glucose control at the time of fracture in the subjects who had sustained fractures was not able to be assessed. Another limitation is that this was a cross sectional study and does not indicate what effect disease duration or long-term glucose control will have on the bones. Despite these limitations this study has merit as it is the 1st case controlled histomorphometric study of Type 1 Diabetes Mellitus patients. As such, it is valuable in determining future directions for research into the pathogenesis
of low-trauma fracture risk in diabetics. Importantly, it also supports the need for larger studies that are carefully designed, and that measure other possibilities for the cause of the low-trauma fractures in diabetics.

CONCLUSION

In summary, this cross-sectional case controlled study indicates that Type 1 Diabetes Mellitus does not have an effect on bone mineral density by DXA and bone histomorphometric characteristics before clinically apparent diabetic complications have manifested. However, this study has opened up other related avenues that can be explored in future studies.
Preliminary Data & Future Research Directions

Although we did not focus on recruiting diabetics with a history of fracture, we did recruit 5 subjects who had previously fractured. This may be a result of recruitment bias, but we examined these subjects and compared them to the nonfracturing diabetics to find any identifiable bone characteristics. Because of the small sample, there were no statistically significant differences in any variables and the following interpretation is preliminary. However, on examination of the variables there are some trends that may be clinically significant. (Table 10) BV/TV was lower (~27%) in fracturing diabetics than in the nonfracturing diabetics. (Figure 7) Other structural variables such as Tb. Th and Tb.N were lower and Tb.Sp was higher by histomorphometric analysis. OS/BS, Ob.S/BS and MS/BS were also lower. As a result, the derived dynamic variables such as BFR/BS, BFR/BV and Ac.F were lower in the fracturing subjects indicating lower remodeling or modeling. Overall the fracturing subjects had less bone structure as indicated by lower BV/TV which may be a result of lower remodeling or modeling seen in some of the subjects. A larger study of diabetic subjects with fractures will be needed to identify particular bone characteristics these fracturing subjects have.

IMPLICATIONS

This study did not show any effect of Type 1 Diabetes Mellitus and its inherent hyperglycemia and insulin deficiency on bone by standard histomorphometry analysis techniques. This implies two things. First, Type 1 Diabetes may not affect bone structure or remodeling directly, it may be a result of microvascular or macrovascular complications. A new study addressing a population of diabetics suffering from these complications could answer that question definitively. Second, the bone intrinsic material
properties could be affected. There are several novel techniques that could be used on the existing biopsy specimens to provide an answer to these questions. Furthermore, the diabetic subjects with fracture may have specific structural bone deficits that may or may not be related to their diabetes. This needs to be explored further in patients suffering from both diabetes and fracture.

Further research is needed in humans with Type 1 Diabetes Mellitus to provide answers to this problem of excessive fracture.
Table 10 Fracturing Diabetics Histomorphometric Variables\textsuperscript{a}

<table>
<thead>
<tr>
<th></th>
<th>Fracturing Diabetics\textsuperscript{b}</th>
<th>Diabetics without Fracture</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>5</td>
<td>13</td>
</tr>
<tr>
<td>BV/TV %</td>
<td>16.04(13.32-21.41)</td>
<td>21.95(18.18-28.08)</td>
</tr>
<tr>
<td>Tb.Th μm</td>
<td>102.0(100.7-134.5)</td>
<td>125.2(112.4-162.2)</td>
</tr>
<tr>
<td>Tb.N #/mm</td>
<td>1.49(1.17-1.79)</td>
<td>1.74(1.54-1.79)</td>
</tr>
<tr>
<td>Tb.Sp μm</td>
<td>660(554-841)</td>
<td>558(544-638)</td>
</tr>
<tr>
<td>OS/BS %</td>
<td>4.1(1.9-10.8)</td>
<td>11.1(6.5-11.8)</td>
</tr>
<tr>
<td>Ob.S/BS %</td>
<td>1.2(0.9-7.9)</td>
<td>4.5(3.6-9.5)</td>
</tr>
<tr>
<td>MS/BS %</td>
<td>2.7(1.7-11.4)</td>
<td>6.9(4.1-10.1)</td>
</tr>
<tr>
<td>BFR/BV %/yr</td>
<td>9.4(7.5-35.9)</td>
<td>26.2(11.6-32.4)</td>
</tr>
<tr>
<td>Ac.F #/yr</td>
<td>0.19(0.11-0.79)</td>
<td>0.46(0.30-0.71)</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Median (Interquartile Range)

\textsuperscript{b} No significant differences were found using Mann Whitney U test
Figure 7 Plot of BV/TV % in 3 groups. (♦ controls, ■ diabetes, ▲ fracturing diabetics). No significant differences between the control and diabetic groups. Fracturing diabetics had a trend towards lower BV/TV % compared to non-fracturing diabetics (P=0.085, Mann Whitney U test).
REFERENCES


disease study equation for estimating glomerular filtration rate. *Annals of Internal Medicine, 145*, 247-254.


APPENDIX
INFORMED CONSENT FORM

Title of Study: Pilot Study - Type 1 Diabetes and Bone Health

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INTRODUCTION
You are being asked to participate in a study to determine the effects of diabetes on bone health. Bone is constantly repairing itself and this study is designed to use various methods to determine if diabetes affects the repair process.

STUDY PROCEDURES
About 30 adults will participate in this part of the study. Requirements to be included in this study include: age ≥ 19 yrs or ≤ 50 yrs. Have a diagnosis of Type 1 diabetes clinically defined as diabetes onset before age 50, acute presentation or diabetic ketoacidosis, with normal weight. If history is equivocal, antibodies for diabetes > 1.45 U/mL will be used to define diagnosis. Diabetic subjects must be on insulin treatment. All subjects must have Body Mass Index (BMI) between 18-30, not on any medications
that are known to interfere with bone metabolism including thiazides such as hydrochlorothiazide, loop diuretics such as Lasix, steroids such as prednisone, anti-seizure medicine, bisphosphonates such as Actonel or Boniva, metformin, glitazones such as Actos or Avandia, have normal or only mildly impaired kidney function, no history of cancer other than skin cancer, no unstable chest pain, heart attack, uncontrolled hypertension, malabsorption, active rheumatoid disease or collagen disease such as Erhlos-Danlos or osteogenesis imperfecta, have not been pregnant or nursing in the last 12 months. We will also recruit 30 age and sex matched controls, and an additional 30 unmatched controls.

**Study Procedures**

1. At the beginning of the study a physical exam will be done including vital signs, height, weight, and a more detailed history including family fracture history, personal fracture history, menstrual history, diabetic history, alcohol and tobacco use. You will be asked to complete a physical activity questionnaire, Vitamin D and calcium intake questionnaires, and sun exposure questionnaire will be done.

2. A measurement of skin color will be taken using an IMS SmartProbe device (similar to a camera). We will make 3 measurements including inner upper arm, flank and forearm.

3. 46 cc of blood (≈ 3 tablespoons) will be drawn. The blood will be used to measure renal status, diabetes diagnosis and glycemic control, Vitamin D status, thyroid function, hormone levels, bone remodeling markers and to exclude malabsorption syndromes, kidney and liver disease.

4. If it is possible that you may be pregnant, a urine pregnancy test will be performed prior to the radiographic procedures to ensure that you are not pregnant at the time of the exam.

5. Bone mineral density testing (known as DXA) will be performed at the first visit. This test involves lying down on a table in different positions while pictures are taken of your bones. This is a painless test that takes about 10 minutes. This test will provide information about your bone health, as well as information about the amount of fat and muscle in your body.

6. The peripheral qCT, which measures the density of bones in your arms or legs, will be done. This test involves holding your forearm and tibia in a machine that takes pictures of the bones. This is a painless test that takes about 10 minutes. This test will provide information about your bone health.

7. You will undergo a single bone biopsy of your hip approximately two months after your first visit. The biopsy will give us detailed information about your bone quality. This will include information about whether you have low bone density because you are breaking down too much bone or not forming enough new bone. The biopsy will also give a detailed look at the three-dimensional structure of your bone and at the way calcium crystals are organized in your bone. The bone biopsy provides detailed information about the quality of your bone that cannot be obtained through other tests like x-rays or blood tests.

**Procedure for Bone Biopsy**
You will be given a package of medication containing an oral antibiotic (tetracycline), which sticks to your bone and helps measure bone formation. You will take the tetracycline in two separate 3-day treatment periods, 14 days apart. During the time you are taking the antibiotic, no supplemental calcium should be taken. Your bone biopsy will be taken between 5 and 14 days after taking your last tablet of tetracycline. We will ask for frequent blood sugar measurements to be recorded during the time you are taking the antibiotic.

On the day of the biopsy, you will go to an outpatient surgery suite or the study clinic approximately 30-60 minutes before the procedure. You will lie down on a table and devices to monitor blood pressure, pulse, heart function, and oxygen content of the blood will be connected to you. Before beginning the biopsy, we will draw 3 cc (≈1/2 teaspoon of blood) for a hemoglobin A1c and you will receive a mild sedative (a drug to help you relax) in your arm vein. The area around your hip will be numbed by an injection of a local anesthetic. A small incision (approximately 2 cm or ¾ inch) will be made in the skin over your hip bone. A needle will then be inserted into the hip bone. You will feel some pressure. A biopsy (piece of bone about the size of a pea) will be taken from the bone through the needle. The incision will be closed with 3 stitches and a bandage will be placed over the wound until it is healed.

RISKS
The potential risks of this study are related to the blood draws, radiation exposure from the bone density test and the peripheral QCT and the bone biopsy.

The risks from the blood draw include pain, bleeding, bruising, infection and inflammation at the site.

The amount of radiation from the bone density test is 24.8 microsieverts (μSv) which is less than the radiation received during a transatlantic airplane flight (40μSv) or from a chest x-ray (40 μSv). The peripheral qCT has 2.46 microsieverts (μSv) of radiation.

The risks of the bone biopsy include those related to the antibiotics (tetracyclines) administered before the biopsy, the risks of medications given for relaxation and pain relief during the procedure and the risk of the biopsy itself. The tetracycline antibiotics may cause stomach upset. Skin rashes may develop if you are exposed to sunlight while on the antibiotics. You will be advised to stop the tetracycline if stomach upset develops. You will also be advised to avoid sunlight exposure during the 6 days you are taking the medication. Medication will be offered to you during the bone biopsy in order to reduce anxiety and provide pain relief. People who receive these medications may have brief and temporary loss of protective reflexes (the ability of your body to react to dangerous situations), but you will be watched closely and at regular intervals during and after the procedure. Complications during the biopsy itself are very unusual. Occasionally there will be a bruise at the biopsy site or discomfort that can last a few days. There may be some minor temporary discomfort when the intravenous catheter (a hollow tube inserted into the vein) is introduced into your arm to administer the sedative, and when the local anesthetic is initially injected into the skin at your hip. There is a risk of infections at the site of the biopsy. The risk of infection will be kept to a minimum by using sterile...
technique. The incision will be sutured and a pressure-dressing bandage applied to prevent bleeding. Every precaution will be taken to avoid any complications.

**Risk to a pregnant female.**
Tetracycline crosses the placenta and enters the baby’s blood stream. This may cause permanent discoloration of teeth if used during the last half of pregnancy. Because of the potential risk of harm to an unborn fetus, all sexually-active women who are able to have children must use a medically acceptable method of birth control while taking the study drug. Women may not breastfeed while taking the tetracycline. There is still a risk that pregnancy could occur despite the responsible use of a reliable method of birth control. You agree to notify the investigator as soon as possible of any failure of proper use of your birth control method, or if you become pregnant, either of which will result in your being withdrawn from the study.

If you have any injury, bad effect, or any unusual health experience during this study, make sure that you immediately tell Dr Armas at 402-679-5821. You can call at any time, day or night, to report such health experiences.

**BENEFITS**
The potential benefits to you are possibly: knowledge gained about the nature of how minerals are processed in your bones, knowledge about your bone mass, and the results of tests done on biopsy specimens. On a wider scale, there may be general benefits to society because the knowledge gained from this study may help better understand the effects of diabetes on bone health.

**STORAGE OF UNUSED SERUM OR BONE SAMPLES**
We will store leftover serum for future lab measurements related to diabetes and bone health. No genetic studies will be done on these samples. Please check the appropriate box to indicate your agreement with storing your serum for future lab measurements.

☐ Yes  ☐ No

After testing has been done on the bone biopsy specimen, we may have some leftover specimen. We will store leftover bone specimens for future measurements related to diabetes and bone health. No genetic studies will be done on these samples. Please check the appropriate box to indicate your agreement with storing your bone biopsy specimen for future measurements.

☐ Yes  ☐ No

**ADDITIONAL COSTS**
There are no costs to you for participating in this study.

**COMPENSATION**
You will be offered a total compensation of $350 for completing the study. Compensation will be prorated if you do not complete the study. Reimbursement will be allotted in the following schedule: Visit 1: Screening: $50, Visit 2: Bone biopsy: $300.
Payment in the form of a check will be sent to you approximately 6-8 weeks after study completion.

CONFIDENTIALITY
We will not release any information about you without your permission, except as may be required by law. Please note, however, that representatives of the Creighton University Institutional Review Board, other internal departments that provide support and oversight at Creighton University/Creighton University Medical Center and the U.S. Food and Drug Administration may legally review research records which may identify you by name. When we publish or present the results of this scientific work, we will not reveal your identity.

VOLUNTARY PARTICIPATION
Participation in this study is voluntary. Refusal to participate will involve no penalty or loss of benefits to which you are otherwise entitled. Likewise, if you elect to participate in this study, you may discontinue your participation at any time without penalty or loss of benefits to which you are otherwise entitled. You are encouraged to ask questions at any time during the study. If you have a problem, have more questions about the study or in the event of a study-related injury please call Dr. Armas at 402-280-4470. If you have any questions about your rights as a subject in this study, you may also contact the Creighton University Institutional Review Board Office at 402-280-2126.

ALTERNATIVE PROCEDURES
The alternative is not to participate in this research study. Your decision whether or not to participate in this study will have no effect on your medical care at this hospital.

RESEARCH-RELATED INJURY
The investigator will make every effort to prevent study-related injuries and illnesses. If you are injured or become ill while you are in the study and the illness or injury is due to your participation in this study, you will receive necessary medical care at the usual charge. The costs of this care that are not covered by the sponsor will be charged to you or to your health insurer. No funds are available from Creighton University or Creighton University Medical Center to repay you or compensate you for a study-related injury or illness. There is also no compensation available for payment of your lost wages or other losses.

By signing this consent form you will not be waiving any of your legal rights which you otherwise would have as a subject in a research study.

CONSENT STATEMENT
You are free to refuse to participate in this research project or to withdraw your consent and discontinue participation in the project at any time without penalty, loss of benefits to which you are otherwise entitled or effect on your relationship to the institution(s) involved in this research project.
My signature below indicates that all my questions have been answered. I agree to participate in the project as described above.

__________________________________  __________________
Signature of Subject Date Signed

If you are not satisfied with the manner in which this study is being conducted, you may report (anonymously if you so choose) any complaints to the Institutional Review Board by calling (402) 280-2126, or addressing a letter to the Institutional Review Board, Office of Grants Administration, Creighton University, 2500 California Plaza, Omaha, NE 68178.

A copy of this form has been given to me. __________ Subject’s Initials

For the Research Investigator—I have discussed with this subject the procedure(s) described above and the risks involved; I believe he/she understands the contents of the consent document and is competent to give legally effective and informed consent.

__________________________________  __________________
Signature of Responsible Investigator Date Signed

We would appreciate your feedback on your experience as a research participant at Creighton University; please fill out our survey at http://www.creighton.edu/participantsurvey.
BONE HISTOMORPHOMETRY NOMENCLATURE AND FORMULAE

1XL = sum length of regions bearing single fluorochrome label measured on unstained slides

2XL = sum length of regions bearing double fluorochrome label measured on unstained slides

2XTh = inter label thickness

Ac.F = activation frequency, BFRBS/0.001*W.Th

Aj.AR = adjusted apposition rate, MAR*MSOS/100

BFR/BS = bone formation rate (bone surface referent), 365.25*MAR*

\[(MSBS)*1000/100,000\]

BFR/BV = bone formation rate (bone volume referent),

\[(BSTV*MSBS*MAR*365.25)*100/1000*BVTV\]

BV/TV = bone volume, (100*TBArG)/TtArG

ErL = sum length of all eroded surface measured on Goldner stained slide

ES/BS = eroded surface (bone surface referent), 100*(ErL+OcL)/TSG

FP = formation period, W.Th*OSBS/365.25*MSBS*MAR

GrLG = edge of the counting window, is subtracted to get TSLG

GrLU = edge of the counting window, is subtracted to get TSLU

ILD = inter label time

MAR = mineral apposition rate, (1000*2XTh*π)/(ILD*#2XTh)*4

Mlt = mineralization lag time, O.Th*OSBS/MSBS*MAR

MS/BS = mineralizing surface (bone surface referent), 100*2XL+(1XL/2)/TSU

MS/OS = mineralizing surface (osteoid surface referent), 100*MSBS/OSBS
ObL = sum length of all osteoid surface that is covered with osteoblasts measured on
Goldner stained slide
Ob.S/BS=osteoblast surface (bone surface referent), 100*ObL/TSG
OcL= sum length of all eroded surface that is covered with osteoclasts measured on
Goldner stained slide
OS/BS=osteoid surface (bone surface referent), 100*(OsL+ObL)/TSG
OsL = sum length of all osteoid surface measured on Goldner stained slide
O.Th=osteoid thickness, (1000*OsTh*π)/#OsTh*4
PcTh=sum thickness of all measurements from bone marrow interface to cement
line measured on Toluidine blue stained slide
Rm.P=remodeling period, FP+RP
RP= FP*ESBS/OSBS
TBArG=All trabecular bone tissue area measured on Goldner stained slide
Tb.N=trabecular number, 10*BV/TV/Tb.Th
Tb.Sp=trabecular separation, (1000/Tb.N)-(Tb.Th/10)
Tb.Th=trabecular thickness, 20*BVTV/BSTV
TSG=Total surface on Goldner slide, TSLG-GrLG
TSLG=Length of all bone surfaces measured on Goldner slide
TSLU= Length of all bone surfaces measured on unstained slide
TSU = Total Surface on unstained slide, TSLU-GrLU
TtArG=All trabecular tissue area measured on Goldner stained slide
W.Th=wall thickness, (1000*PcTh*π)/[#(PcTh)*4]
## Appendix Table 1 – Individual Patient Demographics

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Preparation of Reagents Involved in Sectioning and Staining

1. Haupt's Gelatin Adhesive
   (Expiration Date is 12 months from Date of Preparation)
   1. Add 3g gelatin to 300ml dH₂O in a 500ml flask.
   2. Dissolve on a heated stir plate.
   3. Add and dissolve 6g of phenol crystals.
   4. Add 45ml of glycerol; stir well and filter
   5. Store at room temperature
   The Expiration Date for Haupt’s is 1 year).

2. Goldner Staining Reagents:
   All prepared stains are stored at room temperature. Expiration Dates for each are
   listed below. All solutions that are made with distilled water will be made with fresh
   distilled water from the distilled water tap.

A. Weighert’s Hematoxylin - modified

1) Solution A
   Expiration Date is 12 months from Date of Preparation
   a) 4.9g of Hematoxylin crystals
   b) 490ml 95% ethanol
   c) Filter
   d) Store tightly capped at room temperature

2) Solution B
   Expiration Date is 12 months from Date of Preparation
   a) 20ml 62% FeCl₃
   b) 475ml of distilled water
   c) 5ml of HCl
   d) May be filtered
   e) Store tightly capped at room temperature

B. Weighert's Hematoxylin (Working Solution)
   1. Combine 200ml each of Solutions A and B for each tray of slides
      IMMEDIATELY before use.
   2. Do not store; dispose of at close of each day.
C. Masson's Ponceau-fuchsin
   a. **Ponceau-Fuchsin G, R, 2R 1% aqueous solution (A)**.
      Prepare fresh. Discard excess. Do not store.
      Add 0.75g of Ponceau G, R, 2R to 75ml dH₂O.
   b. **Acid Fuchsin, 1% aqueous solution (B)**
      Prepare fresh. Discard excess.
      Add 0.25g of Acid Fuchsin to 25ml dH₂O.
   a. Stock Solution
      Add 3 parts Solution A to 1 part Solution B
      0.2% Acetic Acid
      Add 200ml of 1% acetic acid to 800ml dH₂O.
   b. Working Solution
      Expiration Date is 12 months from Date of Preparation
      1. Dilute Stock Solution 1:10 with 0.2% acetic acid for staining.
      2. Add 100ml of solution C to 900ml of solution D.
      3. Date and store tightly capped at room temperature

D. Phosphotungstic-Phosphomolybdic Solution
   Expiration Date is 6 months from Date of Preparation
   a. Add 12.5g of Phosphomolybdic Acid (refrigerated at 4°C).
      (DO NOT USE a metal spatula with the acid), plus 12.5g of
      Phosphotungstic Acid to 495ml dH₂O.
   b. May be filtered.
   c. Date and store at 4°C.

E. 1% Naphthol Green B Solution
   Expiration Date is 6 months from Date of Preparation
   a. Add 4.8g of Naphthol Green B to 480 ml dH₂O
   b. Add 4.8ml of Acetic Acid
   c. Stir on the mechanical stirrer then filter
   d. Date and store at 4°C.

F. Alcoholic Safranine Solution
   Expiration Date is 6 months from Date of Preparation
   a. 4g of Safranine du Gatinais
   b. 400ml of Absolute ethyl alcohol
   c. Place in stoppered flask in a 50°C-60°C oven for 2 days.
   d. Agitate periodically. Cool and filter.
   e. Date and store at room temperature.
2. Toluidine Blue Reagents

**Toluidine Blue Buffer**

Expiration Date is 12 months from Date of Preparation

a. Buffer Mixture pH 6.8 (Order from Orbeco Analytical Systems, Inc.; 185 Marine Street; Farmingdale, NY 11735; 516-293-4110 #R-3693P)

Dissolve thoroughly one vial in 100ml dH$_2$O. Date and store in tightly capped jar at room temperature.

b. Buffer Mixture pH 5.8 (Order from Orbeco Analytical Systems, Inc.; 185 Marine Street; Farmingdale, NY 11735; 516-293-4110 #R-3693P)

Dissolve thoroughly one vial in 100ml of dH$_2$O. Date and store in a tightly capped jar at room temperature.

**Working Buffer Solution**

pH 6.5 IS CRITICAL FOR WORKING SOLUTION.

1) 10ml Buffer pH 6.8 add to 475ml dH$_2$O.

2) 10ml Buffer pH 5.8 add to above solution.

3) Adjust pH as necessary. Use the pH meter to test the solution.

4) Date and store in tightly capped jar at 4°C.

**1% T.B. Stain**

Expiration Date is 3 months from Date of Preparation

1) 0.25g toluidine blue O

2) q.s. to 25ml with dH$_2$O

3) Date and store at room temperature